Regulatory Control of the Amidotransferase Domain of Carbamoyl Phosphate Synthetase†

Bryant W. Miles, Jennifer A. Banzon, and Frank M. Raushel*

Department of Chemistry, Texas A&M University, College Station, Texas 77843

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ABSTRACT: Carbamoyl phosphate synthetase catalyzes the hydrolysis of glutamine by the nucleophilic attack of an active site cysteine residue through a mechanism that requires the formation of a γ-glutamyl thioester intermediate. The steady-state mole fraction of the thioester intermediate was determined to be 0.23 in the presence and absence of ATP and bicarbonate. The kinetics of formation and hydrolysis of the γ-glutamyl thioester intermediate during CPS catalyzed hydrolysis of glutamine were determined. When ATP and bicarbonate are added to CPS and glutamine, the k_{cat} for glutamine hydrolysis increases from 0.17 to 150 min^{-1}. The observed rate constant for thioester intermediate formation increases from 18 to 580 min^{-1}, and the microscopic rate constant for hydrolysis of the intermediate increases from 0.15 to 460 min^{-1}. These results demonstrate the kinetic competence of the thioester intermediate during glutamine hydrolysis. The rate-determining step changes from the hydrolysis of the intermediate when ATP and bicarbonate are absent to the formation of the intermediate upon the addition of ATP and bicarbonate. The 3 order of magnitude increase in the rate of glutamine hydrolysis upon the addition of ATP and bicarbonate is indicative of the allosteric communication between two of the three reaction centers of CPS. These sites are physically separated by ∼45 Å.

Carbamoyl phosphate synthetase (CPS)[1] from *E. coli* is composed of two distinct subunits. The smaller subunit (42 kDa) is required for the hydrolysis of glutamine, while the larger subunit (118 kDa) catalyzes the synthesis of carbamoyl phosphate from bicarbonate, ammonia, and two molecules of ATP (*1*). The overall reaction mechanism is initiated by the activation of bicarbonate through phosphorylation with ATP. The carboxy phosphate intermediate subsequently reacts with ammonia to form carbamate. The second molecule of ATP then phosphorylates the unstable carbamate intermediate to generate the final product, carbamoyl phosphate (*2*).

The X-ray crystal structure of CPS has revealed that the active site for glutamine hydrolysis is ∼45 Å away from the ATP binding site required for the phosphorylation of bicarbonate. This nucleotide site is an additional ∼45 Å away from the second ATP binding site that is required for the phosphorylation of carbamate (*3*). A long interior tunnel that is protected from the external solvent connects the three reaction centers. The ammonia produced within the small subunit must thus diffuse through this tunnel to the large subunit where it reacts with the carboxy phosphate intermediate to form carbamate. The carbamate then migrates to the second ATP binding site where it is phosphorylated to form the final product, carbamoyl phosphate.

CPS from *E. coli* is a member of the glutamine amidotransferases. This is a diverse family of biosynthetic enzymes that utilize glutamine as a source of ammonia for the synthesis of products with an amino group functionality. CPS belongs to the TrpG family of amidotransferases whose other members include GMP synthetase, CTP synthetase, FGAM synthetase, anthranilate synthetase, and PABA synthetase (*4*). All of these enzymes contain a specific domain that catalyzes the hydrolysis of glutamine to ammonia and glutamate. The glutaminase domain contains a conserved triad of residues consisting of cysteine, histidine, and glutamate (*5*−*8*). The recent crystal structure of the *E. coli* CPS has demonstrated that His-353 and Cys-269 are within hydrogen bonding distance of each other and that Glu-355 is hydrogen bonded to His-353 (*3*).

Although each subunit of CPS has its own distinct function, the two subunits have substantial interactions with one another where the binding of ligands to one subunit modulates the activity of the other subunit. For example, the binding of ATP and bicarbonate to the large subunit substantially increases the rate of the glutaminase activity. Chemical reagents that modify the essential cysteine residue of the amidotransferase subunit destroy the glutaminase activity while dramatically increasing the rate of the bicarbonate-dependent ATPase activity of the large subunit (*9*−*14*). In addition, mutations within the large subunit can affect the glutaminase activity of the small subunit (*15*−*17*), and mutations in the amidotransferase subunit can affect the kinetic parameters of the large subunit (*7*, *18*).

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* To whom correspondence should be addressed (FAX, 409-845-9452; E-mail, raushel@tamu.edu).

[1] Abbreviations: CPS, carbamoyl phosphate synthetase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TCA, trichloroacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; APAD, 3-acetylpyrimidine adenine nucleoside; CAD, the trifunctional enzyme consisting of carbamoyl phosphate synthetase, aspartate transcarbamoylase, and dihydroorotase.

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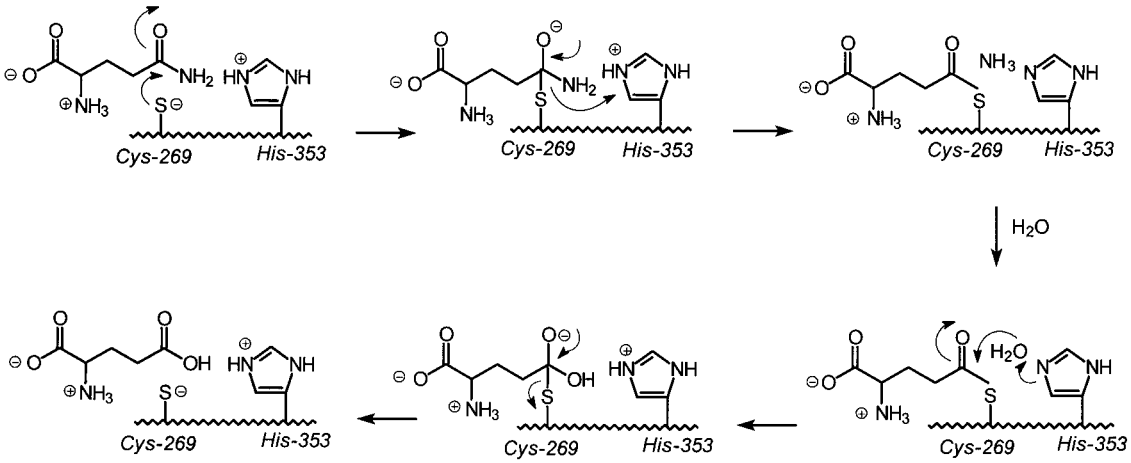
Scheme 1



The working model for the chemical mechanism of the glutaminase reaction is shown in Scheme 1. In this mechanism, His-353 acts as a general base to assist Cys-269 during the nucleophilic attack on the amide carbonyl of glutamine. The transient tetrahedral intermediate collapses to produce a covalent γ-glutamyl thioester intermediate and ammonia. His-353 subsequently assists the nucleophilic attack of water to regenerate the thiolate anion of Cys-269. The salient feature of this reaction mechanism is the formation of the γ-glutamyl enzyme intermediate. Wellner et al. observed that incubation of CPS from *E. coli* with L-[^{14}C]glutamine resulted in the formation of an enzyme−[^{14}C]glutamine complex (*19*). The isolated complex produced glutamate upon the addition of MgATP and bicarbonate. Chaparian and Evans (*6*) established the existence of an acid-stable glutamine−enzyme complex for the multifunctional CAD protein. Lusty (*20*) showed that a CPS−[^{14}C]glutamine complex could be isolated by incubating L-[^{14}C]glutamine with the enzyme and precipitating the protein with TCA. Lusty further established that this acid-stable form of CPS-bound glutamine reacts with hydroxylamine to produce γ-glutamyl hydroxamate and its cyclic derivative, pyrrollidonecarboxylic acid. The X-ray crystal structure of the H353N mutant of the small subunit of CPS (*21*) has directly demonstrated the existence of the thioester intermediate and the role of Cys-269 in thioester formation.

Carbamoyl phosphate synthetase has evolved to conserve glutamine in the absence of bicarbonate and ATP such that the rate of glutamine hydrolysis is very low when ATP and bicarbonate are absent, thus conserving glutamine. However, the mechanism of communication between the two active sites separated by ~45 Å has yet to be determined. The objective of this investigation is to firmly establish the kinetic competence of the γ-glutamyl enzyme intermediate during glutamine hydrolysis in both the presence and absence of ligands bound to the large subunit. These studies have demonstrated that the hydrolysis of glutamine by the small subunit is enhanced by nearly 3 orders of magnitude when ATP is hydrolyzed by the large subunit.

MATERIALS AND METHODS

Materials. All chemicals and coupling enzymes were purchased from either Aldrich or Sigma unless otherwise stated. L-[U-^{14}C]Glutamine was purchased from Amersham. Nitrocellulose filters with a diameter of 2.5 cm and a pore size of 0.45 μm were obtained from Sigma. The purification of wild-type CPS has been described previously (*17*).

Enzyme Assays. A continuous spectrophotometric assay was used to follow the production of glutamate during the CPS-catalyzed hydrolysis of glutamine. The assay solutions were composed of 0.1 M HEPES, pH 6.8, 0.1 M KCl, 20 units of glutamate dehydrogenase (GDH), and 1.0 mM 3-acetylpyrimidine adenine nucleoside (APAD). CPS (0.3−20 nmol) was added to initiate the reaction in a volume of 1.0 mL. The hydrolysis of glutamine was followed by coupling the formation of glutamate using the GDH reduction of APAD while measuring the increase in absorbance at 363 nm with an extinction coefficient of 8.3 mM^{-1} cm^{-1}. This procedure was also used to measure glutamate formation in the presence of ATP and bicarbonate.

Formation of Thioester Intermediate in the Absence of ATP and Bicarbonate. The reaction was initiated by the addition of CPS (10−60 μM) to a solution containing 0.1 M HEPES buffer, pH 6.8, with various concentrations (0.05−5 mM) of [^{14}C]glutamine (1000−4000 cpm/nmol) in a final volume of 100 μL. The solutions were incubated at 25 °C until a 200 μL aliquot of 20% TCA was added to the reaction mixture at various time points ranging from 2 s to 5 min to quench the reaction and precipitate the protein. The quenched enzyme was then chilled on ice for 15 min. The precipitated protein was added to the nitrocellulose filter and washed with 10 mL of ice-cold 1 M HCl. The filter was removed and placed in a scintillation vial containing 600 μL of 1 M KOH. The vial was shaken overnight or until the nitrocellulose filter completely dissolved. The solution was then neutralized by adding 200 μL of glacial acetic acid followed by the addition of 20 mL of scintillation fluid. A blank was prepared by adding an aliquot of the assay mixture directly to the 200 μL of 20% TCA prior to the addition of CPS. The blank was subtracted from the experimental values to account for any nonspecific glutamine binding.

Decay of the Thioester Intermediate in the Absence of ATP and Bicarbonate. CPS (3 mg/mL) was incubated with 2.0 mM [^{14}C]glutamine in 0.1 M HEPES buffer, pH 6.8 at 25 °C. Aliquots of 100 μL were removed and quickly eluted through a Penefsky column (*24*). The eluates were quenched at incremental time points (1−60 min) with 100 μL of 20% TCA. After a 15 min incubation on ice, the samples were

filtered, washed, and counted as previously described. The rate of decay was also determined using a pulse−chase technique similar to the procedure developed by Chaparian and Evans (*6*). CPS (3 mg/mL) was incubated with 1.0 mM [^{14}C]glutamine in 0.1 M HEPES buffer, pH 6.8 at 25 °C. After 2 min of incubation, unlabeled glutamine was added to produce a final glutamine concentration of 100 mM. The specific activity was reduced from 2000 to 50 cpm/nmol. Aliquots of 100 μL were removed at time points between 1 and 60 min and quenched with 100 μL of 20% TCA. The samples were precipitated on ice, filtered, washed, and counted as described previously.

Formation and Decay of Thioester Intermediate in the Presence of ATP and Bicarbonate. The rate of formation and decay of the glutamyl-thioester intermediate in the presence of ATP and bicarbonate was too rapid to be determined manually. The measurements were made with the aid of a Bio Logic quenched-flow apparatus at 25 °C. Three solutions were prepared: (a) CPS (20 μM) in 0.1 M HEPES buffer, pH 6.8; (b) radiolabeled glutamine (0.025−5.0 mM; 2000 cpm/nmol) in 0.1 M HEPES buffer, pH 6.8, with 5 mM ATP, 10 mM $MgCl_2$, 50 mM KCl, 10 mM ornithine, and 20 mM bicarbonate; and (c) 0.7 M HCl. The reaction was initiated by mixing equal volumes of substrate and enzyme, and then the reaction was quenched with 0.7 M HCl. The concentration of the acid quench was chosen so that the reaction would be rapidly stopped, but the enzyme would not precipitate. The samples collected were subsequently precipitated with 20% TCA and treated in the same manner as described above.

RESULTS

Determination of the Steady-State Concentration of the Protein-Bound Thioester Intermediate. The steady-state concentration of the γ-glutamyl thioester intermediate formed during the incubation of CPS with [^{14}C]glutamine was determined as a function of the initial glutamine concentration (pH 6.8, 25 °C). The fraction of labeled CPS was plotted versus the glutamine concentration, and the data were fitted to eq 1. In this equation, F equals the fraction of labeled enzyme, F_{max} represents the maximum fraction of CPS labeled, [A] is the glutamine concentration, and $K_{1/2}$ is the concentration of glutamine where the fraction of labeled enzyme is equal to $^1/_2$ F_{max}. For this set of experiments, F_{max} is 0.21 ± 0.02 and $K_{1/2}$ is 17 ± 2 μM. These data are shown in Figure 1A.

$$F = F_{max}[A]/(K_{1/2} + [A]) \quad (1)$$

The steady-state concentration of the γ-glutamyl thioester intermediate formed during incubation of CPS in the presence of 5.0 mM ATP, 10 mM $MgCl_2$, 40 mM bicarbonate, and [^{14}C]glutamine was also determined as a function of glutamine concentration (pH 6.8, 25 °C). In this set of experiments, F_{max} equals 0.23 ± 0.03, and $K_{1/2}$ equals 80 ± 11 μM. These data are shown in Figure 1B.

Formation of the γ-Glutamyl Thioester Intermediate in the Absence of ATP and Bicarbonate. The rate of formation of the putative thioester intermediate was slow enough that the rates could be measured manually. CPS and radiolabeled glutamine were incubated together for variable amounts of time and then quenched with TCA. The time courses for intermediate formation were fit to a single exponential (eq 2), where α is the mole fraction of enzyme labeled and λ is the observed rate constant. The results are shown in Figure 2A. A plot of the observed rate constants for intermediate formation versus the initial glutamine concentration produced a straight line as shown in Figure 2B. The apparent second-order rate constant obtained from the slope of this plot was determined to be 3400 ± 200 M^{-1} min^{-1}.

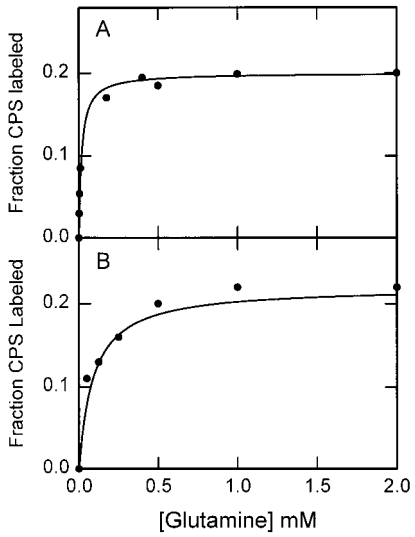


FIGURE 1: Mole fraction of [^{14}C]glutamine−CPS intermediate as a function of glutamine concentration. The enzyme (20 μM) was incubated with various concentrations of [^{14}C]glutamine (2000 cpm/nmol) for 2 min. The data were fit to eq 1 as shown by the solid lines. (A) Mole fraction of CPS labeled with glutamine in the absence of ATP and bicarbonate. The maximum mole fraction of enzyme labeled was 0.21 ± 0.02 with an apparent $K_{1/2}$ of 17 ± 2 μM. (B) Concentration of intermediate formed in the presence of 5.0 mM ATP, 40 mM HCO_3^-, and 10 mM $MgCl_2$. The maximum fraction of labeled intermediate was 0.23 ± 0.03, and the apparent $K_{1/2}$ for glutamine was 80 ± 11 μM.

$$y = \alpha(1 - e^{-\lambda t}) \quad (2)$$

Decay of the Thioester Intermediate in the Absence of ATP and Bicarbonate. The rate of decay of the thioester intermediate was determined by incubation of the enzyme with radiolabeled glutamine followed by rapid elution through a Penefsky column to separate the free from the enzyme-bound radiolabel (*22*). The time course for the hydrolysis of the intermediate was fit to a single-exponential decay. In the absence of ATP and bicarbonate, the first-order rate constant was determined to be 0.16 ± 0.02 min^{-1}. This experiment was repeated using the pulse−chase method of Chaparian and Evans (*6*). This method yielded a rate constant of 0.15 ± 0.01 min^{-1} as shown in Figure 3.

Formation of the γ-Glutamyl Thioester Intermediate in the Presence of MgATP and Bicarbonate. In the presence of ATP and bicarbonate, the rate of formation of the thioester intermediate is too rapid to measure manually. The rate measurements were made using rapid quench techniques. The time courses for intermediate formation were fit to a first-order exponential equation as the starting point for the analysis. The results are shown in Figure 4. The observed first-order rate constants were plotted as a function of the initial glutamine concentration (Figure 5). The data were fit to eq 3 where the maximum rate constant for thioester

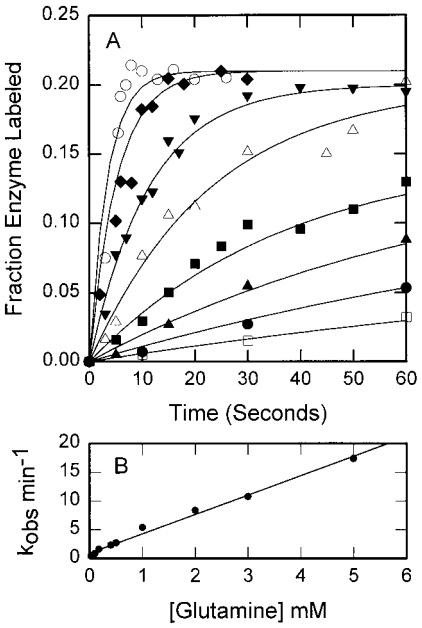


FIGURE 2: Rate of intermediate formation in the absence of ATP and bicarbonate as a function of glutamine concentration. The experiments were conducted at 25 °C and pH 6.8 as described in the text. (A) The time courses for the intermediate formation at glutamine concentrations of (□) 0.025 mM, (●) 0.05 mM, (▲) 0.10 mM, (■) 0.175 mM, (△) 0.50 mM, (▼) 1.0 mM, (◆) 3.0 mM, and (○) 5.0 mM glutamine. The solid lines are the single-exponential fits of the data at each glutamine concentration. (B) Plot of the pseudo-first-order rate constant versus glutamine concentration. The solid line is the linear regression. The apparent second-order rate constant for thioester formation is 3400 ± 200 M^{-1} min^{-1}.

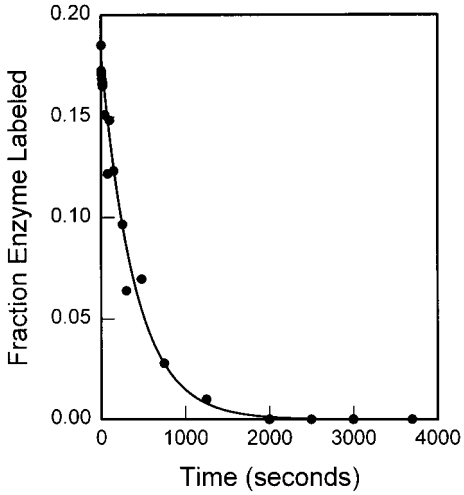


FIGURE 3: Hydrolysis of the covalent intermediate determined by the pulse−chase method (pH 6.8, 25 °C). The enzyme (20 μM) was incubated with 1.0 mM [^{14}C]glutamine (2000 cpm/nmol) for 2 min, at which time an excess of unlabeled glutamine was added such that the specific radioactivity was reduced to 50 cpm/nmol. The loss of label was fit to a single-exponential decay yielding a first-order rate constant of 0.15 ± 0.01 min^{-1}.

formation is 580 ± 20 min^{-1} and the K_a for glutamine is 80 ± 20 μM. The rate of hydrolysis of the thioester intermediate is also very fast in the presence of ATP and bicarbonate. To determine the rate of thioester hydrolysis with ATP and bicarbonate present, the amount of the thioester intermediate was also determined using lower ratios of glutamine to enzyme. Under such conditions, the concentration of the intermediate rises to a maximum value and then decays as

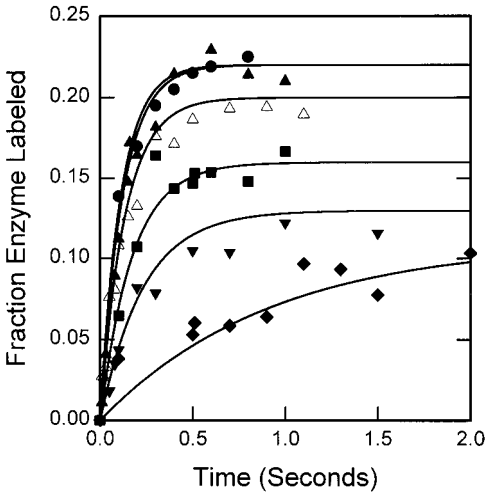


FIGURE 4: Formation of thioester intermediate in the presence of 5.0 mM ATP, 10 mM $MgCl_2$, and 20 mM bicarbonate. The experiments were done at 25 °C and pH 6.8 as described in the text. The measured time courses for the intermediate formation at glutamine concentrations of (◆) 0.054 mM, (▼) 0.125 mM, (■) 0.25 mM, (△) 0.50 mM, (●) 2.0 mM, and (▲) 4.0 mM glutamine. The solid lines are fits of the data to a single exponential.

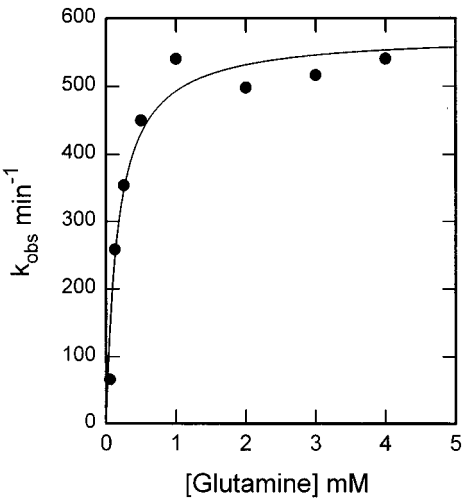


FIGURE 5: Plot of the observed first-order rate constants versus the initial glutamine concentration for the formation of the intermediate in the presence of ATP and bicarbonate. The solid line is a fit of the data to eq 3 yielding a K_d of 80 ± 20 μM and a k_{max} of 580 ± 30 min^{-1}.

the glutamine is consumed (Figure 6).

$$k_{obs} = k_{max}A/(K_a + A) \quad (3)$$

Steady-State Kinetic Parameters. The values for k_{cat} and k_{cat}/K_m for the hydrolysis of glutamine in the presence and absence of ATP and bicarbonate were determined at pH 6.8 and 25 °C. In the absence of ATP and bicarbonate, the K_m for glutamine is 180 ± 30 μM and the k_{cat} is 0.17 ± 0.01 min^{-1}. In the presence of ATP and bicarbonate, the k_{cat} value increases by nearly 3 orders of magnitude to 150 ± 15 min^{-1} while the K_m value is 70 ± 10 μM.

DISCUSSION

A working model for the carbamoyl phosphate synthetase catalyzed hydrolysis of glutamine is presented in Scheme 1. The central feature of this chemical mechanism is the

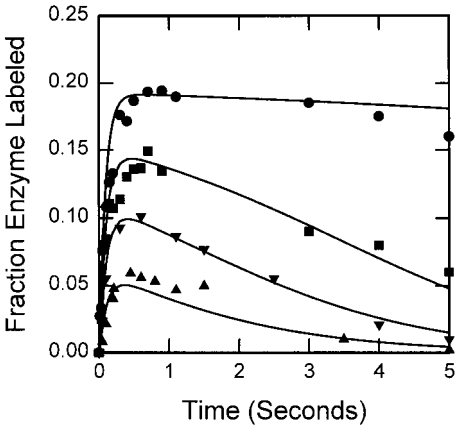


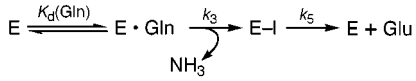
FIGURE 6: Rate of intermediate formation and decay in the presence of 5.0 mM ATP, 10 mM $MgCl_2$, and 40 mM bicarbonate when the initial substrate concentration approaches the enzyme concentration. The experiments were conducted at 25 °C and pH 6.8 as described in the text. The enzyme (50 μM) was incubated with 500 μM (●), 250 μM (■), 125 μM (◆) and 50 μM (▲) glutamine. The solid lines are the simulations of the time courses using the mechanism shown in Scheme 2 where $K_d = 150$ μM, $k_3 = 140$ min^{-1}, and $k_5 = 462$ min^{-1}.

formation of a thioester intermediate following the nucleophilic attack of the thiolate anion of Cys-269 upon the carbonyl group of the glutamine side chain. Previous studies have demonstrated the stoichiometric binding of glutamine to *E. coli* CPS by gel filtration in the absence of ATP and bicarbonate (19). An acid-stable glutamine−CPS complex can be isolated by precipitating CPS with trichloroacetic acid in the absence of ATP and bicarbonate (*6*, *20*). The chemical stability of the isolated complex led to the hypothesis that this adduct is the γ-glutamyl thioester intermediate depicted in Scheme 1. Experimental support for this conjecture has most recently been provided by the X-ray crystal structure of the H353N mutant of the *E. coli* enzyme in the presence of glutamine (*21*).

Evans and co-workers have investigated the occurrence of the thioester intermediate with the hamster CAD enzyme (*6*). The steady-state mole fraction of the thioester intermediate for the hamster CAD enzyme was reported to be 0.86. However, they were unable to isolate the intermediate when ATP and bicarbonate were present. The rate constant for the decay of the thioester intermediate in the absence of ATP and bicarbonate was measured by pulse−chase techniques to be 0.17 s^{-1}. This value is comparable to the k_{cat} for glutamine hydrolysis of 0.14 s^{-1}. For the *E. coli* CPS enzyme, the steady-state mole fraction of the thioester intermediate has been determined to be between 0.25 and 0.38 (*20*, *24*), but the rate of breakdown of the intermediate has not been measured. Moreover, the kinetic competence of thioester formation in CPS has not been directly demonstrated for either the hamster CAD or the *E. coli* enzyme nor have the effects of ATP and bicarbonate on the kinetics of formation and decay of this intermediate been measured.

The minimal kinetic mechanism for the hydrolysis of glutamine by CPS is presented in Scheme 2. This mechanism is a simplification of the chemical transformation shown in Scheme 1 where the two tetrahedral intermediates on the reaction pathway have been merged into the intermediate complexes for E·Gln and E−I, respectively. In this scheme, E·Gln represents the Michaelis complex for the binding of

Scheme 2



glutamine to the small subunit of CPS, and E−I represents the thioester intermediate.

The time courses for formation of the thioester intermediate in the presence of ATP and bicarbonate are shown in Figures 4 and 6. To obtain a better understanding of the mechanism for thioester formation and decay, the concentration dependence exhibited by glutamine on the rates of formation and fraction of CPS labeled was measured. To evaluate these data in terms of the specific kinetic mechanism shown in Scheme 2, the differential eqs describing the time-dependent changes in enzyme intermediates were solved. The integrated rate equation describing the fraction of thioester intermediate as a function of time is presented in eq 2 where α and γ are defined below. The fraction of thioester intermediate (α) is given by eq 4 while the observed rate constant (λ) for the formation of the thioester intermediate as a function of the glutamine concentration is given by eq 5.

$$\alpha = k_3[\mathrm{Gln}]/((k_3 + k_5)[\mathrm{Gln}] + K_d k_5) \quad (4)$$

$$\lambda = k_3[\mathrm{Gln}]/(K_d + [\mathrm{Gln}]) + k_5 \quad (5)$$

To obtain reliable estimates for the values of K_d, k_3, and k_5 for the mechanism depicted in Scheme 2, the kinetic data were further analyzed by simulations of the time courses for the formation and decay of the thioester intermediate using the KINSIM program (*23*). The rates of thioester formation and decay were determined under conditions where the concentrations of glutamine were allowed to approach the carbamoyl phosphate synthetase concentration. Under these conditions, there is a rapid formation of the thioester intermediate followed by hydrolysis (Figure 6). The minimal kinetic mechanism shown in Scheme 2 can be utilized to fit the experimental time courses under all experimental conditions (Figures 4 and 6) to yield the microscopic rate and equilibrium constants. The values for K_d, k_3, and k_5 were determined to be 150 ± 20 μM, 140 ± 10 min^{-1}, and 462 ± 25 min^{-1}, respectively. The simulated time courses (shown in Figure 6) fit the experimental data quite well. These results demonstrate that in the presence of ATP and bicarbonate, the hydrolysis of glutamine is primarily limited by the rate of formation of the thioester intermediate since $k_3 < k_5$.

In the absence of ATP and bicarbonate, the rate of hydrolysis of glutamine by CPS is substantially slower than when these two substrates are added to the reaction mixture. The turnover number for glutamine hydrolysis in the absence of these additional ligands for CPS is 0.17 min^{-1}. This rate constant increases by nearly 3 orders of magnitude to 150 min^{-1} when the levels of ATP and bicarbonate are saturating. Therefore, the binding of ATP and the consequent reaction with bicarbonate within the large subunit of CPS must somehow transmit conformational or dynamic changes to the small subunit. These structural alterations substantially modulate the ability of the active site to hydrolyze glutamine. It is curious to note that the steady-state levels of the thioester

Scheme 3

$$E \overset{K_d(Gln)}{\rightleftharpoons} E \cdot Gln \underset{k_4(NH_3)}{\overset{k_3}{\rightleftharpoons}} E\text{–}I \xrightarrow{k_5} E + Glu$$

intermediate are essentially the same, whether ATP and bicarbonate are bound to the protein. In the absence of ATP/bicarbonate, the rate constant (0.16 min^{-1}) for the disappearance of the thioester intermediate is identical to the steady-state turnover for glutamine hydrolysis (0.17 min^{-1}). These results clearly demonstrate that the rate-limiting step for the overall hydrolysis of glutamine in the absence of other ligands is the hydrolysis of the thioester intermediate.

The time courses for the formation of the thioester intermediate as a function of the initial glutamine concentration, in the absence of the other substrates required for carbamoyl phosphate synthesis, are shown in Figure 2. The observed rates of intermediate formation versus the initial glutamine concentration do not exhibit saturation kinetics within the range from 0 to 5 mM glutamine. For technical reasons, it was not possible to obtain rate data above 5 mM glutamine. The lack of curvature at high glutamine concentrations suggests an initial weak binding of glutamine prior to thioester formation. The rate of intermediate formation at 5 mM glutamine provides the lower limit of 18 min^{-1} for the rate constant of thioester formation. The slope of the linear least-squares fit (Figure 2B) is equal to the second-order rate constant for thioester intermediate formation, 3400 M^{-1} min^{-1}. Since the decay of the intermediate is equal to the steady-state value of k_{cat} for the overall reaction, the breakdown of the acid-stable complex is the rate-determining step for the CPS-catalyzed hydrolysis of glutamine in the absence of ATP and bicarbonate.

It was not possible to fit the experimental data for glutamine hydrolysis in the absence of ATP and bicarbonate to the simple model that appears in Scheme 2. The lower limit for the rate constant of thioester formation, obtained from exponential fits of the data presented in Figure 2, is 18 min^{-1}. This rate constant is 2 orders of magnitude greater than the decay rate constant of 0.16 min^{-1}. Since the rate of formation of the intermediate is significantly greater than the rate of decay, then the fraction of the protein covalently attached to the glutamate moiety is expected to be near unity. However, the mole fraction of the intermediate was experimentally found to be 0.23. This discrepancy suggests that the simple mechanism for glutamine hydrolysis in the absence of ATP and bicarbonate is more complicated than the mechanism proposed in Scheme 2. A scenario that would resolve this conflict is the chemical reversibility of the step for the formation of the thioester intermediate rather than the irreversible step as depicted in Scheme 2. In the absence of ATP and bicarbonate, the ammonia, produced from the hydrolysis of glutamine, may initially be sequestered from the bulk solvent and available to react with the thioester. This would make the re-formation of glutamine readily reversible as illustrated in Scheme 3. Alternatively, a significant fraction of the enzyme may exist as one of the tetrahedral complexes, which may then partition toward glutamine formation after the acid-quench.

Carbamoyl phosphate synthetase has apparently evolved to minimize the wasteful hydrolysis of glutamine when the other substrates required for the synthesis of carbamoyl phosphate are not present. However, when ATP and bicarbonate are added to a mixture of CPS and glutamine, the turnover number for glutamine hydrolysis increases from 0.17 to 150 min^{-1}. There is a comparable increase in the rate of formation and decay of the γ-glutamyl enzyme intermediate. The rate constant for the decay of the intermediate increases from 0.15 to 460 min^{-1} upon the addition of ATP and bicarbonate, a 3000-fold increase. The rate-determining step is the formation of the thioester intermediate when ATP and bicarbonate are present, but the limiting step is the hydrolysis of the thioester intermediate in the absence of these other ligands. Carbamoyl phosphate synthetase has three reaction centers connected by a channel running through the interior of the protein. The three active sites must work synchronously to orchestrate the synthesis of carbamoyl phosphate. This feat requires allosteric communication between the distant active sites.

The chemical accessibility of Cys-248 from the small subunit of CPS to the thiol reagent *N*-ethylmaleimide (NEM) may reflect the magnitude of the conformational changes that must occur within CPS during catalysis (*18*). This cysteine residue, located in the small subunit, is labeled by NEM only when MgATP and bicarbonate are present. Modification of this cysteine residue *enhances* the glutaminase activity by 20−30-fold while simultaneously *decreasing* the carbamoyl phosphate synthesis rate (*10*, *25*, *26*). Modification of this cysteine residue thus uncouples glutamine hydrolysis from bicarbonate activation. Site-directed mutagenesis of Cys-248 with an aspartate results in a mutant enzyme that has similar kinetic properties as the NEM-labeled protein (*18*). This cysteine residue is 55 Å away from the closest ATP binding site on the large subunit of CPS (*3*). It appears that the protein with a modified or mutated Cys-248 is sterically locked into an activated conformation for glutamine hydrolysis.

Two other enzymes have been reported to contain molecular tunnels through which intermediates are channeled between active sites. The best characterized of these proteins is tryptophan synthetase. The channel in this enzyme provides a mechanism through which the enzyme coordinates the reactions at each active site and protects the indole intermediate from diffusion into the bulk solvent (*27*, *28*). More recently, the crystal structure of glutamine phosphoribosyl pyrophosphate amidotransferase with a carbocyclic analogue of phosphoribosyl pyrophosphate (PRPP) was reported (*29*). This enzyme is a glutamine amidotransferase, which couples the hydrolysis of glutamine to the production of phosphoribosylamine. The enzyme has a glutamine amidotransferase domain at the N-terminal end of the protein and a PRPP binding site 17 Å away. The binding of PRPP causes a structural transformation that completely shields the bound PRPP from solvent. This conformational change also produces a long hydrophobic channel connecting the two active sites. The reactions at each active site are coupled together by conformational changes that occur during the catalytic cycles of the enzyme.

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