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Detection of Intermediates in the Carbamoyl Phosphate Synthetase Reaction

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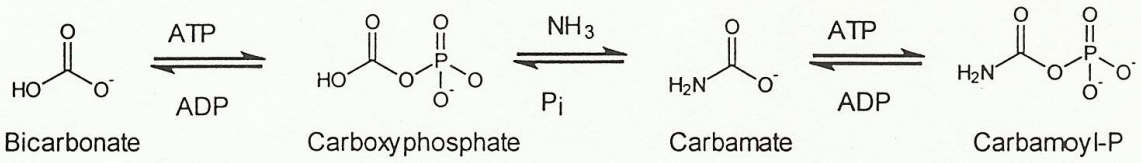
The reaction mechanism of carbamoyl phosphate synthetase was examined for the formation of several transient intermediates. The time course for the change in $[H^+]$ during the bicarbonate-dependent ATPase reaction is consistent with a mechanism in which carboxy phosphate, but not CO_2, is formed and released from the enzyme. The time course for the change in $[H^+]$ during the formation of ATP from ADP and carbamoyl-P is consistent with the initial decomposition of carbamate to CO_2 and NH_4^+. A bicarbonate-water oxygen exchange reaction is catalyzed during the bicarbonate-dependent ATPase reaction. This exchange reaction is suppressed during the synthesis of carbamoyl-P. These results are inconsistent with the nucleotide switch mechanism. However, all of these experiments are consistent with the formation of carboxy phosphate and carbamate during the reaction mechanism of CPS.

1. Introduction

Carbamoyl phosphate synthetase (CPS) from *Escherichia coli* catalyzes the ATP-dependent formation of carbamoyl phosphate, a metabolite common to both the arginine biosynthetic pathway and the *de novo* route for pyrimidine biosynthesis [1]. This reaction is shown in Equation 1. The CPS from *E. coli* is a heterodimeric protein composed of a small (42 kDa) and a large (118 kDa) subunit [2]. The small subunit functions as an amidotransferase domain to produce NH_3 for the active site of the large subunit. The large subunit thus serves as the site for the formation of carbamoyl phosphate, using ammonia derived from either the hydrolysis of glutamine or the external medium.

$$2\ MgATP + HCO_3^- + Gln + H_2O \leftrightarrows NH_2CO_2PO_3^{=} + 2\ MgADP + P_i + Glu \quad (1)$$

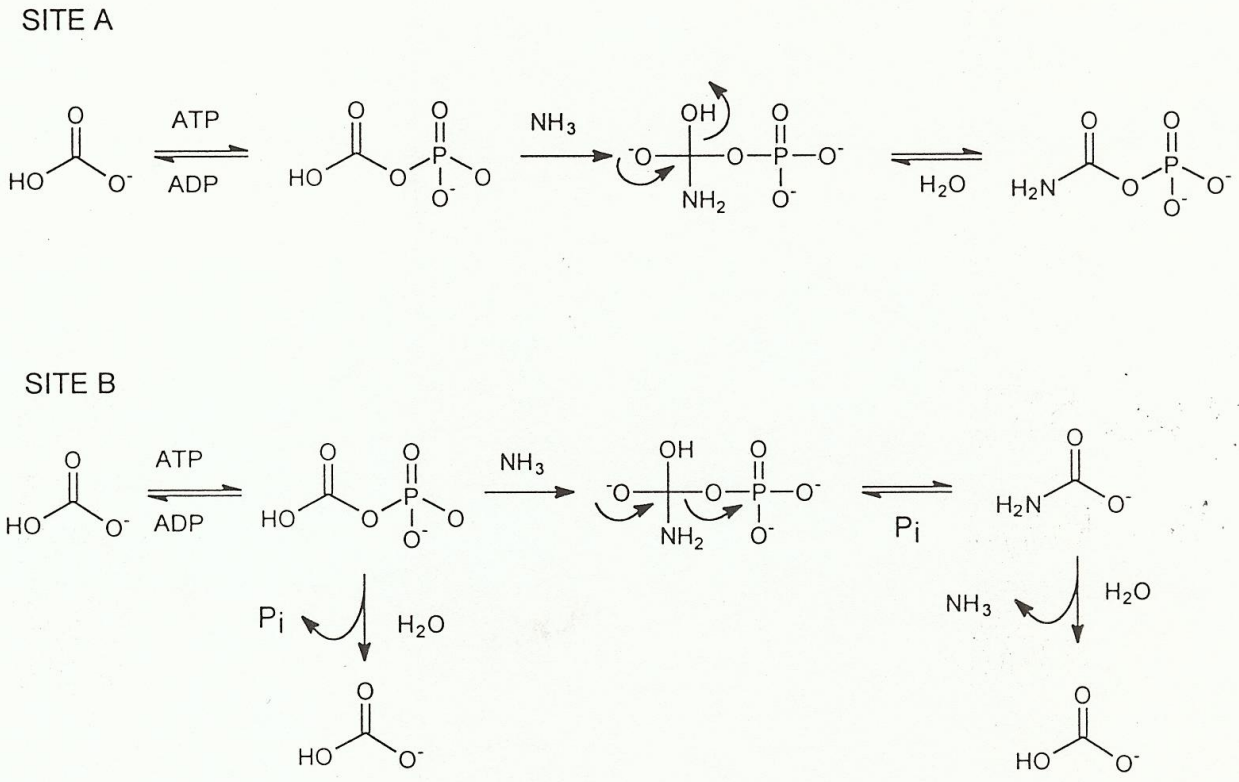
The X-ray crystal structure of CPS from *E. coli* has been solved to a resolution of 2.8 Å by Thoden *et al.* [3]. In this structure, the nucleotide binding sites for each of the two molecules of ATP used in the formation of carbamoyl-P have been localized from the position of ADP bound to the protein. The two nucleotide binding sites are found within two homologous domains. In the *E. coli* protein, the nucleotide binding domain from the N-terminal half of the large subunit is composed of amino acid residues 1-400 while that from the C-terminal half ranges from amino acid residues 553-933. Lusty has shown that these two regions of the protein are approximately 40% identical in amino acid sequence, and thus she has proposed that the modern-day enzyme arose via a gene duplication event followed by fusion and subsequent specialization of the individual domains [4].



Scheme 1

The chemical reaction mechanism for the synthesis of carbamoyl phosphate is currently thought to proceed through a sequence of three reactions involving carboxy phosphate and carbamate as key intermediates as illustrated in Scheme 1 [5]. In support of the proposed reaction mechanism, CPS has been shown to catalyze a bicarbonate-dependent ATPase reaction in the absence of a nitrogen source and an ATP synthesis reaction from ADP and carbamoyl phosphate [6]. Further evidence in support of the kinetic competence of carboxy phosphate and carbamate in the reaction mechanism for CPS has been obtained using positional isotope exchange (PIX) and chemical labeling experiments [7-9]. It has been proposed that the N-terminal domain is utilized for the phosphorylation of bicarbonate while the C-terminal domain functions to phosphorylate carbamate in the reaction mechanism presented above [10]. These functional assignments for the two homologous halves of CPS are supported by the differential effects on the two partial reactions upon mutagenesis of critical amino acid residues in either of the two domains [10-13]. Surprisingly, the two nucleotide binding sites are positioned ~35 Å apart from one another in the X-ray crystal structure [3]. Since carbamate has been proposed to be synthesized within the N-terminal domain and then phosphorylated by the ATP bound within the C-terminal domain, this reaction intermediate must then migrate (diffuse) from one site to the other in order to complete the reaction sequence. Support for this unusual event has come from the finding of a molecular tunnel that interconnects the two active sites contained within the large subunit of CPS [3].

The classical view of the chemical and mechanistic events during the synthesis of carbamoyl-P has recently been challenged by Kothe *et al.* [14]. An alternative mechanism has been put forth which proposes that the synthesis of carbamoyl-P utilizes a parallel pathway to drive conformational changes that enables the synthesis of carbamoyl-P to proceed entirely within a single catalytic domain of the large subunit. In this novel proposal, one nucleotide binding domain serves to phosphorylate bicarbonate by ATP with the generation of carboxy phosphate. Ammonia then reacts with the carboxy phosphate intermediate to form carbamoyl-P through the direct elimination of hydroxide (or water) from the tetrahedral intermediate. This thermodynamically unfavorable transformation is apparently driven by conformational changes induced through the bicarbonate-dependent hydrolysis of ATP at the other nucleotide binding domain. At this second site, ATP is proposed to phosphorylate bicarbonate to form carboxy phosphate and then this intermediate reacts with ammonia (or water in cases where glutamine is used as a nitrogen source). The tetrahedral adduct formed at this site then collapses to carbamate (or bicarbonate) through the direct elimination of phosphate. The overall substrate and product stoichiometry is identical for the nucleotide switch mechanism to that of the sequential mechanism. However, in the original sequential chemical mechanism for the formation of carbamoyl-P, the two phosphorylation domains are coupled through the migration of carbamate while in the mechanism proposed by Kothe *et al.* [14], the two

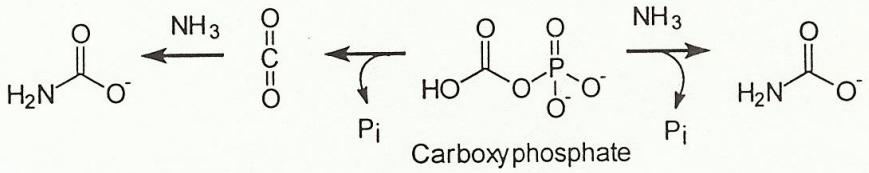


Scheme 2

sites are linked solely via conformational changes induced through the hydrolysis of ATP. This alternative mechanism is graphically illustrated in Scheme 2.

Currently, there is no experimental evidence to unequivocally permit a distinction between these two mechanistic proposals for the synthesis of carbamoyl-P. However, an unambiguous choice can be made between these two mechanisms if the incorporation of oxygen-18 from the solvent into the residual bicarbonate (or the exchange of oxygen-18 from the bicarbonate to the solvent) is monitored in the presence and absence of a nitrogen source. In both mechanisms, one atom of oxygen from the solvent is transferred directly to bicarbonate during each turnover in the absence of a nitrogen source. Upon inclusion of a nitrogen source, no bicarbonate-oxygen exchange with solvent is possible with the sequential mechanism since the carboxy phosphate and carbamate intermediates are converted directly to carbamoyl-P. However, in the parallel mechanism a molecule of bicarbonate must exchange with solvent during every turnover, whether or not a nitrogen source is present.

One of the other unanswered questions about the proposed sequential reaction mechanism for CPS is whether the carboxy phosphate intermediate reacts directly with NH_3 to form carbamate or, alternatively, if this unstable intermediate dissociates to CO_2 and phosphate prior to the reaction with NH_3 [15]. These two alternative reaction mechanisms for the formation of carbamate are illustrated in Scheme 3. The existence of CO_2 and carbamate in the reaction sequence has been probed by measuring the transient uptake and liberation of protons during the bicarbonate-dependent ATPase reaction and the ATP synthesis reaction from ADP and carbamoyl-P.

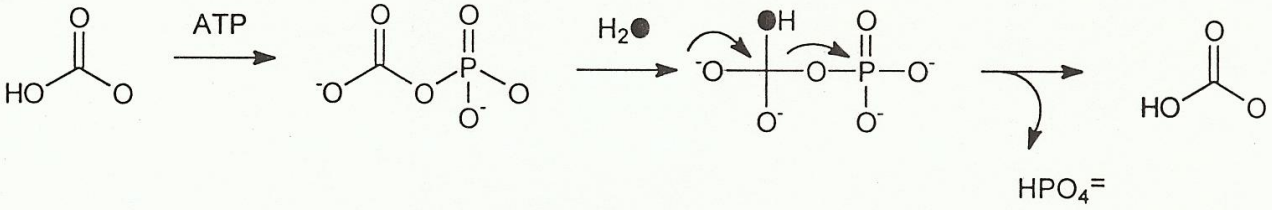


Scheme 3

2. Results

The sequential chemical mechanism (Scheme 1) and the parallel mechanism (Scheme 2) for the operation of CPS differ significantly in how the two nucleotide binding sites are utilized for the coordinated synthesis of carbamoyl-P. In the sequential mechanism the bicarbonate is first phosphorylated, amidated, and then rephosphorylated in a linear fashion for the ultimate construction of carbamoyl-P. The two nucleotide binding domains are linked through the physical migration of carbamate from the site of synthesis to the site of phosphorylation. In contrast, the parallel mechanism postulates that one site (labeled in Scheme 2 as site **A**) is able to construct carbamoyl-P from a single phosphorylation event. The companion site (labeled in Scheme 2 as site **B**) apparently serves to drive conformational changes at Site **A** through the bicarbonate-dependent hydrolysis of a second molecule of ATP. The two mechanisms can, however, be differentiated by measuring the rate of bicarbonate-water exchange in the presence and absence of a nitrogen source. In the sequential mechanism there is no opportunity for an enzyme-catalyzed exchange of solvent water with the substrate bicarbonate during the synthesis of carbamoyl-P. In this mechanism the carboxy phosphate intermediate is captured directly by the attack of ammonia and then the second molecule of ATP phosphorylates the carbamate. However, the sequential mechanism predicts that in the absence of a nitrogen source a bicarbonate-water exchange reaction will occur with each turnover as shown in Scheme 4. When there is no ammonia for reaction with the carboxy phosphate, this reaction intermediate will form bicarbonate and inorganic phosphate. This partial reaction has been shown to occur with exclusive cleavage of the carbon-oxygen bond [5]. The decomposition of carboxy phosphate may occur either through the intermediacy of carbon dioxide or through the direct attack of water (as shown in Scheme 4) at the carbonyl carbon of the intermediate. Nevertheless, in either case, one atom of oxygen from water will be incorporated into the recycled bicarbonate after every turnover of the bicarbonate-dependent ATPase reaction.

The parallel mechanism also predicts that in the absence of a nitrogen source that oxygen from solvent will be incorporated into the bicarbonate pool after every turnover. Moreover, this mechanism also *requires* that in the presence of a nitrogen source there will be an exchange of solvent with the bicarbonate pool within site **B** concurrent with the formation of carbamoyl-P at site **A**. This exchange reaction is dictated by the required formation of either carbamate (as shown in Scheme 5) or carboxy phosphate that is eventually hydrolyzed by water. Therefore, the two competing chemical mechanisms for the synthesis of carbamoyl-P can be differentiated by measuring the rate of water-bicarbonate isotope exchange in the presence and absence of a nitrogen source. The sequential mechanism requires that *no exchange* be observed in the presence of a nitrogen



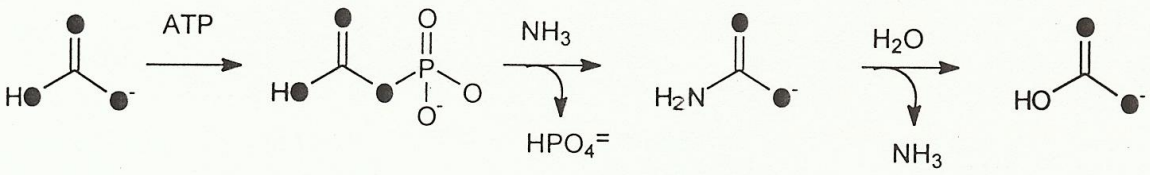
Scheme 4

source whereas the parallel mechanism requires that an exchange of oxygen from water into the bicarbonate pool will occur during *every turnover*.

Measurement of Water-Bicarbonate Isotope Exchange. The rate of isotopic exchange between solvent water and bicarbonate was measured by monitoring the incorporation of oxygen-18 from the solvent into the bicarbonate. The bicarbonate was labeled with carbon-13 and NMR spectroscopy was used to follow the exchange reaction by measuring the small chemical shift difference when an oxygen-18 is directly bonded to the carbon relative to when an oxygen-16 is bonded to the carbon. The difference in chemical shift between each of these resonances is 0.017 ppm. The relative ratios of the peak heights for these four resonances after equilibration with 30 atom % oxygen-18 are 32:44:21:3. These values agree quite closely with the values expected from a simple statistical distribution of oxygen-18 and oxygen-16 within the three oxygens bonded directly to carbon (34:44:19:3) when the mole fraction of oxygen-18 is 0.30.

Water-Bicarbonate Isotope Exchange During the Bicarbonate-Dependent ATPase Reaction. Both reaction mechanisms predict that an isotope exchange will occur between water and bicarbonate during the bicarbonate-dependent ATPase reaction. Therefore, measurement of the exchange of label from the solvent water into the bicarbonate pool during the bicarbonate-dependent ATPase reaction serves as a good control for the development of a method for monitoring this reaction. The exchange reaction was conducted at pH 8.5 to minimize the non-enzymatic exchange of solvent water with bicarbonate. In order to effectively stop the exchange reaction after a fixed period of time, the incubation mixture was quenched by raising the pH above 12 with the addition of KOH. When 6.7 mM [^{13}C]-HCO_3^- is incubated with 20 mM ATP at pH 8.5 in a volume of 1.0 mL (33 atom % oxygen-18 H_2O) in the presence of 9.2 mg CPS for 30 minutes there is a significant amount of oxygen exchange between the solvent water and the bicarbonate pool. The relative amounts of bicarbonate containing 0, 1, and 2 atoms of oxygen-18 are 42:46:12 (23 $\pm$ 1 atom % ^{18}O) while in the control reaction in the absence of enzyme the ratios are 69:27:4 (11 $\pm$ 1 atom % ^{18}O).

The combined enzymatic plus non-enzymatic first-order rate constant for the water-bicarbonate exchange during the bicarbonate-dependent ATPase reaction of CPS was found to be 0.12 $\pm$ 0.01 min^{-1}. The observed rate of oxygen exchange in the non-enzymatic control was 0.044 $\pm$ 0.004 min^{-1}. Therefore, the net rate for the CPS catalyzed exchange of water and bicarbonate during the bicarbonate-dependent ATPase reaction was 0.076 $\pm$ 0.011 min^{-1} (0.12 – 0.044 min^{-1}). Quantitation of the ATP/ADP ratio via ^{31}P NMR spectroscopy at the end of the 30 minute reaction period showed that 13.2 $\pm$ 0.6 μmoles of ADP were formed. Thus, the average rate for the enzymatic production of ADP during the 30 minute incubation period was 0.44 $\pm$ 0.02 μmoles min^{-1}. This reaction rate conforms reasonably well with the initial rate for the CPS catalyzed water-bicarbonate exchange of 0.51 $\pm$ 0.07 μmoles min^{-1} (6.7 μmoles x 0.076 min^{-1}). This result thus confirms that an

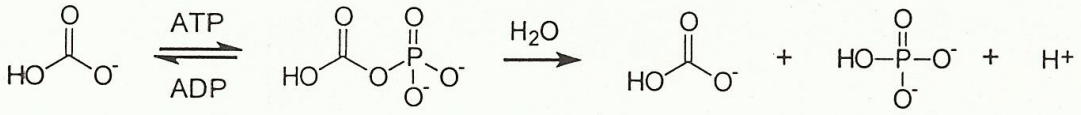


Scheme 5

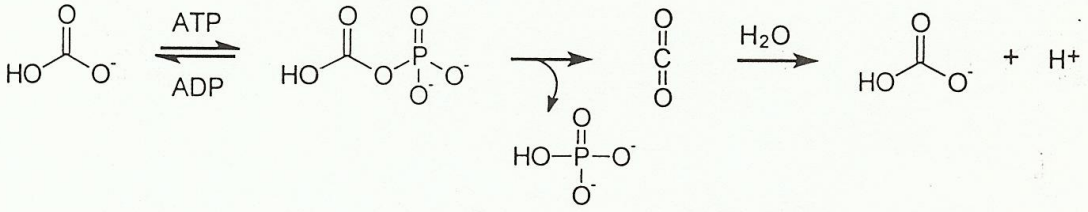
exchange of an oxygen atom from solvent water into the bicarbonate pool occurs with every turnover of the bicarbonate-dependent ATPase reaction of CPS.

Water-Bicarbonate Exchange During Formation of Carbamoyl Phosphate. Exchange reactions during the formation of carbamoyl-P were followed by monitoring the migration of an oxygen-18 from labeled bicarbonate into the solvent water pool. Ornithine transcarbamoylase and ornithine were added to the reaction mixture in order to convert the unstable carbamoyl-P to the more stable citrulline and to prevent reversal of the reaction. Pyruvate kinase and PEP were added to keep the ATP levels relatively constant during the incubation period. The ratios of bicarbonate species containing 0, 1, 2, and 3 atoms of oxygen-18 are 2:9:38:51 (79 $\pm$ 3 atom % ^{18}O). When 15 mM of the labeled bicarbonate was incubated with MgATP and glutamine in the presence or absence of CPS in a volume of 1.0 mL for 5 minutes there was no significant difference in the final ratios of the bicarbonate species containing variable amounts of oxygen-18. The net turnover of ATP in the presence of CPS was determined to be 13.0 $\pm$ 0.6 μmoles based upon the ratio of the ^{31}P resonances for P_i and PEP after the reaction was quenched with KOH. This value corresponds to 6.5 $\pm$ 0.3 μmoles of carbamoyl-P since two molecules of ATP are consumed for every carbamoyl-P produced when the overall reaction is fully coupled. For the control reaction the final ratios of the various species of bicarbonate containing 0, 1, 2, or 3 atoms of oxygen-18 were found to be 2:16:47:35 (72 $\pm$ 3 atom % ^{18}O). These values correspond to a rate constant of 0.061 $\pm$ 0.011 min^{-1} for the nonenzymatic exchange reaction. In the presence of CPS the ratios of the various species of bicarbonate containing 0, 1, 2, or 3 atoms of oxygen-18 were found to be 2:19:46:33 (70 $\pm$ 3 atom % ^{18}O). These values correspond to a rate constant for water-bicarbonate exchange of 0.075 $\pm$ 0.012 min^{-1}. Subtraction of the rate constant for the observed water-bicarbonate exchange in the absence of added enzyme from the value obtained in the presence of added CPS provides the net rate constant for water- bicarbonate exchange during the enzymatic synthesis of carbamoyl-P. This value is 0.014 $\pm$ 0.012 min^{-1} (0.075 - 0.061 min^{-1}). The upper limit for the initial rate of exchange would be 0.21 μmoles min^{-1} (0.014 min^{-1} x 15 μmoles bicarbonate).

Intermediacy of CO_2. If a carboxy phosphate intermediate were to form CO_2 prior to the addition of a nitrogen source to the active site, then the transient existence of CO_2 may be established through the requisite changes in the pH of the medium upon non-enzymatic rehydration of the CO_2 to bicarbonate. For example, if the carboxy phosphate intermediate were to decompose during the bicarbonate-dependent ATPase reaction through the direct attack of an activated water molecule, then the overall proton flux through such a pathway would liberate one proton for every turnover as shown in Scheme 6. However, if the carboxy phosphate intermediate were to dissociate directly to CO_2 and phosphate (either on or off the enzyme surface) then the transient proton flux would be quite different as illustrated in Scheme 7.



Scheme 6



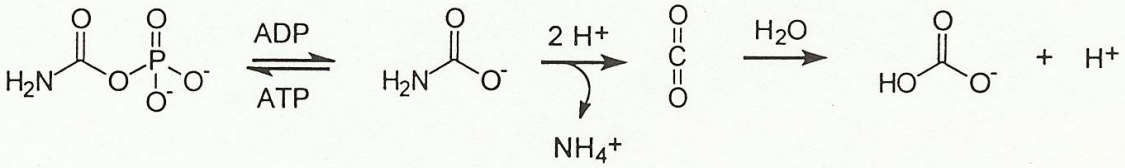
Scheme 7

Any reaction mechanism involving the intermediacy of CO_2 would be characterized by a measurable lag in the overall time course for proton production if the bicarbonate-dependent ATPase reaction was made to proceed faster than the non-enzymatic hydration of CO_2 back to bicarbonate. Thus, the existence of CO_2 during the reaction cycle can be established experimentally.

Spectrophotometric Assay of the Bicarbonate-Dependent ATPase Reaction. The bicarbonate-dependent ATPase reaction of CPS was monitored at 570 nm using the cresol red indicator assay. The results are shown in Figure 1. During the first 30 seconds of the reaction the absorbance change is nearly linear with time, and the initial velocity of the reaction was determined to be 0.26 μmoles H^+ min^{-1} mg^{-1} CPS. This value is in agreement with the initial rate of ADP formation using the pyruvate kinase/lactate dehydrogenase coupled assay system. The total absorbance change corresponded to ~10 μmoles of ATP hydrolyzed during the incubation period. Also presented in Figure 1 is a theoretical curve of the time course for a reaction mechanism where CO_2 is the initial product of the reaction rather than bicarbonate (Scheme 7).

During the partial back reaction of CPS, the initial formation of carbamate from ADP and carbamoyl phosphate would not involve net proton uptake or release, but the subsequent decomposition of carbamate to CO_2 and NH_4^+ would involve a net uptake of two protons as shown in Scheme 8. The decomposition of carbamate into NH_4^+ and CO_2 at neutral pH is very rapid [16]. The subsequent hydration of CO_2 is much slower and occurs with the release of 1 equivalent of H^+. The rapid production and decomposition of carbamate, followed by the slower nonenzymatic hydration of CO_2, would result in a biphasic time course for the change in proton concentration.

Spectrophotometric Assay of the ATP Synthesis Reaction. The time course for the uptake and release of protons during the ATP synthesis reaction of CPS was monitored at 570 nm using a stopped-flow spectrophotometer. The results are shown in Figure 2. The change in absorbance as a function of time was monophasic when the reaction was initiated with 1.8 mg/mL of CPS. The total absorbance change corresponded to 1.1 equivalents of H^+ taken up during the reaction (Figure 2). A fit of these data to a single



Scheme 8

exponential gave a first-order rate constant of 0.009 ± 0.001 s^{-1}. When 18 mg/mL of CPS was used to initiate the reaction, the absorbance change was biphasic with time. After 80 seconds, 1.5 equivalents of H^+ were taken up in the first phase of the reaction followed by the release of 0.4 equivalents of H^+ during the slower phase of the reaction. A fit of the data from the slower phase to a single exponential gave a first-order rate constant of 0.010 ± 0.001 s^{-1}.

3. Discussion

The nucleotide switch mechanism requires that a water-bicarbonate exchange reaction occurs in concert with carbamoyl-P synthesis. However, in the presence of glutamine the rate of carbamoyl-P formation (1.3 μmoles min^{-1}) is greater than six times faster than the rate of water-bicarbonate exchange. The experimental results clearly demonstrate that there is no significant exchange of oxygen from the bicarbonate to solvent water during the coupled formation of carbamoyl phosphate by CPS. This result is fully consistent with the requirements of the sequential reaction mechanism but it is not consistent with the constraints posed by the parallel mechanism [14] as originally formulated. If the nucleotide switch mechanism was fully functional, then the expected ratios of labeled bicarbonate at the end of the incubation period would have been 6:26:44:24 (62 atom % ^{18}O). The predicted values for relative amounts of the various bicarbonate species are clearly inconsistent with the experimentally determined observations and thus the nucleotide switch mechanism for the synthesis of carbamoyl-P by CPS from *E. coli* is inappropriate.

For the bicarbonate-dependent ATPase reaction it is uncertain whether the ultimate enzyme-catalyzed product is carboxy phosphate or CO_2 [15]. Either of these two compounds could potentially react with ammonia to form carbamate as shown in Scheme 3. If the carboxy phosphate intermediate were to be attacked directly by water to form bicarbonate and phosphate, then there will be a linear production of one proton for every ATP hydrolyzed as illustrated in Scheme 6. However, if carboxy phosphate were to dissociate unimolecularly to CO_2 and P_i during the bicarbonate-dependent ATPase reaction, then there would be no net change in H^+ concentration until after the CO_2 was non-enzymatically hydrated to form bicarbonate and one proton. This mechanism is presented in Scheme 7. If the enzyme-catalyzed hydrolysis of ATP can be made to proceed faster than the uncatalyzed hydration of CO_2, then the time course for the liberation of the single proton per catalytic cycle would exhibit a lag. The initial velocity data obtained in the present study clearly shows that the initial rate of H^+ production during the bicarbonate-dependent ATPase reaction of CPS was linear with time (Figure 1) with no detectable lag due to the slow hydration of CO_2. Moreover, the initial rate of H^+ formation was identical to the rate of ADP formation. These results are thus consistent with the formation of carboxy phosphate, but not CO_2, during the bicarbonate-dependent ATPase reaction of CPS.

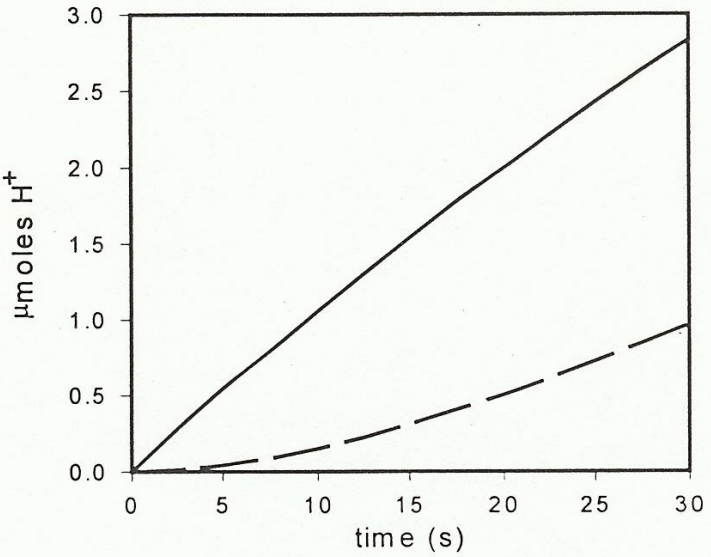


Figure 1. Spectrophotometric pH indicator assay for the bicarbonate-dependent ATPase reaction. The experimental time course for H^+ production when 15 mg CPS was mixed with 10 mM MgATP and excess bicarbonate at pH 8.0 is shown as the solid line. The initial rate of H^+ production is 0.26 µmol min^{-1} mg^{-1} CPS. The theoretical time course if CO_2 was the initial reaction product (Scheme 7) is shown as the dashed line. Additional details are given in the text.

The stopped-flow studies were consistent with the intermediacy of carbamate during the partial back reaction of CPS from *E. coli*. The experiments using the spectrophotometric pH indicator assay with carbamate kinase (data not shown) have shown that the technique can provide clear qualitative evidence for the existence of carbamate as an intermediate, provided that enough enzyme can be used so that the initial enzymatic step is significantly faster than the non-enzymatic hydration of CO_2. At low enzyme concentrations the phosphoryl transfer from carbamoyl phosphate to ADP (i.e., the enzymatic step) would be the rate-determining step, allowing the subsequent hydration reaction of CO_2 to achieve equilibrium. Therefore, at low enzyme concentrations the net uptake of only a single proton per catalytic cycle is observed (Scheme 8). However, when higher amounts of carbamate kinase are used, the phosphoryl transfer step, coupled with the dissociation of carbamate to CO_2 and ammonium ion, now become faster than the uncatalyzed hydration of the CO_2 to bicarbonate. Under these conditions there is predicted to be a rapid uptake of two protons per catalytic cycle, followed by the slower release of 1 proton as the CO_2 is hydrated. The time courses for the change in H^+ concentration during the carbamate kinase reaction clearly show this expected phenomenon and the second phase is dictated by the non-enzymatic hydration rate of CO_2.

The stopped-flow data for CPS during the partial back reaction (Figure 2) are qualitatively the same as for carbamate kinase. When 18 mg/mL of CPS were used to initiate the reaction, there was an increase in OD_{570} due to the uptake of approximately 1.5 equivalents of H^+, followed by a slower decrease in OD_{570} giving a final net uptake of 1.1 equivalents of H^+. The relatively low specific activity for ATP synthesis by *E. coli* CPS (0.4 µmol min^{-1} mg^{-1}) limits the feasibility of increasing the rate of the enzymatic step to the point of being significantly faster than the rate of CO_2 hydration. Therefore, the uptake of 2 full equivalents of H^+ is not observed in the fast phase of the time course using CPS as found in the time course using carbamate kinase (Figure 2), an enzyme that has a much

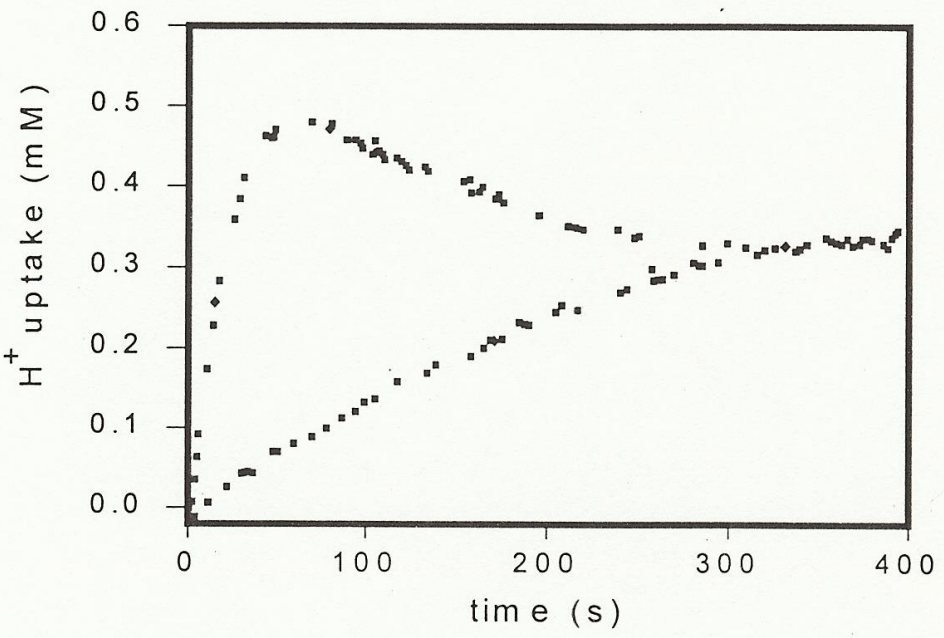


Figure 2. Stopped-flow spectrophotometric pH indicator assay for the ATP synthesis reaction of CPS using either 1.8 mg/mL (lower curve) or 18 mg/mL of enzyme (upper curve). The initial carbamoyl phosphate concentration was 0.4 mM. A fit of the lower curve to a single exponential gives a first-order rate constant of $0.009 \pm 0.001\ s^{-1}$. The upper curve shows an initial uptake of 1.5 equivalents of H^+, followed by a slower release of 0.4 equivalents of H^+. A fit of the slower phase for this curve to a single exponential gives a first order rate constant of $0.010 \pm 0.001\ s^{-1}$. Additional details are given in the text.

higher specific activity for carbamate synthesis (725 μmol min^{-1} mg^{-1}). Only 1.5 equivalents of H^+ are taken up in the initial phase of the reaction because the enzymatic synthesis of carbamate is only slightly faster than the CO_2 hydration rate. The enzyme-catalyzed step slows down as the substrate is removed, while the amount of CO_2 undergoing hydration increases until it becomes as fast as the enzymatic step. However, these data are fully consistent with the formation of carbamate as a reaction intermediate in the partial back reaction of CPS.

Acknowledgement

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