

Carbamoyl Phosphate Synthetase from *Escherichia coli* Does Not Catalyze the Dehydration of Bicarbonate to Carbon Dioxide¹

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The reaction catalyzed by carbamoyl phosphate synthetase (CPS) from *Escherichia coli* was examined for the formation of several transient intermediates. The chemical mechanism for CPS has been previously postulated to involve the formation of carboxy phosphate and carbamate as transient intermediates. However, it is uncertain whether the carbamate arises from the reaction of ammonia with the carboxy phosphate intermediate or whether the carboxy phosphate must first dissociate to carbon dioxide prior to the attack by ammonia. A spectrophotometric pH indicator assay was used to show that during the bicarbonate-dependent ATPase partial reaction, the initial rate of H⁺ production is linear and equivalent to the rate of ADP formation. The time course for H⁺ production is consistent with a mechanism in which carboxy phosphate, but not CO₂, is formed and released from the enzyme. No ¹³C-NMR signal is observed for CO₂ during the ATPase reaction at either low or high concentration of CPS. Isotope exchange experiments using [γ-¹⁸O₄] ATP as the initial substrate in the ATPase reaction produced P_i without the detectable loss of 18-oxygen, which is inconsistent with the formation of CO₂ during the bicarbonate-dependent ATPase reaction. For the partial back reaction catalyzed by CPS, a pH indicator assay was utilized to test for the initial production of carbamate and ATP from carbamoyl phosphate and ADP. When the rate of ATP synthesis was made relatively fast, the time course for H⁺ production was biphasic. These results are consistent with the rapid uptake of H⁺ during the breakdown of carbamate to CO₂ and NH₄⁺, followed by the slower release of protons during the nonenzymatic hydration of CO₂. Overall, these results are consistent with the formation of carbamate during the partial back reaction of CPS but no evidence could be obtained for the intermediacy of CO₂ during the bicarbonate-dependent ATPase reaction.

INTRODUCTION

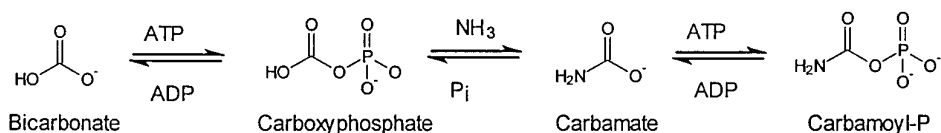
Carbamoyl phosphate synthetase (CPS,³ EC 2.7.2.5) from *Escherichia coli* catalyzes the ATP-dependent formation of carbamoyl phosphate, a metabolite common to both the arginine biosynthetic pathway and the *de novo* route for pyrimidine biosynthesis (*I*). This reaction is shown below:



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³ Abbreviations used: CPS, carbamoyl phosphate synthetase; PIX, positional isotope exchange.

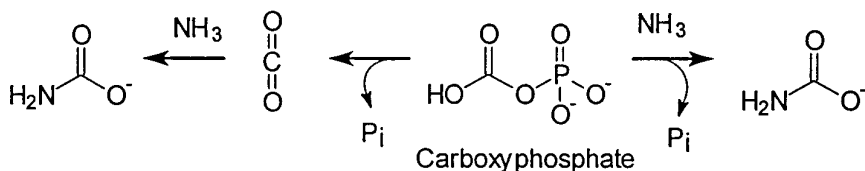


SCHEME 1

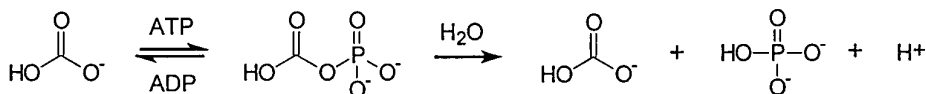
The CPS from *E. coli* is a heterodimeric protein composed of a small (42 kDa) and a large (118 kDa) subunit (2). The small subunit functions as an amidotransferase domain to produce nascent NH_3 for the active site of the large subunit. The large subunit thus serves as the site for the formation of carbamoyl phosphate, using ammonia either derived from the hydrolysis of glutamine or added exogenously to the external medium.

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 (3). 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 (4). 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 (5–7). Wimmer *et al.* (5) used PIX to demonstrate that carboxy phosphate is formed as a kinetically competent intermediate in the CPS reaction. In addition, the PIX experiments of Raushel and Villafranca (6) were consistent with the formation of carbamate during the reaction pathway of the partial back reaction. However, Kothe *et al.* (8) have recently proposed a slightly modified version of this reaction mechanism for CPS that awaits experimental verification.

One of the unanswered questions about the reaction mechanism for CPS is whether the putative 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 (9). Raushel and Villafranca (10) were unable to detect any loss of labeled oxygen within $[\gamma\text{-}^{18}\text{O}]\text{ATP}$ that would be consistent with the direct formation of CO_2 and P_i from the carboxy phosphate



SCHEME 2

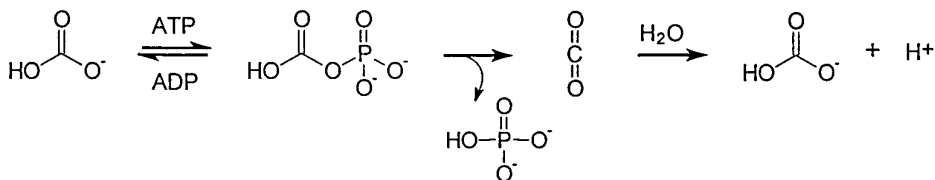


SCHEME 3

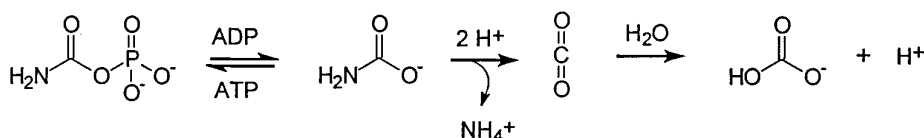
intermediate. However, if the formation of CO_2 from carboxy phosphate was an irreversible step in the reaction mechanism, then no exchange of label would have been observed, regardless of the intermediacy of CO_2 . These two alternative reaction mechanisms for the formation of carbamate are illustrated in Scheme 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 by direct observation of this compound using NMR spectroscopy, or indirectly through the requisite changes in the pH of the medium upon nonenzymatic 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 3. 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 4. 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 nonenzymatic hydration of CO_2 back to bicarbonate. Moreover, the ratio of the enzymatic bicarbonate-dependent ATPase reaction rate relative to the nonenzymatic rate of CO_2 hydration can be readily manipulated simply by increasing the concentration of CPS. Thus, the existence of CO_2 during the reaction cycle can be established experimentally.

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 5. The decomposition of carbamate into NH_4^+ and CO_2 at neutral pH is very rapid (11). The subsequent hydration of CO_2 is



SCHEME 4



SCHEME 5

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.

In the present investigation, the two partial reactions of CPS were monitored spectroscopically using a pH indicator to follow the time course for the uptake and release of protons during the catalytic cycle. The relatively rapid transformation of CO_2 and carbamate, which makes both of these species difficult to detect, can be easily manipulated to aid in the analysis of these intermediates during the reactions catalyzed by CPS. These studies have demonstrated that CO_2 is not the initial product of the bicarbonate-dependent ATPase but that carbamate is the initial product of the partial ATP synthesis reaction.

MATERIALS AND METHODS

Chemicals and enzymes. Wild-type *E. coli* CPS was prepared as previously described by Mareya *et al.* (12). $[\text{^{18}O}]\text{H}_2\text{O}$ (97 at.%) was purchased from Cambridge Isotope Laboratories. $[\gamma\text{-}^{18}\text{O}_4]\text{ATP}$ was prepared as previously described (6). All other chemicals and enzymes were purchased from Sigma.

Spectrophotometric pH assay. The changes in the concentration of H^+ during the bicarbonate-dependent ATPase reaction of CPS were monitored continuously using the pH indicator method of Hall *et al.* (13). The colorimetric indicator used in this assay was cresol red ($\text{pK}_a = 8.3$, $\lambda_{\text{max}} = 570$) where a typical reaction mixture contained 2% cresol red, 25 mM Tris-HCl (pH 8.0), 15 mg of CPS (94 μM), 100 mM KCl, 20 mM MgCl_2 , 10 mM ATP, and 40 mM KHCO_3 in a total volume of 1.0 mL. The reaction was initiated by the addition of the ATP, and the decrease in absorbance at 570 nm was monitored for 3 min, until essentially all of the ATP was consumed. In order to convert the observed change in absorbance into concentration of H^+ , the extinction coefficient (0.11 mM^{-1}) for the colorimetric change under the specific reaction conditions used in these experiments was determined by titration of an incomplete reaction mixture (lacking ATP) with HCl. A pyruvate kinase/lactate dehydrogenase-coupled activity assay of the bicarbonate-dependent ATPase reaction in the presence of 2% cresol red showed that the pH indicator does not affect the rate of the enzyme reaction. An additional control experiment was carried out to test for any contaminating carbonic anhydrase activity in the CPS preparation. A cresol red assay that monitors the hydration of 1.0 mM CO_2 in the presence of 15 mg (94 μM) of CPS gave a rate of CO_2 hydration identical to the noncatalyzed hydration of CO_2 , indicating that no carbonic anhydrase activity was present in the CPS preparation.

Stopped-flow experiments. The uptake and release of H^+ during the ATP synthesis reactions of both carbamate kinase and CPS were monitored using the cresol red spectrophotometric assay with a stopped-flow instrument from Hi-Tech Ltd. The experimental conditions were initially optimized using the enzyme carbamate kinase, an enzyme known to catalyze the formation of carbamate from ADP and carbamoyl phosphate (11). The technique was then applied to the CPS-catalyzed reaction. In a typical stopped-flow experiment, syringe **A** contained 25 mM Tris-HCl, pH 8.0, 2% cresol red, 100 mM KCl, 20 mM $MgCl_2$, 10 mM ADP, and variable amounts of enzyme, while syringe **B** contained 25 mM Tris-HCl, pH 8.0, 2% cresol red, 100 mM KCl, 20 mM $MgCl_2$, and either 0.8 or 1.0 mM carbamoyl phosphate. In order to calibrate the absorbance changes as μmol of H^+ per unit time, a standard curve was generated using the assay conditions described above with 5.3 $\mu\text{g/mL}$ of carbamate kinase in syringe **A** and variable amounts of carbamoyl phosphate in syringe **B**. The overall change in OD_{570} after 250 s was plotted versus the initial concentration of carbamoyl phosphate. The slope of the resulting straight line is equal to the extinction coefficient for the expected optical changes under the reaction conditions. Each stopped-flow reaction profile represents the average of at least three runs.

Nuclear magnetic resonance spectroscopy. All NMR experiments were carried out using a Varian Unity 500 multinuclear spectrometer operating either at 125 MHz for ^{13}C or 205 MHz for ^{31}P . The typical acquisition parameters for the ^{13}C experiments were 25,000 Hz sweep width, 2.5 s acquisition time, and 4 μs pulse width with a 30-s delay between transients. ^{13}C spectra were referenced to bicarbonate (160.9 ppm). Typical acquisition parameters for the ^{31}P experiments were 2.5 s acquisition time, 10,000 Hz sweep width, and 10 μs pulse width with a 10-s delay between transients. ^{31}P spectra were referenced to inorganic phosphate at pH 7.5.

^{18}O exchange using $[\gamma\text{-}^{18}\text{O}_4]\text{ATP}$. The reaction mixtures contained 50 mM Hepes, pH 7.5, 1.0 mg CPS (9.0 μM), 12 mM $MgCl_2$, 100 mM KCl, 10 mM HCO_3^- , 10 mM $[\gamma\text{-}^{18}\text{O}_4]\text{ATP}$, and 10% D_2O , in a total volume of 0.7 mL. The samples were allowed to react at 25°C for 40 min and then the reaction was quenched by the addition of 100 μmol of EDTA. The ^{18}O distribution among the phosphorus-containing species was analyzed using ^{31}P NMR spectroscopy according to the method of Cohn and Hu (14).

^{13}C NMR experiments. The bicarbonate-dependent ATPase reaction using $[^{13}\text{C}]\text{HCO}_3^-$ as the substrate was monitored by ^{13}C NMR spectroscopy during catalytic turnover to test for the appearance of any new carbon-containing species. A reaction mixture containing 12 mg CPS (75 μM), 25 mM Hepes, pH 8.0, 12 mM $MgCl_2$, 100 mM KCl, 10 mM $[^{13}\text{C}]\text{HCO}_3^-$, 100 units pyruvate kinase, 50 mM PEP, 1.0 mM ATP, and 10% D_2O in a total volume of 1.0 mL was allowed to react at 25°C while measuring the ^{13}C NMR spectrum. A control experiment using only 10 mM $\text{H}^{13}\text{CO}_3^-$ and 25 mM Hepes, pH 8.0, showed only a ^{13}C -singlet for bicarbonate at 160.9 ppm along with several other peaks from the buffer in the region of 60–80 ppm. A second experiment was carried out using a higher enzyme concentration. The reaction mixture contained 50 mg CPS (312 μM), 100 mM KH_2PO_4 , pH 7.6, 1.0 mM $\text{NaH}^{13}\text{CO}_3$, 1.0 mM ATP, 5.0 mM $MgCl_2$, 100 mM KCl, 50 units pyruvate kinase, 50 mM PEP, and 10% D_2O in a total volume of 1.0 mL and allowed to react at 5°C while the ^{13}C NMR spectrum was obtained.

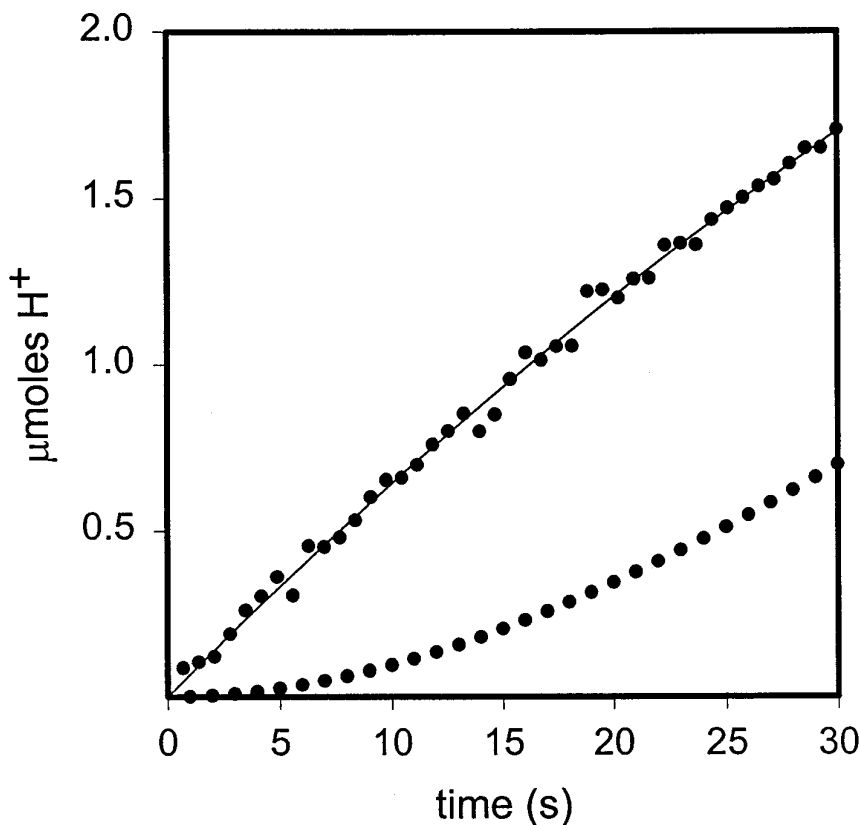


FIG. 1. Spectrophotometric pH indicator assay for the bicarbonate-dependent ATPase reaction. The experimental time course for H^+ production when 15 mg CPS (final concentration of $94 \mu\text{M}$) was mixed with 10 mM MgATP and excess bicarbonate at pH 8.0 is shown as the solid line with filled circles. The initial rate of H^+ production is $0.26 \mu\text{mol min}^{-1} \text{mg}^{-1}$ CPS. The theoretical time course if CO_2 was the initial reaction product (Scheme 4) is shown as the filled symbols with no line. This time course was generated with Eq. [2] where $\nu_e = 0.26 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and $k_h = 0.036 \text{ s}^{-1}$. Additional details are given in the text.

RESULTS

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 in the absence and presence of added carbonic anhydrase. The results are shown in Fig. 1. During the first 30 s of the reaction the absorbance change is nearly linear with time, and the initial velocity of the reaction was determined to be $0.26 \mu\text{mol H}^+ \text{min}^{-1} \text{mg}^{-1}$ 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 (data not shown) corresponded to $\sim 10 \mu\text{mol}$ of ATP hydrolyzed during the incubation period. An identical time

course was obtained in the presence of 500 units of carbonic anhydrase (data not shown). Also presented in Fig. 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 4). This time course was generated using Eq. [2], where ν_e is the measured initial rate of ADP formation and k_h is the known rate constant (0.036 s^{-1}) for the hydration of CO_2 at pH 8.0, 25°C (15):

$$[\text{H}^+] = \nu_e t - \nu_e (1/k_h)(1 - e^{-k_h t}). \quad [2]$$

Spectrophotometric assay of the ATP synthesis reaction. The time courses for the uptake and release of protons during the ATP synthesis reactions of both carbamate kinase and CPS were monitored at 570 nm using a stopped-flow spectrophotometer. The results are shown in Figs. 2 and 3. For the carbamate kinase reaction (Fig. 2), the absorbance change was monophasic with time when $0.60 \mu\text{g/mL}$ of enzyme was utilized to initiate the reaction. The final absorbance change corresponded to 1.1 equivalents of H^+ taken up per mole of carbamoyl phosphate in the initial reaction mixture. A fit of these data to a single exponential function gave a first-order rate constant of $0.022 \pm 0.001 \text{ s}^{-1}$. When the carbamate kinase concentration was increased to $6.0 \mu\text{g/mL}$ of enzyme, the absorbance change was biphasic with time. There was a rapid uptake of 2.2 equivalents of H^+ ($<10 \text{ s}$) followed by a slower release of 1.0 equivalent of H^+ over the next 2 min. The first-order rate constant for the slow phase was determined to be $0.043 \pm 0.001 \text{ s}^{-1}$. This rate constant is consistent with the value reported for the hydration of CO_2 (0.036 s^{-1}) under similar reaction conditions (15).

Time courses similar to those observed with carbamate kinase for the change in H^+ concentration were also obtained when CPS was added to a solution containing an excess of MgADP and 0.40 mM carbamoyl phosphate. The change in absorbance as a function of time was monophasic when the reaction was initiated with 1.8 mg/mL of CPS ($11 \mu\text{M}$). The total absorbance change corresponded to 1.1 equivalents of H^+ per mole of carbamoyl phosphate in the initial reaction mixture (Fig. 3). A fit of these data to a single exponential gave a first-order rate constant of $0.009 \pm 0.001 \text{ s}^{-1}$. When 18 mg/mL of CPS ($113 \mu\text{M}$) was used to initiate the reaction, the absorbance change was biphasic with time. After 80 s, 1.5 equivalents of H^+ was 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 $0.010 \pm 0.001 \text{ s}^{-1}$.

Isotope exchange experiments. $[\gamma\text{-}^{18}\text{O}_4]\text{ATP}$ was incubated with CPS until greater than 98% of the nucleotide was hydrolyzed to ADP. The ^{31}P NMR spectrum of the γ -phosphoryl group of the labeled ATP used for this experiment is shown in Fig. 4A. Integration of the various resonances corresponding to the species containing either 4 (88%), 3 (11%), or 2 (1%) atoms of ^{18}O indicated that the overall ^{18}O content for the four oxygens of the γ -phosphoryl group was 96%. The ^{31}P NMR spectrum of the inorganic phosphate produced during the bicarbonate-dependent ATPase reaction is shown in Fig. 4B. Integration of the various resonances for the inorganic phosphate containing either 3 (87%), 2 (10%), or 1 (3%) atoms of ^{18}O indicated that the overall ^{18}O content was 71%. The most downfield resonance signal for the phosphate containing no ^{18}O was introduced as a contaminant with the enzyme preparation. If the labeled ATP was hydrolyzed without washout of