Access to the carbamate tunnel of carbamoyl phosphate synthetase

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Abstract

The X-ray crystal structure of carbamoyl phosphate synthetase (CPS) from Escherichia coli revealed the existence of a molecular tunnel that has been proposed to facilitate the translocation of reaction intermediates between remotely located active sites. Five highly conserved glutamate residues, including Glu-25, Glu-383, Glu-577, Glu-604, and Glu-916, are close together in two clusters in the interior wall of the molecular tunnel that enables the intermediate carbamate to migrate from the site of synthesis to the site of utilization. Two arginines, Arg-306 and Arg-848, are located at either end of the carbamate tunnel and participate in the binding of ATP at each of the two active sites within the large subunit of CPS. The mutation of Glu-25 or Glu-577 results in a diminution in the overall rate of carbamoyl phosphate formation. Similar effects are observed upon mutation of Arg-306 and Arg-848 to alanine residues. The conserved glutamate and arginine residues may function in concert with one another to control entry of carbamate into the tunnel prior to phosphorylation to carbamoyl phosphate. The electrostatic environment of tunnel interior may help to stabilize the tunnel architecture and prevent decomposition of carbamate through protonation.

Keywords: Carbamoyl phosphate synthetase; Carbamate tunnel

Carbamoyl phosphate synthetase (CPS) catalyzes the assembly of carbamoyl phosphate from ATP, bicarbonate, and glutamine/ammonia (Eq. (1)). CPS from Escherichia coli is a heterodimeric protein of two unequal sized subunits. The small subunit (~42 kDa) catalyzes the hydrolysis of glutamine, whereas the large subunit (~118 kDa) contains two homologous ATP binding sites that are responsible for the phosphorylation of bicarbonate and carbamate. The generally accepted chemical mechanism for the biosynthesis of carbamoyl phosphate by CPS is shown in Scheme 1 [1]. In addition to the overall synthesis of carbamoyl phosphate, CPS is known to catalyze three partial reactions as presented in Eqs. (2)–(4) [2]:

\[
\begin{align*}
2\text{MgATP} + \text{HCO}_3^- + \text{Gln} & \rightarrow 2\text{MgADP} + \text{Glu} + \text{P}_i + \text{Carbamoyl-P} & (1) \\
\text{glutamine} + \text{H}_2\text{O} & \rightarrow \text{glutamate} + \text{NH}_3 & (2) \\
\text{MgATP} + \text{H}_2\text{O} & \rightarrow \text{MgADP} + \text{P}_i & (3) \\
\text{MgADP} + \text{Carbamoyl-P} & \rightarrow \text{MgATP} + \text{NH}_2\text{CO}_2^- & (4)
\end{align*}
\]

The X-ray structure of CPS revealed the locations of the three distinct active sites within the protein framework (Fig. 1) [3]. Remarkably, these active sites are linked to one another via a long molecular tunnel, which extends nearly 100 Å from one end of the protein to the other. The molecular tunnel functions in the translocation of reaction intermediates between active sites, as originally postulated in the reaction catalyzed by tryptophan synthase [4]. Ammonia, produced from the hydrolysis of glutamine in the small subunit, moves approximately 45 Å to the active site embedded in the N-terminal half of the large subunit where it reacts with the intermediate carboxy phosphate to form carbamate. This intermediate then migrates to the structurally homologous site in the C-terminal domain to generate carbamoyl phosphate upon phosphorylation by ATP. Kinetic evidence for the direct channeling of ammonia through the molecular tunnel was provided by the competitive incorporation of isotopically labeled ammonia into carbamoyl phosphate.
glutamine and ammonia into the final product, carbamoyl phosphate [5]. The internally produced NH₃ from the hydrolysis of glutamine does not equilibrate with the ammonia in the bulk solvent but is apparently sequestered and channeled directly to the large subunit.

Further experimental support for the channeling of ammonia through the ammonia tunnel was provided by the three-dimensional structure and kinetic properties of channel-impaired mutant enzymes [6,7]. These mutants were constructed with the original intent of physically clogging the ammonia tunnel by replacing amino acid residues that form the inner surface of the molecular tunnel with ones possessing bulkier side chains. The altered kinetic behavior of two mutants, G359F and G359Y, was consistent with the postulated role of the ammonia tunnel. These mutants were not capable of using glutamine as a nitrogen source but retained full activity when ammonia was provided. However, the X-ray structure of G359F revealed that the introduction of a phenylalanine at residue 359 forced a change in the local protein structure of the small subunit, which led to the rearrangement of a loop that extends from Glu-355 to Ala-364 [8]. This unanticipated conformational change caused the formation of a hole in the ammonia tunnel, which served as an escape route for ammonia directly to the bulk solvent.

The direct channeling of the second intermediate, carbamate, has been challenged by an alternative mechanism proposed by Kothe et al. [9]. In the nucleotide switch mechanism, the active site within the N-terminal domain of CPS catalyzes the formation of carbamoyl phosphate through the direct elimination of water (or hydroxide) from a tetrahedral intermediate. The active site found within the C-terminal half of the large subunit was proposed to facilitate this reaction through the induction of a protein conformational change mediated by the bicarbonate-dependent hydrolysis of ATP. This unconventional mechanism specifically excludes the involvement of carbamate as a direct precursor to the enzymatic synthesis of carbamoyl phosphate. However, the nucleotide switch mechanism was experimentally dismissed due to the lack of an isotope exchange reaction between bicarbonate and solvent water in the presence of glutamine [10]. The classical sequential mechanism requires that no exchange of ¹⁸O between bicarbonate and water be observed in the presence of a nitrogen source, whereas the nucleotide switch or parallel mechanism requires that the incorporation of oxygen from water into the bicarbonate pool occur during every turnover. The rate of this exchange reaction was fully suppressed upon the addition of glutamine. This observation is, therefore, fully consistent with the mechanism that requires the channeling of carbamate through the molecular tunnel. Mutants designed to contain a blockage within the carbamate tunnel exhibited kinetic properties that were in good agreement with the proposed role of the tunnel [11]. These mutants hampered the overall rate of carbamoyl phosphate synthesis, whereas the other partial reactions remained close to what was observed with the wild type enzyme. A quantitative assessment of the disruptive communication between the two phosphorylation sites correlated with the putative size of the blockage within the tunnel.

The amino acid composition of the carbamate tunnel is shown in Table 1. Interestingly, seven out of thirty residues are hydrophilic. Five of these residues are glutamates, which are invariably conserved in all other prokaryotic CPS enzymes. Moreover, they are all located on a specific side of the carbamate tunnel in the three-dimensional representation presented in Fig. 2. The side chain carboxyl groups of all five glutamates point toward the inside of the carbamate tunnel, covering the inner surface of the tunnel in the vicinity of the two ATP binding sites at either end. In addition, the carbamate tunnel is capped with two other hydrophilic residues, Arg-306 and Arg-848, which are also highly conserved among 61 prokaryotic organisms.

<table>
<thead>
<tr>
<th>N-terminal domain</th>
<th>C-terminal domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ile18</td>
<td>Asn570</td>
</tr>
<tr>
<td>Val19</td>
<td>Arg571</td>
</tr>
<tr>
<td>Ile20</td>
<td>Ile572</td>
</tr>
<tr>
<td>Gly21</td>
<td>Gly573</td>
</tr>
<tr>
<td>Gln22</td>
<td>Gln574</td>
</tr>
<tr>
<td>Ala23</td>
<td>Gly575</td>
</tr>
<tr>
<td>Cys24</td>
<td>Ile576</td>
</tr>
<tr>
<td>Glu25</td>
<td>Glu577</td>
</tr>
<tr>
<td>Phe26</td>
<td>Phe578</td>
</tr>
<tr>
<td>Ala52</td>
<td>Glu604</td>
</tr>
<tr>
<td>Met378</td>
<td>Met911</td>
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<tr>
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<td>Ser913</td>
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<td>Val381</td>
<td>Thr914</td>
</tr>
<tr>
<td>Gly382</td>
<td>Gly915</td>
</tr>
<tr>
<td>Gln383</td>
<td>Gln916</td>
</tr>
</tbody>
</table>

*Residues in bold are over 96% conserved among 61 prokaryotic organisms.
Fig. 1. A trace of α-carbons of the wild type CPS [3]. The small subunit is represented in pink, and the large subunit of four different domains is displayed according to the following color scheme; the N-terminal domain in green, the C-terminal in blue, the oligomerization domain in black, and the allosteric domain in red. The carbamate tunnel is highlighted in red and the ammonia tunnel is in gray. Three active sites are indicated in circles.

Fig. 2. A surface representation of an inner wall of the carbamate tunnel [3]. A surface is colored according to the electrostatic charges. Red represents negative charge and blue for positive charge. Clipping planes were adjusted to view the inside profile of the protein matrix using the program Web Lab Viewer Pro. The size of the probe atom was set to 1.4 Å that has been used to describe the Connolly surface.
conserved. Each of these arginines is in direct contact with the γ-phosphoryl group of the ATP analogue, AMPPNP, bound within the two active sites as illustrated in Fig. 3 [12]. The overall shape of the carbamate tunnel is nearly linear between these two arginines, which extends symmetrically from one ATP binding site in the N-terminal domain to the C-terminal domain. These two arginines may function to control access to the carbamate tunnel, based upon their orientation and participation in nucleotide binding (Fig. 4). The formation and consumption of carbamate near Arg-306 and Arg-848 could serve as the key for the initiation of conformational changes that enable the entry or exit of ligands in the carbamate tunnel. In this study, the functional role of these conserved glutamates and arginines in the catalytic operation of CPS is investigated.

Materials and methods

Materials

All chemicals and coupling enzymes were purchased from either Sigma or Aldrich, unless otherwise stated. Restriction enzymes were purchased from New England Biolabs and pfu DNA polymerase was acquired from Promega. The clone for ornithine transcarbamoylase was a generous gift from the laboratory of Dr. N. Allewell.

Bacterial strains and plasmids

Site-directed mutagenesis of CPS was performed as described previously [13]. The E. coli strains used for this
study were RC50 and XL-Blue [14]. The RC50 strain used for protein expression was a generous gift from Dr. Carol J. Lusty. All plasmids used in this project were derived from pMS03 [13]. Site-directed mutagenesis was performed using the polymerase chain reaction and the overlap extension method of Ho et al. [15]. All of the site-directed changes made to the wild type CPS were confirmed by DNA sequencing of the modified plasmids. Oligonucleotide synthesis and DNA sequencing were performed by the Gene Technology Laboratory, Texas A&M University.

Expression and purification of mutant proteins

The plasmids containing the carAB genes were transformed in the RC50 cell line of E. coli for expression of the wild type and mutant forms of CPS. The wild type and mutants of CPS were purified as previously described [16]. The mutants E383Q, E577Q, E604Q, E916Q, E383Q/E916Q, E25Q/E383Q, E25Q/E383Q/E604Q, R306A, and R848A were expressed and purified to greater than 95% homogeneity, as judged by SDS–polyacrylamide gel electrophoresis.

Kinetic measurements and analysis

The rate of glutamine hydrolysis was determined by coupling the formation of glutamate to the production of α-ketoglutarate with L-glutamate dehydrogenase and 3-acylpyridine adenine dinucleotide (APAD) [16]. The reaction mixtures contained 50 mM Hepes (pH 7.6), 20 mM MgCl₂, 100 mM KCl, 12 U OTCase, and 10 mM glutamine. The kinetic parameters were determined by fitting the experimental data to Eq. (5), where $k_{cat}$ is the turnover number, $K_m$ is the Michaelis constant, $A$ is the substrate concentration, and $E_i$ is the enzyme concentration. The data for the enhancement of ATP hydrolysis in the presence of a nitrogen source were fitted to Eq. (6) [18].

$$v/E_i = k_{cat}A/(K_m + A),$$

$$v = V_o(K_a + \alpha)/(K_a + I).$$

Results and discussion

The carbamate tunnel of CPS connects the two homologous active sites contained within the amino- and carboxy-terminal domains of the large subunit. The 30 amino acid residues that constitute the interior walls of the carbamate tunnel are listed in Table 1. Half of these residues are located in the N-terminal domain while the other half originates from the C-terminal domain. These residues are not in direct physical contact with the nucleotide analogue bound to the two active sites but they serve primarily as the building blocks for the assembly of the carbamate tunnel [12]. Seven of these residues possess side chains that can ionize. The guanidino group of the lone arginine (Arg-571) is pointing away from the interior wall of the carbamate tunnel, whereas the side chains of all five glutamates are positioned inside the tunnel. In addition to these residues, the poles of the carbamate tunnel are capped with two other arginine residues (Arg-306 and Arg-848) that make direct molecular contact with the bound nucleotide analogue, AMPPNP (Fig. 3).

The distribution of the five glutamates within the carbamate tunnel is illustrated in Fig. 2. Most of the tunnel is hydrophobic, as expected from the amino acid composition, except for two clusters of glutamic acid residues. Glu-25, Glu-383, and Glu-604 are located near the carboxy phosphate binding site, whereas Glu-577 and Glu-916 are positioned at the opposite end of the carbamate tunnel near the site for the synthesis of carbamoyl phosphate. The two capping residues, Arg-306 and Arg-848, are located on the opposite face of the carbamate tunnel relative to the position of the conserved glutamates. At one end of the carbamate tunnel, Glu-604 bridges Glu-25 and Glu-383 at a distance of 2.8...
and 3.0 Å, respectively (Fig. 5). The only nearby positive charge is the guanidino group of Arg-306, which is ~4 Å away from the carboxyl group of Glu-25. There are also four ordered water molecules that are capable of forming hydrogen bonds with this trio of glutamates. One of these water molecules interacts with Glu-25 and is also 2.7 Å away from Ne of Arg-306. Similarly, the guanidino side chain of Arg-848 and the carboxyl group of Glu-577 are linked by a bound water molecule in the domain for the synthesis of carbamoyl phosphate. However, at a distance of 4.7 Å, the side chains of Glu-577 and Glu-916 are not within hydrogen bonding distance to one another. There are no other residues with a positive charge near the cluster of Glu-577 and Glu-916. Two water molecules are found within 3.5 Å of Glu-916 and one water molecule is within this same distance to Glu-577.

**Mutation of conserved glutamates**

The five conserved glutamates found within the carbamoyl tunnel were mutated to glutamine (except for Glu-25) in an attempt to determine the role of these residues toward the maintenance of structure and function in CPS. The mutation of either Glu-383 or Glu-916 causes a moderate change in the bicarbonate-dependent ATPase, glutaminase, and ATP synthesis reactions (Table 2). In addition, E916Q exhibits a ~10-fold decrease in the rate of carbamoyl phosphate synthesis compared to the wild type enzyme, whereas E383Q possesses nearly wild type level of activity. The effect of E916Q on the synthesis of carbamoyl phosphate may originate from an inefficient delivery of carbamate, since the rate of the bicarbonate-dependent ATPase reaction is normal relative to the wild type enzyme. The small perturbation on the rate of the partial ATP synthesis reaction indicates that the catalytic machinery at the carbamoyl phosphate binding site is not seriously affected. When Glu-604 was changed to glutamine, the overall catalytic activity is diminished more profoundly than for the mutant E383Q. The rate of ATP hydrolysis is elevated compared to the wild type enzyme and is not further activated by the presence of a nitrogen source. This may indicate that the mutant allows more ready access of water to the active site, possibly due to a change in local structure. The glutaminase and ATP synthesis rates for E604Q are comparable to the wild type enzyme.

Glu-577 and Glu-25 are a homologous pair of residues within the carbamate tunnel of CPS. All of the partial reactions and the synthesis of carbamoyl phosphate were attenuated to the greatest extent for E577Q among all other single site mutants. The bicarbonate-dependent ATPase activity at the carboxy phosphate binding site is diminished significantly, even though the site of mutation is distant from the catalytic site that is most affected. The reduction in the rate of glutamine hydrolysis is a direct consequence of the perturbation to the catalytic properties of the carboxy phosphate domain. Glu-577 may thus play a central role as part of the conduit for relaying communication among the three active sites.

**Double glutamate mutations**

The role of Glu-25 was examined in the context of the double mutant E25Q/E383Q, since there were relatively minor effects on the catalytic activity by E383Q alone. The carbamoyl phosphate synthetase activity of E25Q/E383Q was diminished 50-fold. In addition, the bicarbonate-dependent ATPase activity of this mutant is more than 10-fold lower than the wild type enzyme, with virtually no activation by either glutamine or ammonium ion. The rate of the partial ATP synthesis reaction

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Fig. 5. Distances among glutamates and an arginine in (A) the N-terminal end synthetase domain and (B) the C-terminal end synthetase domain. Coordinates taken from pdb file 1BXR.
Table 2
Kinetic constants for CPS and various mutations of conserved glutamate and arginine residues

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Glutaminase&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HCO&lt;sub&gt;3&lt;/sub&gt;-dependent ATP hydrolysis</th>
<th>Glutamine-dependent ATP hydrolysis</th>
<th>NH&lt;sub&gt;4&lt;/sub&gt;Cl-dependent ATP hydrolysis</th>
<th>ATP synthesis</th>
<th>Carbamoyl-P synthesis&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>$k_{cat}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>$K_m$ (mM)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>$k_{cat}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>$K_m$ (mM)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>$k_{cat}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>WT</td>
<td>2.0 ± 0.1</td>
<td>0.14 ± 0.01</td>
<td>4.0 ± 0.1</td>
<td>0.12 ± 0.01</td>
<td>3.9 ± 0.1</td>
<td>10 ± 13</td>
</tr>
<tr>
<td>E383Q</td>
<td>0.84 ± 0.08</td>
<td>0.62 ± 0.07</td>
<td>3.2 ± 0.1</td>
<td>0.15 ± 0.03</td>
<td>1.7 ± 0.1</td>
<td>110 ± 23</td>
</tr>
<tr>
<td>E604Q</td>
<td>0.81 ± 0.09</td>
<td>1.3 ± 0.01</td>
<td>1.5 ± 0.1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>1.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>E577Q</td>
<td>0.091 ± 0.001</td>
<td>0.12 ± 0.01</td>
<td>0.17 ± 0.01&lt;sup&gt;f&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.12 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>E916Q</td>
<td>1.5 ± 0.02</td>
<td>0.60 ± 0.03</td>
<td>4.2 ± 0.2</td>
<td>0.11 ± 0.01</td>
<td>1.3 ± 0.1</td>
<td>160 ± 30</td>
</tr>
<tr>
<td>E25Q/E383Q</td>
<td>0.96 ± 0.11</td>
<td>0.31 ± 0.02</td>
<td>0.32 ± 0.3&lt;sup&gt;f&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.51 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>E383Q/E25Q</td>
<td>1.5 ± 0.1</td>
<td>0.27 ± 0.002</td>
<td>4.0 ± 0.03</td>
<td>0.14 ± 0.02</td>
<td>ND&lt;sup&gt;g&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>E916Q</td>
<td>0.42 ± 0.04</td>
<td>0.061 ± 0.003</td>
<td>0.15 ± 0.01&lt;sup&gt;f&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.097 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>E25Q/E383Q</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E604Q</td>
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<tr>
<td>R306A</td>
<td>ND</td>
<td>0.086 ± 0.011</td>
<td>0.10 ± 0.01</td>
<td>0.22&lt;sup&gt;b&lt;/sup&gt; ± 0.03</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>R484A</td>
<td>ND</td>
<td>0.60 ± 0.03</td>
<td>0.95 ± 0.04</td>
<td>0.40&lt;sup&gt;b&lt;/sup&gt; ± 0.07</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reaction conditions are described in Materials and methods.

<sup>b</sup> 10 mM glutamine.

<sup>c</sup> $K_m$ for glutamine.

<sup>d</sup> $K_m$ for ammonium.

<sup>e</sup> $K_m$ for ADP.

<sup>f</sup> Rate measured at 10 mM glutamine.

<sup>g</sup> Not applicable.

<sup>b</sup> Rate measured at 300 mM NH<sub>4</sub>Cl.

<sup>h</sup> Not determined.

<sup>i</sup> Detection limit.

<sup>k</sup> $K_m$ for ATP.
is reduced, but the glutaminase reaction is not affected significantly. It appears that these mutations reduce the enhancement of the bicarbonate-dependent ATPase reaction by glutamine/ammonia, without affecting the other two active sites. The lowered rate of carbamoyl phosphate synthesis can be rationalized by a diminished rate of formation of carbamate within the active site for the synthesis of carboxy phosphate. The kinetic behavior of the double mutant, E383Q/E916Q, is almost indistinguishable from that of E916Q; an indication that the mutation of E383 to glutamine is not deleterious to the catalytic properties of CPS.

**Triple glutamate mutation**

A mutant in which all three glutamates near the carboxy phosphate binding domain end of the carbamate tunnel was constructed. In this mutant, E25Q/E383Q/E604Q, the glutaminase activity is decreased about fivefold, and the bicarbonate-dependent ATPase activity is diminished at least 20-fold. However, the partial ATP synthesis reaction catalyzed within the C-terminal domain is not changed substantially. The cumulative alteration of the three glutamate residues in this part of the carbamate tunnel was catastrophic for the bicarbonate-dependent ATP hydrolysis and the subsequent generation of carbamate, resulting in a very low rate of carbamoyl phosphate production.

**Mutation of conserved arginine residues**

The guanidino group of Arg-848 interacts with the γ-phosphoryl group of AMPPNP, in conjunction with Arg-845 (Fig. 3). Based on the ATP synthesis reaction catalyzed by R848A, the $k_m$ for MgADP is about two orders of magnitude higher (6.9 mM) than the wild type value, although $k_{cat}$ is only fivefold smaller (0.087 s$^{-1}$). The overall rate of carbamoyl phosphate synthesis is substantially reduced. This result appears to originate from a perturbation to the binding of MgADP in the active site within the C-terminal domain. When Arg-306 was mutated to alanine, the bicarbonate-dependent ATPase activity is reduced by an order of magnitude, whereas R848A exhibits a slightly decreased $k_{cat}$ for the same reaction (Table 2). In addition, the ATPase reaction is not activated by the addition of glutamine or ammonia for R306A, although there is about a twofold increase with the addition of glutamine but not with ammonia for R848A. It is evident that R306A is unable to synthesize carbamoyl phosphate, most likely due to a diminished net production of carbamate in the active site responsible for the synthesis of carboxy phosphate. Since the side chain of Arg-306 is apparently utilized to anchor the γ-phosphoryl group of the nucleotide, this loss of activity may result from the unfavorable binding of MgATP at this site.

**Gatekeepers to the carbamate tunnel**

The conserved glutamate and arginine residues found at the ends and in the interior of the carbamate tunnel of CPS are important for the proper functioning of this enzyme. The mutation of these residues results in the diminution of carbamoyl phosphate formation. The mutation of the two glutamate residues (Glu-25 and Glu-577) that ion pair with the two arginine residues (R306 and R848) is more disruptive to the catalytic than the mutation of the other conserved glutamates found within the carbamate tunnel. This result suggests that these two glutamates may function in conjunction with the two arginine residues to control access of carbamate into the molecular tunnel. The mutation of either arginine to alanine also disrupts substantially the ability of CPS to catalyze the synthesis of carbamoyl phosphate. In addition, the half life of carbamate is dependent on pH and temperature and this reactive intermediate becomes thermodynamically unstable at lower pH [19]. Therefore, the enzyme must prevent the protonation of this intermediate to facilitate the delivery between the two reaction centers. The five glutamate residues spread over the interior surface wall of the carbamate tunnel may provide a basic environment for the trapping of protons.

Molecular dynamics simulations have indicated that changes in the hydrophobicity of a protein tunnel can prevent the penetration by water or ions into a channel [20,21]. Furthermore, a closed hydrophobic nanopore can be opened by adding dipoles to its lining [21]. Therefore, carbamate, with a net negative charge, may be blocked from entering the carbamate tunnel if the lining at the entrance region is too hydrophobic. The two clusters of glutamates are positioned at the ends of the carbamate tunnel, which is also consistent with a role as a gatekeeper. Although the side chains for the other amino acids within the carbamate tunnel are hydrophobic, the backbone carbonyl groups from 11 of them are oriented toward the inside layer of the carbamate tunnel. Therefore, the overall electrostatic environment of the interior wall of the carbamate tunnel may be delicately tuned to enable the most efficient passage of carbamate from one end of the tunnel to the other.

**References**