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Restricted Passage of Reaction Intermediates through the Ammonia Tunnel of Carbamoyl Phosphate Synthetase*

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The x-ray crystal structure of the heterodimeric carbamoyl phosphate synthetase from *Escherichia coli* has identified an intermolecular tunnel that connects the glutamine binding site within the small amidotransferase subunit to the two phosphorylation sites within the large synthetase subunit. The tunneling of the ammonia intermediate through the interior of the protein has been proposed as a mechanism for the delivery of the ammonia from the small subunit to the large subunit. A series of mutants created within the ammonia tunnel were prepared by the placement of a constriction via site-directed mutagenesis. The degree of constriction within the ammonia tunnel of these enzymes was found to correlate to the extent of the uncoupling of the partial reactions, the diminution of carbamoyl phosphate formation, and the percentage of the internally derived ammonia that is channeled through the ammonia tunnel. NMR spectroscopy and a radiolabeled probe were used to detect and identify the enzymatic synthesis of *N*-amino carbamoyl phosphate and *N*-hydroxy carbamoyl phosphate from hydroxylamine and hydrazine. The kinetic results indicate that hydroxylamine, derived from the hydrolysis of γ-glutamyl hydroxamate, is channeled through the ammonia tunnel to the large subunit. Discrimination between the passage of ammonia and hydroxylamine was observed among some of these tunnel-impaired enzymes. The overall results provide biochemical evidence for the tunneling of ammonia within the native carbamoyl phosphate synthetase.

Carbamoyl phosphate synthetase (CPS)[1] from *Escherichia coli* catalyzes the formation of carbamoyl phosphate from bicarbonate, glutamine, and two molecules of ATP (Equation 1) (1). When one or more of these substrates are not present, CPS catalyzes three partial reactions: (*a*) the hydrolysis of glutamine, (*b*) the bicarbonate-dependent hydrolysis of ATP, and (*c*) the formation of ATP from carbamoyl phosphate and ADP (Equations 2–4) (2). Ammonia can substitute for glutamine in the overall reaction as an alternative source of nitrogen (Equation 5). The enzyme is composed of a small amidotransferase subunit and a large synthetase subunit (1, 3, 4). The synthesis of carbamoyl phosphate has been postulated to occur via four distinct chemical steps within the active sites of CPS (Scheme 1) (2). The x-ray structure of CPS from *E. coli* has confirmed that the binding site for glutamine is contained within the small subunit, whereas the catalytic sites for the phosphorylation of bicarbonate and carbamate are located within the large subunit (5, 6). However, the most intriguing feature of the CPS structure is that the three active sites located within the small and large subunits are physically separated in three-dimensional space by nearly 100 Å (5, 6). The channeling of ammonia and carbamate through the interior of the protein has been proposed as a mechanism to deliver these two labile intermediates from one active site to the next (7–9). For recent reviews on substrate channeling, see Refs. 10–12.

$$2\ MgATP + HCO_3^- + Gln + H_2O \rightarrow 2\ MgADP + P_i + Glu + carbamoyl\text{-}P \quad \text{(Eq. 1)}$$

$$Gln + H_2O \rightarrow Glu + NH_3 \quad \text{(Eq. 2)}$$

$$MgATP + H_2O \rightarrow MgADP + P_i \quad \text{(Eq. 3)}$$

$$MgADP + carbamoyl\text{-}P \rightarrow MgATP + NH_2CO_2^- \quad \text{(Eq. 4)}$$

$$2\ MgATP + HCO_3^- + NH_3 \rightarrow 2\ MgADP + P_i + carbamoyl\text{-}P \quad \text{(Eq. 5)}$$

Utilizing a visual inspection of the CPS model and a computational search with the software package GRASP, Thoden *et al.* (5, 6) identified a molecular tunnel within the interior of the heterodimeric protein, which leads from the glutamine binding site within the small subunit toward the two phosphorylation sites within the large subunit. Consequently, the ammonia produced within the active site of the small subunit must transverse the first half of this molecular tunnel in order to react with the carboxy phosphate intermediate formed at the site of bicarbonate phosphorylation within the large subunit. The carbamate intermediate must then diffuse through the second half of the molecular tunnel to be phosphorylated by the ATP bound to the other phosphorylation site of the large subunit. A ribbon representation of CPS with the relative locations of the three active sites and the intermolecular tunnel is shown in Fig. 1 (5).

Biochemical results are fully consistent with the tunneling of the ammonia and carbamate intermediates. Isotope competition experiments with $^{15}NH_3$ and unlabeled glutamine have demonstrated that the *internal* ammonia, derived directly from the hydrolysis of glutamine, does not dissociate from the small subunit and then reassociate to the large subunit (13). The pH activity profiles also confirm that the enzyme-bound NH_3 must be sequestered from the bulk solvent because NH_4^+ is not a substrate for the synthetase reaction (14, 15). These results are thus consistent with a mechanism that requires the direct tunneling of ammonia between the two subunits. The lack of an ^{18}O isotope exchange reaction between solvent water and bicarbonate during the overall synthesis of carbamoyl phosphate

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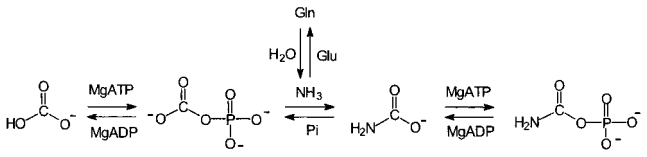
[1] The abbreviations used are: CPS, carbamoyl phosphate synthetase; CHES, 2-(cyclohexylamino)ethanesulfonic acid.



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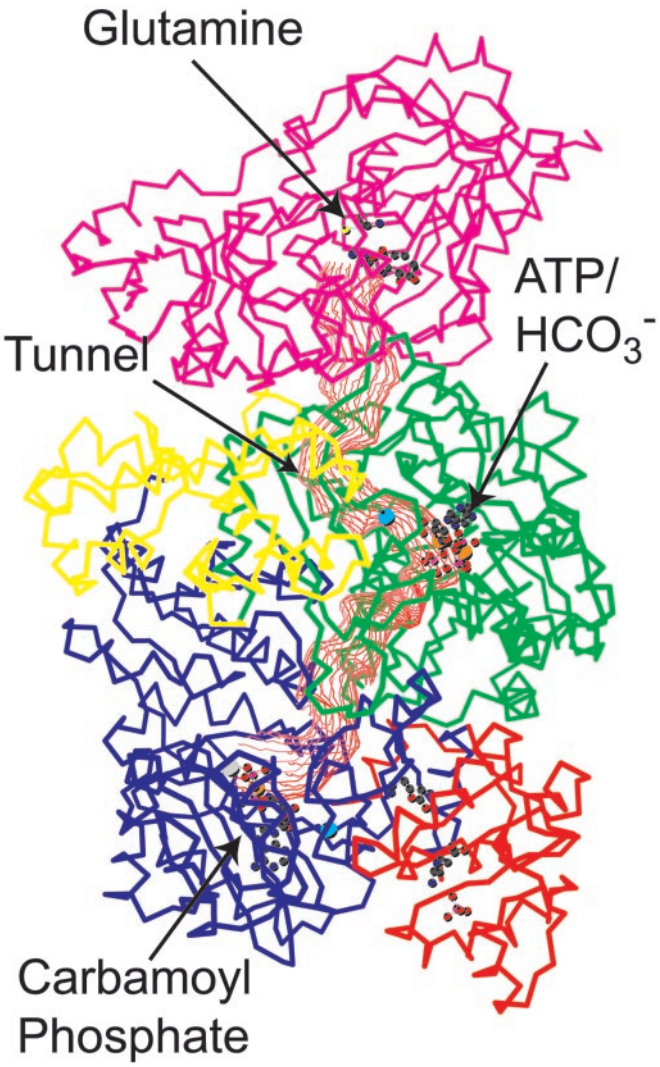


FIG. 1. **An α-carbon trace of the three-dimensional structure of CPS from *E. coli* (taken from Ref. 5).** The binding sites for glutamine, ATP/bicarbonate, and carbamoyl phosphate are *highlighted*. The molecular tunnel connecting the three binding sites is *outlined* in *red*.

suggests that all of the carbon-containing intermediates (carboxy phosphate and carbamate) are fully committed to the formation of carbamoyl phosphate and not subjected to hydrolysis (16). Moreover, the half-life of 70 ms for carbamate at neutral pH (17) renders it highly improbable that carbamate would be able to dissociate from the first phosphorylation site and then reassociate to the second phosphorylation site within the large subunit. These results thus support the tunneling of carbamate between the two active sites contained within the large subunit.

We have previously altered some of the residues that define the interior walls of the *ammonia tunnel* within the small subunit with the intention of providing more direct experimental support for the functional significance of the molecular tunnel within CPS (18). Two mutant proteins (G359Y and G359F) displayed kinetic properties consistent with a constriction or blockage of the ammonia tunnel. With the wild-type CPS, the hydrolysis of ATP and glutamine is fully coupled to one another such that one equivalent of carbamoyl phosphate is formed when two equivalents of ATP and one equivalent of glutamine are hydrolyzed. However, with both of these mutants, the hydrolysis of ATP and glutamine became uncoupled from the synthesis of carbamoyl phosphate. However, these mutants were fully functional when *external* ammonia was utilized as the nitrogen source, even though these proteins were unable to use glutamine for the synthesis of carbamoyl phosphate. These results also suggested the existence of an alternate route to the bicarbonate phosphorylation site when ammonia is provided as an external nitrogen source (18).

In order to more fully substantiate the direct tunneling of the ammonia intermediate as a mechanistic link within the CPS heterodimer, we have now made further refinements to the architecture of the ammonia tunnel. In the first approach, we modulated the size of the physical constriction within the ammonia tunnel located in the small subunit of carbamoyl phosphate synthetase. This was attempted through the placement of hydrophobic side chains of varying sizes within the walls of the ammonia tunnel. In the second approach, hydroxylamine and hydrazine were utilized as alternative nitrogen sources of greater bulk. If hydroxylamine and hydrazine are able to be transported through the ammonia tunnel of the wild-type CPS, then the variable physical constrictions within the ammonia tunnel may differentially affect the transport rate from one active site to the next.

EXPERIMENTAL PROCEDURES

Materials—All chemicals and coupling enzymes used for kinetic measurements were purchased from either Sigma or Aldrich unless otherwise stated. Ammonium chloride (^{15}N, ≥98%), sodium bicarbonate (^{13}C, ≥99%), hydroxylamine HCl (^{15}N, 98%+) and hydrazine sulfate ($^{15}N_2$, ≥98%) were purchased from Cambridge Isotope Laboratories. Sodium [^{14}C]bicarbonate was purchased from Amersham Pharmacia Biotech. Restriction enzymes, Vent® DNA polymerase, and *Pfu* DNA polymerase were purchased from Promega. Oligonucleotide synthesis and DNA sequencing were performed by the Gene Technologies Laboratory, Texas A&M University. The *E. coli* RC50 cell line, which does not express CPS (21), was a generous gift of Dr. Carol J. Lusty.

Mutagenesis, Expression, and Purification of the Mutant Proteins—Site-directed mutagenesis was performed as described previously (22) using the polymerase chain reaction and the overlap extension method of Ho *et al.* (23). The mutant plasmids were transformed in the *E. coli* RC50 cell line for the expression of the modified proteins. The wild-type and mutant proteins were purified as described by Mareya and Raushel (24).

Kinetic Measurements—The synthesis of carbamoyl phosphate was determined by measuring the rate of citrulline formation in a coupled assay containing ornithine transcarbamoylase and ornithine (25). The rate of ATP hydrolysis was determined by assaying the formation of MgADP using a pyruvate kinase/lactate dehydrogenase coupling system (24). The rates of hydrolysis of glutamine, γ-glutamyl hydroxamate, and γ-glutamyl hydrazide were determined by coupling the formation of glutamate to the production of α-ketoglutarate with L-glutamate dehydrogenase and 3-acetylpyridine adenine dinucleotide (24).

Statistical Analysis of Kinetic Data—The kinetic parameters, V_{max} and K_m, were determined by fitting the experimental data to Equation 6, where v is the initial velocity, V_{max} is the maximal velocity, K_m is the Michaelis constant, and A is the substrate concentration. The data for the enhancement of ATP hydrolysis in the presence of a nitrogen source were fitted to Equation 7 (26). In this equation, V_o is the initial enzyme velocity in the absence of a nitrogen source (I) (ammonia or glutamine), K_a is the apparent activation constant, and α is the ratio of the velocities at saturating and zero concentration of the nitrogen source. In this case, V_{max} is expressed as αV_o.

$$v = V_{max}A/(K_m + A) \quad \text{(Eq. 6)}$$

$$v = V_o(K_a + \alpha I)/(K_a + I) \quad \text{(Eq. 7)}$$

Detection of Carbamoyl Phosphate Analogs—The formation of carbamoyl phosphate analogs from the nonnatural nitrogen sources, hy-

TABLE I

Kinetic parameters for the ATPase reactions of the wild-type and mutant enzymes

Shown are kinetic constants for ADP formation monitored for the bicarbonate-dependent ATPase (Equation 3), ammonia-dependent ATPase (Equation 5), or glutamine-dependent ATPase reactions (Equation 1).

Enzymes	HCO_3^--dependent[a]	NH_3-dependent[b]		Gln-dependent[c]	
	k_{cat}	k_{cat}	K_m (NH_4^+)	k_{cat}	K_m (Gln)
	s^{-1}	s^{-1}	mM	s^{-1}	mM
Wild-type	0.39 ± 0.01	7.0 ± 0.6	250 ± 32	5.8 ± 0.1	0.098 ± 0.004
S35F	0.49 ± 0.02	5.4 ± 0.2	231 ± 25	5.7 ± 0.1	0.46 ± 0.01
G359S	1.2 ± 0.1	12 ± 0.2	34 ± 2	3.2 ± 0.3	20 ± 6
G359L	1.2 ± 0.1	10.5 ± 0.3	31 ± 3	1.5[d]	NA[e]
G359F[f]	1.2 ± 0.1	12 ± 0.2	34 ± 1	1.5[d]	NA

[a] Reaction conditions for the bicarbonate-dependent ATPase reaction: pH 7.6, 25 °C, 5.0 mM ATP, 40 mM bicarbonate, 20 mM $MgCl_2$, 100 mM KCl, and 10 mM ornithine.
[b] Reaction conditions: the same as above except for variable amounts of NH_4Cl.
[c] Reaction conditions: the same as above except for variable amounts of glutamine.
[d] Rate constant at a glutamine concentration of 40 mM.
[e] NA, not applicable.
[f] Data from Ref. 15.

droxylamine and hydrazine, was probed using [^{14}C]bicarbonate. Each reaction mixture contained 50 mM HEPES (pH 7.6), 10 mM ornithine, 40 mM $MgCl_2$, 100 mM KCl, 30 mM ATP, 5 mM [^{14}C]bicarbonate (specific activity of 24 μCi/μmol), and 16 μM CPS. The concentration of the nitrogen sources were 300 mM for NH_4Cl, NH_2OH or NH_2NH_2. A control reaction, which contained no CPS, was carried out in parallel. Aliquots of the reaction mixture were quenched after 5 and 10 min with 2 volumes of 5% trichloroacetic acid. The quenched samples were incubated on ice for 5 min and then centrifuged at 14,000 rpm for 3 min to remove precipitated protein. Powered dry ice was added to the supernatant solutions to remove the unreacted bicarbonate. The amount of the nonvolatile products was determined by liquid scintillation counting. The same experiments were repeated using 20 mM glutamine, 20 mM γ-glutamyl hydroxamate, or 20 mM γ-glutamyl hydrazide as the nitrogen source.

Identification of Carbamoyl Phosphate Analogs by ^{13}C and ^{31}P NMR—The synthesis of the carbamoyl phosphate analogs using hydroxylamine or hydrazine as nitrogen sources was investigated by NMR spectroscopy. The reactions were buffered by 100 mM HEPES (pH 7.6) or 100 mM CHES (pH 9.0). Each reaction mixture contained 10 mM ornithine, 20 mM ATP, 40 mM $MgCl_2$, 100 mM KCl, 20 mM [^{12}C]bicarbonate or [^{13}C]bicarbonate, 30 mM phosphoenolpyruvate, 120 units pyruvate kinase, 30 μM CPS, and 10% D_2O. For the synthesis of carbamoyl phosphate, 300 mM NH_4Cl (or $^{15}NH_4Cl$) was included as the nitrogen source. For the formation of the two carbamoyl phosphate analogs, 200 mM NH_2OH (or $^{15}NH_2OH$) or 200 mM NH_2NH_2 (or $^{15}NH_2{}^{15}NH_2$) was utilized as the sole nitrogen source. A reaction containing no CPS was used as a control. ^{31}P and ^{13}C NMR experiments were conducted on a Unity Plus-300 NMR spectrometer. ^{31}P spectra were obtained at a frequency of 121.4 MHz. Typical acquisition parameters were 10,000 Hz sweep width, 2 μs acquisition time, no delay between pulses, and 6 μs (or 37.2°) pulse width. The ^{31}P spectra were internally referenced to phosphate. ^{13}C spectra were obtained at a frequency of 75.4 MHz. Typical acquisition parameters were 16,000 Hz sweep width, 2 μs acquisition time, no delay between pulses, and 2.8 μs (or 30.5°) pulse width.

RESULTS

The previous investigation of the ammonia tunnel of CPS demonstrated that the passage of ammonia could be restricted within the tunnel via the substitution of specific residues, which constitute part of the tunnel wall, with bulkier hydrophobic residues (18). In order to more fully substantiate the tunneling of the ammonia intermediate within CPS, a new series of mutants with different degrees of potential constriction within the ammonia tunnel was created. Three mutants, G359S, G359L, and S35F, were constructed and purified in addition to the wild-type CPS and the previously prepared G359F. The wild-type and mutant enzymes were expressed and purified to greater than 95% homogeneity, as judged by SDS-polyacrylamide gel electrophoresis. The effects of these modifications on the catalytic properties of CPS were determined for each mutant by measuring the rate of carbamoyl phosphate synthesis and the rates of the partial reactions. The kinetic parameters, K_m and k_{cat}, obtained for the wild-type and the mutant enzymes, are summarized in Tables I–III.

Kinetic Properties of S35F—The substitution of Ser-35 with a bulky phenylalanine was intended to block or constrict the passage of ammonia to the large subunit within the ammonia tunnel. The catalytic properties of the S35F mutant are very similar to the wild-type enzyme, with two notable exceptions. During the overall synthesis of carbamoyl phosphate with glutamine as the nitrogen source, the K_m values for glutamine are elevated by 5–6-fold (Tables I–III), and the maximal rate of glutamine hydrolysis is about twice the wild-type value (Table II). Consequently, the partial reactions within the heterodimer of S35F are mildly uncoupled from one another. An estimate of reaction stoichiometry is obtained from the ratio of the maximal rate of the formation of ADP (Table I), glutamine (Table II), and carbamoyl phosphate (Table III). For the synthesis of one equivalent of carbamoyl phosphate, 2.4 equivalents of ATP and 2.3 equivalents of glutamine are consumed.

Kinetic Properties of G359S—The replacement of Gly-359 with a serine residue yields a mutant of CPS with properties somewhat between the wild-type protein and G359F. With G359F, the hydrolysis of ATP and glutamine became almost completely uncoupled from the synthesis of carbamoyl phosphate (18). With G359S, it is apparent that these two partial reactions are still coupled to one another to a significant extent, as evidenced by the formation of carbamoyl phosphate and the mutual stimulation of the bicarbonate-dependent ATPase and glutaminase reactions. In all of the glutamine-dependent reactions, the K_m values for glutamine are elevated by 200–400-fold (Tables I–III). During the synthesis of carbamoyl phosphate, the rate of glutamine hydrolysis is enhanced by 150-fold (Table II) relative to the absence of ATP and bicarbonate. The mutant also has an elevated bicarbonate-dependent ATPase activity, which is further stimulated about 3-fold in the presence of glutamine (Table I). When glutamine is used as the nitrogen source, the maximal rate of carbamoyl phosphate formation is one-third of the wild-type value (Table III), and 3 equivalents of ATP and 5 equivalents of glutamine are utilized for the production of one equivalent of carbamoyl phosphate.

Kinetic Properties of G359L—Gly-359 was also mutated to a leucine residue. Although G359L retains the ability to enhance the basal glutaminase activity in the presence of ATP and bicarbonate (88-fold), it no longer stimulates the bicarbonate-dependent hydrolysis of ATP in the presence of glutamine (Tables I and II). This uncoupling between the partial reactions is also reflected in the inability to produce significant amounts

TABLE II

Kinetic parameters for the glutaminase reaction of the wild-type and mutant enzymes

Shown are kinetic constants for glutamate formation in the absence (Equation 2) or presence (Equation 1) of ATP and bicarbonate.

Enzymes	Partial reaction[a]		Overall reaction[b]	
	k_{cat}	K_m (Gln)	k_{cat}	K_m (Gln)
	s^{-1}	mM	s^{-1}	mM
Wild-type	0.0040 ± 0.0001	0.083 ± 0.006	2.9 ± 0.1	0.069 ± 0.007
S35F	0.0015 ± 0.0001	0.084 ± 0.013	5.6 ± 0.1	0.44 ± 0.03
G359S	0.033 ± 0.001	22 ± 2	5.0 ± 0.2	29 ± 2
G359L	0.028 ± 0.001	24 ± 2	2.5 ± 0.1	24 ± 2
G359F[c]	0.028[d]	>40	2.4[d]	>40

[a] Reaction conditions: pH 7.6, 25 °C, variable amounts of glutamine, 100 mM KCl.
[b] Reaction conditions: pH 7.6, 25 °C, variable amounts of glutamine, 5.0 mM ATP, 40 mM bicarbonate, 20 mM $MgCl_2$, 100 mM KCl, and 10 mM ornithine.
[c] Data from Ref. 15.
[d] Rate constant at 40 mM glutamine.

TABLE III

Kinetic parameters for carbamoyl phosphate synthesis by the wild-type and mutant enzymes

Shown are kinetic constants for carbamoyl phosphate formation (Equations 5 and 1).

Enzymes	NH_3-dependent[a]		Gln-dependent[b]	
	k_{cat}	K_m (NH_4Cl)	k_{cat}	K_m (Gln)
	s^{-1}	mM	s^{-1}	mM
Wild-type	2.9 ± 0.1	211 ± 23	3.2 ± 0.1	0.075 ± 0.003
S35F	3.2 ± 0.2	265 ± 29	2.4 ± 0.1	0.43 ± 0.02
G359S	6.3 ± 0.2	28 ± 2	1.1 ± 0.1	27 ± 6
G359L	5.6 ± 0.1	25 ± 2	0.14 ± 0.02	30 ± 10
G359F[c]	4.8 ± 0.2	27 ± 3	0.049[d]	>40

[a] Reaction conditions: pH 7.6, 25 °C, variable amounts of NH_4Cl, 5 mM ATP, 40 mM bicarbonate, 20 mM, $MgCl_2$, 100 mM, KCl, 10 mM ornithine.
[b] Reaction conditions: pH 7.6, 25 °C, variable amounts of glutamine, 5.0 mM ATP, 40 mM bicarbonate, 20 mM $MgCl_2$, 100 mM KCl, and 10 mM ornithine.
[c] Data from Ref. 15.
[d] Rate constant at 40 mM glutamine.

of carbamoyl phosphate. The rate of carbamoyl phosphate formation with G359L is less than 5% that of the wild-type CPS (Table III), and 11 equivalents of ATP and 18 equivalents of glutamine are required for the synthesis of one equivalent of carbamoyl phosphate. The overall reaction stoichiometry of this series of tunnel mutants is summarized in Fig. 2. The mutants at position-359 are fully functional when external ammonia is provided as the alternate nitrogen source. It is unclear why the k_{cat} values are slightly higher than the wild-type value. Moreover, the K_m values for NH_4^+/NH_3 are reduced by 8–11-fold, whereas the wild-type reaction stoichiometry is preserved (Table III).

Enzymatic Formation of N-Hydroxy Carbamoyl Phosphate and N-Amino Carbamoyl Phosphate—An alternative approach to the placement of a constriction within the ammonia tunnel of CPS is the utilization of nucleophiles other than ammonia as the nitrogen source. Both hydroxylamine and hydrazine are bulkier than ammonia. Attempts were made to detect the formation of carbamoyl phosphate analogs from these two alternative nitrogen sources. Hydroxylamine, hydrazine, or ammonia was incubated with MgATP, [^{14}C]bicarbonate, and CPS. The reactions were quenched at various times, and the unreacted bicarbonate was removed. The amount of nonvolatile ^{14}C-containing product(s) was estimated by liquid scintillation counting (Table IV). For the reaction with ammonia, this method provides a quantitative assay for carbamoyl phosphate (27). The results here are thus consistent with the formation of a carbamoyl phosphate-like product in the enzymatic reaction using hydroxylamine or hydrazine as the nitrogen source. Similar results were also obtained when γ-glutamyl hydroxamate or γ-glutamyl hydrazide was provided as the nitrogen source (Table IV). These carbamoyl phosphate-like products have been identified as *N*-hydroxy carbamoyl phosphate (Scheme 2, *panel 2*) and *N*-amino carbamoyl phosphate (Scheme 2, *panel 3*) by NMR spectroscopy using hydroxylamine and hydrazine as nitrogen sources. The ^{13}C and ^{31}P NMR spectra of these two carbamoyl phosphate analogs are very similar to those of carbamoyl phosphate (Fig. 3). For all three compounds, the coupling constants between the carbonyl carbon and the phosphorus atom are close to 5 Hz, and the coupling constants between the carbonyl carbon and the amide nitrogen are about 25–26 Hz (Fig. 3). In every case, the phosphorus atom did not appear coupled to the amide nitrogen. For *N*-amino carbamoyl phosphate (3), no coupling between the carbonyl carbon and the terminal amino group was observed. It has been reported that the coupling constant between the methyl carbon and the terminal amino nitrogen in *N,N*-[^{13}C]dimethyl[^{15}N]hydrazine is less than 1 Hz (28). Attempts to obtain the ^{15}N NMR spectra of the two carbamoyl phosphate analogs were unsuccessful be-

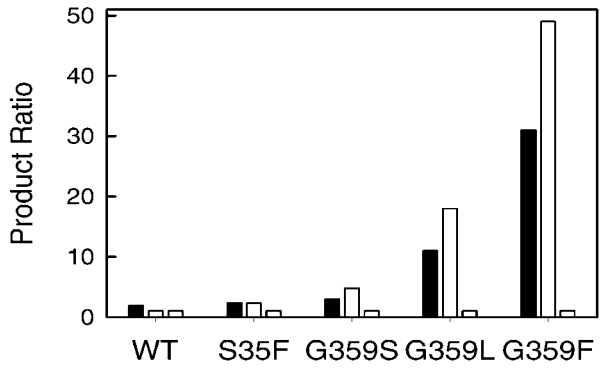
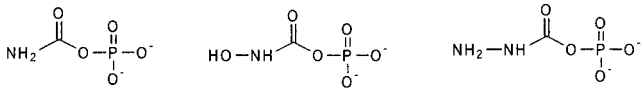


FIG. 2. **The reaction stoichiometry of the wild-type and mutant enzymes.** The products, ADP, glutamate, and carbamoyl phosphate, are represented by the *black, white,* and *gray bars,* respectively. The amount of carbamoyl phosphate is normalized as 1 for each enzyme. The reaction stoichiometry is obtained from the ratio of the maximal rate of the formation of ADP (Table I), glutamine (Table II), and carbamoyl phosphate (Table III). The reaction stoichiometry of the wild-type (*WT*) CPS is 2 ATP:1 glutamine:1 carbamoyl phosphate.

TABLE IV
Enzymatic formation of nonvolatile ^{14}C-containing products from various nitrogen sources, [^{14}C]bicarbonate, and ATP

The formation of carbamoyl phosphate or analogs from various nitrogen sources was probed using [^{14}C]bicarbonate. Each reaction mixture contained 30 mM ATP, 5 mM [^{14}C]bicarbonate (specific activity of 24 μCi/μmol) and 16 μM CPS. The nitrogen sources were 300 mM NH_4Cl, 300 mM NH_2OH, 300 mM NH_2NH_2, 20 mM glutamine, 20 mM γ-glutamyl hydroxamate, or 20 mM γ-glutamyl hydrazide. A control reaction containing 300 mM NH_4Cl, which contained no CPS, was carried out in parallel. Aliquots of the reaction mixtures were acid quenched after 5 and 10 min. The unreacted bicarbonate was removed by the addition of powered dry ice. The amount of the nonvolatile product was determined by liquid scintillation counting.

Nitrogen source	Product	
	5 min incubation	10 min incubation
	μM	
Control	0.4	0.5
NH_4Cl	1194	1851
NH_2OH	49.8	76.8
NH_2NH_2	23.3	51.7
Glutamine	2410	2550
γ-glutamyl hydroxamate	41.6	48.9
γ-Glutamyl hydrazide	15.7	23.9



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cause of the negative nuclear Overhauser effects, proton exchange, and pH fluctuations during the data acquisition. The ^{13}C and ^{31}P signals of the two carbamoyl phosphate analogs were not due to contamination by ammonia in the hydroxylamine or hydrazine. Although carbamoyl phosphate was fully converted to citrulline by ornithine transcarbamoylase according to the ^{13}C and ^{31}P NMR spectra, *N*-hydroxyl carbamoyl phosphate and *N*-amino carbamoyl phosphate were not substrates for ornithine transcarbamoylase. Second, although carbamoyl phosphate was more stable at neutral pH than alkaline pH, both analogs of carbamoyl phosphate appeared more stable at alkaline pH.

γ-Glutamyl Hydroxamate and γ-Glutamyl Hydrazide as Substrates of Wild-type CPS—The hydrolysis of γ-glutamyl hydroxamate and γ-glutamyl hydrazide by CPS has been reported (14, 19). In the absence of ATP and bicarbonate, the kinetic parameters for the hydrolysis of γ-glutamyl hydroxamate and γ-glutamyl hydrazide are very similar to that for glutamine except that the K_m value for the hydrazide is 5-fold higher than the K_m for glutamine (Table V). In the presence of saturating levels of ATP and bicarbonate, the maximal rate for the hydrolysis of γ-glutamyl hydroxamate is enhanced 2700-fold, whereas the K_m value for γ-glutamyl hydroxamate is increased 13-fold relative to the absence of ATP and bicarbonate (Table V). For comparison, the enhancement in the k_{cat} value with glutamine is about 700-fold, whereas the K_m value for glutamine is unchanged (Table II). For the hydrolysis of γ-glutamyl hydrazide in the presence of ATP and bicarbonate, the k_{cat} value is enhanced by more than 100-fold relative to the absence of ATP and bicarbonate. However, there is little net enhancement in k_{cat}/K_m because the K_m value for γ-glutamyl hydrazide is also increased by the same extent.

The bicarbonate-dependent ATPase activity is enhanced 15-fold when glutamine or ammonia is present in the assay solution. This bicarbonate-dependent ATPase activity can also be stimulated by γ-glutamyl hydroxamate (6-fold) or hydroxylamine (5-fold) (Table VI). The stimulation by γ-glutamyl hydrazide or hydrazine is significantly smaller (1.3- and 2.2-fold, respectively) (data not shown).

γ-Glutamyl Hydroxamate as a Substrate of Ammonia Tunnel Mutants—The kinetic parameters for the hydrolysis of γ-glutamyl hydroxamate by S35F are very similar to the wild-type enzyme. In the presence of ATP and bicarbonate, the k_{cat} value is enhanced 4600-fold relative to the absence of ATP and bicarbonate (Table V). The bicarbonate-dependent ATPase activity is mutually enhanced 3.8-fold when γ-glutamyl hydroxamate is hydrolyzed within the small subunit (Table VI). For comparison, the rate increase with the wild-type enzyme is 6.2-fold. For G359S and G359L, the k_{cat} values for the hydrolysis of γ-glutamyl hydroxamate in the presence of ATP and bicarbonate are 3- and 20-fold lower than the wild-type value, whereas the K_m values are elevated substantially. With both enzymes, the ability to stimulate the bicarbonate-dependent ATPase by γ-glutamyl hydroxamate has been lost. However, when hydroxylamine is provided as the direct nitrogen source, the stimulation of the bicarbonate-dependent ATPase activity is observed with both wild-type and the three mutant enzymes (4–8-fold) (Table VI). An overall comparison of the ATPase activation within this series of mutants by different nitrogen sources is summarized in Fig. 4.

DISCUSSION

The most remarkable feature of the x-ray crystal structure of carbamoyl phosphate synthetase is the presence of an extended intermolecular tunnel, which connects the glutaminase active site within the small subunit and the two ATP binding sites within the large subunit of CPS (5, 6). The overall synthesis of carbamoyl phosphate requires three reactive intermediates, ammonia, carboxy phosphate, and carbamate (29, 30). However, contemporary biochemical results provide only indirect support for the tunneling of the two intermediates. An earlier investigation has demonstrated that a constricted ammonia tunnel impedes the passage of ammonia from the small subunit to the large subunit (18). By expanding on this earlier theme, we attempt to provide more direct biochemical evidence for the functional significance of the tunneling of ammonia within CPS.

Gly-359 is one of the amino acid residues within the small subunit of CPS that defines the interior walls of the ammonia tunnel. Previous substitutions with phenylalanine and tyrosine at this position produced two mutant enzymes the kinetic properties of which were consistent with an ammonia tunnel that was almost fully clogged. With G359F and G359Y, the formation of carbamoyl phosphate using glutamine as the nitrogen source was almost completely inhibited, whereas the partial reactions occurring on the small and large subunits were largely uncoupled from one another. In the current study, three additional mutants, G359S, G359L, and S35F, were prepared. G359S and G359L displayed kinetic properties that were intermediate in scope between the wild-type CPS and G359F (Tables I–III). With the wild-type CPS, the hydrolysis of ATP and glutamine is fully coupled such that one equivalent of carbamoyl phosphate is synthesized when two equivalents of ATP and one equivalent of glutamine are consumed. As the side chain of residue 359 is switched from a hydrogen atom to a hydroxymethyl group, an isobutyl group, or a benzyl group, the catalytic properties of the enzyme deviate from full coupling to almost complete uncoupling of the two partial reactions (Tables I–III and Fig. 2). The size of the side chain at position 359 correlates very well with the extent of the diminution of carbamoyl phosphate formation (Table III). It thus appears that with this series of tunnel mutants, the percentage of the *internal* ammonia, derived from the hydrolysis of glutamine, that is able to traverse the full length of the ammonia tunnel is determined by the degree of constriction within this tunnel.

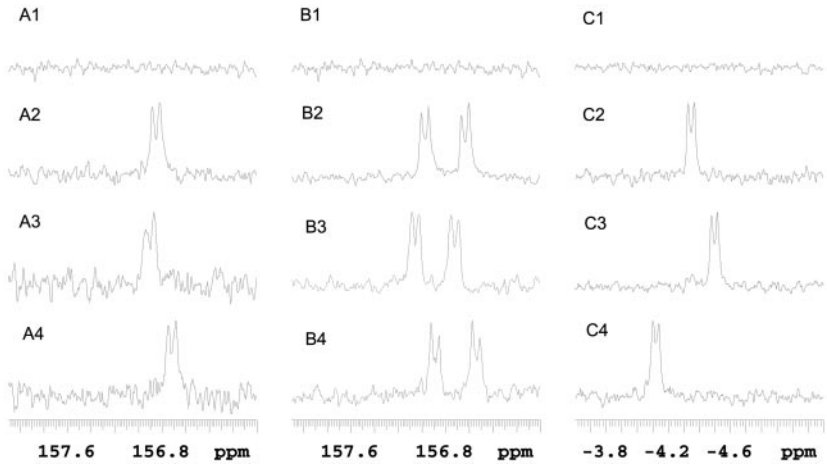

FIG. 3. **NMR spectra of carbamoyl phosphate and analogs.** The ^{13}C and ^{31}P NMR spectra for the control (*1*), carbamoyl phosphate (*2*), *N*-hydroxy carbamoyl phosphate (*3*), and *N*-amino carbamoyl phosphate (*4*) are shown. *A* and *B* present the ^{13}C NMR spectra, and *C* presents the ^{31}P NMR spectra. For *A* and *C,* [^{13}C]bicarbonate and [^{14}N]nitrogen sources were used. For *B,* [^{13}C]bicarbonate and ^{15}N-enriched nitrogen sources were used. The ^{31}P NMR spectra of the doubly enriched products were identical to those shown in panel *C*. Carbamoyl phosphate: δ (^{13}C NMR, D_2O) 156.85, δ (^{31}P NMR, D_2O) −4.35, $J_{C,P}$ = 4.7 Hz, $J_{C,NH2CO}$ = 25.7 Hz; *N*-hydroxy carbamoyl phosphate: δ (^{13}C NMR, D_2O) 156.90, δ (^{31}P NMR, D_2O) −4.50, $J_{C,P}$ = 5.0 Hz, $J_{C,\ NHCO}$ = 25.2 Hz; *N*-amino carbamoyl phosphate: δ (^{13}C NMR, D_2O) 156.72, δ (^{31}P NMR, D_2O) −4.12, $J_{C,P}$ = 4.7 Hz, $J_{C,\ NHCO}$ = 26.3 Hz.

TABLE V

Kinetic parameters for the hydrolysis of γ-glutamyl hydroxamate and γ-glutamyl hydrazide by the wild-type and mutant enzymes

Shown are kinetic constants for glutamate formation in the absence or presence of ATP and bicarbonate.

	γ-Glutamyl hydroxamate				γ-Glutamyl hydrazide			
	Partial reaction[a]		Overall reaction[b]		Partial reaction[a]		Overall reaction[b]	
	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m
	s^{-1}	mM	s^{-1}	mM	s^{-1}	mM	s^{-1}	mM
Wild-type	0.0030	0.10	7.9	1.3	0.0020	0.45	0.19[d]	>40
S35F	0.0014	0.096	6.7	2.1	0.0018	0.60	0.15[d]	>40
G359S	0.022[c]	>40	2.6[c]	>40	ND[d]	ND	ND	ND
G359L	0.012[c]	>40	0.37	29	ND	ND	ND	ND

[a] Reaction conditions: pH 7.6, 25 °C, 100 mM KCl, variable amounts of γ-glutamyl hydroxamate, or γ-glutamyl hydrazide.
[b] Reaction conditions: pH 7.6, 25 °C, variable amounts of γ-glutamyl hydroxamate or γ-glutamyl hydrazide, 5.0 mM ATP, 40 mM bicarbonate, 20 mM $MgCl_2$, 100 mM KCl, and 10 mM ornithine.
[c] Rate constant at 40 mM γ-glutamyl hydroxamate or γ-glutamyl hydrazide.
[d] ND, not determined.

TABLE VI

Stimulation of the bicarbonate-dependent ATPase activity by γ-glutamyl hydroxamate and hydroxylamine

Shown are kinetic constants for ADP formation.

Enzymes	HCO_3^--dependent[a]	γ-glutamyl hydroxamate[b]			Hydroxylamine[c]		
	k_{cat}	k_{cat}	α	K_a	k_{cat}	α	K_a
	s^{-1}	s^{-1}		mM	s^{-1}		mM
Wild-type	0.39	2.4	6.2	1.5	2.0	5.0	68
S35F	0.49	1.9	3.8	1.7	4.0	8.2	168
G359S	1.17	1.5[c]	1.2	>40	4.3	3.7	88
G359L	1.17	1.4[c]	1.2	>40	4.8	4.1	67

[a] Reaction conditions for the bicarbonate-dependent ATPase reaction: pH 7.6, 25 °C, 5.0 mM ATP, 40 mM bicarbonate, 20 mM $MgCl_2$, 100 mM KCl, and 10 mM ornithine.
[b] Reaction conditions: the same as above except for variable amounts of γ-glutamyl hydroxamate.
[c] Reaction conditions: the same as above except for variable amounts of hydroxylamine.
[d] Rate constant at 40 mM γ-glutamyl hydroxamate.

The catalytic properties of S35F differ only slightly from the wild-type CPS (Tables I–III and Fig. 2), and the two partial reactions are mildly uncoupled from one another. These overall kinetic characteristics thus place S35F between the wild-type CPS and G359S.

For each mutant within this series, the hydrolysis of glutamine within the small subunit is still stimulated by the hydrolysis of ATP within the large subunit (Table II). Therefore, a similar conformational change must still be transmitted from the large subunit to the small subunit, as observed with the wild-type CPS. The triggering event for this conformational change has been attributed to the formation of the carboxy phosphate intermediate within the large subunit (27). Each of these tunnel mutants also retains the ability to synthesize carbamoyl phosphate when ammonia is provided as the direct nitrogen source (Table III). This further strengthens the previous argument that ammonia can access an alternate route to the bicarbonate phosphorylation site within the large subunit (18).

The second approach that was undertaken to provide direct support for the tunneling of the ammonia intermediate within CPS was to probe the tunneling of intermediates that are physically larger than ammonia. Hydroxylamine and hydrazine have an overall size that is larger than ammonia. The utilization of a radiolabeled probe and NMR spectroscopy has detected and identified the formation of *N*-hydroxy carbamoyl phosphate and *N*-amino carbamoyl phosphate when hydroxylamine or hydrazine substitutes for ammonia as the external nitrogen source. Earlier, MacKinley *et al.* (31) reported the likely formation of *N*-amino carbamoyl phosphate from hydrazine in a reaction catalyzed by carbamoyl phosphate synthetase I from frog liver. The putative *N*-amino carbamoyl phosphate in that study was more base-stable than carbamoyl phosphate, as similarly observed here with *N*-amino carbamoyl phosphate or

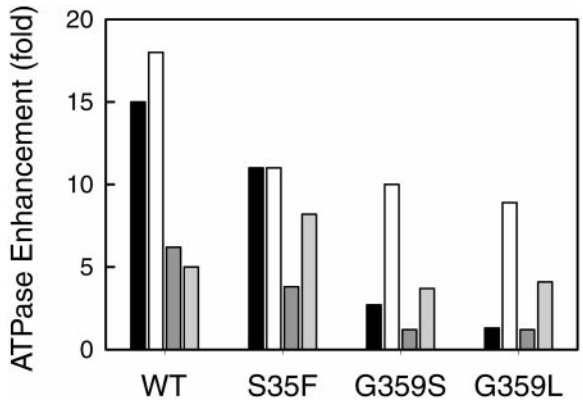

FIG. 4. **Stimulation of ATP hydrolysis by the various sources of nitrogen.** The maximal rate enhancement by glutamine, ammonia, γ-glutamyl hydroxamate, and hydroxylamine is represented by the *black, white, dark gray,* and *light gray bars,* respectively.

N-hydroxyl carbamoyl phosphate formed by the *E. coli* enzyme. Increased base stability of other *N*-substituted derivatives of carbamoyl phosphate has been reported (32). The formation of carbamoyl phosphate analogs by CPS when hydroxylamine or hydrazine is included as the external nitrogen source suggests that the carbamate tunnel within the large subunit is flexible enough to accommodate the passage of the two analogs of the carbamate intermediate.

When γ-glutamyl hydroxamate or γ-glutamyl hydrazide is provided as the nitrogen source, both compounds are hydrolyzed to hydroxylamine or hydrazine and to glutamate, in a reaction analogous to glutamine hydrolysis. For this *glutaminase* partial reaction, the two glutamine analogs display kinetic parameters similar to glutamine. As with glutamine, the hydrolysis of these two glutamine analogs within the small subunit is stimulated by the hydrolysis of ATP within the large subunit. On the other hand, the hydrolysis of ATP within the large subunit is enhanced by the hydrolysis of γ-glutamyl hydroxamate, but not γ-glutamyl hydrazide. The stimulation of the ATPase activity by γ-glutamyl hydroxamate mirrors that by hydroxylamine. With glutamine, the elevation of the steady-state rate of ATP hydrolysis is indicative of an enhanced rate of attack on the carboxy phosphate intermediate by the internally derived ammonia relative to water (27). By analogy, the stimulation of the ATPase activity by γ-glutamyl hydroxamate most likely reflects the reaction between hydroxylamine and the carboxy phosphate intermediate. It is not understood why the hydrolysis of ATP is not stimulated by hydrazine or γ-glutamyl hydrazide. The enzymatic formation of *N*-amino carbamoyl phosphate from hydrazine as the alternative nitrogen source requires the reaction between hydrazine and the carboxy phosphate intermediate. However, the reactions involving hydroxylamine or hydrazine are complicated by various factors. Both of these compounds are weaker nucleophiles than ammonia (based upon the pK_a values), whereas the putative carbamate analog intermediates are less stable than carbamate (33). Unlike the synthesis of carbamoyl phosphate using glutamine as the nitrogen source, the partial reactions within the small subunit and the large subunit are not fully coupled to one another when glutamine is substituted by γ-glutamyl hydroxamate or γ-glutamyl hydrazide.

Although hydroxylamine is bulkier than ammonia, hydroxylamine derived from the hydrolysis of γ-glutamyl hydroxamate still appears to be able to transverse the ammonia tunnel of the wild-type CPS. It was anticipated that some of the mutants within the ammonia tunnel would allow the passage of the ammonia but not the bulkier hydroxylamine. This prediction was confirmed. Based upon the ability to stimulate the ATPase activity in the presence of glutamine or γ-glutamyl hydroxamate, G359S, which contains a partially clogged ammonia tunnel, seems to allow the passage of the internally derived ammonia to a certain degree but completely prevents hydroxylamine from diffusing through the interior ammonia tunnel (Fig. 4). When the degree of constriction within the ammonia tunnel is further increased with G359L, both the ammonia and hydroxylamine are inefficiently channeled (Fig. 4). The overall trend in the enhancement of the ATPase activity by γ-glutamyl hydroxamate for the wild-type CPS, S35F, G359S, and G359L is very similar to the trend displayed by glutamine (Fig. 4). These results are also consistent with the pattern of uncoupling of the partial reactions among these enzymes (Fig. 2). Each of these tunnel-impaired mutants retains the ability to activate the ATPase activity within the large subunit when either ammonia or hydroxylamine is provided as the direct nitrogen source (Fig. 4). This suggests that ammonia or hydroxylamine can bind to the large subunit via an alternative route, other than the ammonia tunnel.

Recently, Anderson and co-workers (20, 34) have reported a pair of "channel-impaired mutants" of tryptophan synthase ($\alpha_2\beta_2$). Tryptophan synthase, which catalyzes the formation of L-tryptophan from indole 3-glycerol phosphate and L-serine, also contains an intermolecular tunnel connecting the active sites of the α and β subunits. Pre-steady-state kinetic analysis and computer simulations suggest that the rate of channeling of indole in βC170F and βC170W mutants is reduced by ≥10- and ≥5000-fold, respectively. However, the βC170F mutant appears to be only mildly affected in terms of overall macroscopic rate constants (20). It is possible that some of the CPS mutants with only slightly altered steady-state parameters may still be significantly impaired in ammonia tunneling. The respective advantage and disadvantage of steady-state and pre-steady-state approaches for evaluation of substrate tunneling have been recently reviewed (12).

In summary, we have prepared a series of ammonia tunnel mutants by the placement of a constriction inside the ammonia tunnel. The degree of constriction within the ammonia tunnel of these enzymes correlates nicely with the extent of the uncoupling of the partial reactions occurring within the small and large subunit, the extent of inhibition of carbamoyl phosphate formation, and the percentage of the internally derived ammonia that is channeled through the ammonia tunnel. By utilizing NMR and a radiolabeled probe, we have also detected and confirmed the enzymatic synthesis of *N*-amino carbamoyl phosphate and *N*-hydroxy carbamoyl phosphate from hydroxylamine and hydrazine as nonnatural nitrogen sources. The kinetic results indicate that hydroxylamine, derived from the hydrolysis of γ-glutamyl hydroxamate, is channeled through the ammonia tunnel to the large subunit. The combination of these two experimental approaches, namely constricting the ammonia tunnel and *enlarging* the intermediate that must migrate from one site to the next, allows us to observe discrimination between the passage of ammonia and hydroxylamine among these tunnel-impaired enzymes. The overall results therefore provide compelling evidence for the tunneling of ammonia within the native structure of CPS.

REFERENCES

1. Anderson, P. M., and Meister, A. (1965) *Biochemistry* **4,** 2803–2809
2. Anderson, P. M., and Meister, A. (1966) *Biochemistry* **5,** 3164–3169
3. Matthews, S. L., and Anderson, P. M. (1972) *Biochemistry* **11,** 1176–1183
4. Trotta, P. P., Burt, M. E., Haschemeyer, R. H., and Meister, A. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 2599–2603
5. Thoden, J. B., Holden, H. M., Wesenberg, G., Raushel, F. M., and Rayment, I. (1997) *Biochemistry* **36,** 6305–6316
6. Thoden, J. B., Raushel, F. M., Benning, M. M., Rayment, I., and Holden, H. M. (1999) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **55,** 8–24
7. Raushel, F. M., Thoden, J. B., Reinhart, G. D., and Holden, H. M. (1998) *Cur. Opin. Chem. Biol.* **2,** 624–632

8. Holden, H. M., Thoden, J. B., and Raushel, F. M. (1998) *Cur. Opin. Struct. Biol.* **8,** 679–685
9. Raushel, F. M., Thoden, J. B., and Holden, H. M. (1999) *Biochemistry* **38,** 7891–7899
10. Ovadi, J. (1991) *J. Theor. Biol.* **152,** 1–22
11. Miles, E. W., Rhee, S., and Davies, D. R. (1999) *J. Biol. Chem.* **274,** 12193–12196
12. Anderson, K. S. (1999) *Methods Enzymol.* **308,** 111–145
13. Mullins, L. S., and Raushel, F. M. (1999) *J. Am. Chem. Soc.* **121,** 3803–3804
14. Rubino, S. D., Nyunoya, H., and Lusty, C. J. (1986) *J. Biol. Chem.* **261,** 11320–11327
15. Cohen, N. S., Kyan, F. S., Jyan, S. S., Cheung, C. W., and Raijman, L. (1985) *Biochem. J.* **229,** 205–211
16. Raushel, F. M., Mullins, L. S., and Gibson, G. E. (1998) *Biochemistry* **37,** 10272–10278
17. Wang, T. T., Bishop, S. H., and Himoe, A. (1972) *J. Biol. Chem.* **247,** 4437–4440
18. Huang, X., and Raushel, F. M. (2000) *Biochemistry* **39,** 3240–3247
19. Anderson, P. M., and Meister, A. (1966) *Biochemistry* **5,** 3157–3163
20. Anderson, K. S., Kim, A. Y., Quillen, J. M., Sayers, E., Yang, X.-J., and Miles, E. W. (1995) *J. Biol. Chem.* **270,** 29936–29944
21. Wellner, V. P., Anderson, P. M., and Meister, A. (1973) *Biochemistry* **12,** 2061–2066
22. 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
23. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77,** 51–59
24. Mareya, S. M., and Raushel, F. M. (1994) *Biochemistry* **33,** 2945–2950
25. Snodgrass, P. J., and Parry, D. J. (1969) *J. Lab. Clin. Med.* **73,** 940–950
26. Cleland, W. W. (1970) *Enzymes* **2,** 1–65
27. Miles, B. W., and Raushel, F. M. (2000) *Biochemistry* **39,** 5051–5056
28. Lichter, R. L., and Roberts, J. D. (1970) *J. Am. Chem. Soc.* **93,** 5218–5224
29. Raushel, F. M., and Villafranca, J. J. (1980) *Biochemistry* **19,** 3170–3174
30. Wimmer, M. J., Rose, I. A., Powers, S. G., and Meister, A. (1979) *J. Biol. Chem.* **254,** 1854–1859
31. McKinley, S., Anderson, C. D., and Jones, M. E. (1967) *J. Biol. Chem.* **242,** 3381–3390
32. Allen, C. M., Jr., Richelson, E., and Jones, M. E. (1966) *Current Aspects of Biochemical Energetics, Fritz Lipmann Dedicatory Volume* (Kaplan, N. D., and Kennedy, E. P., eds), pp. 401–412, Academic Press, New York
33. Fasman, G. D. (ed) (1975) *Handbook of Biochemistry and Molecular Biology*, pp. 307–348, CRC Press, Cleveland, OH
34. Schlichting, I., Yang, X.-J., Miles, Kim, A. Y., and Anderson, K. S. (1994) *J. Biol. Chem.* **269,** 26591–26593