Specificity Determining Residues in Ammonia- and Glutamine-dependent Carbamoyl Phosphate Synthetases*

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Carbamoyl phosphate synthetases (CPSs) utilize either glutamine or ammonia for the ATP-dependent generation of carbamoyl phosphate. In glutamine-utilizing CPSs (e.g. the single Escherichia coli CPS and mammalian CPS II), the hydrolysis of glutamine to yield ammonia is catalyzed at a triad-type glutamine amidotransferase domain. Non-glutamine-utilizing CPSs (e.g. rat and human CPS I), lacking the catalytic cysteine residue, can generate carbamoyl phosphate only in the presence of free ammonia. Frog CPS I (fCPS I), unlike mammalian CPS Is, retains most of the glutamine amidotransferase residues conserved in glutamine-utilizing CPSs, including an intact catalytic triad, and could therefore be expected to use glutamine. To determine why fCPS I is unable to utilize glutamine, we compared sequences of glutamine-using and non-glutamine-using CPSs to identify residues that are present or conservatively substituted in all glutamine-utilizing CPSs but absent in fCPS I. We constructed the site-directed mutants Q273E, L270K, Q273E/N240S, and Q273E/L270K in E. coli CPS and have determined that simultaneous occurrence of the two substitutions, Glu → Glu and Leu → Lys, found in the frog CPS I glutamine amidotransferase domain are sufficient to eliminate glutamine utilization by the E. coli enzyme.

Formation of carbamoyl phosphate (CP) is the first step in the synthesis of arginine and pyrimidines and, in higher organisms, is also critical in nitric oxide formation and in ammonia detoxification via the urea cycle. Carbamoyl phosphate synthetases (CPSs) are made up of a two-domain glutamine amidotransferase (GAT) component and a four-domain synthetase (SYN) component (1–5). The N-terminal domain of the GAT component is required for interaction with the SYN component (6, 7), and the C-terminal domain has a triad-type glutamine amidotransferase structure (5, 8). In glutamine-utilizing CPSs (e.g. the single Escherichia coli CPS and mammalian CPS II), the hydrolysis of glutamine to yield ammonia is catalyzed at the GAT domain, where the cysteine of the Cys-His-Glu triad carries out a nucleophilic attack on the amide carbonyl of glutamine (9–12).

The solved triad GAT structures for E. coli CPS (5) and GMP synthetase (8, 29) reveal a nearly identical topology of β-sheet flanked by α-helices and a β-ribbon. Surprisingly, both structures also display a fairly uniformed glutamine specificity pocket (8). Apart from the essential triad cysteine 269, the only E. coli CPS side chain groups that have been identified as interacting with glutamine are serine 47 and glutamine 273. S47A and Q273A have 13- and 20-fold increased K_m values, respectively, but unchanged rates of glutamine utilization (22). It has been assumed that conformational changes must occur to yield the known tight specificity of triad GATs for glutamine, but no crystals have yet been obtained with alternate conformations (8). In the present work, we have utilized site-directed mutagenesis to further probe the molecular basis for the glutamine specificity inherent in E. coli CPS, for the unexpected inability of frog CPS I to utilize glutamine, and for the dramatic differences in ammonia binding among CPSs. We have targeted for analysis those residues that are conserved in the GAT domains of glutamine-utilizing CPSs but that are not present in frog CPS I.

EXPERIMENTAL PROCEDURES

Materials—Chemicals and coupling enzymes were purchased from Fisher and Sigma Aldrich. 1-[3,4-3H]Glutamine (39.3 Ci/mmol) was
purchased from PerkinElmer Life Sciences. Preformulated bacterial growth media (L-Broth, Terrific Broth II) were purchased from Qiagen.

**Strains and Plasmids—**XLI-Blue E. coli cells were purchased from Stratagene and used for transformation and propagation of plasmid DNA (Stratagene). CPS was cloned into pUC19 (34), which has been described (with the catalytic subunits of CPS and the SYN component) and is defective in the Lon protease, was kindly provided by Dr. Carol Lusty (New York Public Health Research Institute (6)). The plasmid pUCABI (9606 base pairs), encoding both the GAT and SYN components of CPS, was a generous gift of Dr. Mendel Tuchman (Washington Children’s Hospital); in construction of this plasmid, expression of CPS was placed under control of the isopropyl-1-thio-β-D-galactopyranoside-inducible trc promoter, the carA translational start site was changed from TTG to ATG, and a Met → Val substitution was made at amino acid position two (30). For the present study, pUCABI was modified such that it would carry only the carAB genes. The arom (ornithine transcarbamoylase) gene was excised from pUCABI by digestion of the plasmid DNA with BamHI and KpnI. The large fragment was gel-purified, treated with mung bean nuclease to create blunt ends, and recircularized to create pUCAB (8594 base pairs).

**Recombinant DNA Methods—**Bacterial transformations and recombinant DNA techniques were carried out as described in Sambrook et al. (31).Restriction enzymes, nucleases, and ligase were obtained from New England Biolabs. Recombinant Fnu DNA polymerase was from New England Biolabs. Mutagenic oligonucleotides were generated using QuikChange method (Stratagene). Each mutagenesis cassette was verified to identify nucleotide substitutions. Oligonucleotide primers for mutagenesis and sequencing were synthesized at the Tufts University Core Lab Facility. Mutagenesis primers corresponding to the coding strand sequence with mutated residues in bold were as follows: Q273E, 5'-GTTCGTCGACGACTGCTGG-3'; Q273E/270K, 5'-GGTATTCGGCA-GAGTCATCAGCTCCTGG-3'; L270K, 5'-TCGAAGGGAGATG-3'. Mutagenesis primers corresponding to the coding strand sequence were synthesized at the Tufts University Core Lab Facility. Mutagenesis primers corresponding to the coding strand sequence (with mutated residues in bold) were as follows: Q273E, 5'-GTTCGTCGACGACTGCTGG-3'; Q273E/270K, 5'-GGTATTCGGCA-GAGTCATCAGCTCCTGG-3'; L270K, 5'-GGATTTGCGGACCTTCTGAGATCCTACGCTTTCTGG-3'.

**CPS Purification—**Bullfrogs, Rana catesbiana, were obtained from the Lemberger Co. (Wisconsin). Native frog liver CPS I (fCPS I) was prepared as described previously via a protocol including cetyltrimethylammonium bromide and acetone precipitation steps and Ali-Gel Blue (Bio-Rad) chromatography (32).

The purification protocol for recombinant wild type E. coli CPS (eCPS) and for its site-directed mutants was adapted from those previously described for native (33) and recombinant (19) E. coli CPS. E. coli strain L673 transformed with pUCAB was grown to stationary phase at 37°C in Terrific Broth containing 100 mg/l ampicillin, then diluted 100-fold in the same medium and grown to an optical density of about 0.6 at 600 nm. The culture was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, harvested by centrifugation at 5,000 × g for 10 min, and resuspended in 0.2 M potassium phosphate, 1 mM EDTA, 1 mM diethiothreitol, and 0.2 m M phenylmethylsulfonyl fluoride, pH 7.6. The cells were disrupted by sonication (six 30-s pulses, with cooling intervals between pulses), and cell debris was removed by centrifugation at 16,000 × g for 20 min. Cleared lysate (4.100 ml) was applied to a Hi-Prep™16/10 DEAE column (AKTA fast protein liquid chromatography, Amersham Biosciences, Inc.) equilibrated in 0.1 M potassium phosphate and 1 mM EDTA, pH 7.6 (Buffer A). Bound protein was eluted from the column with a 0–50% discontinuous gradient of 1 M KCl (0–10% in 80 ml, 10–55% in 120 ml) in Buffer A. The eCPS peak typically eluted at 250 mM KCl. eCPS-containing fractions were pooled and concentrated by the addition of solid ammonium sulfate to 75%. The eCPS was then loaded onto a 1.5 ml HiLoad Superdex 200 column. eCPS was eluted from this column in Buffer A and stored for further use.

The purity of all protein preparations was at least 95%, as assessed by Coomassie Blue staining of SDS-polyacrylamide (4–15% linear gradient) gels (34). Protein concentration was determined by the dye binding assay using the BCA Protein Assay Kit (Pierce) (35).

**Enzyme Assays and Data Analysis—**CP synthesis was determined in a two-step assay by coupling the CPS reaction to that of ornithine transcarbamoylase and then quantitating the resulting citrulline. The reaction mixtures contained 50 mM HEPES, 100 mM KCl, 10 mM ATP, 20 mM MgCl₂, 20 mM NaHCO₃, 1 mM diithiothreitol, 5 mM ornithine, 0.2 units of ornithine transcarbamoylase, and either 300 mM NH₄Cl or 10 mM glutamine and were initiated by the addition of CPS (37 °C, pH 7.6).

**ATPase activities** were determined in a pyruvate kinase/lactate dehydrogenase coupled assay, essentially as previously described (18). The reaction mixtures contained variable ATP, 30 mM HEPES, 100 mM KCl, 20 mM MgSO₄, 40 mM NaHCO₃, 1 mM sodium phosphoenolpyruvate, 0.2 mM NADH, 18 units of pyruvate kinase and 24 units of lactate dehydrogenase (25°C, pH 7.6). 20 mM ornithine was included where indicated. After initiation by the addition of CPS, the reaction was monitored continuously at 340 nm, and the amount of ADP formed was calculated from the loss of NADH. To determine ammonium-dependent ATPase activity, 300 mM NH₄Cl was included in the reaction mixture, and to determine glutamine-dependent ATPase activity, 10 mM glutamine was included. To determine bicarbonate-dependent ATPase activity, no ammonia source was added to the assay mixture.

Glutamine hydrolysis was determined as previously described (11) by coupling glutamate formation to the glutamate dehydrogenase-catalyzed reduction of 3-acetyl pyridine dinucleotide (ε₆₅₀ = 8.3 mM⁻¹ cm⁻¹). The reaction mixtures contained variable amounts of glutamine, 100 mM HEPES, 10 mM ATP, 20 mM MgCl₂, 40 mM NaHCO₃, 100 mM KCl, 20 mM MgSO₄, 1 mM sodium phosphoenolpyruvate, 0.2 mM NADH, and 20 units of lactate dehydrogenase (25°C, pH 7.6). After initiation by the addition of CPS, the reaction was monitored continuously at 363 nm, and the amount of glutamate formed was calculated from the formation of reduced 3-acetylpyridine nucleotide. For all routine assays involving fCPS I, 5 mM N-acetylglutamate was included in the reaction mixture.

**RESULTS**

**Inability of Frog CPS I (fCPS I) to Utilize Glutamine—**Although early studies on rat and human CPS I established clearly that they were unable to utilize glutamine (24), fCPS I was simply assumed to also not utilize glutamine since its other determined properties were identical to those of the rat and human enzymes. However, the relatively recent determination of the fCPS I sequence revealed that it was the only CPS I that retained the entire GAT catalytic triad and raised the question of whether it might utilize glutamine as an aminating substrate (27). To answer this question, we have purified native fCPS I and determined its ability to utilize glutamine in synthesizing CP. The purified frog enzyme was fully active when free ammonia was the aminating substrate. In the presence of 30 mM NH₄Cl, the specific activity of the enzyme was 0.8 μmol of CP/min/mg. However, in the absence of ammonia and presence of glutamine (up to 135 mM), fCPS I had no detectable activity. We also tested glutamine in the absence of the allosteric activator N-acetylglutamate (39) and again found no detectable activity.

Because fCPS I proved unable to catalyze the overall synthesis of CP from glutamine, there could be an impairment of glutamine recognition/utilization by the CPS I GAT domain, and/or glutamine hydrolysis might have, by some other mechanism, become uncoupled from CP synthesis. To address the first possibility, we undertook experiments to examine both the glutamine-hydrolyzing and glutamine binding abilities of the fCPS I that retained the entire GAT catalytic triad and raised the question of whether it might utilize glutamine as an aminating substrate (27). To answer this question, we have purified native fCPS I and determined its ability to utilize glutamine in synthesizing CP. The purified frog enzyme was fully active when free ammonia was the aminating substrate. In the presence of 30 mM NH₄Cl, the specific activity of the enzyme was 0.8 μmol of CP/min/mg. However, in the absence of ammonia and presence of glutamine (up to 135 mM), fCPS I had no detectable activity. We also tested glutamine in the absence of the allosteric activator N-acetylglutamate (39) and again found no detectable activity.
native enzyme. As a positive control for these studies, we used the glutamine-utilizing eCPS. eCPS, like other glutamine-utilizing CPSs, is able to catalyze the hydrolysis of glutamate at the GAT domain independently of the overall CP synthesis reaction. However, the rate of this uncoupled partial reaction is extremely slow, with a $k_{cat}$ value of 0.25 min$^{-1}$ (22). When both the GAT and SYN active sites are occupied (with glutamine and with ATP/bicarbonate, respectively), the $k_{cat}$ value for the coupled glutaminase increases to 2.9 s$^{-1}$ (22). Our glutaminase assay conditions yielded a $k_{cat}$ for glutamate formation by eCPS very similar to that previously reported (1.5 s$^{-1}$ in the presence of ATP/bicarbonate). However, despite varying assay conditions (10–30 mM glutamine, with and without N-acetylglutamate, with and without ATP/bicarbonate), ICPs did not display any detectable glutamine hydrolysis activity. We also carried out glutamine binding studies on ICPs. As presented below, the binding study protocol yielded significant [$^{3}$H]glutamine binding for wild type eCPS and for several of its mutants. However, with ICPs I, no [$^{3}$H]glutamine binding was detected. Thus, despite the striking presence of an intact catalytic triad, ICPs are unable to bind and utilize glutamine.

Identification of Residues Potentially Critical for GAT Domain Activity—Our initial studies of the native ICPs together with its known sequence established that changes in non-triad residues must be responsible for its inability to utilize glutamine. The Cys-His-Glu catalytic triad residues are indicated by a boldface. Also shown are GAT domain residues for ammonia utilizing CPSs (H.s., Homo sapiens; R.c., Rana catesbiana; frog; R.n., Rattus norvegicus, rat).

**FIG. 1.** Invariant residues in the GAT domains of glutamine utilizing and ammonia utilizing CPSs. Analysis of an amino acid sequence alignment (Munich Information Center for Protein Sequences) revealed 29 residues that are invariant in glutamine-utilizing CPSs from 23 different organisms. These invariant residues are numbered according to eCPS. The nine residues shown in boldface are invariant in all triad GAT domains. The Cys-His-Glu catalytic triad residues are indicated by a boldface. Also shown are GAT domain residues for ammonia utilizing CPSs (H.s., Homo sapiens; R.c., Rana catesbiana; frog; R.n., Rattus norvegicus, rat).

**FIG. 2.** Modeling the N240S, L270K, and Q273E mutations in silico. Swiss-Model, the Glaxo Wellcome automated modeling server, was used to visualize potential spatial consequences of mutating the wild type eCPS residues Asn-240, Leu-270, Gln-273 (Protein Data Bank code 1JDB, shown in black) to their ICPs I counterparts. Mutants modeled were N240S, L270K, and Q273E (shown in gray). Stick models showing the changes in spatial orientation of active site residues resulting from the mutations are superimposed in gray over the black wild type positions.
Glutamine Binding Analysis of Wild Type and Mutant eCPSs—Wild type eCPS and the Q273E, L270K, and Q273E/L270K mutants were purified as described under “Experimental Procedures” and used for [3H]glutamine binding studies to further dissect their interaction with glutamine (Table II). To ensure specificity of binding, glutamine was supplied at a concentration close to its $K_m$ value for eCPS (120 $\mu$M), and the incubation was for only 1 min (19). After removal of free [3H]glutamine by centrifugal desalting (38, 40), wild type eCPS exhibited robust binding (0.147 mol of [3H]Gln/mol of CPS). However, both the Gln $\rightarrow$ Ghu and Leu $\rightarrow$ Lys substitutions made in eCPS to mirror ICPS I residues significantly altered the ability of eCPS to bind this substrate. The Q273E eCPS mutant was >10-fold impaired in its glutamine binding ability as compared with the wild type enzyme, whereas in L270K as well as in the double mutant Q273E/L270K, glutamine binding was almost entirely abolished. The behavior of these mutants suggests that these two substitutions in the ICPS I GAT domain may contribute significantly to this enzyme’s inability to use glutamine as a nitrogen donor despite its retention of the catalytic triad.

Glutamine Hydrolysis Activity of Wild Type and Mutant eCPSs—The glutamine-hydrolyzing activities of purified wild type and mutant eCPSs were determined as previously described (11) by following the glutamate dehydrogenase-mediated reduction of 3-acylpyridine dinucleotide. As discussed above, wild type eCPS carries out the uncoupled glutaminase reaction at an extremely slow rate that would make difficult the accurate determination of even slower mutant rates. However, the glutaminase activity is increased 600-fold when both the GAT and SYN active sites are occupied (with glutamine and presence of ornithine to further dissect the effects of the mutations.

Glutamine Utilization during CP Synthesis by Q273E and L270K—Compared with wild type eCPS, Q273E exhibited only a slight perturbation in its glutamine $K_m$ (0.36 versus 0.16 mM for eCPS), whereas L270K had an ~25-fold increased $K_m$ for this substrate (3.93 mM, Table IV). Both Q273E and L270K exhibited depressed turnover rates, ~4- and 10-fold slower than the wild type enzyme, respectively (Table IV). L270K displayed even greater deterioration in efficiency of glutamine utilization, with $k_{cat}/K_m$ more than 2 orders of magnitude worse than the value for wild type eCPS (0.27 versus 57.59 s$^{-1}$). As expected, given its previous characterization as an effector of $K_m$, ornithine had no noteworthy effect on these parameters for the utilization of glutamine (Table IV).

Q273E/L270K had no detectable glutamine-dependent CP synthetic activity. The double mutant did display bicarbonate-dependent ATPase activity, reflecting uncoupled nonproductive turnover at the first ATP site. However, the addition of glutamine at concentrations up to 20 mM did not cause significant changes in the rate of ATP hydrolysis, with $k_{cat}$ values of 0.70 and 0.78 s$^{-1}$ in the presence and absence of glutamine, respectively (Table V). Interestingly, the bicarbonate-dependent ATPase activity for Q273E/L270K was much greater than wild type. Values for $k_{cat}$ in the absence and presence of ornithine were 0.062 and 0.073 s$^{-1}$ for wild type and 0.78 and 2.11 s$^{-1}$ for the double mutant (Table V). It thus appears that Q273E/L270K has undergone a relaxation of the structural constraints normally limiting ATP hydrolysis in the absence of an ammonia source. In contrast both L270K and Q273E display
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TABLE I

| CPS synthesis by WT and mutant CPS IIIs |
|----------------------------------------|
| % Glutamine-dependent activity | eCPS | Q273E | L270K | Q273E/L270K | Q273E/N240S |
|----------------------------------------|
| % Ammonia-dependent activity | 100 | 110 | 20 | 4 | 85 |
|----------------------------------------|

TABLE II

| [3H]Glutamine binding by CPSs |
|-------------------------------|
| [3H]Glutamine binding studies for purified WT eCPS and mutant CPSs were performed with non-saturating glutamine. Binding was for 1 min at 25 °C using 100 μg of CPS in 143 mM potassium phosphate buffer, pH 6.8, 5 mM EDTA, and 120 μM [3H]glutamine (100 μl total volume). Enzyme-bound radioactivity was recovered in the effluent after centrifugation through pre-packaged spin columns and was added to 10 ml of scintillation fluid to determine cpm. In addition to the no enzyme control, the SYN component of eCPS (120 kDa) was included as a control for non-specific binding of [3H]glutamine to CPS. |
|--------------------------|
| CPS | cpm counted | [3H]Glutamine/CPS | mol/mol |
|--------------------------|
| fCPS I | 51 | 0 | 0 |
| eCPS | 35497 | 0.147 | 0.0005 |
| Q273E | 2556 | 0.0095 | 0.0005 |
| Q273E/L270K | 235 | 0.0005 | 0.0005 |
| L270K | 174 | 0.0001 | 0.0001 |
| eCPS SYN only | 70 | 0 | 0 |
| No enzyme | 29 | 0 | 0 |

TABLE III

| Glutamine- and ammonia-dependent CP synthesis by purified WT and mutant CPSs |
|---------------------------------------------------------------------------|
| CP synthesis was performed using cleared lysates. eCPS concentrations were normalized by densitometric analysis of a Western blot. Assays were carried out at 37 °C for 10 min as described under "Experimental Procedures." eCPS generated 0.11 and 0.14 μmol of citrulline in the glutamine- and ammonia-dependent assays, respectively. These values were used to represent 100% activity. Corresponding activity values were calculated for each of the four mutants. |
|--------------------------|
| CPS | 3 min | 7 min | 10 min |
|--------------------------|
| eCPS, Glu | 2.58 | 5.17 | 7.28 |
| eCPS, NH₃ | 3.03 | 6.42 | 10.47 |
| Q273E, Glu | 1.70 | 5.12 | 7.44 |
| Q273E, NH₃ | 2.62 | 5.02 | 10.35 |
| L270K, Glu | 0.32 | 0.66 | 1.05 |
| L270K, NH₃ | 1.86 | 7.05 | 10.62 |
| Q273E/L270K, Glu | 0 | 0 | 0 |
| Q273E/L270K, NH₃ | 3.46 | 9.69 | 12.46 |
| fCPS I, Glu | 0 | 0 | 0 |
| fCPS I, NH₃ | 2.48 | 7.66 | 11.05 |

Ammonia Utilization during CP Synthesis by Q273E, L270K, and Q273E/L270K—Ammonia-dependent ATPase assays were performed to assess the functioning of the SYN component of the eCPS mutants. Because the mutations were all in the GAT component and the ammonia-dependent reaction occurs only on the SYN component, any effects on this activity should reflect conformational changes in the SYN component caused by interaction with the GAT mutants. The primary effect of the mutations was on the K₉ for ammonia, whereas the turnover numbers were similar to those of wild type eCPS (Table IV). Although uncharged NH₃ is known to be the actual CPS substrate (28), the data are presented in terms of NH₄⁺ since it is the level of NH₄Cl that is varied during the experiment; under the conditions of the experiment, NH₃ represents about 4% of the total NH₄⁺ added to the solution (28). Surprisingly, Q273E displayed an ~6-fold increase in ammonium K₉ (615 versus 96.1 mM for eCPS). However, L270K had a slightly lower ammonium K₉ (64 mM) than wild type eCPS, and Q273E/L270K had a 4-fold lower ammonium K₉ (26 mM). An enhanced interaction with ammonia in the mutants is consistent with the very low fCPS I K₉ for ammonia. As expected, given its previous characterization as an effector of K₉, the addition of ornithine had no noteworthy effect on the utilization of ammonia (Table IV).

ATP and Allosteric Effector Interaction in Q273E, L270K, and Q273E/L270K—ATP utilization was probed in three assays as follows. Glutamine-dependent ATPase activity reflects cleavage at both ATP sites of the SYN component coupled to glutamine cleavage at the GAT domain. Ammonia-dependent ATPase activity reflects cleavage at both sites of the SYN component, and bicarbonate-dependent ATPase activity reflects uncoupled nonproductive cleavage at only the first of the two SYN ATP sites. There were no striking effects of the mutations on any of the K₉ values for ATP. For Q273E in all three assays, the K₉ for ATP was similar to that for wild type eCPS (Table V). For L270K, the K₉ for ATP was decreased 4-fold in the glutamine-dependent assay, increased 3-fold in the ammonia-dependent assay, and unchanged in the bicarbonate-dependent assay. As discussed above, the glutamine-dependent assay for Q273E/L270K reflected only the basal uncoupled ATP hydrolysis activity. The Q273E/L270K K₉ for ATP was increased 3-fold in the ammonia-dependent assay with values of 0.18 and 0.06 mM, respectively, for the double mutant and the wild type enzymes.

When glutamine-dependent CP synthesis was determined in the presence of the positive allosteric effector ornithine, wild type eCPS exhibited the expected 9-fold decrease in its K₉ for ATP (0.053 versus 0.444 mM) and a corresponding 9-fold increase in efficiency (Table V). L270K showed a similar ornithine response, with a 10-fold decrease in its K₉ for ATP, whereas Q273E showed only a 1.7-fold decrease in its K₉ for ATP (0.278 versus 0.465 mM).

In addition to serving as a substrate, ammonia is also a positive allosteric effector for eCPS. Like ornithine, ammonia acts to decrease the K₉ for ATP (21). However, the details of this interaction are not defined. Neither the allosterically active molecular species (ammonia and/or ammonium) nor the localization of the binding site are known. In contrast to its abnormally small response to ornithine, Q273E showed a wild type-like decrease in the K₉ for ATP when ammonia was present (Table V). For wild type eCPS, the K₉ for ATP in the ammonia-dependent ATPase reaction was 0.062 mM (versus 0.444 mM in the glutamine-dependent reaction), and for Q273E, the K₉ values were 0.092 and 0.465 mM. As an additional probe for the Q273E allosteric response, we tested the effect of UMP, which serves as a feedback inhibitor for eCPS. In the presence of 0.1 mM UMP, the Q273E mutant and eCPS exhibited similar decreases in glutamine-dependent ATPase-specific activity (8 and 3%, respectively, of the activity observed in the absence of UMP).
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**TABLE IV**

Kinetic parameters for utilization of glutamine and ammonia by WT and mutant CPSs.

ATPase assays were performed in a 500-μl volume at 25 °C, pH 7.6, in 50 mM HEPES, 40 mM NaHCO₃, 20 mM MgSO₄, 100 mM KCl, 5 mM ATP and in the absence and presence of the allosteric activator ornithine (20 mM). The rate of ADP formation was monitored in the presence of glutamine (0.08–10 mM; glutamine-dependent ATPase) and NH₄Cl (0.04–540 mM; ammonia-dependent ATPase) as described under "Experimental Procedures." Glutamine-dependent activity was not detected for Q273E/L270K (NA). S.E. of the kinetic parameters was determined (GraFit, version 5.01) from nonlinear regression curve fitting and was within ±10%.

| Orinine    | Glutamine-dependent ATPase | Ammonia-dependent ATPase |
|------------|-----------------------------|--------------------------|
|            | Gln Kₘ | k₅ | k₅/Kₘ | NH₄⁺ Kₘ | k₅ | k₅/Kₘ |
| eCPS       |         |     |       |         |     |       |
| –          | 0.16   | 9.1 |        | 96.1    | 4.3 | 0.045 |
| +          | 0.17   | 9.4 |        | 111     | 5.1 | 0.046 |
| Q273E      | –      | 3.6 |       | 5.28    | 615 | 3.7  |
| +          | 0.24   | 2.4 |        | 10.09   | 681 | 4.25 |
| L270K      | –      | 3.93 |       | 0.27    | 64.0 | 2.7 |
| +          | 5.09   | 1.1 |        | 0.21    | 72.0 | 2.2 |
| Q273E/L270K| –      | NA  | NA     | NA      | NA  | 0.20  |
| +          | NA     | NA  | NA     | 28.0    | 5.0 | 0.18  |

**TABLE V**

Kinetic parameters for utilization of ATP by WT and mutant CPSs.

ATPase assays were performed in a 500-μl volume at 25 °C, pH 7.6, in 50 mM HEPES, 40 mM NaHCO₃, 20 mM MgSO₄, 100 mM KCl and in the absence and presence of ornithine (20 mM). ATP concentrations were varied from 0.002 to 1 mM. Rates of ADP formation were monitored as described under "Experimental Procedures." The rate of ADP formation was monitored in the absence of a nitrogen donor (bicarbonate-dependent ATPase) or in the presence of NH₄Cl (300 mM; ammonia-dependent ATPase) or glutamine (10 mM; glutamine-dependent ATPase). Glutamine-dependent k₅ values determined for the Q273E/L270K mutant reflect only the basal bicarbonate-dependent ATPase activity. S.E. of the kinetic parameters was determined (GraFit, version 5.01) from nonlinear regression curve fitting and was within ±25%.

| Orinine    | Bicarbonate-dependent ATPase | Ammonia-dependent ATPase | Glutamine-dependent ATPase |
|------------|-------------------------------|--------------------------|-----------------------------|
|            | ATP Kₘ | k₅ | k₅/Kₘ | ATP Kₘ | k₅ | k₅/Kₘ | ATP Kₘ | k₅ | k₅/Kₘ |
| eCPS       |         |     |       |         |     |       |         |     |       |
| –          | 0.013  | 0.62 | 46.4  | 0.062 | 1.6 | 25.30 | 0.444 | 6.4 | 14.40 |
| +          | 0.011  | 0.73 | 6.72   | 0.057 | 2.2 | 38.44 | 0.053 | 6.8 | 128.2 |
| Q273E      | –      | 0.005 | 0.039 | 7.89   | 0.092 | 1.5 | 16.03 | 0.465 | 3.45 | 7.42 |
| +          | 0.003  | 0.038 | 14.18  | 0.058 | 1.4 | 23.90 | 0.278 | 12.4 | 44.51 |
| L270K      | –      | 0.015 | 0.096 | 6.40   | 0.21  | 3.0 | 14.26 | 0.113 | 1.1 | 10.03 |
| +          | 0.017  | 0.18 | 10.57  | 0.043 | 1.5 | 35.05 | 0.011 | 0.7 | 60.36 |
| Q273E/L270K| –      | 0.054 | 0.78  | 14.37  | 0.18  | 5.3 | 29.14 | 0.019 | 0.7 | 36.21 |
| +          | 0.038  | 2.11 | 55.58  | 0.12  | 8.0 | 66.44 | 0.017 | 1.0 | 58.35 |

**DISCUSSION**

One major goal of the present work was to determine why frog CPS I is unable to utilize glutamine even though it retains the GAT catalytic triad and most of the GAT residues conserved in glutamine-utilizing CPSs. As a prelude to addressing this question, we used native ICPs I to demonstrate for the first time that the enzyme is unable to use glutamine, in keeping with the earlier findings for CPS I from rat and human tissue (24). It thus appears that the ability of CPS to bind ammonia tightly, as has been observed only for CPS I, is consistently accompanied by the inability to utilize glutamine. We have determined that simultaneous occurrence of two substitutions found in the ICPs I GAT domain, Q273E and L270K, are sufficient to eliminate glutamine utilization by E. coli CPS. The Q273E/L270K mutant showed negligible glutamine binding, no detectable glutamine hydrolysis, and no detectable glutamine-dependent CP synthesis. In striking contrast, the double mutant had an unchanged k₅ for ammonia-dependent CP synthesis relative to that for wild type eCPS and displayed tighter binding of ammonia.

Of the two single mutations, L270K had a much greater impact on the ability of eCPS to utilize glutamine with negligible glutamine binding under the selective binding conditions, 25-fold higher k₅ for glutamine, and 10-fold slower rate of carrying out glutamine-dependent CP synthesis (Table IV). The Q273E mutant showed more moderate effects, with a 10-fold reduction in glutamine binding under selective binding conditions, a 2-fold higher k₅ for glutamine, and a 4-fold slower rate of carrying out glutamine-dependent CP synthesis (Table IV). Interestingly, the sole effect of the more charge-conservative Q273A mutation was to increase the k₅ for glutamine by 20-fold (22). For wild type eCPS, Q273A, and Q273E, respectively, the k₅ values for glutamine were 0.16, 2.1, and 0.24 mM, and the k₅ values were 9.1, 9.9, and 1.9 s⁻¹. Like Q273E/L270K, both Q273E and L270K displayed wild type rates for ammonia-dependent CP synthesis, indicating no major disruption of the SYN component in any of the mutants. However, the synergistic effect of the double mutation on glutamine usage, with Q273E/L270K completely incapable of utilizing glutamine, whereas the two component mutations were much less crippled, suggests a significant disruption of GAT active site structure when both changes occur simultaneously in the glutamine binding pocket.

As a first step toward assessing the structural effects of the present mutations, we have modeled these changes in silico using the wild type eCPS structure as a scaffold (Swiss-Model, Glaxo Wellcome; Fig. 2) (42, 43). In this model, substitution of the leucine residue at position 270 with the longer lysine side chain results in a more constrictive architecture imposed on the glutamine binding pocket. It appears that glutamine could be partially occluded, the thioester intermediate could be positioned in a manner unfavorable for efficient hydrolysis, and/or the channel connecting the GAT active site to the SYN component could be blocked. Based on x-ray crystallography data, Huang and Raushel (44) suggest the involvement of 10 residues, Ser-35, Met-36, Asp-45, Lys-202, Gly-293, Ala-309, Asn-311, His-353, Pro-358, Gly-359, in formation of this channel. Site-directed mutagenesis analysis of these residues primarily implicated Gly-359, with kinetic parameters for the G359Y and G359F proteins indicating impaired glutamine utilization. The spatial clustering of the three residues targeted for mutagenesis in our study is distinct from the previously identified
channel residues and may impact glutamine utilization at the point of entry of glutamine into the channel as opposed to constricting the diameter of the passage farther down (Fig. 4). Precluding entry of glutamine would be an efficient mechanism for non-glutamine utilizing CPS I to adopt and could explain why fCPS I remains unable to utilize this substrate despite retaining the appropriate catalytic machinery.

In wild type eCPS, the amide nitrogen of Gln-273 appears to form a hydrogen bond with the \( \alpha \)-carboxylate of glutamine and thereby helps to correctly position both the substrate and the intermediates derived from it (12, 22). The Q273E mutation would alter this interaction since it replaces the hydrophilic amide with a negatively charged oxygen and might well result in a more extended alteration of the wild type pattern of hydrogen bonds and salt bridges since the model predicts that the orientation of the mutant side chain is very different from that of the wild type.

These mutants also provide some insight into the molecular basis for the varying ammonia selectivity that occurs in CPSs. In mammals, the hepatic [NH\(_4\)\(^+\) + NH\(_3\)] is maintained at about 0.5 mM, and coma and death result when this level is exceeded (45). CPS I from various sources has an ammonium \( K_m \) of 1–2 mM (equivalent to 40 \( \mu \)M ammonia) that allows it to effectively detoxify excess ammonia, but the enzyme is completely unable to utilize glutamine. In contrast, eCPS has a \( K_m \) for ammonium of 96 mM that would preclude serving in ammonia detoxification but can efficiently utilize glutamine, with a \( K_m \) of 0.2 mM. In addition to mirroring CPS I in its inability to utilize glutamine, the Q273E/L270K double mutant of eCPS has a 4-fold decrease in ammonium \( K_m \) and a corresponding 4-fold increase in its efficiency for utilizing ammonium. Similarly, the G359Y and G359F mutants designed to block the ammonia channel (44) displayed 8–11-fold decreases in the ammonium \( K_m \). Clearly, additional structural differences in eCPS and CPS I must contribute to their much larger difference in ammonium \( K_m \). However, this movement of the double mutant toward a smaller \( K_m \) is more significant than the actual measured value since the double mutant must “offset” the 6-fold increase in ammonium \( K_m \) observed for the Q273E single mutant.

Because ammonia-dependent CP synthesis occurs entirely on the SYN component, and since this activity is altered in Q273E and Q273E/L270K, structural effects of these mutations must be communicated across the GAT domain interface to the SYN component. This cross-subunit communication is also evidenced in the alteration of uncoupled ATP hydrolysis that is localized to the first ATP site of the SYN component. Q273E/L270K shows a 10-fold rate increase, suggesting that there has been a relaxation of the structural constraints normally limiting ATP hydrolysis in the absence of an ammonia source.

These studies have also provided the first suggestion of communication between the GAT and allosteric domains. In wild type eCPS, ornithine (4, 15) and UMP (16) bind at the allosteric domain, and this occupancy is communicated to alter utilization of ATP, primarily at the second ATP-utilizing domain and slightly at the first ATP-utilizing domain (21). It has also been well established for wild type eCPS that there is two-way communication of occupancy of the GAT and first ATP-utilizing domains. However, Q273E has a greatly decreased response to the activator ornithine while retaining responsiveness to the inhibitor UMP. Therefore, a structural change resulting from the Q273E mutation must be communicated to the allosteric domain itself and/or to the second ATP-utilizing domain. Q273E/L270K displays enhanced responsiveness to ornithine at the first ATP-utilizing domain, as evidenced by a 30-fold increase in the uncoupled ATPase activity when ornithine is present. It thus appears that the structural effects of the Q273E mutation are communicated throughout all or most of the SYN component. Whereas the single Q273E mutation serves as a long range effector of binding site linkages but has only a small effect on glutamine utilization, the single L270K mutation shows wild type communication among domains but provides a significant loss of glutamine utilization. A detailed elucidation of how these two mutations combine to yield a eCPS mutant incapable of utilizing glutamine will most likely require solution of CPS I crystal structures as well as structures of eCPS mutants incorporating CPS I features.

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