Gain of Glutaminase Function in Mutants of the Ammonia-specific Frog Carbamoyl Phosphate Synthetase*

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Depending on their physiological role, carbamoyl phosphate synthetases (CPSs) use either glutamine or free ammonia as the nitrogen donor for carbamoyl phosphate synthesis. Sequence analysis of known CPSs indicates that, regardless of whether they are ammonia- or glutamine-specific, all CPSs contain the structural equivalent of a triad-type glutamine amidotransferase (GAT) domain. In ammonia-specific CPSs, such as those of rat or human, the catalytic inactivity of the GAT domain can be rationalized by the substitution of the Triad cysteine residue by serine (1). The ammonia-specific CPS of Rana catesbeiana (fCPS) presents an interesting anomaly in that, despite its retention of the entire catalytic triad (2) and almost all other residues conserved in Triad GATs, it is unable to utilize glutamine as a nitrogen-donating substrate (3). Based on our earlier work with the glutamine-utilizing E. coli CPS (eCPS), we have targeted residues Lys258 and Glu261 in the fCPS GAT domain as critical for preventing GAT function. Previously we have shown that substitution of the corresponding residues in eCPS by their fCPS counterparts (Leu → Lys and Gln → Glu) resulted in complete loss of GAT function in eCPS (3). To examine the role of these residues in the fCPS GAT component, we have cloned the full-length fCPS gene from R. catesbeiana liver. Here we report the first heterologous expression of an ammonia-specific CPS and show that a single mutation of the frog enzyme, K258L, yields a gain of glutaminase function.

The evolution of a urea cycle that effectively removes excess, potentially neurotoxic ammonia was critical for the adaptation of life to a terrestrial rather than aquatic habitat (4–6). Arginine biosynthetic pathways were most likely the evolutionary precursors of the urea cycle, with very few changes needed for pathway transformation (6). Four of these changes occurred in the enzyme that catalyzes the entry and rate-limiting step of the urea cycle, carbamoyl phosphate synthetase (CPS), and they were as follows: (i) a decrease in $K_m$ for ammonia to $\sim 1$ from $\sim 100$ mM; (ii) a loss of interaction with glutamine to avoid competition with the preferred substrate ammonia; (iii) localization to the hepatic mitochondrial matrix to allow independent regulation and avoid futile cycling; and (iv) gain of communication with a sensor of excess amino acids, N-acetylglutamate (AGA, which serves as an essential allosteric activator only for urea-synthesizing CPSs). In addition to the ammonia-specific CPS required for urea synthesis, most organisms also express a cytosolic glutamine-specific CPS that is involved in pyrimidine biosynthesis. In Escherichia coli, a single, glutamine-specific CPS (eCPS) participates in both arginine and pyrimidine synthetic pathways (7, 8). Glutamine-utilizing CPSs, e.g. eCPS, bind and cleave glutamine at a glutamine amidotransferase (GAT) domain and channel the resulting free ammonia, sequestered within the enzyme, to a synthetase (SYN) domain where all other ligands are bound and all other reactions take place (7, 8). Ammonia can substitute for glutamine in eCPS, but with a much higher $K_m$ (111 versus 0.17 mM, Ref. 3). The contrasting properties of ammonia-specific CPSs are even more intriguing when considered in the broader context of the GAT family (comprising the Triad and Ntn subfamilies) that participates in biosynthetic pathways for amino acids, amino sugars, coenzymes, and purine and pyrimidine nucleotides (9). Of the hundreds of GAT family members characterized in various organisms and tissues, these CPSs are the only enzymes that share the family-defining sequence motifs but have lost the ability to utilize glutamine and gained the ability to scaveng low levels of ammonia. For rat and human ammonia-specific CPSs, loss of glutamine usage is explained, at least in part, by substitution of serine for the cysteine of the catalytic triad (1, 10). However, the ammonia-specific CPS of Rana catesbeiana (fCPS) retains the entire catalytic triad (2) and almost all of the other amino acids conserved in Triad GATs (3) and, thus, is an ideal candidate for detailed elucidation of the molecular basis for glutamine discrimination in CPSs. Here we report that the present day frog ammonia-specific CPS retains an unexpectedly close link to glutamine-utilizing CPSs, with only a single mutation required for gain of glutaminase function.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Recombinant DNA Methods—**The ESP® yeast protein expression and purification system, including the Schizosaccharomyces pombe host strain SP-Q01 and Edinburgh minimal medium, was from Stratagene. The S. pombe expression plasmid pESP-S (11) was the generous gift of Quinn Lu (GlaxoSmithKline). pCR2.1, used for cloning of PCR products, was from Invitrogen. S. pombe transformations were carried out according to the supplier (Stratagene). QuikChange™ was used for site-directed mutagenesis (Stratagene), and fidelity was verified by sequencing. Mutagenesis primers (mutated codons set in boldface) were as follows for K258L, E261Q, and K258L/E261Q, respectively: 5’-TTGCGCATCTGTCCGGAGATGAAATTGCAGCTTTGGC-3’; 5’-TTTGGCACTGTTAAAGGAAATCCAAATTTGCGAGCTTGGC-3’; and 5’-TTTGGCACTGTTCTCGGGAATGAAATTGCAGCTTTGGC-3’. Cloning of Full-length fCPS cDNA—R. catesbeiana total RNA was prepared from frozen liver with the SV total RNA isolation system (Promega) for amplification of full-length fCPS in reverse transcription PCR reactions (RNA LA PCR Kit, Version 1.1; Takara Bio Inc.) with the supplied oligo(dT)-adapter primer and the gene-specific primer pair,


### TABLE 1

|            | Ammonia-dependent ADP formation | Glutamine-dependent ADP formation |
|------------|--------------------------------|-----------------------------------|
|            | \( k_{\text{cat}} \) \( s^{-1} \) | \( k_{\text{cat}} / K_m \) \( s^{-1} \) | \( K_m \) \( m\text{M} \) | \( k_{\text{cat}} \) \( s^{-1} \) | \( k_{\text{cat}} / K_m \) \( s^{-1} \) |
| Native fCPS | 0.42                            | 1.8                              | 4.4                          | 0.27                         | 2.4                          | 8.8                          |
| WT fCPS    | 0.39                            | 1.8                              | 4.5                          | 0.27                         | 2.1                          | 7.8                          |
| K258L      | 0.7                             | 1.9                              | 2.7                          | 0.29                         | 2.4                          | 8.2                          |
| E261Q      | 0.48                            | 0.6                              | 1.3                          | 1.6                          | 0.5                          | 0.3                          |
| K258L/E261Q| 0.56                            | 1.6                              | 2.9                          | 0.26                         | 2.2                          | 8.5                          |
| eCPS       | 111                             | 5.1                              | 0.05                         | 0.06                         | 2.2                          | 38.4                         |

*For determination of ammonia parameters, [ATP] was held constant at 10 mM, and [NH₄Cl] varied from 0.2 to 30 mM.

*For determination of ATP parameters, [NH₄Cl] was held constant at 30 mM, and [ATP] varied from 0.1 to 10 mM. S.E. of the kinetic parameters was determined to be within \( \pm 11\% \) by nonlinear regression curve fitting.

**RESULTS AND DISCUSSION**

Cloning, Expression, and Characterization of Wild Type fCPS—Despite the critical role of ammonia-specific liver CPS,
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| Glutamine-dependent ADP formation | | Glutaminase$^\dagger$ |
|----------------------------------|-----------------|-----------------|
| Gln $K_m$ $^{\dagger}$ | $k_{cat}$ | $K_m$ | ATP $K_m$ | $k_{cat}$ | $K_m$ | Gln $K_m$ | $k_{cat}$ | $K_m$ |
| (mM) | (s$^{-1}$) | (mM$^{-1}$ s$^{-1}$) | (mM) | (s$^{-1}$) | (mM$^{-1}$ s$^{-1}$) | (mM) | (s$^{-1}$) | (mM$^{-1}$ s$^{-1}$) |
| K258L | ND | ND | ND | 0.08 | 0.2 | 2.3 | 3.45 | 0.2 | 0.05 |
| K258L/E261Q | 0.17 | 0.24 | 1.4 | 0.07 | 0.3 | 4.5 | 0.2 | 0.8 |
| eCPS | 0.17 | 9.4 | 53.7 | 0.05 | 6.8 | 128.2 | 0.15 | 1.5 | 9.8 |

$^\dagger$For determination of glutamine parameters, [ATP] was held constant at 10 mM, and [glutamine] varied from 0.04 to 10 mM.

For determination of ATP parameters, [glutamine] was held constant at 10 mM, and [ATP] varied from 0.02 to 10 mM. ND, not determinable.

*For glutaminase assays, [glutamine] was varied from 0.1 to 20 mM. S.E. of the kinetic parameters was determined to be within $\pm$12% by nonlinear regression curve fitting.

this enzyme has not been as extensively studied as the glutamine-dependent CPSs, primarily due to the absence of a recombinant expression system. Previously, our laboratory has tried unsuccessfully to express ammonia-dependent CPSs in several bacterial expression systems, with inclusion bodies resulting in all cases. However, we have been able to express fCPS as a soluble, active protein from the expression vector pESP-5 (11) in the fission yeast S. pombe. Full-length fCPS was cloned from R. catesbeiana liver and inserted in pESP-5 as described under “Experimental Procedures.” fCPS was expressed as the mature protein (1463 amino acid residues), with the 33 N-terminal residues of the fCPS precursor replaced by a methionine (set in boldface) and the His$_6$-FLAG$^\dagger$ fusion tag (HHHHHHHDYKDDDKHASH). The N-terminal residues that were replaced serve as a mitochondrial matrix-targeting signal and are normally cleaved as the fCPS precursor crosses the inner mitochondrial membrane (2). A two-step purification protocol, with nickel affinity and size exclusion chromatography, yielded 10–15 mg of pure soluble fCPS per liter of yeast culture medium. Because our previous attempts to remove fusion partners by protease treatment revealed additional proteolysis sites in fCPS, presumably at the links between domains that are extremely susceptible to proteolysis (13–16), we retained the tag in all fCPS constructions.

The kinetic and physical properties of recombinant wild type fCPS were very similar to those observed for the native frog enzyme, indicating that the structure of the recombinant protein mirrors that of the native protein and further indicating that the fusion tag is a functionally neutral modification. Recombinant and native fCPSs exhibited comparable ammonia-dependent CP synthesis activities (1.17 and 1.12 $\mu$mol CP/min/mg, respectively), similar $k_{cat}$ and $K_m$ values for ammonia and ATP (Table I), and both required the presence of the essential allosteric activator, AGA. SDS-PAGE analysis confirmed that recombinant fCPS was the expected size (162 kDa), and recombinant and native fCPSs yielded essentially identical gel filtration profiles (data not shown).

Gain of Glutaminase Function in fCPS Mutants—Based on analysis of GAT that are conserved in glutamine-utilizing CPSs but not ammonia-specific CPSs (Fig. 1), we have identified Lys$_{258}$ and Glu$_{261}$ as residues critical for preventing glutamine usage by fCPS (3). Additional rationale for targeting these residues was provided by our previous demonstration that simultaneous occurrence of the corresponding substitutions in eCPS (Leu $\rightarrow$ Lys and Gln $\rightarrow$ Glu) prevent it from using glutamine (3).

Here we have constructed in fCPS the reverse mutants K258L, E261Q, and K258L/E261Q. Both K258L and K258L/E261Q could synthesize CP in the presence of glutamine, whereas E261Q could not (Fig. 2). K258L, K258L/E261Q, fCPS, and eCPS had similar rates for ammonia-dependent CP synthesis (1.05–1.17 $\mu$mol/min/mg), whereas E261Q functioned at about 60% of this rate. AGA was required for CP synthesis by all fCPS constructs, with either glutamine or ammonia as the nitrogen source. The absence of a solved structure for fCPS prevents detailed structure/function analysis of the mutants. However, our findings clearly demonstrate that the presence of lysine at position 258 precluded use of glutamine and confirmed the critical role of leucine at this position.

To further define the interaction of the mutants with glutamine, we directly measured glutamine hydrolysis (Table II). Like other members of the GAT family, eCPS can catalyze glutamine hydrolysis in the absence of additional substrates (7), although the rate of this uncoupled partial reaction is extremely slow ($k_{cat}$ 0.24 min$^{-1}$; Ref. 20). This activity increases dramatically ($k_{cat}$ 1.5 s$^{-1}$; Table II) when glutamine is held constant at 10 mM. Glutamine hydrolysis is coupled to CP synthesis on the SYN domain (7) by the addition of ATP and bicarbonate. Neither fCPS nor E261Q exhibited detectable hydrolysis or even binding of glutamine under any of the conditions tested. When saturating amounts of ATP and bicarbonate were present, both K258L and K258L/E261Q had robust glutaminase activities (Table II), although the $k_{cat}$ values were 7.5-fold lower than that of eCPS. It should be noted that the eCPS glutaminase $k_{cat}$ value of 1.5 s$^{-1}$ was
lower than the value of 3.4–4.7 s⁻¹ predicted by the ADP formation kcat values (6.8 and 9.4 s⁻¹; Table I). Presumably, these kcat differences reflect the different coupling systems used in the assays. The double mutant exhibited a Km for glutamine that was about equal to that of eCPS (0.20 μM), whereas K258L had a 23-fold elevated Km for glutamine (3.45 μM), indicating that the E261Q mutation facilitated interaction with glutamine. Surprisingly, given the well-established effect on eCPS behavior (7, 20), elimination of coupling with CP synthesis had no effect on the glutaminase activity of K258L and K258L/E261Q. When ATP was omitted from the glutaminase mix, kcat values remained unchanged (0.2 s⁻¹ for both mutants), and the Km values showed little change (6.57 mM for K258L and 0.74 mM for K258L/E261Q). This lack of response to SYN substrates suggested that the fCPS mutants did not communicate occupancy of the SYN active site to the GAT active site but, rather, had GAT active sites that were permanently in the high activity conformation. It is also noteworthy that, for both K258L and K258L/E261Q, AGA had no effect on glutaminase activity.

Ability of fCPS Mutants to Synchronize Catalysis at the Multiple Active Sites—Next, we assessed the effects of the GAT mutants on SYN domain function via ADP formation assays. Formation of the high energy intermediate CP requires concomitant cleavage of two molecules of ATP to form two ADPs (19, 21). In ammonia-dependent ADP formation assays, K258L and K258L/E261Q displayed wild type kinetic parameters for ammonia and ATP usage (Table I). The E261Q mutation yielded modest changes in interaction with ATP, possibly reflecting some long-range structural perturbation in this mutant. In glutamine-dependent ADP formation assays (Table II), we could not detect any activity with the native, wild type, or E261Q fCPSs, whereas K258L/E261Q did exhibit substantial ADP formation activity. The K258L/E261Q parameters were consistent with those determined in the glutaminase assay, with the Km for glutamine about equal to that of eCPS, and the kcat being somewhat lower. Although K258L displayed Michaelis-Menten behavior in the glutaminase assay and when ATP was the variable substrate in the glutamine-dependent ADP formation assay, it failed to do so when glutamine was the variable substrate in the latter assay (Table II). Instead, production of ADP by K258L was undetectable below a plateau glutamine concentration of ~1 mM, and, as glutamine concentration was incrementally increased to 20 mM, the rates measured did not show the expected correlation with substrate concentration (i.e. the apparent Vmax increased as the glutamine concentration ranged from 1 to 20 mM). This kinetic behavior suggested that the GAT domain of K258L was acting independently of the SYN domain and was not coupling glutamine cleavage to CP synthesis. The anomalous kinetic data further suggested that the K258L GAT domain, rather than channeling the ammonia sequestered within the protein, was releasing it into bulk solution so that CP could be formed only when the solution ammonia concentration was equivalent to that required for ammonia-dependent CP formation.

As an additional probe for synchronization between the GAT and SYN domains, we determined the relative rates of product formation for fCPSs (Fig. 3). When ammonia was the aminating substrate, the production of CP and ADP increased steadily with time of incubation and was consistently at or near the expected 1:2 ratio for wild type fCPS and all three of the mutants. With glutamine as the aminating substrate, the behavior of K258L and K258L/E261Q was markedly different, with the production of CP lagging behind production of glutamate in ratios as high as 1:13 and 1:10 for the double and single mutants, respectively. The ratio of CP/glutamate became larger with increasing time of incubation but remained far from the 1:1 ratio of a coupled system. Additionally, K258L and K258L/E261Q formed excess ADP relative to CP, with respective ratios of 6:1 and 8:1 early in the incubation and 3:1 ratios for both at 15 min. The uncoupling of ADP formation from CP formation most likely reflects the nonproductive turnover at the first ATP site that is known to occur when the intermediate carbonyl phosphate reacts with water rather than ammonia (19–23). Together, these findings indicated that both K258L and K258L/E261Q failed to channel ammonia directly from the GAT to the SYN domain, thereby making CP formation dependent on sufficient buildup of the ammonia released into solution. The uncoupled character of K258L/E261Q was presumably masked in the assay for glutamine-dependent ADP formation, whereas that of K258L was apparent (Table II), because the double mutant has a relatively low Km for glutamine.

Potential Roles for the GAT Domain—We conclude that the GAT domain must play a critical role in ammonia-specific fCPS, because it has been retained so faithfully that a single mutation, K258L, was sufficient to enable glutamine utilization. Occurrence of two simultaneous mutations (K258L/E261Q) was even more effective, whereas the E261Q mutation conferred no detectable gain of function, suggesting that the latter mutation provided compensatory structural stabilization to the Triad scaffolding. The GAT domain of present-day ammonia-specific CPSs might well serve an entirely structural role but might also contribute to the lower Km for ammonia relative to other amidotransferases. No ammonia site has yet been identified for any GAT, nor has it been determined whether there is an alternative to the tunnel for external ammonia entry to the SYN active site, possibly sharing access with ATP and bicarbonate (9, 20).

Our present findings clearly show that the cross-talk between the GAT and SYN domains that occurs in eCPS was not established in the fCPS mutants K258L and K258L/E261Q and that the ammonia derived from glutamine is not sequestered within the tunnel. It is not yet clear how extensive the underlying l changes are (relative to a functional tunnel), whether they are confined to one or both domains, or what path is taken by ammonia between the GAT and SYN active sites. The molecular basis for coordination of the GAT and SYN active sites connected by a channel has been elucidated for two other GATs, glutamine phosphoribosylpyrophosphate amidotransferase (24, 25) and imidazole glycerol phosphate synthase (26–28), and is based on a cycle of conformational changes that control access of substrates, intermediates, and bulk solvent to the active sites and/or the tunnel. Thus far, data for CPS are limited to a single solved conformation of eCPS and identification of 10 GAT residues that appear to line the interior of the tunnel (20). It is noteworthy that fCPS has retained seven of these ten residues and has conservative substitutions for the other three. Availability of a robust expression system for fCPS, the first reported for any ammonia-specific CPS, will greatly facilitate determination of the detailed molecular mechanism for present-day CPSs and should also further elucidate the evolution of both the CPS and GAT families.

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REFERENCES
1. Rubino, S. D., Nyunoya, H. & Lusty, C. J. (1986) J. Biol. Chem. 261, 11320–11327
2. Helling, C. C. & Atkinson, B. G. (1994) J. Biol. Chem. 269, 11743–11750
3. Saeed-Kothe, A. & Powers-Lee, S. G. (2002) J. Biol. Chem. 277, 7231–7238
4. Mommsen, T. P. & Walsh, P. J. (1988) Science 243, 72–75
5. Randall, D. J., Wood, C. M., Perry, S. F., Bergman, H., Malay, G. M., Mommsen, T. P. & Wright, P. A. (1989) Nature 337, 165–166
6. Paulus, H. (1983) Curr. Top. Cell. Regul. 22, 177–200
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7. Meister, A. (1989) Adv. Enzymol. Relat. Areas Mol. Biol. 62, 315–374
8. Thoden, J. B., Holden, H. M., Wesenberg, G., Raushel, F. M. & Rayment, I. (1997) Biochemistry 36, 6305–6316
9. Zalkin, H. & Smith, J. L. (1998) Adv. Enzymol. Relat. Areas Mol. Biol. 72, 87–144
10. Haraguchi, Y., Uchino, T., Takiguchi, M., Endo, F., Mori, M. & Matsuda, I. (1991) Gene 107, 335–340
11. Hosfield, T. & Lu, Q. (1999) BioTechniques 27, 58–60
12. Mori, M. & Cohen, P. P. (1978) J. Biol. Chem. 253, 8337–8339
13. Powers-Lee, S. G. & Corina, K. (1986) J. Biol. Chem. 261, 15349–15352
14. Guadalajara, A., Grisolia, S. & Rubin, V. (1987) Eur. J. Biochem. 165, 163–169
15. Evans, D. R. & Balon, M. A. (1988) Biochim. Biophys. Acta. 953, 185–196
16. Marshall, M. & Fahien, L. A. (1988) Arch. Biochem. Biophys. 262, 455–470
17. Anderson, P. M. & Meister, A. (1966) Biochemistry 5, 3157–3163
18. Cohen, N. S., Kyan, F. S., Kyan, S. S., Cheung, C. W. & Rajman, L. (1985) Biochem. J. 229, 205–211
19. Miles, B. W. & Raushel, F. M. (2000) Biochemistry 39, 5051–5056
20. Huang, X. & Raushel, F. M. (2000) Biochemistry 39, 3240–3247
21. Jones, M. E. & Lipmann, F. (1960) Proc. Natl. Acad. Sci. U. S. A. 46, 1194–1205
22. Kethe, M., Eroglu, B., Mazza, H., Samudera, H. & Powers-Lee, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12348–12353
23. Powers, S. G. & Meister, A. (1978) J. Biol. Chem. 253, 1258–1265
24. Krahn, J. M., Kim, J. H., Burns, M. R., Parry, R. J., Zalkin, H. & Smith, J. L. (1997) Biochemistry 36, 11061–11068
25. Chen, S., Burgner, J. W., Krahn, J. M., Smith, J. L. & Zalkin, H. (1999) Biochemistry 38, 11659–11669
26. Beismann, D. & Sterner, R. (2001) J. Biol. Chem. 276, 20387–20396
27. Korolev, S., Starina, T., Evdokimova, E., Beasley, S., Edwards, A., Joachimiak, A. & Sarchenke, A. (2002) Proteins 49, 420–422
28. Omi, R., Minaguchi, H., Goto, M., Miyahara, I., Hayashi, H., Kagamiyama, H. & Hirotsu, K. (2002) J. Biochem. (Tokyo) 132, 759–765
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