Synchronization of the Three Reaction Centers within Carbamoyl Phosphate Synthetase†

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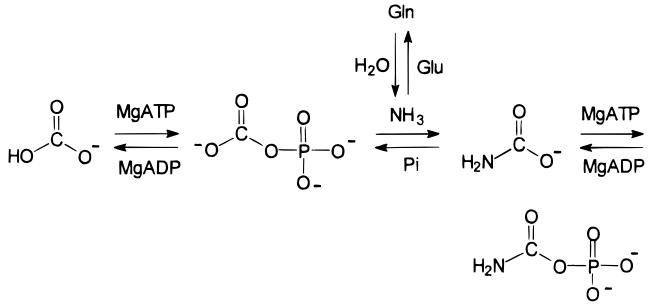
ABSTRACT: Carbamoyl phosphate synthetase from *E. coli* catalyzes the synthesis of carbamoyl phosphate through a series of four reactions occurring at three active sites connected by a molecular tunnel of 100 Å. To understand the mechanism for coordination and synchronization among the active sites, the pre-steady-state time courses for the formation of phosphate, ADP, glutamate, and carbamoyl phosphate were determined. When bicarbonate and ATP were rapidly mixed with CPS, a stoichiometric burst of acid-labile phosphate and ADP was observed with a formation rate constant of 1100 min^{-1}. The burst phase was followed by a linear steady-state phase with a rate constant of 12 min^{-1}. When glutamine or ammonia was added to the initial reaction mixture, the magnitude and the rate of formation of the burst phase for either phosphate or ADP were unchanged, but the rate constant for the linear steady-state phase increased to an average value of 78 min^{-1}. These results demonstrate that the initial phosphorylation of bicarbonate is independent of the binding or hydrolysis of glutamine. The pre-steady-state time course for the hydrolysis of glutamine in the absence of ATP exhibited a burst of glutamate formation with a rate constant of 4 min^{-1} when the reaction was quenched with base. In the presence of ATP and bicarbonate, the rate constant for the formation of the burst of glutamate was 1100 min^{-1}. The hydrolysis of ATP thus enhanced the hydrolysis of glutamine by a factor of 275, but there was no effect by glutamine on the initial phosphorylation of bicarbonate. The pre-steady-state time course for the formation of carbamoyl phosphate was linear with an overall rate constant of 72 min^{-1}. The absence of an initial burst of carbamoyl phosphate formation eliminates product release as a rate-determining step for CPS. Overall, these results have been interpreted to be consistent with a mechanism whereby the phosphorylation of bicarbonate serves as the initial trigger for the rest of the reaction cascade. The formation of the carboxy phosphate intermediate within the large subunit must induce a conformational change to the active site of the small subunit that enhances the hydrolysis of glutamine. Thus, ammonia is not released into the molecular tunnel until the activated bicarbonate is ready to form carbamate. The rate-limiting step for the steady-state assembly of carbamoyl phosphate is either the formation, migration, or phosphorylation of the carbamate intermediate.

Carbamoyl phosphate synthetase (CPS)[1] from *E. coli* catalyzes the assembly of carbamoyl phosphate from two molecules of ATP, one molecule of bicarbonate, and one molecule of glutamine (*1*). The proposed chemical mechanism for this transformation is presented in Scheme 1. In this reaction mechanism, the first ATP phosphorylates bicarbonate to form the unstable intermediate, carboxy phosphate. Glutamine is hydrolyzed, and then the ammonia reacts with the carboxy phosphate to generate a third intermediate, carbamate. In the last step, the second ATP phosphorylates carbamate to yield the final product, carbamoyl phosphate. Evidence in support of this reaction mechanism has been obtained by the observation of three partial reactions (*2*), positional isotope exchange (*3*−*5*), and isotope labeling experiments (*6*, *7*).

Scheme 1



The CPS from *E. coli* is found as a heterodimer (*8*, *9*). There is a small subunit with a molecular weight of ∼42 000 and a large subunit of molecular weight ∼118 000. The small subunit has been shown to be responsible for the hydrolysis of glutamine via a thioester intermediate (*10*−*15*) while the large subunit catalyzes two separate phosphorylation events (*2*). The three-dimensional crystal structure of CPS has been determined to high resolution (*15*−*18*). This remarkable

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[1] Abbreviations: CPS, carbamoyl phosphate synthetase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IGP, indole 3-glycerol phosphate; G3P, glyceraldehyde 3-phosphate; CAD, trifunctional enzyme composed of carbamoyl phosphate synthetase, aspartate transcarbamoylase, and dihydroorotase; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

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structure has identified the precise location of three distinct active sites contained within the heterodimeric protein. The site for glutamine hydrolysis is found at the interface of the two subdomains that comprise the N-terminal and C-terminal halves of the small subunit. The binding site for the ATP that is required for the phosphorylation of bicarbonate is situated within the N-terminal half of the large subunit (*19*−*21*) while the ATP binding site that is needed for the phosphorylation of carbamate is localized within the C-terminal half of this same subunit (*19*, *20*, *22*). Amazingly, these three active sites are separated in three-dimensional space by a linear distance of nearly 100 Å (*16*−*18*).

The physical separation of the three active sites within the three-dimensional framework of CPS requires that two of the reaction products produced within one active site must be subsequently translocated to the adjacent active site where they can then serve as substrates for the next reaction. Thus, the ammonia formed within the small subunit must react with the carboxy phosphate intermediate that is synthesized within the large subunit. In addition, the carbamate intermediate that is initially formed within the N-terminal half of the large subunit is phosphorylated by the ATP bound to the C-terminal half of the large subunit. Isotope labeling experiments with $^{15}NH_3$ have demonstrated that the ammonia produced from the hydrolysis of glutamine does not dissociate from the small subunit and then reassociate to the large subunit (*23*). At neutral pH, carbamate has a measured half-life of 70 ms (*24*), and thus it is unlikely that a carbamate intermediate would be physically able to be released into solution and then recaptured during each catalytic cycle. Therefore, it is highly probable that NH_3 and carbamate must diffuse from their respective sites of synthesis to their sites of utilization through a molecular passageway that extends from one active site to the next. A molecular tunnel that runs through the interior of the large and small subunits has been identified and mapped within the X-ray crystal structure (*16*, *17*).

In the chemical mechanism that is presented in Scheme 1, there are four separate reactions that must occur within three distinct active sites. What is missing from this reaction mechanism is a physical description of how CPS is able to function as a finely tuned molecular machine and coordinate these four reaction cycles with one another. Analysis of the reaction stoichiometry has confirmed the ratio of products as indicated by the chemical events depicted in Scheme 1 (*1*). These results thus require that the individual reactions are synchronized and fully coupled with one another. However, it is not intuitively obvious how the hydrolysis of glutamine can occur at precisely the same rate as the phosphorylation of bicarbonate. Do these reactions fortuitously occur at the same rate or are they coordinated with one another by some allosteric signaling process? If these reactions are coupled to one another by induced conformational changes, then it is important to understand how this information is transmitted among the multiple active sites. In this investigation we have measured the pre-steady-state time courses for the formation of ADP, inorganic phosphate, carboxy phosphate, carbamoyl phosphate, and glutamate. The results are consistent with a mechanism whereby the phosphorylation of bicarbonate serves as the initial trigger for the rest of the reaction cascade.

MATERIALS AND METHODS

Materials. Carbamoyl phosphate synthetase from *E. coli* was isolated using the procedure of Mareya and Raushel (*25*). [γ-^{32}P]ATP, [α-^{32}P]ATP, [^{14}C]glutamine, and [^{14}C]bicarbonate were all obtained from Amersham.

Rapid Quench Experiments. The rapid quench experiments were conducted with a Kintek RQF-3 rapid quench instrument. All of the experiments were done at 25 °C, pH 7.6, with reaction concentrations of 50 mM HEPES, 100 mM KCl, 10 mM $MgCl_2$, 10 mM ornithine,[2] and 20 mM bicarbonate. The ATP and glutamine concentrations were held fixed at 1.0 mM, unless otherwise noted. The reactions were initiated by mixing 15 μL of 120 μM CPS with 15 μL of the radiolabeled substrate. The reaction was subsequently quenched with 1.0 M HCl. The samples were then centrifuged for 3 min at 14 000 rpm.

Product Assays. The rate of formation of acid-labile phosphate was measured using [γ-^{32}P]ATP with a specific radioactivity of 89 000 cpm/nmol. One microliter of the quenched reaction was spotted on a polyethylenimine TLC plate (Selecto Scientific) and then developed using 0.75 M phosphate buffer, pH 3.6. The formation of ADP was followed using [α-^{32}P]ATP with a specific radioactivity of 89 000 cpm/nmol. One microliter of the quenched reaction was spotted on a polyethylenimine TLC plate and developed using 0.3 M phosphate buffer, pH 7.0. The rate of carbamoyl phosphate formation was measured using 20 mM [^{14}C]-bicarbonate with a specific radioactivity of 94 000 cpm/nmol. One microliter of the quenched reaction was spotted on a Whatman CEL 300 DEAE TLC plate and developed using 0.5 M $LiCl_2$, pH 5.5 (*26*). Alternatively, all of the unreacted bicarbonate could be removed from the quenched reaction solution by the addition of powdered dry ice. The nonvolatile [^{14}C]carbamoyl phosphate was determined by liquid scintillation counting. Both of these methods yielded identical results. All of the TLC plates were thoroughly dried and analyzed using a phosphor imaging plate (Molecular Dynamics). For ^{32}P-radiolabels, the plates were developed for 12 h while ^{14}C-radiolabels required a minimum of 48 h of incubation. The imaged plates were then quantified by phosphorimage analysis using a Molecular Dynamics Storm 860 PhosphorImager System with ImageQuant software. The production of glutamate was measured using the HPLC assay described by Chaparian and Evans after quenching the reaction with hydroxide (*10*). Glutamine and glutamate were derivatized with *o*-phthalaldehyde/β-mercaptoethanol, separated with a Gilson HPLC system using a Partisil 5 ODS-3, 4.5 × 100 mm column, and detected with a Gilson fluorimeter with a 305−395 nm excitation filter and 430−470 nm emission filter.

Data Analysis. The time courses that exhibited burst kinetics were fitted to eq 1. In this equation, P is the amount of product formed at time t, E_t is the total initial enzyme concentration, λ is the first-order rate constant for the burst phase, β is the amplitude of the burst phase, and k_{cat} is the rate constant for the steady-state phase of the reaction. Those time courses with a linear increase in product were fitted to eq 2. The standard errors are reported for the fits to the

[2] Ornithine is an allosteric ligand of CPS that activates the enzyme by lowering the Michaelis constant for MgATP.

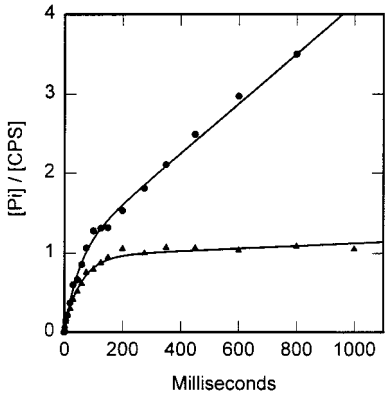


FIGURE 1: Time course for the formation of acid-labile phosphate catalyzed by CPS in the presence (●) and absence (▲) of 1.0 mM glutamine. These data were fitted to eq 1. In the presence of 1.0 mM glutamine, the burst amplitude (β) was 1.00 ± 0.05, and the rate constant for the appearance of the burst (λ) was 1200 ± 200 min^{-1}. In the absence of glutamine, the burst amplitude (β) was 0.93 ± 0.04, and the rate constant for the formation of the burst (λ) was 1100 ± 100 min^{-1}. The linear steady-state rate constant increased from 12 ± 1 to 180 ± 10 min^{-1} when glutamine was added. In the absence of glutamine, the time course represented the sum of carboxy phosphate and inorganic phosphate while in the presence of glutamine the appearance of carbamoyl phosphate was included. Additional details are provided in the text.

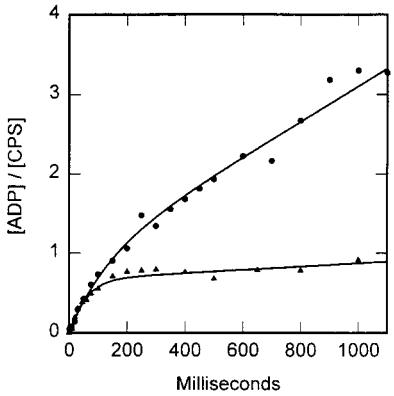


FIGURE 2: Time course for the formation of ADP catalyzed by CPS in the presence (●) and absence (▲) of 1.0 mM glutamine. These data were fitted to eq 1. In the presence of 1.0 mM glutamine, the burst amplitude (β) was 0.90 ± 0.10 enzyme equiv, and the rate constant for the appearance of the burst (λ) was 1100 ± 100 min^{-1}. In the absence of glutamine, the burst amplitude (β) was 0.70 ± 0.10 enzyme equiv, and the rate constant for the appearance of the burst (λ) was 1100 ± 200 min^{-1}. The linear steady-state rate constant increased from 12 ± 1 to 132 ± 6 min^{-1} upon the addition of glutamine. Additional details are provided in the text.

appropriate equation of the data presented in Figures 1−4.

$$P/E_t = \beta(1 - e^{-\lambda t}) + (k_{cat})t \quad (1)$$

$$P/E_t = (k_{cat})t \quad (2)$$

RESULTS

Product Formation within the Large Subunit. The pre-steady-state time courses for the formation of phosphate, carbamoyl phosphate, and ADP were determined. For direct comparison, all of the experiments were conducted using a final concentration of 60 μM CPS, 0.05 M HEPES buffer, pH 7.6, 25 °C, containing 10 mM ornithine, 10 mM $MgCl_2$, 20 mM HCO_3^-, and 0.1 M KCl. The time courses for the formation of acid-labile phosphate in the presence and absence of 1.0 mM glutamine are shown in Figure 1. In the experiments conducted in the absence of glutamine, the acid quench rapidly decomposed the carboxy phosphate intermediate, and thus the measurement of acid-labile phosphate was a direct quantitation of the time course for the formation of the carboxy phosphate intermediate. Moreover, carbamoyl phosphate and phosphate were not separated from one another by thin-layer chromatography under the conditions employed for these investigations. Therefore, the time courses for the formation of acid-labile phosphate, when either glutamine or ammonia was included in the reaction mixture, represented the overall sum of carboxy phosphate, phosphate, and carbamoyl phosphate. In the absence of glutamine, there was an exponential burst of approximately 1 enzyme equiv of acid-labile phosphate (0.93 ± 0.04) that was followed by a linear steady-state rate. The rate constant for the initial burst of acid-labile phosphate was 1100 ± 100 min^{-1} (Figure 1). In the presence of 1.0 mM glutamine, there was also an exponential burst of approximately 1 enzyme equiv of acid-labile phosphate (1.00 ± 0.05) that was formed with a rate constant equal to 1200 ± 200 min^{-1} (Figure 1). Similarly, when 300 mM ammonia was used as the nitrogen source, the amplitude of the burst phase was 0.87 ± 0.07 enzyme equiv and was formed with a rate constant of 1200 ± 100 min^{-1} (data not shown). The steady-state rate constant for acid-labile phosphate formation (P_i and carbamoyl-P) increased from 12 ± 1 to 180 ± 10 min^{-1} in the absence and presence of glutamine, respectively. The steady-state rate constant for formation of acid-labile phosphate in the presence of 300 mM ammonia was 158 ± 6 min^{-1}. For the experiments conducted in the presence of either glutamine or ammonia, the steady-state rate constant must be divided by a factor of 2 in order to correct for the fact that the sum of two separate products was being measured (P_i and carbamoyl-P). Therefore, the steady-state rate constants in the presence of glutamine and ammonia were 90 ± 5 and 79 ± 3 min^{-1}, respectively. Preincubation of the enzyme with glutamine prior to the addition of ATP and bicarbonate did not change the time course for formation of acid-labile phosphate (data not shown).

The time courses for the formation of ADP in the absence and presence of 1.0 mM glutamine are shown in Figure 2. In either case, there was an exponential burst of approximately 1 enzyme equiv of ADP followed by a linear steady-state rate. The rate constant for the initial phase in the presence and absence of glutamine was determined to be 1080 ± 100 and 1100 ± 200 min^{-1}, respectively. The amplitude of the burst phase was 0.90 ± 0.10 enzyme equiv in the presence of glutamine and 0.70 ± 10 in the absence of glutamine. The steady-state rate constant for ADP formation increased from 12 ± 1 to 132 ± 6 min^{-1} (66 ± 3 min^{-1} after correction was made for the two molecules of ADP formed per turnover) upon the addition of 1.0 mM glutamine. The time course for carbamoyl phosphate formation (Figure 3) was linear with an observed rate constant of 72 ± 6 min^{-1}.

Product Formation within the Small Subunit. The pre-steady-state time courses for the hydrolysis of glutamine have been previously determined at pH 6.8 (*14*). However, most of the kinetic analyses on CPS have been conducted at pH 7.6, and thus the pre-steady-state kinetics were remeasured

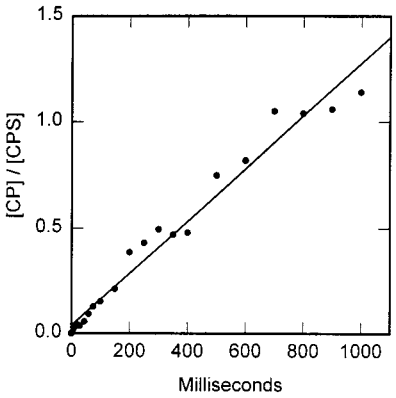


FIGURE 3: Time course for the formation of carbamoyl phosphate catalyzed by CPS. These data were fitted to eq 2. The rate constant was determined to be 72 ± 6 min^{-1}. Additional details are provided in the text.

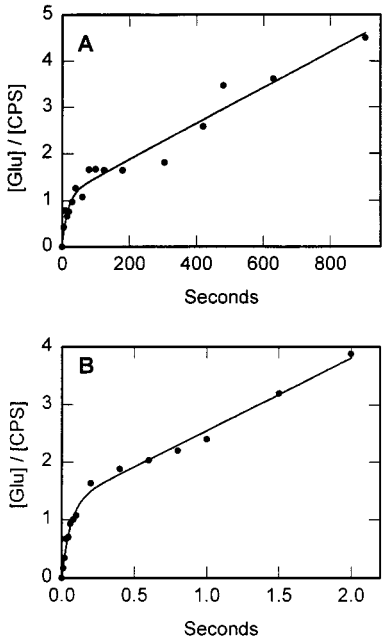
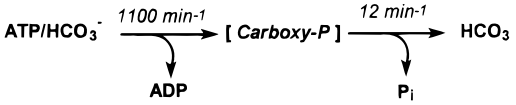


FIGURE 4: Time course for the formation of glutamate catalyzed by CPS in the presence and absence of added ATP/bicarbonate. These data were fitted to eq 1. (A) In the absence of ATP, the burst amplitude (β) was 1.0 ± 0.2 enzyme equiv, and the rate constant for the appearance of the burst (λ) was 4 ± 1 min^{-1}. The steady-state rate constant for the linear phase was 0.24 ± 0.02 min^{-1}. (B) In the presence of 1.0 mM ATP, the burst amplitude (β) for glutamate formation was 1.2 ± 0.1 enzyme equiv, and the rate constant for formation of the burst (λ) was 1100 ± 200 min^{-1}. The steady-state rate constant was 76 ± 4 min^{-1}. These reactions were quenched with the addition of hydroxide, and thus the appearance of glutamate also includes the formation of the thiol-ester intermediate. Additional details are provided in the text.

at this pH so that a direct comparison of the time courses could be made for all products under identical conditions. In the absence of ATP, the time course for the hydrolysis of glutamine showed a burst of approximately 1 enzyme equiv (1.0 ± 0.1) that was formed with a rate constant of 4 ± 1 min^{-1}. The rate constant for the linear steady-state phase was 0.24 ± 0.02 min^{-1} (Figure 4A). In the presence of ATP/HCO_3^-, the amplitude of the burst phase was 1.2 ± 0.1 enzyme equiv that was formed with a rate constant of 1100 ± 200 min^{-1}. The rate constant for the steady-state portion of the time course was 76 ± 4 min^{-1} (Figure 4B). Since the thiol ester intermediate is unstable under basic conditions

Scheme 2



(*14*), these time courses represent the sum of the formation of glutamate and the thiol ester intermediate and are thus equivalent to the time courses for the formation of ammonia.

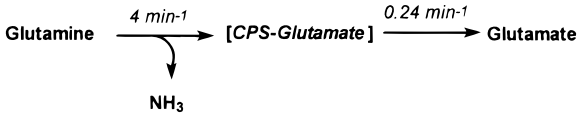
DISCUSSION

Carbamoyl phosphate synthetase is a complex assemblage of three active sites strung together by a molecular tunnel that spans a distance of nearly 100 Å (*16*, *17*). This protein has evolved to synthesize carbamoyl phosphate via a reaction mechanism that requires four chemical steps and three reactive intermediates. These reactions occur as parallel and serial events during each catalytic cycle. The apparent mechanistic advantage that can be envisioned from this type of protein architecture is that catalytic modules can be arranged in a linear sequence with one another to enable products from one active site to be passed directly to a subsequent active site. Moreover, the protective environment of the protein interior can stabilize highly reactive intermediates. However, optimization of molecular machines of this type would appear to require structural and kinetic controls that will enable the synchronization of one active site with another. Otherwise, an uncoupling of the parallel reactions from one another will severely cripple the overall catalytic process. The mechanistic constraints exhibited by CPS have been addressed by measuring the pre-steady-state time courses for product and intermediate formation.

When ATP and bicarbonate are mixed with CPS, approximately 1 enzyme-equiv each of acid-labile phosphate and ADP is rapidly formed (Figures 1 and 2). The time courses for the formation of these two products represent the rate at which the intermediate, carboxy phosphate, is formed in the absence of either glutamine or ammonia as a source of nitrogen. The initial burst of product formation proceeds with an average rate constant of 1100 min^{-1} and is followed by a much slower steady-state process with a rate constant of 12 min^{-1}. The second phase of this time course is apparently dominated by the slow hydrolytic decay of the thermodynamically unstable carboxy phosphate intermediate. The kinetic relationships are summarized in Scheme 2. Previous studies of ATP hydrolysis by CPS have also reported a stoichiometric burst of acid-labile phosphate (*27*, *28*).

Glutamine is slowly hydrolyzed to glutamate and ammonia when this substrate is mixed with CPS in the absence of ATP/bicarbonate (Figure 4). When the reaction is quenched with base, there is a burst of approximately 1 enzyme equiv of glutamate. The burst phase represents the time course for the formation of the thioester intermediate and any subsequent enzyme forms that contain glutamate. Therefore, this time course serves as an indirect measurement for the formation of ammonia within the small subunit of CPS. The kinetic constants for the glutaminase reaction in the absence of substrates bound to the large subunit are summarized in Scheme 3. The pre-steady-state time courses for glutamate and ammonia production for the hamster CAD enzyme in the absence of ATP have been measured (*13*). A lag in

Scheme 3



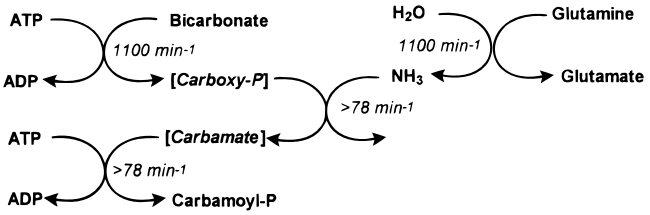
glutamate production and a 0.4 mol equiv burst of ammonia were observed when the reaction was quenched with acetonitrile.

When ATP and bicarbonate are allowed to react with CPS in the presence of either glutamine or ammonia, the rate constant for the fast phase of product formation (carboxy phosphate and ADP) is not changed from the value obtained in the absence of a nitrogen source (Figures 1 and 2). These results clearly indicate that the initial phosphorylation of bicarbonate by the ATP bound to the active site within the N-terminal half of the large subunit of CPS is kinetically independent of processes occurring within the small subunit. The catalytic reaction that follows the formation of the carboxy phosphate intermediate is, however, significantly faster when either glutamine or ammonia is added to the reaction mixture since the average value for the macroscopic rate constant obtained from the steady-state phase is increased to 78 min^{-1} from a basal value of 12 min^{-1}.

The size of the burst phase for the formation of acid-labile phosphate and ADP does not change significantly in the presence or absence of a nitrogen source (Figures 1 and 2). These results indicate that a subsequent catalytic event must therefore limit the overall steady-state formation of carbamoyl phosphate by CPS. The possibilities for this rate-determining step include the following: (a) hydrolysis of glutamine and migration of ammonia; (b) formation of the carbamate intermediate; (c) migration of the carbamate intermediate; (d) phosphorylation of carbamate; or (e) dissociation of the products. If the release of any of the various products were rate-limiting for the overall reaction of CPS, then a burst of 1 enzyme equiv of carbamoyl phosphate would have been detected. However, the time course for the formation of carbamoyl phosphate is linear (Figure 3), and thus the release of products cannot be rate-limiting under these reaction conditions. This conclusion is also supported by the observation that only 1 equiv of ADP is formed in the rapid phase of the time course when glutamine is included in the reaction mixture.

The time course for the formation of glutamate in the presence of ATP/bicarbonate shows a burst of 1 enzyme equiv. It is interesting to note that the rate constant for the fast phase of this time course is identical to the rapid phase of the time courses when the formation of either acid-labile phosphate or ADP is monitored. This result dramatically illustrates the tight coupling between the lone reaction center in the small subunit and the active site found within the N-terminal half of the large subunit of CPS. It also demonstrates that the molecular tunnel that connects these two active sites does not act as an "ammonia pipeline" by filling up with ammonia as a mechanism for the supply of NH_3 to the large subunit. Since there is a burst of 1 enzyme equiv of glutamate that is formed at a rate identical to the rate of formation of the carboxy phosphate intermediate, then the rate of formation of ammonia cannot be rate-limiting for the overall reaction. This conclusion is confirmed by the observation that the time courses for acid-labile phosphate formation are identical, whether the nitrogen source is supplied via glutamine or provided directly as ammonia from bulk solution.

Scheme 4



The time course for the formation of glutamate is very much dependent on the inclusion of ATP/bicarbonate to the reaction mixture. When ATP/bicarbonate is included in the reaction mixture, the rate constant for the burst of formation of glutamate increases from 4 to 1100 min^{-1}. This is a 275-fold rate enhancement. These results demonstrate that the phosphorylation of bicarbonate dominates the subsequent reaction cascade. The phosphorylation of bicarbonate is required for the efficient hydrolysis of glutamine, but the hydrolysis of glutamine is not required for the rapid phosphorylation of bicarbonate. Therefore, a molecule of ammonia from the hydrolysis of glutamine is not injected into the molecular tunnel linking the large and small subunits until an activated bicarbonate is ready for the subsequent reaction to form carbamate. The rate enhancement observed for the hydrolysis of glutamine that is driven by catalytic events within the large subunit must be transmitted by conformational changes to the catalytic residues that reside in the small subunit. This reciprocal coupling of the reaction centers thus ensures that the catalytic events are coupled to one another. These results are summarized in Scheme 4.

The results reported here do not identify the specific rate-limiting step in the overall synthesis of carbamoyl phosphate by CPS. It remains to be determined whether the phosphorylation, migration, or formation of carbamate is the rate-limiting step. However, these experiments do not reveal what, if any, coupling occurs between the two reaction centers contained within the large subunit of CPS. Experiments to address these remaining issues are in progress.

Tryptophan synthase was the first enzyme identified to contain a molecular tunnel through which intermediates are channeled between active sites (*29*). The enzyme is an $\alpha_2\beta_2$ heterodimer that catalyzes the synthesis of tryptophan from indole 3-glycerol phosphate (IGP) and serine. The α-subunit catalyzes the cleavage of IGP into indole and glyceraldehyde 3-phosphate (G3P). The β-subunit catalyzes the condensation of indole with serine to form tryptophan through a pyridoxal phosphate mediated reaction. The indole produced at the α-site is channeled ~25 Å to the active site in the β-subunit where it reacts with the aminoacrylate intermediate of serine and pyridoxal phosphate (*29*−*32*). Similar to CPS, the two reactions are coupled together such that the reactants are stoichiometrically converted into product (*33*). The chemical events at the β-subunit regulate the activity of the α-subunit in a manner similar to the carboxy phosphate domain of CPS controlling the rate of glutamine hydrolysis. The addition of serine produces a conformational change in the enzyme, which produces a 20-fold enhancement in the velocity of the reaction at the α-subunit (*34*, *35*). The formation of the

aminoacrylate intermediate thus initiates conformational changes that activate the cleavage of indole 3-glycerol phosphate (*33*, *36*, *37*). The formation of the tryptophan external aldimine intermediate then induces a conformational change to open the structure, allowing the release of products and the binding of substrates for the next round of catalysis (*38*). The conformational change in tryptophan synthetase that activates the α-site upon the addition of serine could be observed by a change in the intrinsic fluorescence of the protein (*33*). No changes in the intrinsic protein fluorescence have been detected for ligand-mediated conformational changes in CPS.

Glutamine phosphoribosyl pyrophosphate amidotransferase has recently been shown to form a molecular conduit for the delivery of ammonia produced from the hydrolysis of glutamine (*39*). Tryptophan residues have been cleverly placed within this protein via site-directed mutagenesis to monitor conformational changes that occur during substrate binding and catalysis. It has been determined that the binding of phosphoribosyl pyrophosphate initiates the catalytic cycle of the enzyme by enabling the binding of glutamine and the subsequent hydrolytic reaction (*40*). It is highly likely that other proteins with multiple reaction centers will be found to have evolved with conformational coupling and synchronization mechanisms.

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