An Engineered Blockage within the Ammonia Tunnel of Carbamoyl Phosphate Synthetase Prevents the Use of Glutamine as a Substrate but Not Ammonia†

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ABSTRACT: The heterodimeric carbamoyl phosphate synthetase (CPS) from *Escherichia coli* catalyzes the formation of carbamoyl phosphate from bicarbonate, glutamine, and two molecules of ATP. The enzyme catalyzes the hydrolysis of glutamine within the small amidotransferase subunit and then transfers ammonia to the two active sites within the large subunit. These three active sites are connected via an intermolecular tunnel, which has been located within the X-ray crystal structure of CPS from *E. coli*. It has been proposed that the ammonia intermediate diffuses through this molecular tunnel from the binding site for glutamine within the small subunit to the phosphorylation site for bicarbonate within the large subunit. To provide experimental support for the functional significance of this molecular tunnel, residues that define the interior walls of the "ammonia tunnel" within the small subunit were targeted for site-directed mutagenesis. These structural modifications were intended to either block or impede the passage of ammonia toward the large subunit. Two mutant proteins (G359Y and G359F) display kinetic properties consistent with a constriction or blockage of the ammonia tunnel. With both mutants, the glutaminase and bicarbonate-dependent ATPase reactions have become uncoupled from one another. However, these mutant enzymes are fully functional when external ammonia is utilized as the nitrogen source but are unable to use glutamine for the synthesis of carbamoyl-P. These results suggest the existence of an alternate route to the bicarbonate phosphorylation site when ammonia is provided as an external nitrogen source.

Carbamoyl phosphate synthetase (CPS)[1] from *Escherichia coli* catalyzes the first committed step in the biosynthetic pathways for arginine and pyrimidine nucleotides (*1*). The heterodimeric protein is composed of a small amidotransferase subunit (∼42 kDa) and a large synthetase subunit (∼118 kDa) (*2*−*4*). The enzyme assembles carbamoyl phosphate from bicarbonate, glutamine, and two molecules of ATP as presented in eq 1 (*2*). In addition to the overall reaction, CPS also catalyzes three partial reactions when one or more of the substrates are absent from the reaction mixture (*5*). These partial reactions are summarized in eqs 2−4.

$$2\text{MgATP} + \text{HCO}_3^- + \text{Gln} + \text{H}_2\text{O} \rightarrow 2\text{MgADP} + \text{P}_i + \text{Glu} + \text{carbamoyl-P} \quad (1)$$

$$\text{Gln} + \text{H}_2\text{O} \rightarrow \text{Glu} + \text{NH}_3 \quad (2)$$

$$\text{MgATP} + \text{H}_2\text{O} \rightarrow \text{MgADP} + \text{P}_i \quad (3)$$

$$\text{MgADP} + \text{carbamoyl-P} \rightarrow \text{MgATP} + \text{NH}_2\text{CO}_2^- \quad (4)$$

The overall synthesis reaction has been proposed to occur within the three active sites of CPS via four distinct chemical steps as illustrated in Scheme 1 (*5*). The large subunit contains separate active sites for the phosphorylation of bicarbonate and carbamate, whereas the small subunit catalyzes the hydrolysis of glutamine through the intermediacy of a thiol ester with Cys-269 (*6*−*8*). However, the most remarkable feature of this enzyme is the fact that the three active sites contained within the heterodimer are separated in three-dimensional space by nearly 100 Å (*9*, *10*). This observation strongly suggests that two of the unstable reaction intermediates, ammonia and carbamate, must diffuse through the interior of the protein from their site of formation to their site of utilization. This proposal has been bolstered by the identification of an intermolecular tunnel in CPS, which leads from the active site within the small subunit toward the two active sites contained within the large subunit (*9*, *10*). A ribbon representation of CPS with the relative locations of the three active sites and the intermolecular tunnel is shown in Figure 1 (*10*). Measurement of the competition between *internal* (derived from the hydrolysis of glutamine) and *external* ammonia during the formation of carbamoyl phosphate is fully consistent with a mechanism that requires the direct tunneling of ammonia between the two subunits when glutamine is used as the nitrogen source (*11*). The tunneling of carbamate between the two active sites contained within the large subunit is supported by the lack of an isotope exchange reaction between bicarbonate and solvent water in the presence of glutamine (*12*). Moreover, the observed reaction stoichiometry dictates that the ammonia and carbamate intermediates must be efficiently transported from one active site to the next to maintain the precise coupling of the individual chemical events during the assembly of carbamoyl phosphate.

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[1] Abbreviations: CPS, carbamoyl phosphate synthetase; PCR, polymerase chain reaction; PEP, phosphoenolpyruvate; OTCase, ornithine transcarbamoylase; GPATase, glutamine phosphoribosylpyrophosphate amidotransferase; IGP, indole 3-glycerol phosphate.

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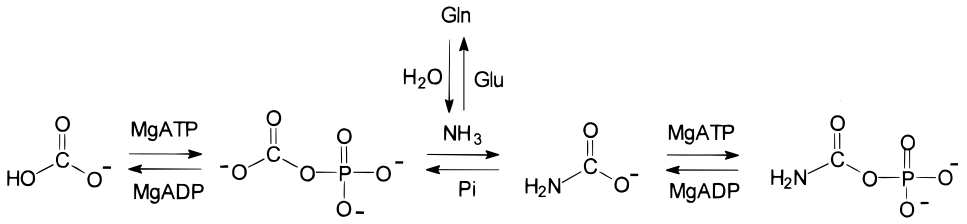
Scheme 1



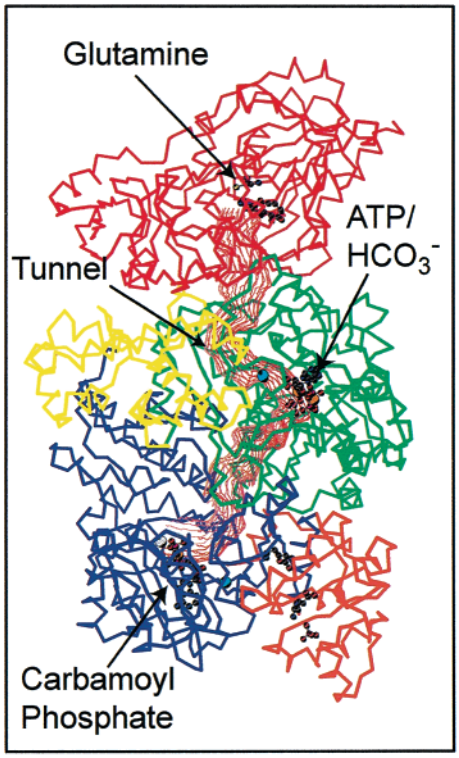


FIGURE 1: α-Carbon trace of the three-dimensional structure of CPS from *E. coli* (taken from ref *9*). The binding sites for glutamine, ATP and/or bicarbonate, and carbamoyl phosphate are highlighted. The molecular tunnel connecting the three binding sites is outlined in red.

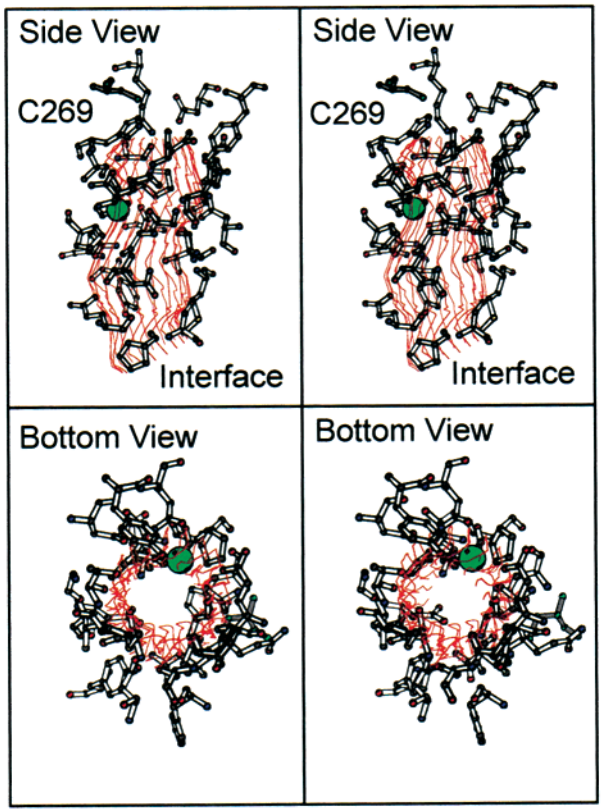


FIGURE 2: Stereoviews of a ball-and-stick representation of the ammonia tunnel from the small subunit of CPS. The top panel is the side view. The bottom panel is the view looking up from the interface of the small and large subunits. The tunnel as illustrated in red was originally described in refs *9* and *10*. The amino acids shown in this representation are those positioned within 3.5 Å of the center of the proposed tunnel. The α-carbon of Gly-359 is highlighted in green.

The small subunit of CPS, which houses the active site for the hydrolysis of glutamine, has dimensions of approximately 75 Å × 60 Å × 60 Å (*9*). The "ammonia tunnel" provides a direct mechanistic link between the binding site for glutamine and the site for formation of the carboxy phosphate intermediate within the large subunit. The average radius of this molecular passageway is 3.2 Å, which appears sufficiently large to permit the facilitated diffusion of ammonia from one site to the next (*9*). The segment of the ammonia tunnel contained within the small subunit of CPS extends ~20 Å in length from the active site cysteine to the molecular interface between the two subunits. A schematic representation of this section of the ammonia tunnel is shown in Figure 2.

The amino acid residues that define the interior walls of the ammonia tunnel within the small subunit of CPS were targeted for site-directed mutagenesis. These structural modifications were intended to either block or impede the passage of ammonia toward its ultimate destination within the large subunit. If the molecular tunnel within the small subunit of CPS functions as a conduit for the delivery of ammonia to the large subunit, then the reshaping of this architectural feature will either severely inhibit the formation of carbamoyl phosphate or uncouple the partial reactions from one another. Experiments with mutant enzymes with clogged or constipated tunnels will also address whether externally added ammonia must traverse the same intersubunit tunnel prior to the reaction with the carboxy phosphate intermediate. In this paper, we describe the kinetic properties of CPS mutants that are consistent with a constriction or impairment of the ammonia tunnel. These mutants are unable to catalyze the formation of carbamoyl phosphate using glutamine as a substrate but are relatively unaffected when external ammonia is used as the nitrogen source. These results provide the first experimental support for the functional significance of the molecular tunnel, which has been located within the X-ray crystal structure of CPS from *E. coli*. These results also suggest the existence of an alternate route to the carboxy phosphate intermediate when ammonia is provided directly from the bulk solvent.

MATERIALS AND METHODS

Materials. All chemicals and coupling enzymes used for kinetic measurements were purchased from either Sigma or Aldrich unless otherwise stated. Restriction enzymes, Vent^R DNA polymerase, and Pfu DNA polymerase were purchased from Promega. Oligonucleotide synthesis and DNA sequencing were performed by the Gene Technologies Laboratory, Biology Department, Texas A&M University. The clone for ornithine transcarbamoylase was a generous gift from the laboratory of N. Allewell.

Mutagenesis, Expression, and Purification of the Mutant Proteins. Site-directed mutagenesis was performed as previously described with the wild-type CPS (*13*) using the polymerase chain reaction and the overlap extension method of Ho et al. (*14*). The mutant plasmids were transformed in the RC50 cell line (a generous gift of C. J. Lusty) for the expression of modified proteins. The wild-type and mutant proteins were purified as described by Mareya et al. (*15*).

Kinetic Measurements and Statistical Analysis of Kinetic Data. Assay conditions for monitoring the rate of formation of ADP, glutamate, and carbamoyl phosphate were carried out as previously described (*6*). The kinetic parameters, V_{max} and K_m, were determined by fitting the experimental data to eq 5

$$\nu = V_{max}A/(K_m + A) \quad (5)$$

where ν 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 eq 6 (*16*).

$$\nu = V_0(K_a + \alpha I)/(K_a + I) \quad (6)$$

where V_0 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 concentrations of and without the nitrogen source. In this case, V_{max} is expressed as αV_0.

Time Course for Carbamoyl Phosphate Formation. The formation of carbamoyl phosphate was assessed by measuring the amount of citrulline formed in a coupled assay with ornithine transcarbamoylase (OTCase) and ornithine (*17*). The reaction mixture contained 50 mM HEPES (pH 7.6), 100 mM KCl, 40 mM $KHCO_3$, 10 mM ATP, 20 mM $MgCl_2$, 10 mM ornithine, 50 mM glutamine, 96 units of pyruvate kinase, 20 mM PEP, and 200 units of OTCase in a final volume of 8.0 mL. Pyruvate kinase and PEP were included for the purpose of recycling of the ATP. Aliquots of 0.75 mL were quenched with 2.25 mL of a citrulline assay cocktail at times ranging from 2 to 60 min. The samples were centrifuged for 5 min at 14 000 rpm before quantitation of the citrulline concentration (*17*).

Time Course for Glutamate Formation. The formation of glutamate was assessed using a glutamate dehydrogenase coupled assay (*15*). The reaction mixture contained 50 mM HEPES (pH 7.6), 100 mM KCl, 40 mM $KHCO_3$, 10 mM ATP, 20 mM $MgCl_2$, 10 mM ornithine, 50 mM glutamine, 96 units of pyruvate kinase, and 20 mM PEP in a final volume of 8.0 mL. Aliquots of 0.75 mL were quenched with an equal volume of 1.0 M HCl. The quenched samples were incubated on ice for 20 min, and then centrifuged for 5 min to remove precipitated proteins. The supernatant was neutralized with 1.0 M Tris (pH 10.5) to a final pH of 7.5 before determination of the glutamate concentration.

RESULTS

Amino acid residues lining the ammonia tunnel within the small subunit of CPS were mutated with the intention of restricting the free passage of ammonia from the site of generation to the site of utilization in the large subunit. An alternative strategy was to introduce acidic residues in an attempt to protonate the ammonia intermediate to the less reactive ammonium cation. The mutants D45E, S35C, A309S, A309C, A309C/S35C, M36K, G293A, G293S, G293I, G293F, G359F, and G359Y 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 formation of ADP, glutamate, and carbamoyl phosphate under a variety of assay formats. The kinetic parameters, K_m and k_{cat}, obtained for the wild-type and mutant enzymes, are summarized in Tables 1−3.

Kinetic Properties of D45E. Asp-45 is situated 5−7 Å directly across from the triad of catalytic residues within the small subunit of CPS. The side chain of Asp-45, and the side chains of Lys-202 and His-353, form the entrance to the ammonia tunnel. The lengthening of the side chain was intended to block or impede ammonia from entering the intermolecular tunnel. For the glutaminase partial reaction, the K_m value for glutamine is elevated by 2 orders of magnitude relative to that of the wild-type enzyme. In the overall synthesis of carbamoyl phosphate, using glutamine as the nitrogen source, there is a 3−4-fold reduction in k_{cat} while there is essentially no change in the K_m value for glutamine. This particular mutation does not appear to affect the function of the large subunit since D45E displays unaltered bicarbonate-dependent ATPase, ammonia-dependent ATPase, and carbamoyl phosphate synthesis activities.

Kinetic Properties of M36K. The backbone and side chain of Met-36 form part of the interior wall of the ammonia tunnel near the interface between the small and large subunits of CPS. In simple computer-generated models, the replacement of Met-36 with a lysine residue can be accommodated without causing significant steric interference with neighboring residues. The nearest charged residue is >9 Å away. The M36K mutant was designed with the anticipation that the ϵ-amino group of the lysine side chain might be able to protonate the ammonia intermediate as it passes through the molecular tunnel. The ammonium cation is incapable of reacting with the carboxy phosphate intermediate (*18*, *19*), and as a result, the chemical events at the two active sites connected by the ammonia tunnel would become uncoupled from one another. However, for the partial glutaminase reaction and the overall synthesis of carbamoyl phosphate, the effect of this modification is limited to a 3−5-fold change in the K_m value for glutamine.

Kinetic Properties of Ala-309 and Ser-35 Mutants. Residues Ala-309 and Ser-35 are located across from each other within the ammonia tunnel of CPS. The methyl side chain of Ala-309 is 6.8 Å from the hydroxyl group of Ser-35. If

Table 1: Kinetic Parameters for the ATPase Reactions of the Wild-Type and Mutant Enzymes[a]

	HCO_3^- -dependent[b]	NH_3-dependent[c]		Gln-dependent[d]	
enzyme	k_{cat} (s^{-1})	k_{cat} (s^{-1})	$K_m(NH_4^+)$ (mM)	k_{cat} (s^{-1})	K_m(Gln) (mM)
WT	0.20 ± 0.01	8.0 ± 0.6	250 ± 32	6.9 ± 0.08	0.098 ± 0.004
D45E	0.37 ± 0.02	5.3 ± 0.1	262 ± 11	2.7 ± 0.02	0.117 ± 0.006
M36K	0.36 ± 0.02	6.3 ± 0.2	380 ± 25	4.1 ± 0.03	0.53 ± 0.01
A309C/S35C	0.57 ± 0.02	5.7 ± 0.2	175 ± 17	5.5 ± 0.02	0.075 ± 0.002
A309C	0.67 ± 0.04	7.2 ± 0.3	190 ± 20	6.5 ± 0.09	0.063 ± 0.004
A309S	0.35 ± 0.01	6.0 ± 0.2	237 ± 22	5.1 ± 0.2	0.18 ± 0.02
S35C	0.60 ± 0.03	6.5 ± 0.3	144 ± 22	4.0 ± 0.1	0.064 ± 0.007
G293A	0.45 ± 0.01	6.2 ± 0.3	208 ± 21	6.1 ± 0.03	0.095 ± 0.003
G293S	0.68 ± 0.05	8.3 ± 0.3	265 ± 19	5.3 ± 0.8	0.067 ± 0.005
G293I	0.39 ± 0.04	5.7 ± 0.2	301 ± 28	5.9 ± 0.05	0.12 ± 0.005
G293F	0.14 ± 0.01	3.8 ± 0.2	403 ± 52	5.2 ± 0.03	0.356 ± 0.008
G359Y	1.2 ± 0.04	11 ± 0.09	26 ± 0.5	1.4[e]	NA
G359F	1.2 ± 0.07	12 ± 0.2	34 ± 1	1.5[e]	NA

[a] Rate constants for ADP formation monitored for the bicarbonate-dependent ATPase, ammonia-dependent ATPase, or glutamine-dependent ATPase reactions. [b] 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. Ornithine is included in all assays as an allosteric activator. [c] Reaction conditions are the same as above except for variable amounts of NH_4Cl. [d] Reaction conditions are the same as above except for variable amounts of glutamine. [e] Rate constant at a glutamine concentration of 40 mM.

Table 2: Kinetic Parameters for the Glutaminase Reaction of the Wild-Type and Mutant Enzymes[a]

	partial reaction[b]		overall reaction[c]	
enzyme	k_{cat} (min^{-1})	K_m(Gln) (mM)	k_{cat} (s^{-1})	K_m(Gln) (mM)
WT	0.24 ± 0.005	0.083 ± 0.006	2.9 ± 0.05	0.069 ± 0.007
D45E	0.12 ± 0.01	11 ± 2	1.1 ± 0.03	0.12 ± 0.01
M36K	0.54 ± 0.02	0.029 ± 0.005	1.5 ± 0.03	0.22 ± 0.02
A309C/S35C	0.16 ± 0.002	0.072 ± 0.008	2.6 ± 0.1	0.11 ± 0.009
A309C	ND[d]	ND[d]	3.1 ± 0.1	0.095 ± 0.009
A309S	0.21 ± 0.002	0.055 ± 0.002	2.0 ± 0.06	0.11 ± 0.01
S35C	0.12 ± 0.002	0.080 ± 0.005	1.8 ± 0.05	0.095 ± 0.010
G293A	0.22 ± 0.005	0.069 ± 0.008	2.2 ± 0.04	0.082 ± 0.007
G293S	0.10 ± 0.002	0.069 ± 0.006	1.9 ± 0.06	0.074 ± 0.011
G293I	ND[d]	ND[d]	2.5 ± 0.2	0.089 ± 0.010
G293F	ND[d]	ND[d]	3.5 ± 0.06	0.49 ± 0.03
G359Y	3.0[e]	>40	2.7[e]	>40
G359F	1.7[e]	>40	2.4[e]	>40

[a] Rate constant for glutamate formation in the absence or presence of ATP and bicarbonate. [b] Reaction conditions: pH 7.6, 25 °C, variable amounts of glutamine, and 100 mM KCl. [c] 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. [d] Not determined. [e] Rate constant at 40 mM glutamine. The k_{cat}/K_m values for G359Y are 2.4 and 125 M^{-1} s^{-1} for the partial and overall reactions, respectively. The k_{cat}/K_m values for G359F are 1.2 and 85 M^{-1} s^{-1} for the partial and overall reactions, respectively. For comparison, the k_{cat}/K_m values for the wild-type CPS are 50 and 4.2 × 10^4 M^{-1} s^{-1} for the partial and overall reactions, respectively.

Table 3: Kinetic Parameters for the Carbamoyl-P Synthesis Reaction of the Wild-Type and Mutant Enzymes[a]

	NH_3-dependent[b]		Gln-dependent[c]	
enzyme	k_{cat} (s^{-1})	$K_m(NH_4Cl)$ (mM)	k_{cat} (s^{-1})	K_m(Gln) (mM)
WT	2.9 ± 0.1	211 ± 23	3.2 ± 0.02	0.075 ± 0.003
D45E	1.7 ± 0.1	155 ± 25	0.80 ± 0.009	0.11 ± 0.005
M36K	2.6 ± 0.2	463 ± 59	1.7 ± 0.01	0.41 ± 0.01
A309C/S35C	3.0 ± 0.1	184 ± 22	2.6 ± 0.04	0.093 ± 0.006
A309C	ND[d]	ND[d]	2.2 ± 0.1	0.090 ± 0.005
A309S	2.5 ± 0.08	175 ± 16	1.8 ± 0.03	0.12 ± 0.008
S35C	2.9 ± 0.1	119 ± 15	1.6 ± 0.03	0.077 ± 0.006
G293A	2.5 ± 0.1	177 ± 20	1.9 ± 0.04	0.096 ± 0.010
G293S	2.6 ± 0.05	172 ± 7	1.5 ± 0.02	0.075 ± 0.005
G293I	ND[d]	ND[d]	2.1 ± 0.1	0.087 ± 0.012
G293F	ND[d]	ND[d]	1.8 ± 0.02	0.33 ± 0.01
G359Y	5.0 ± 0.1	24 ± 2	0.18[e]	>40
G359F	4.8 ± 0.2	27 ± 3	0.049[e]	>40

[a] Rate constant for carbamoyl phosphate formation. [b] Reaction conditions: pH 7.6, 25 °C, variable amounts of NH_4Cl, 5.0 mM ATP, 40 mM bicarbonate, 20 mM $MgCl_2$, 100 mM KCl, and 10 mM ornithine. [c] 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. [d] Not determined. [e] Rate constant at 40 mM glutamine. The k_{cat}/K_m values are 5.7 and 3.3 M^{-1} s^{-1} for G359Y and G359F, respectively. For comparison, the k_{cat}/K_m value for the wild-type CPS is 4.3 × 10^4 M^{-1} s^{-1}.

these two residues are mutated to cysteine, then the closest calculated distance between the two new thiol groups would be ~5.8 Å if it is assumed that there are no other compensating movements of the backbone atoms. Since the overall protein structure is not rigid, conformational changes may enable the two thiol groups to move within bonding distance of one another. A cross-linked tunnel could seriously impede the facilitated diffusion of ammonia from one active site to the next. If the disulfide bond cannot form, then the thiol groups of the cysteine residues may still be able to disrupt the function of the tunnel via protonation of the ammonia intermediate to an ammonium cation. The double mutant A309S/S35C was prepared, and single mutations to cysteine or serine were introduced for purposes of comparison. Unfortunately, all four mutants (S35C, A309S, A309C, and A309C/S35C) display kinetic properties similar to those of the wild-type enzyme. Characterization of the oxidation state of the cysteines within the A309C/S35C mutant was not experimentally pursued.

Kinetic Properties of Gly-293 Mutants. Gly-293, whose backbone atoms form part of the wall within the ammonia tunnel, is located between Ala-309 and Ser-35 in the small subunit of CPS. This residue was mutated to alanine, serine, isoleucine, and phenylalanine. However, the replacement of Gly-293 with these bulkier residues did not significantly alter the kinetic properties of the mutant enzymes relative to those of the wild-type protein. Except for small fluctuations in the K_m value for glutamine, these mutants appear to be very similar to the wild-type protein.

Kinetic Properties of Gly-359 Mutants. Gly-359 is ~5 Å away from Gly-293. Like Gly-293, Gly-359 is situated between Ala-309 and Ser-35. The relative position of the α-carbon of Gly-359 within the small subunit of CPS is illustrated in Figure 2 as a green sphere. Mutant proteins

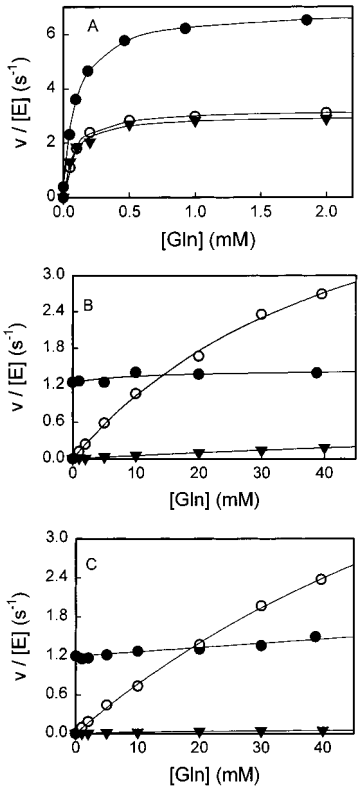


FIGURE 3: Initial rate plots for the glutamine-dependent reactions: (A) wild-type CPS, (B) G359Y CPS, and (C) G359F CPS. The formation of ADP (●) and glutamate (○) is shown along with the synthesis of carbamoyl phosphate (▼), in the overall synthetic reaction using glutamine as the nitrogen source. Additional details are provided in the text.

G359Y and G359F were constructed with the aim of blocking the passage of ammonia within the ammonia tunnel. The kinetic properties of the two mutants are similar. For the glutaminase partial reaction, the K_m values for glutamine are elevated by >400-fold while the k_{cat}/K_m values are reduced by 20−40-fold, compared to that of the wild-type enzyme (Table 2). In the presence of ATP and bicarbonate, the rates of glutamine hydrolysis are enhanced by 50−70-fold, relative to those in the absence of ATP and bicarbonate. In the wild-type enzyme, this enhancement is 670-fold. Both mutants have an elevated bicarbonate-dependent ATPase activity, which is not further stimulated by the hydrolysis of glutamine (Table 1). For the synthesis of carbamoyl phosphate using glutamine as the nitrogen source, the values of k_{cat}/K_m are reduced by 7500−13000-fold, relative to that of the wild-type protein (Table 3). It is apparent that the original reaction stoichiometry is not maintained with these two mutants, and thus, the catalytic reactions at the individual reaction centers have become uncoupled from one another. At a glutamine concentration of 40 mM, 8 equiv of ATP and 15 equiv of glutamine are consumed for the synthesis of 1 equiv of carbamoyl phosphate in G359Y, whereas 30 equiv of ATP and 48 equiv of glutamine are hydrolyzed for every 1 equiv of carbamoyl phosphate formed with G359F (Figure 3). However, the two mutants did not lose their capability of synthesizing carbamoyl phosphate using external ammonia as the source of nitrogen. Moreover, the K_m values for NH_4^+/NH_3 are decreased 8−11-fold, relative to the K_m value of the wild-type CPS, and the wild-type reaction stoichiometry is retained (Table 3 and Figure 4).

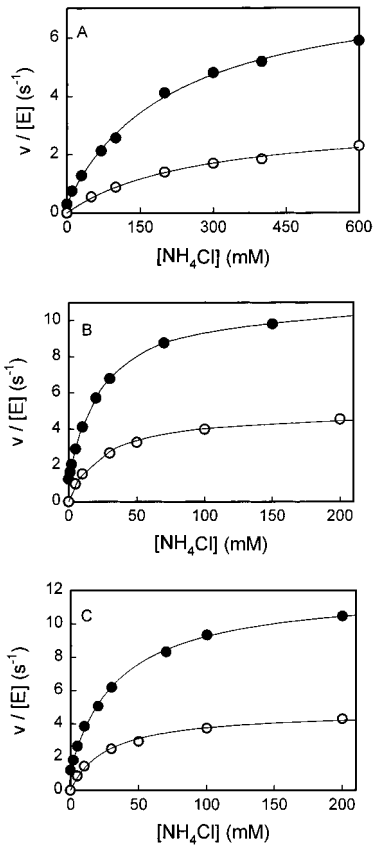


FIGURE 4: Initial rate plots of the ammonia-dependent reactions: (A) wild-type CPS, (B) G359Y CPS, and (C) G359F CPS. The formation of ADP (●) was monitored along with the synthesis of carbamoyl phosphate (○). Additional details are described in the text.

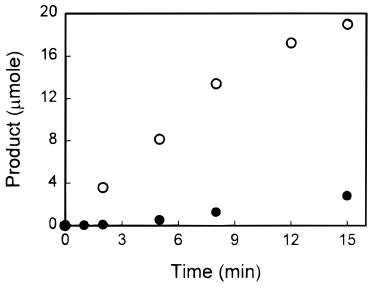


FIGURE 5: Time courses for the formation of carbamoyl phosphate (●) and glutamate (○) in the G359F mutant. Glutamine was provided as the initial nitrogen source. The observed rate enhancement after an initial lag in the formation of carbamoyl phosphate indicates the reutilization of internally derived ammonia after dissociation into the bulk solution. Additional details are provided in the text.

Time Courses for Carbamoyl Phosphate and Glutamate Formation in G359F. On the basis of the observed kinetic properties of G359F, a lag for the time course of carbamoyl phosphate formation was predicted and observed (Figure 5). Carbamoyl phosphate formation accelerates as ammonia, which is derived from the hydrolysis of glutamine, accumulates in the bulk solution. It is apparent that the initial low rate of carbamoyl phosphate formation with this mutant is due to the intrinsic activity using glutamine as the nitrogen source and the latter elevated rate is the result of utilization of ammonia from the bulk solution.

DISCUSSION

The channeling of ammonia through an intermolecular tunnel in CPS has been proposed as a mechanism to account for the apparent migration of this reaction intermediate from the site of glutamine hydrolysis to the site of bicarbonate activation within the large subunit (*9*, *10*). Evidence supporting this proposal includes the physical identification of a well-defined molecular passageway from X-ray crystallographic data and the demonstration of the lack of mixing of *external* and *internal* ammonia during the synthesis of carbamoyl phosphate (*9*−*11*). This study aims to provide additional experimental support for the functional significance of the molecular tunnel in CPS and its role in the coordination of catalytic activity among the three active sites. To address these issues, amino acid residues which coalesce in the folded structure of the small subunit to form the interior walls of the ammonia tunnel were mutated with the intention of blocking or impeding the migration of ammonia through this molecular corridor.

A total of six amino acids within the confines of the small subunit were mutated in an effort to disrupt the function of the ammonia tunnel. It is highly unlikely that the ammonia intermediate is protonated to an ammonium cation during its migration from the small subunit to the large subunit in the wild-type enzyme. The M36K mutant was designed to place a cationic side chain in position to transfer a proton from the lysine to the ammonia intermediate. This particular alteration to the molecular environment of the ammonia tunnel appears to be ineffective, since the catalytic properties of this mutant are very similar to those of the wild-type enzyme. The A309C/S35C mutant was constructed in an attempt to cross-link the interior walls of the ammonia tunnel through the formation of a disulfide bond. This mutant also exhibits kinetic constants very similar to those of the wild-type enzyme. However, it was not ascertained whether the disulfide bond was actually formed, but the added steric congestion of the two thiol groups was apparently insufficient to disrupt the migration of ammonia to any measurable extent. Similarly, the D45E mutant was designed to constrict the size of the entrance to the ammonia tunnel. With this mutation, the maximal rate of carbamoyl phosphate synthesis is reduced 3−4-fold when glutamine is utilized as the source of internal ammonia. However, the rates of glutamate and ADP formation are decreased by the same amount. These results are thus consistent with a slight perturbation to the catalytic residues by the mutated glutamate due to their relative proximity. The modifications to Gly-293 and Gly-359 were also designed to physically block the passage of ammonia through the tunnel. The four mutations of Gly-293 were ineffective in this regard.

The insertion of aromatic substituents in place of the glycine residue at position 359 causes significant perturbations to the catalytic properties of CPS. With these mutations (G359Y and G359F), the amidotransferase activity is almost completely uncoupled from the ATPase activity and, thus, only very small amounts of carbamoyl phosphate are formed. The observed rate of glutamine hydrolysis is greater than 1 order of magnitude faster than the rate of carbamoyl phosphate formation for each of these mutants. The mutants G359Y and G359F also display an elevated bicarbonate-dependent ATPase activity, indicating an enhanced rate of decomposition of the carboxy phosphate intermediate by water in the absence of an added nitrogen source. This result is likely due to an induced conformational change in the structure of the large subunit. The inability of glutamine to stimulate the hydrolysis of ATP is fully consistent with the retardation of the movement of ammonia through the interior tunnel. For the wild-type CPS, the steady-state rate of ATP hydrolysis is enhanced by about 1 order of magnitude when glutamine is hydrolyzed by the small subunit (*1*). However, this stimulation in the steady-state rate is not the result of an increase in the microscopic rate constant for the phosphorylation of bicarbonate. Rather, it is due to the enhanced rate of attack by ammonia on the carboxy phosphate intermediate relative to that observed for attack by water.[2] Since the steady-state rate of ATP hydrolysis within the large subunit is not stimulated by the hydrolysis of glutamine within the small subunit of either G359Y or G359F, then the internally produced ammonia must not be available for nucleophilic attack on the carboxy phosphate intermediate. These results are thus consistent with a physical blockage of the ammonia tunnel that prevents the ammonia from migration to the large subunit.

The hydrolysis of glutamine is stimulated 50−70-fold when ATP is hydrolyzed within the large subunit of G359Y and G359F. Therefore, a conformational change must be transmitted from the large subunit to the small subunit within these two mutants, similar to what is observed with the wild-type enzyme. These rate enhancements compare favorably with the ∼700-fold stimulation found with the wild-type enzyme (*7*). However, the less robust stimulation with the two mutants can be ascribed, in part, to the higher basal rate of glutamine hydrolysis and an elevated K_m value for glutamine. The results strongly suggest that the machinery or conduit for signal transduction between the two subunits is largely intact, although localized regions of structure of the enzyme must be perturbed to some extent.

When ammonia is provided as an external nitrogen source to the wild-type enzyme, it is unclear whether this substrate must diffuse through the same molecular path as the ammonia derived from the hydrolysis of glutamine. Since the mutants G359Y and G359F work perfectly well using external ammonia as a nitrogen source for the assembly of carbamoyl phosphate, external ammonia must bind to the large subunit via a route that detours around the blocked ammonia tunnel. This conclusion is also supported by the observation that glutamine can be used as a nitrogen source for these mutants only after the glutamine has been largely hydrolyzed and the ammonia product released into the bulk solution.

Recently, a 20 Å tunnel has been identified in glutamine phosphoribosylpyrophosphate amidotransferase (GPATase), which is a member of the *Ntn* class of amidotransferases (*20*). Zalkin, Smith, and colleagues have proposed that external ammonia enters the ammonia tunnel via the glutamine site of GPATase (*21*). This latter proposal is based largely on the comparative catalytic properties of the A82W and C1S/A82W mutants coupled with an assumption that the hydrogen bond between the hydroxyl group of Ser-1 and the backbone amide nitrogen of Tyr-74 would be unable to

[2] B. W. Miles and F. M. Raushel, unpublished results.

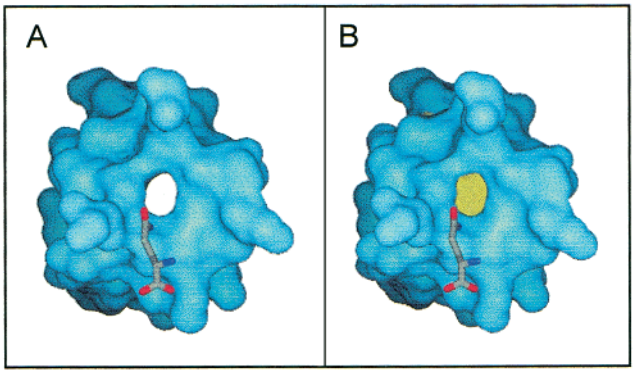


FIGURE 6: Surface model of the ammonia tunnel within the small subunit of CPS prepared with the computer program SPOCK. The models were prepared using only the residues within 3.5 Å of the proposed ammonia tunnel within the small subunit, excluding the bound substrate. A default water radius of 1.4 Å was used. The left panel (A) shows the view of the ammonia tunnel in C269S CPS, containing bound glutamine (*23*). The right panel (B) illustrates the potential blockage within the tunnel. This image was constructed using coordinates from an unrefined computer model using INSIGHT in which Gly-359 of the C269S mutant was replaced with a tyrosine residue.

form in the C1S/A82W mutant (*21*). This conclusion is apparently inconsistent with the rather broad specificity of GPATases. A series of nucleophiles, including propanol, *tert*-butyl alcohol, hydroxylamine, hydrazine, and phenylhydrazine, have been shown to react with phosphoribosylpyrophosphate in avian GPATase (*22*). It is not clear how all of these substrates can be accommodated by the tunnel identified in the bacterial GPATase.

We have successfully constructed constipated mutants of the ammonia tunnel within the small subunit of CPS. These mutants (G359F and G359Y) impede the passage of ammonia within the ammonia tunnel. A total of 10 residues have been identified from the X-ray crystal structure which help form the ammonia tunnel within the small subunit of CPS from *E. coli* (*10*). All of these residues are located within 3.5 Å of the center of the molecular tunnel. Of these 10 residues, three are located within the N-terminal domain of the small subunit (Ser-35, Met-36, and Asp-45), which is structurally unique when compared to other proteins with known three-dimensional structures. The other seven (Lys-202, Gly-293, Ala-309, Asn-311, His-353, Pro-358, and Gly-359) reside within the C-terminal domain of the small subunit, which is homologous to the other members of the *Triad* class of amidotransferases. Except for Gly-293, all of these residues are either strictly conserved or replaced with residues of comparable size and chemical reactivity, among the known carbamoyl phosphate synthetases, including the type I ammonia-specific CPS in which the catalytic cysteine is substituted with a serine within a nonfunctional amidotransferase domain. A surface rendition of the ammonia tunnel showing a portion of the small subunit with bound glutamine from the C269S mutant [the X-ray structure of this mutant has been determined (*23*)] constructed using the SPOCK molecular graphics program[3] using water as the probe molecule is presented in Figure 6A. The tunnel is clearly visible, and the side chain amide nitrogen of the bound glutamine is pointing toward the mouth of the tunnel. The surface model of the same tunnel, except for the replacement of Gly-359 with a tyrosine, clearly shows the blockage of the tunnel leading toward the large subunit (Figure 6B). However, actual structural confirmation awaits an X-ray crystallographic analysis of G359Y and G359F, which is currently underway. Nevertheless, the overall conservation of amino acids residues that line the ammonia tunnel in all of the known CPS enzymes indicates that similar tunnels, as shown here, are likely to be found in carbamoyl phosphate synthetases from other sources. In contrast, none of the seven amino acid residues that form the bulk of the ammonia tunnel that are located in the C-terminal domain of the small subunit (except for the catalytic His-353) is conserved among other *Triad* amidotransferases. Clearly, the molecular architecture employed by other *Triad* amidotransferases for the delivery of ammonia must be different from the one developed for CPS.

Recently, Anderson and Miles have reported a pair of "channel-impaired mutants" of tryptophan synthase (*24*, *25*). Tryptophan synthase, an $\alpha_2\beta_2$ tetrameric enzyme complex, catalyzes the biosynthesis of L-tryptophan from indole 3-glycerol phosphate (IGP) and L-serine, in a reaction mediated by pyridoxal phosphate. The crystal structure of tryptophan synthase from *Salmonella typhimurium* has revealed a tunnel of 25 Å connecting the active sites of the α and β subunits (*26*). It is reported that the transient formation of indole, which is not detectable in the wild-type enzyme, is observed in the βC170F and βC170W mutants, in single-turnover experiments. This result is consistent with a partial blockage for the passage of indole through this tunnel. However, computer simulations of the reaction time course for these mutants place the observed indole inside the tunnel, and the rate of channeling is reduced by >10- and >5000-fold in βC170F and βC170W mutants, respectively. It also appears that intersubunit communication is impaired in the βC170W mutant since the cleavage of IGP is no longer stimulated by the presence of serine. Preliminary structural analysis of the βC170W mutant has indicated that certain residues, which are known to be important for intersubunit communication, may move relative to the wild-type structure (*25*).

It is obvious that the two mutants of CPS reported here with a blockage in the ammonia tunnel share common characteristics with the tunnel-impaired mutants of tryptophan synthase. However, the mutants G359Y and G359F of CPS are different, since the internally derived ammonia is primarily released into the bulk solution, and then utilized as a nitrogen source through a detour to the active site. The fact that the βC170F mutant of tryptophan synthase may have a reduced rate of tunneling and condensation, and yet appears to be only mildly affected in terms of the overall macroscopic rate constants (*25*), cautions us that some of the CPS mutants with wild-type steady-state parameters may still impair the migration of ammonia from one active site to the next. A crude measure for the mean diffusion time (τ) for a one-dimensional 50 Å pass through the ammonia tunnel has been estimated to be $\sim 10^{-7}$ s (*12*). Even if the diffusion rate through this tunnel is inaccurate by several orders of magnitude, it would appear that the rate of diffusion through this tunnel does not limit the rate of carbamoyl phosphate formation in the wild-type enzyme. Therefore, some of the

[3] SPOCK molecular graphics program (v1.0b160p3, copyright 1998, Jon A. Christopher, Texas A&M University).

mutants constructed for this investigation may have significantly reduced migration rates for internal ammonia but no measurable effects on the steady-state kinetic parameters. Efforts to block the passage of intermediates within the tunnel found in the large subunit of CPS are in progress.

REFERENCES

1. Meister, A. (1989) *Adv. Enzymol. Relat. Areas Mol. Biol. 62*, 315−374.
2. Anderson, P. M., and Meister, A. (1965) *Biochemistry 4*, 2803−2809.
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. Anderson, P. M., and Meister, A. (1966) *Biochemistry 5*, 3164−3169.
6. Post, L. E., Post, D. J., and Raushel, F. M. (1990) *J. Biol. Chem. 265*, 7742−7747.
7. Miles, B. W., Banzon, J. A., and Raushel, F. M. (1998) *Biochemistry 37*, 16773−16779.
8. Thoden, J. B., Miran, S. G., Phillips, J. C., Howard, A. J., Raushel, F. M., and Holden, H. M. (1998) *Biochemistry 37*, 8825−8831.
9. Thoden, J. B., Holden, H. M., Wesenberg, G., Raushel, F. M., and Rayment, I. (1997) *Biochemistry 36*, 6305−6316.
10. Thoden, J. B., Raushel, F. M., Benning, M. M., Rayment, I., and Holden, H. M. (1999) *Acta Crystallogr. D55*, 8−24.
11. Mullins, L. S., and Raushel, F. M. (1999) *J. Am. Chem. Soc. 121*, 3803−3804.
12. Raushel, F. M., Mullins, L. S., and Gibson, G. E. (1998) *Biochemistry 37*, 10272−10278.
13. 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.
14. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 51−59.
15. Mareya, S. M., and Raushel, F. M. (1994) *Biochemistry 33*, 2945−2950.
16. Cleland, W. W. (1970) *Enzymes (3rd Ed.) 2*, 1−65.
17. Snodgrass, P. J., and Parry, D. J. (1969) *J. Lab. Clin. Med. 73*, 940−950.
18. Rubino, S. D., Nyunoya, H., and Lusty, C. J. (1986) *J. Biol. Chem. 261*, 11320−11327.
19. Cohen, N. S., Kyan, F. S., Jyan, S. S., Cheung, C. W., and Raijman, L. (1985) *Biochem. J. 229*, 205−211.
20. Krahn, J. M., Kim, J. H., Burns, M. R., Parry, R. S., Zalkin, H., and Smith, J. L. (1997) *Biochemistry 36*, 11061−11068.
21. Chen, S., Burgner, J. W., Krahn, J. M., Smith, J. L., and Zalkin, H. (1999) *Biochemistry 38*, 11659−11669.
22. Hartman, S. C. (1963) *J. Biol. Chem. 238*, 3024−3035.
23. Thoden, J. B., Huang, X., Raushel, F. M., and Holden, H. M. (1999) *Biochemistry 38*, 16158−16166.
24. Schlichting, I., Yang, X.-J., Miles, E. W., Kim, A. Y., and Anderson, K. S. (1994) *J. Biol. Chem. 269*, 26591−26593.
25. 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.
26. Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., and Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857−17871.

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