# Deconstruction of the Catalytic Array within the Amidotransferase Subunit of Carbamoyl Phosphate Synthetase†

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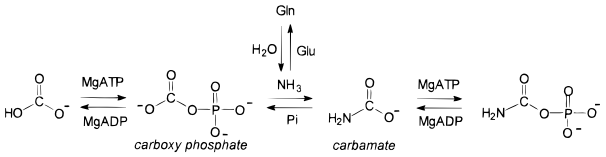
ABSTRACT: Carbamoyl phosphate synthetase from *Escherichia coli* catalyzes the formation of carbamoyl phosphate from bicarbonate, glutamine, and two molecules of ATP. The enzyme consists of a large synthetase subunit, and a small amidotransferase subunit, which belongs to the *Triad* family of glutamine amidotransferases. Previous studies have established that the reaction mechanism of the small subunit proceeds through the formation of a $\gamma$-glutamyl thioester with Cys-269. The roles in the hydrolysis of glutamine played by the conserved residues, Glu-355, Ser-47, Lys-202, and Gln-273, were determined by mutagenesis. In the X-ray crystal structure of the H353N mutant, Ser-47 and Gln-273 interact with the $\gamma$-glutamyl thioester intermediate [Thoden, J. B., Miran, S. G., Phillips, J. C., Howard, A. J., Raushel, F. M., and Holden, H. M. (1998) *Biochemistry 37*, 8825−8831]. The mutants E355D and E355A have elevated values of $K_m$ for glutamine, but the overall carbamoyl phosphate synthesis reaction is unperturbed. E355Q does not significantly affect the bicarbonate-dependent ATPase or glutaminase partial reactions. However, this mutation almost completely uncouples the two partial reactions such that no carbamoyl phosphate is produced. The partial recovery of carbamoyl phosphate synthesis activity in the double mutant E355Q/K202M argues that the loss of activity in E355Q is at least partly due to additional interactions between Gln-355 and Lys-202 in E355Q. The mutants S47A and Q273A have elevated $K_m$ values for glutamine while the $V_{max}$ values are comparable to that of the wild-type enzyme. It is concluded that contrary to the original proposal for the catalytic triad, Glu-355 is not an essential residue for catalysis. The results are consistent with Ser-47 and Gln-273 playing significant roles in the binding of glutamine.

Carbamoyl phosphate synthetase (CPS)[1] from *Escherichia coli* catalyzes the formation of carbamoyl phosphate from bicarbonate, glutamine, and two molecules of ATP. The enzyme contains a large synthetase subunit (∼118 kDa) and a small amidotransferase subunit (∼42 kDa) (*1*−*3*). Based on isotopic labeling studies and discovery of three partial reactions, it has been proposed that carbamoyl phosphate is constructed within the active site(s) of CPS by four distinct chemical reactions as illustrated in Scheme 1 (*4*). The recently determined X-ray crystal structure of CPS from *E. coli* is fully consistent with this reaction mechanism (*5*). The large subunit has been shown to contain distinct active sites for the phosphorylation of bicarbonate and carbamate by ATP, while the small subunit contains the active site for the hydrolysis of glutamine. Remarkably, the three active sites are linearly connected by an interior tunnel spanning nearly 100 Å. Presumably, the ammonia produced within the small subunit diffuses through this tunnel to the large subunit where it reacts with the carboxy phosphate intermediate to form the carbamate intermediate. The carbamate subsequently migrates to the second phosphorylation site where the final product, carbamoyl phosphate, is produced.

Scheme 1




The reaction mechanism of CPS is very complex since multiple reactions are catalyzed via linear and parallel processes. In the absence of a nitrogen source, CPS catalyzes a slow bicarbonate-dependent hydrolysis of ATP. The hydrolysis of glutamine by the small subunit increases the steady-state rate for ATP turnover. Similarly, CPS displays a low rate of glutamine hydrolysis in the absence of ATP or bicarbonate while the presence of both ATP and bicarbonate enhances the rate of the glutamine hydrolysis considerably. In addition, mutations within the large subunit can affect the glutaminase activity of the small subunit (*6*, *7*), and mutations within the small subunit can affect the kinetic parameters of the large subunit (*8*, *9*).

CPS from *E. coli* has been classified as a member of the *Triad* glutamine amidotransferases, which also include anthranilate synthase, GMP synthetase, CTP synthetase, PABA synthetase, and aminodeoxychorismate synthase, among others (*10*). The active site of the *Triad* glutamine amidotransferases contains a conserved Cys-His-Glu triad (*10*, *11*). Based upon sequence and structural comparisons, the catalytic triad in CPS from *E. coli* is Cys-269, His-353, and Glu-355. The recent crystal structure of CPS from *E.*

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[1] Abbreviations: CPS, carbamoyl phosphate synthetase; CAD, the trifunctional mammalian enzyme consisting of carbamoyl phosphate synthetase, aspartate transcarbamoylase, and dihydroorotase; PABA, *p*-aminobenzoate; PCR, polymerase chain reaction.

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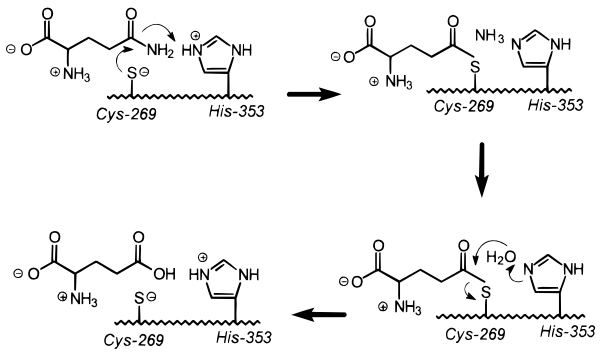
Scheme 2




*coli* has demonstrated that His-353 is within hydrogen bonding distance of Cys-269 while Glu-355 is within hydrogen bonding distance of His-353 (*5*). Previous mutagenesis studies of CPS from *E. coli* have identified Cys-269 and His-353 as the active site cysteine and histidine, respectively (*8*, *12*). The most recent crystallographic study of the CPS mutant H353N, determined in the presence of glutamine, has unequivocally demonstrated that the chemical mechanism for the hydrolysis of glutamine involves a covalent $\gamma$-glutamyl thioester intermediate with Cys-269 (*13*). Moreover, the $\gamma$-glutamyl thioester intermediate has been shown to be kinetically competent by rapid quench experiments (*14*). Together, these studies have firmly established the roles of Cys-269, His-353, and the $\gamma$-glutamyl thioester intermediate in catalysis. However, the role of Glu-355, the third residue of the trio, has not been adequately addressed. A working model for the chemical mechanism of the glutaminase reaction is shown in Scheme 2.

The X-ray crystal structure of the thioester intermediate has identified three additional residues that may play major roles in the binding and hydrolysis of glutamine in CPS. The $\epsilon$-amino group of Lys-202 is within hydrogen bonding distance of the carboxylate group of Glu-355 in the structures of the wild-type and H353N mutant of CPS. This residue is strictly conserved in the amidotransferase domain of CPS, but it does not appear to be conserved in other members of the *Triad* amidotransferase family. In addition to this residue, the hydroxyl group of Ser-47 is within hydrogen bonding distance of the carbonyl oxygen of the $\gamma$-glutamyl thioester intermediate (*13*). This hydroxyl group may function to position the carbonyl carbon of the glutamine carboxamide group for nucleophilic attack by the thiolate anion of Cys-269 and to stabilize the developing oxyanion intermediate. The structural architecture of the small subunit of CPS is distinctly bilobal with the entire catalytic triad located in the C-terminal domain while Ser-47 is located in the N-terminal domain. Nonetheless, Ser-47 and the active site residues are near the interface of the two domains of the small subunit. The only other side-chain group making molecular contact with the $\gamma$-glutamyl thioester intermediate is that of Gln-273 (*13*). Here we probe the catalytic and structural roles of Ser-47 Lys-202, Gln-273, and Glu-355. The spatial relationship of these residues along with residues Cys-269 and His-353 in the wild-type and H353N CPS is shown in Figure 1.

## MATERIALS AND METHODS

*Materials*. All chemicals and coupling enzymes used for activity measurements were purchased from either Sigma or Aldrich unless otherwise stated. Restriction enzymes and Vent$^R$ DNA polymerase were purchased from Promega. Oligonucleotides were synthesized by the Gene Technologies Laboratory, Biology Department, Texas A&M University. The *E. coli* strains used for this study were XL1-Blue and RC50 (*15*).

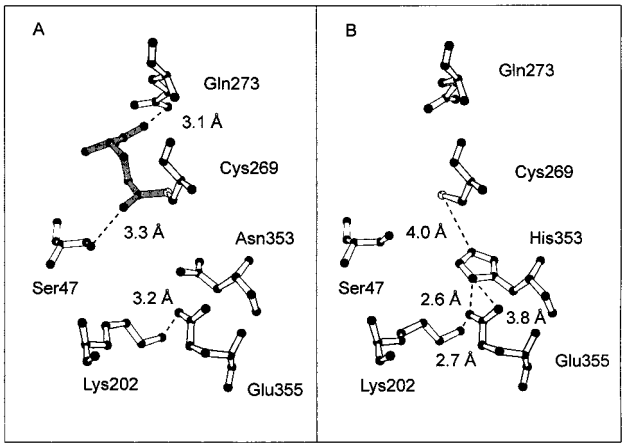



FIGURE 1: Close-up view of the active site for the small subunit of the wild-type and H353N mutant of CPS. (A) H353N CPS (coordinates taken from ref *13*). (B) Wild-type CPS (coordinates taken from ref *5*).

*Methods*. Site-directed mutagenesis was performed as previously described with the wild-type CPS (*15*) using the polymerase chain reaction and the overlap extension method of Ho et al. (*16*). The mutant plasmids were transformed in the RC50 cell line (a generous gift of Carol J. Lusty) for expression and purification of mutant proteins (*9*). Assay conditions for monitoring the rate of formation of ADP, glutamate, and carbamoyl-P were carried out as previously described (*17*). The kinetic parameters, $V_{max}$ and $K_m$, were determined by fitting the experimental data to eq 1, 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 fit to eq 2 (*18*). In this equation, $V_0$ is the initial enzyme velocity in the absence of the nitrogen source $I$ (ammonia or glutamine), $K_a$ is the apparent activation constant, and $\alpha$ is the ratio of the velocities at saturating and zero concentration of the nitrogen source. In this case, $V_{max}$ is expressed as $\alpha V_0$.

$$v = V_{max}A/(K_m + A) \quad (1)$$

$$v = V_0(K_a + \alpha I)/(K_a + I) \quad (2)$$

## RESULTS

Four conserved residues, Ser-47, Lys-202, Gln-273, and Glu-355, located within the active site of the small subunit of CPS, were mutated in order to determine the functional roles of these residues in binding and catalysis during the hydrolysis of glutamine. The mutants S47A, K202M, Q273A, E355A, E355D, E355Q, E355A/K202M, and E355Q/K202M were expressed and purified to greater than 95% homogeneity as judged by SDS−polyacrylamide gel electrophoresis. The effects of these modifications on the catalytic constants were determined for each mutant by measuring the rate of formation of ADP, glutamate, and carbamoyl phosphate

Table 1: Kinetic Parameters for the ATPase Reaction of the Wild-Type and Mutant Enzymes

| | $HCO_3^-$-dependent ATPase[a] | $NH_3$-dependent ATPase[b] | | Gln-dependent ATPase[c] | |
|---|---|---|---|---|---|
| | $V_{max}$ ($s^{-1}$) | $V_{max}$ ($s^{-1}$) | $K_m(NH_4^+)$ (mM) | $V_{max}$ ($s^{-1}$) | $K_m$(Gln) (mM) |
| WT | 0.45 | 9.1 ± 0.5 | 296 ± 32 | 7.7 ± 0.08 | 0.098 ± 0.0004 |
| E355D | 0.56 | 11 ± 0.3 | 210 ± 10 | 7.7 ± 0.05 | 0.087 ± 0.004 |
| E355A | 0.67 | 6.4 ± 0.3 | 174 ± 16 | 6.4 ± 0.05 | 0.61 ± 0.03 |
| E355Q | 1.3 | 8.8 ± 0.3 | 128 ± 12 | 1.6[d] | >45[d] |
| K202M | 0.17 | 11 ± 1.6 | 388 ± 83 | 7.2 ± 0.3 | 1.5 ± 0.2 |
| E355A/K202M | 1.3 | 11 ± 0.3 | 111 ± 8 | 3.5 ± 0.03 | 0.13 ± 0.01 |
| E355Q/K202M | 1.1 | 13 ± 0.5 | 66 ± 8 | 4.0 ± 0.08 | 0.034 ± 0.005 |
| Q273A | 0.69 | 13 ± 0.7 | 185 ± 23 | 9.9 ± 0.1 | 2.1 ± 0.07 |
| S47A | 0.22 | 8.0 ± 0.5 | 640 ± 42 | 8.5 ± 0.1 | 1.3 ± 0.07 |

[a] Total rate of ADP formation monitored for the bicarbonate-dependent ATPase, ammonia-dependent ATPase, or glutamine-dependent ATPase reactions. 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, 10 mM ornithine. [b] 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. [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, 10 mM ornithine. [d] Rate at a glutamine concentration of 45 mM.

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

| | glutaminase in absence of ATP and $HCO_3^-$ [a] | | glutaminase in presence of saturating ATP and $HCO_3^-$ [b] | |
|---|---|---|---|---|
| | $V_{max}$ ($min^{-1}$) | $K_m$(Gln) (mM) | $V_{max}$ ($s^{-1}$) | $K_m$(Gln) (mM) |
| WT | 0.25 ± 0.005 | 0.083 ± 0.006 | 2.9 ± 0.05 | 0.069 ± 0.007 |
| E355D | 0.13 ± 0.02 | 11 ± 3 | 2.9 ± 0.05 | 0.071 ± 0.007 |
| E355A | 0.43 ± 0.03 | 3.8 ± 0.7 | 2.7 ± 0.03 | 0.65 ± 0.04 |
| E355Q | 0.69 ± 0.16 | 56 ± 24 | <0.02[c] | ND |
| K202M | 0.21 ± 0.03 | 4.9 ± 1.9 | 3.7 ± 0.05 | 1.1 ± 0.06 |
| E355A/K202M | 0.24 ± 0.03 | 24 ± 6 | 1.3 ± 0.02 | 0.12 ± 0.01 |
| E355Q/K202M | 0.17 ± 0.02 | 15 ± 3 | 1.3 ± 0.03 | 0.059 ±0.006 |
| Q273A | 1.3 ± 0.08 | 3.7 ± 0.5 | 3.2 ± 0.2 | 1.3 ± 0.3 |
| S47A | ≤0.008[d] | NA | 3.5 ± 0.1 | 0.87 ± 0.09 |

[a] Total rate of glutamate formation for the glutaminase reaction in the absence or presence of ATP and bicarbonate. 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 ATP, 40 mM bicarbonate, 20 mM $MgCl_2$, 100 mM KCl, 10 mM ornithine. [c] Rate at a glutamine concentration of 60 mM. [d] Lower detection limit.

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

| | $NH_3$-dependent[a] | | Gln-dependent[b] | |
|---|---|---|---|---|
| | $V_{max}$ ($s^{-1}$) | $K_m(NH_4Cl)$ (mM) | $V_{max}$ ($s^{-1}$) | $K_m$(Gln) (mM) |
| WT | 2.9 ± 0.1 | 211 ± 23 | 3.2 ± 0.02 | 0.075 ± 0.003 |
| E355D | 3.2 ± 0.3 | 150 ± 34 | 2.3 ± 0.03 | 0.076 ± 0.005 |
| E355A | 2.5 ± 0.1 | 145 ± 20 | 2.1 ± 0.05 | 0.73 ± 0.05 |
| E355Q | 2.9 ± 0.2 | 83 ± 21 | ≤0.01[c] | NA |
| K202M | 4.3 ± 0.3 | 313 ± 56 | 2.3 ± 0.05 | 1.4 ± 0.09 |
| E355A/K202M | 3.7 ± 0.2 | 67 ± 11 | 0.80 ± 0.02 | 0.15 ± 0.01 |
| E355Q/K202M | 5.3 ± 0.1 | 56 ± 4 | 0.64 ± 0.01 | 0.077 ±0.007 |
| Q273A | 4.0 ± 0.2 | 106 ± 18 | 3.2 ± 0.05 | 1.9 ± 0.08 |
| S47A | 2.9 ± 0.1 | 860 ± 44 | 2.9 ± 0.03 | 1.1 ± 0.05 |

[a] Rate of carbamoyl phosphate formation. 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, 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, 10 mM ornithine. [c] Lower detection limit.

under a variety of assay formats. The kinetic parameters, $K_m$ and $V_{max}$, obtained for the wild-type and the mutant enzymes are summarized in Tables 1−3.

*Kinetic Properties of Ser-47*. Ser-47 was identified as a prime target for site-directed mutagenesis after the crystal structure determination of the CPS mutant, H353N (*13*). In this structure, glutamine was found trapped as a covalent $\gamma$-glutamyl thioester intermediate with Cys-269. The hydroxyl group of Ser-47 was within hydrogen-bonding distance of the carbonyl group of the thioester intermediate, and thus this residue is potentially important for stabilization of the oxyanion adduct during the formation and subsequent hydrolysis of the thioester intermediate. In the absence of ATP and bicarbonate, the mutant S47A is unable to hydrolyze glutamine. However, in the presence of ATP and bicarbonate, this mutant hydrolyzes glutamine as well as the wild-type enzyme, but the $K_m$ for glutamine is elevated by about an order of magnitude.

*Kinetic Properties of Gln-273*. In the crystal structure of H353N, the side chain carboxamide of Gln-273 was found to be hydrogen bonded to the α-carboxyl group of the $\gamma$-glutamyl thioester intermediate (*13*). When the side chain of this residue is truncated to an alanine, the $K_m$ values for glutamine are increased between 20- and 40-fold. The effects on the maximal rate of formation of glutamate, ADP, and carbamoyl-P are significantly smaller.

*Kinetic Properties of Glu-355*. The glutamate residue at position 355 is an integral member of the putative catalytic triad with Cys-269 and His-353 (*10*). Surprisingly, the catalytic properties of the three mutations made at this position are all somewhat different from one another. The conservative replacement of Glu-355 with an aspartate results in relatively minor effects on the steady-state kinetic parameters of the overall reaction in the presence of ATP and bicarbonate. However, in the absence of added ATP and bicarbonate, there is a 2 orders of magnitude increase in the Michaelis constant for glutamine. Similarly, removal of the side-chain carboxylate altogether (E355A) does not affect the maximal rate of the partial reactions or the overall

synthesis of carbamoyl phosphate. However, this modification to the active site of the small subunit significantly increases the $K_m$ values for glutamine in all of the glutamine-dependent reactions. The alanine mutation does not appear to affect the synthetase subunit since E355A displayed an unaltered bicarbonate-dependent ATPase activity, ammonia-dependent ATPase reaction, and carbamoyl phosphate synthesis activities.

The conservative replacement of Glu-355 with a glutamine unexpectedly abolishes the ability of this mutant to catalyze carbamoyl phosphate formation. For the partial glutaminase reaction, there is a 2−3-fold increase in the value for $V_{max}$ and a 600-fold increase in the $K_m$ value for glutamine. Moreover, there is no stimulation of the glutaminase activity upon the further addition of ATP and bicarbonate. The ability to synthesize carbamoyl phosphate using ammonia as the nitrogen source is unchanged.

*Kinetic Properties of Lys-202*. Since Lys-202 is ion-paired with Glu-355, this residue was mutated to a methionine in order to identify additional interactions that may be critical for the proper functioning of the catalytic triad. The catalytic properties of K202M are very similar to those of E355A. Removal of the positive charge has minimal effect on the maximal rates of either the partial reactions or the overall synthesis of carbamoyl phosphate. However, this modification does elevate the Michaelis constants for glutamine in all of the glutamine-dependent reactions (15−60-fold).

*Kinetic Properties of E355A/K202M and E355Q/K202M*. The methionine substitution for Lys-202 was paired with the E355A and E355Q mutations in an attempt to maintain the charge balance in the wild-type protein at this particular location in the small subunit. For the E355A/K202M double mutant, the Michaelis constants for glutamine are diminished relative to the values exhibited by the E355A mutant alone. In nearly every case, the $K_m$ values are very close to the wild-type values. However, for the glutaminase partial reaction, there is a 290-fold increase in the $K_m$ value, but the maximal rate of glutamine hydrolysis is unaltered. The catalytic properties of the E355Q/K202M mutant are very similar to the wild-type enzyme with two notable exceptions. In the absence of ATP and bicarbonate, the $K_m$ for the hydrolysis of glutamine is 15 mM compared to a value of ~0.1 mM for the wild-type enzyme. The maximal velocity for the formation of carbamoyl phosphate is reduced about 5-fold relative to that found for the wild-type enzyme when glutamine is used as the nitrogen source.

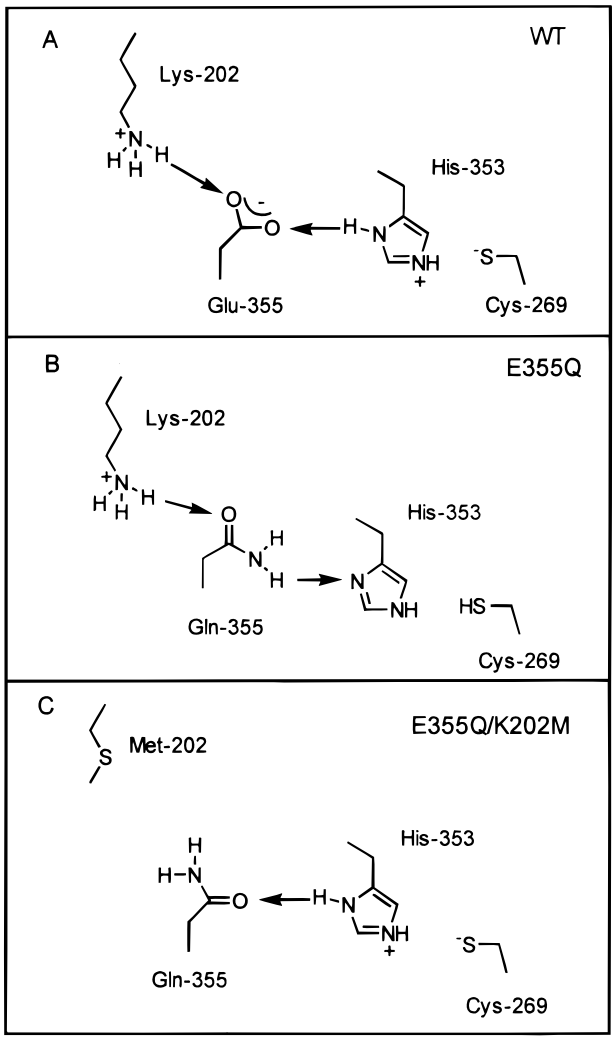
## DISCUSSION

Carbamoyl phosphate synthetase from *E. coli* belongs to the *Triad* family of glutamine amidotransferases, which was named based largely upon sequence comparisons and the observation of a papain-like catalytic triad containing Cys-His-Glu in the crystal structure of GMP synthetase from *E. coli* (*10*, *11*). The concept of a catalytic triad has also been proposed for anthranilate synthase and hamster CAD carbamoyl phosphate synthetase (*19*, *20*). Ample biochemical and structural studies have since provided evidence for the formation of a $\gamma$-glutamyl thioester intermediate and essential roles of the catalytic Cys and His residues during catalysis (*8*, *12*, *13*, *21*). However, the role of the catalytic Glu residue has not been adequately defined. In this study, the function of Glu-355 within the amidotransferase subunit of CPS from *E. coli* was examined by site-directed mutagenesis. Three other strictly conserved residues, Lys-202, Ser-47, and Gln-273, were also mutated in order to more fully elaborate their roles in catalysis.

Glu-355 was changed to aspartate, alanine, and glutamine, and each mutant has distinct characteristics. E355D only affected the $K_m$ value for glutamine in the glutaminase partial reaction. Wild-type kinetic parameters were preserved for the overall synthesis of carbamoyl phosphate. E355A displayed elevated $K_m$ values for both the glutaminase partial reaction and the overall carbamoyl phosphate synthesis reaction. However, the maximal rates were unaltered. Together, the catalytic properties of E355D and E355A argue against an essential role for Glu-355 in catalysis. E355Q is abnormal in this series. Although the modification from Glu to Gln does not significantly affect the two partial reactions (namely, the glutaminase- and bicarbonate-dependent ATPase reactions), it almost completely uncouples these reactions such that no carbamoyl phosphate is produced. Moreover, there is no enhancement of the rate of ATP hydrolysis in the presence of glutamine, and no enhancement of the rate of glutamine hydrolysis in the presence of ATP (Tables 1 and 2). One possible explanation for the aberrant properties of E355Q is that an additional interaction is created between Gln-355 and an unknown residue(s) in the mutant enzyme that disrupts the proper functioning of this active site and coupling with the large subunit. It is observed that the $\epsilon$-amino group of the conserved Lys-202 hydrogen bonds to the $\gamma$-carboxylate group of Glu-355 in X-ray structures of both the wild-type and H353N CPS. To determine if Lys-202 was contributing to the loss of catalytic activity, the mutants K202M, E355A/K202M, and E355Q/K202M were prepared. K202M exhibited a maximal rate of carbamoyl phosphate synthesis comparable to that of the wild-type enzyme and a 15−18-fold increase in the $K_m$ values for glutamine. This result argues against an essential role in catalysis for Lys-202. This is somewhat expected as Lys-202 is only conserved among carbamoyl phosphate synthetases but not with the other *Triad* glutamine amidotransferases. E355Q/K202M did recover some ability to catalyze carbamoyl phosphate formation from glutamine. This observation was supported by significant coupling between the two partial reactions, since approximately six molecules of ATP and two molecules of glutamine were utilized for every molecule of carbamoyl phosphate formed. The result is consistent with the hypothesis that an extra interaction between Lys-202 and the mutated Gln-355 is partly responsible for the lack of carbamoyl phosphate synthesis activity exhibited by E355Q. For E355A/K202M, the enzyme remained active, although the maximal rate for the overall synthesis of carbamoyl phosphate is reduced by 2−3-fold.

A working model for the diminution of the overall catalytic activity exhibited by the E355Q enzyme is presented in Scheme 3.[2] In panel A, the ionic interaction between the carboxylate of Glu-355 and His-353 of the wild-type enzyme would help to enhance the ionization of Cys-269 through polarization of the imidazole side chain. In contrast, replacement of the carboxylate side chain of Glu-355 with the carboxamide group of glutamine (in E355Q) serves to promote the formation of a hydrogen bond between the $\epsilon$-amino group of Lys-202 and the carbonyl oxygen of the

Scheme 3




mutated residue as illustrated in panel B. This interaction would force a reversal in the polarity of the hydrogen bond network to the imidazole side chain and thus diminish the reactivity of Cys-269. The mutation of Lys-202 to methionine may enable the carbonyl oxygen of the carboxamide side chain of the mutated glutamine to form a hydrogen bond to the imidazole that is of the same polarity as in the wild-type structure (panel C). A similar explanation has been proposed and confirmed by X-ray crystallography to rationalize the catalytic properties for a double mutant, D102N/S195C, of trypsin (*22*). A structural analysis of the E355Q and E355Q/K202M mutants of CPS is being pursued.

Walsh and co-workers have mutated the glutamate in the active site of PABA synthetase, another member of the *Triad* amidotransferases (*21*). The corresponding residue, Glu-170, was replaced by aspartate, alanine, and glutamine. The $k_{cat}/K_m$ values of E170D and E170A were reduced by 4-fold and 150-fold, respectively, while E170Q had no detectable activity. The properties of E170Q, however, are complicated by the perturbed folding of this protein since most of the enzyme was found aggregated in the cell pellet (*21*). Nonetheless, the results do not support Glu-170 as an essential catalytic residue. However, our result is in apparent contradiction to a recent analysis of the CAD CPS which concludes that the corresponding residue, Glu-338, in the CPS amidotransferase domain from the trifunctional protein is essential for maintaining His-336 in the optimal orientation for catalysis (*23*). The study utilized a hybrid CPS consisting of the wild-type or mutant mammalian amidotransferase domain and the *E. coli* CPS synthetase subunit to monitor the glutaminase activity. The second-order rate constant $k_{cat}/K_m$ of the hybrid mutant E338G was reduced respectively by $10^4$-fold and $10^5$-fold for the glutaminase reaction in the absence and presence of ATP and bicarbonate, compared to the wild-type hybrid protein. Although it seems unlikely that the *E. coli* CPS and CAD CPS operate through distinct mechanisms to hydrolyze glutamine, we cannot explain the apparent loss of activity of this CAD mutant. One possible explanation is that the drastic replacement of Glu-338 with a glycine may well alter the local secondary structure surrounding this residue. The fact that the *E. coli* CPS mutants E355A, E355D, and E355Q display distinct properties nonetheless underscores the sensitive environment surrounding this residue in CPS from *E. coli*. This may be true for the CAD CPS as well.

Two other mutants constructed for this investigation are S47A and Q273A. The hydroxyl group of Ser-47 appears to form part of the oxyanion hole while Gln-273 clearly contributes to part of the binding pocket for glutamine. S47A had no detectable activity in the partial glutaminase reaction. However, the maximal rate of the overall synthetic reaction for carbamoyl phosphate with this mutant is unaltered. While this result rules out Ser-47 as an essential element of the oxyanion hole, it does suggest that the catalytic coupling of the glutaminase reaction and the ATPase reaction forces the small subunit to adopt a conformation that is different from the one available in the absence of ATP. A similar finding has been reported by Hewagama et al. (*24*). The mutant Q273A had an elevated $K_m$ value for glutamine while the $V_{max}$ value is comparable to that of the wild-type enzyme. Therefore, it appears that Gln-273 contributes to the binding and recognition of the substrate glutamine to the active site of the small subunit.

In summary, our results convincingly argue against Glu-355 as an essential catalytic residue for the hydrolysis of glutamine. The data presented here are fully consistent with Ser-47, Gln-273, and Lys-202 playing a significant role in the ability of the small subunit to generate and deliver ammonia to the large subunit.

[2] The $V_{max}$ for the glutaminase partial reaction of the E355Q mutant is near normal, relative to the wild-type enzyme. The working model that appears in Scheme 3 for the diminution of the glutaminase activity in the presence of ATP does not explain why the partial glutaminase activity in the absence of ATP does not diminish as well. However, it should be noted that the hydrolysis of glutamine for the wild-type enzyme is nearly 3 orders of magnitude slower in the absence of ATP than in the presence of ATP.

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