A Stringent Test for the Nucleotide Switch Mechanism of Carbamoyl Phosphate Synthetase†

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ABSTRACT: Carbamoyl phosphate synthetase (CPS) catalyzes the formation of carbamoyl phosphate from bicarbonate, glutamine, and two molecules of MgATP. The X-ray crystal structure of the enzyme has revealed that the two nucleotide binding sites are separated by approximately 35 Å. Isotopic oxygen exchange of ^{18}O and ^{16}O between solvent water and [^{13}C]bicarbonate was measured using ^{13}C NMR spectroscopy during substrate turnover in the presence and absence of glutamine as a nitrogen source. In the absence of added glutamine, CPS catalyzed the exchange of one oxygen atom from bicarbonate with solvent water during every turnover of the bicarbonate-dependent ATPase reaction. In the presence of added glutamine, there was no exchange of solvent water with bicarbonate during the enzymatic synthesis of carbamoyl phosphate, indicating that any carbon-containing intermediate in the reaction mechanism is committed to the formation of carbamoyl phosphate and is not subject to hydrolysis. These results are fully consistent with a chemical mechanism that requires the physical migration of the carbamate intermediate from the site of its formation within one of the nucleotide binding domains to the other nucleotide binding domain for subsequent phosphorylation by the second MgATP. These results are not compatible with a nucleotide switch mechanism. The nucleotide switch mechanism includes the synthesis of carbamoyl phosphate entirely within a single nucleotide binding domain and concurrent conformational changes driven by the bicarbonate-dependent hydrolysis of MgATP at the second nucleotide binding domain.

Carbamoyl phosphate synthetase (CPS)[1] catalyzes the following reaction:

$$2ATP + HCO_3^- + \text{glutamine} + H_2O \rightarrow 2ADP + \text{glutamate} + P_i + \text{carbamoyl-P} \quad (1)$$

This remarkably complex protein requires five substrates for enzymatic activity and produces up to five separate reaction products. In some forms of the enzyme, the nitrogen source can be provided directly by ammonia (*1*). In addition to the overall biosynthetic reaction presented above, the enzyme from a variety of organisms will also catalyze two additional partial reactions (*2*). For example, in the absence of a nitrogen source, the enzyme will catalyze a slow bicarbonate-dependent ATPase reaction. CPS will also promote the phosphorylation of ADP using carbamoyl-P as a phosphoryl donor. These two partial reactions are shown below:

$$ATP + H_2O \xrightarrow{HCO_3^-} ADP + P_i \quad (2)$$

$$ADP + \text{carbamoyl-P} \rightarrow ATP + \text{carbamate} \quad (3)$$

On the basis of these partial reactions, and other properties of the enzyme, a sequential chemical mechanism has been proposed for the formation of carbamoyl-P (*3*). In the initial step, bicarbonate is phosphorylated by the first ATP to form a carboxy phosphate intermediate. In the subsequent step, carboxy phosphate reacts with ammonia to form carbamate and then this intermediate is ultimately phosphorylated by the second ATP to produce the final product, carbamoyl-P. The kinetic competence of the two unstable intermediates, carboxy phosphate and carbamate, has been supported by positional isotope exchange (*4, 5*), rapid quench experiments (*6*), and the transfer of an ^{18}O from bicarbonate to the phosphate product during the overall biosynthetic reaction (*4*). This reaction mechanism is illustrated in Scheme 1.

The X-ray crystal structure of CPS from *Escherichia coli* has been recently solved to a resolution of 2.8 Å by the laboratories of Holden and of Rayment (*7*). 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 residue 553 to 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 (*8*).

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[1] Abbreviations: CPS, carbamoyl phosphate synthetase; carbamoyl-P, carbamoyl phosphate.

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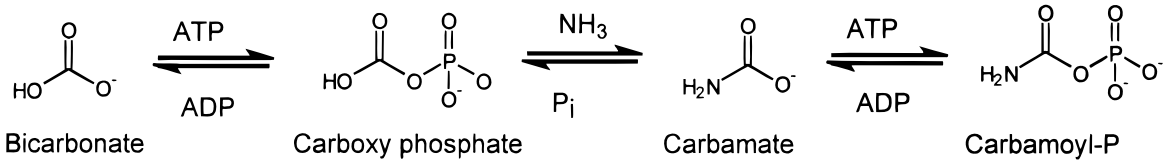
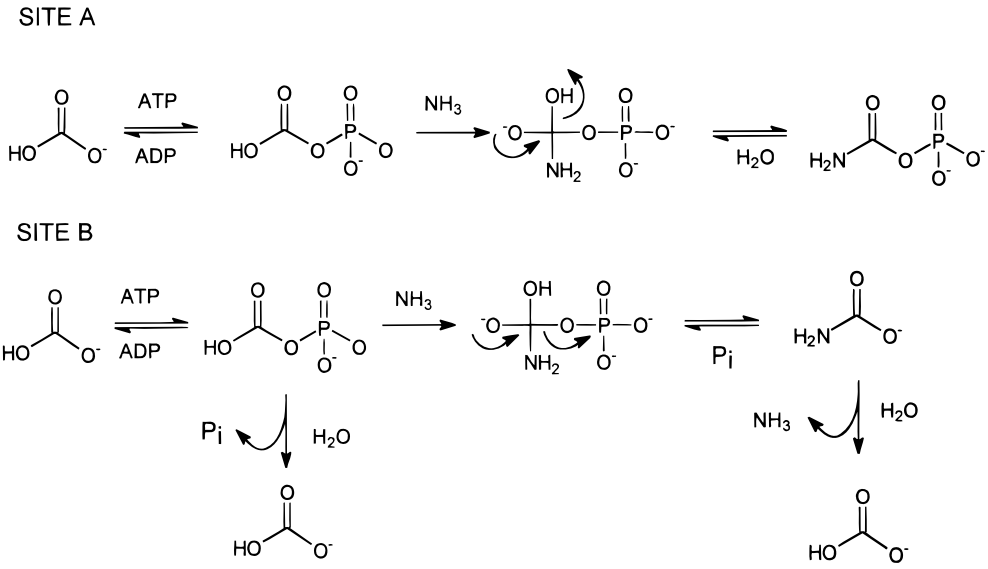
Scheme 1



Scheme 2



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 (*9*). 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 (*9*−*12*). For example, mutagenesis of residues in the N-terminal half of the large subunit predominantly affects the partial ATPase reaction, while the corresponding mutations in the C-terminal half of the large subunit primarily affect the partial back reaction (*11*, *12*). Surprisingly, the two nucleotide binding sites are positioned ∼35 Å apart from one another in the X-ray crystal structure (*7*). 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 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 (*7*).

This view of the chemical and mechanistic events during the synthesis of carbamoyl-P has recently been challenged by Kothe et al. (*13*). 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 for the nucleotide switch mechanism is identical 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. (*13*), the two sites are linked solely via conformational changes induced by 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 ^{18}O from the solvent into the residual bicarbonate (or the exchange of ^{18}O 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 a nitrogen source is present. In this report, evidence is presented to demonstrate that during the synthesis of carbamoyl-P by the CPS from *E. coli* there is no significant exchange of solvent water with the residual bicarbonate. The nucleotide switch mechanism is thus incompatible with direct experimental observations.

MATERIALS AND METHODS

Materials. Carbamoyl phosphate synthetase from *E. coli* was purified following the procedure of Mareya et al. (*14*). At pH 8.5, the specific activity of the CPS preparation was found to be 3.0 μmol of ADP formation min^{-1} mg^{-1} when assayed in the presence of 15 mM glutamine and 0.11 μmol of ADP formation min^{-1} mg^{-1} in the absence of a nitrogen source. ADP formation was measured spectrophotometrically using a pyruvate kinase−lactate dehydrogenase coupling system as described by Mareya et al. (*14*) at 25 °C. ^{18}O-labeled water (95 at. % ^{18}O) was purchased from Aldrich Chemical Co. The ^{13}C-labeled bicarbonate (99 at. % ^{13}C) was obtained from Cambridge Isotope Laboratories. The clone for ornithine transcarbamoylase was a generous gift from the laboratory of N. Allewell (University of Minnesota, Minneapolis, MN). Unless otherwise stated, all other chemicals and coupling enzymes were supplied by either Sigma or Aldrich.

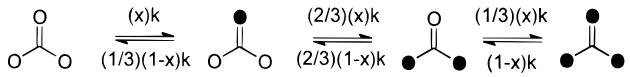
NMR Measurements. ^{13}C and ^{31}P NMR spectra were obtained on a Varian Unity 500 spectrometer utilizing a 5 mm broad-band probe operating at a frequency of either 126 or 202 MHz, respectively. The spectral parameters for the carbon spectra were as follows: 2500 Hz sweep width, 25.6 s acquisition time, 35 s acquisition delay, and 45° pulse width. The ^{31}P spectra were obtained with the following spectral parameters: 10 000 Hz sweep width, 2.5 s acquisition time, 5 s acquisition delay, and 45° pulse width.

Synthesis of [^{18}O,^{13}C]Bicarbonate. The doubly labeled bicarbonate was prepared by mixing 85 mg of [^{13}C]$NaHCO_3$ with 0.90 mL of ^{18}O-labeled water (95 at. % ^{18}O) and 0.10 mL of 1.0 M TAPS buffer (pH 8.5). The reaction vessel was sealed, and the water and bicarbonate were allowed to equilibrate for a period of 2 weeks. Analysis of the ^{13}C NMR spectrum at pH 12 indicated that the ratio of carbonate species containing zero to three atoms of ^{18}O was 2:9:38:51.

Bicarbonate−Water Exchange. The CPS-catalyzed solvent oxygen exchange with bicarbonate was monitored using ^{13}C NMR spectroscopy. This method relies on the small difference in chemical shift induced by ^{18}O relative to ^{16}O within the bicarbonate. The magnitude of the chemical shift difference among the four possible species of either bicarbonate or carbonate (containing zero to three atoms of ^{18}O) was measured by incubating a sample of 10 mM [^{13}C]-bicarbonate in 100 mM TAPS buffer containing 30 at. % [^{18}O]H_2O for 24 h at pH 8.5. The ^{13}C NMR spectrum was acquired, and then the pH was raised to 12 with the addition of KOH and the spectrum reacquired.

The exchange of ^{18}O from water into bicarbonate during the bicarbonate-dependent ATPase reaction was measured at pH 8.5. The sample contained 100 mM TAPS buffer (pH 8.5), 20 mM ATP, 6.7 mM [^{13}C]HCO_3^-, 20 mM $MgCl_2$, 33 at. % [^{18}O]H_2O, 100 mM KCl, and 9.2 mg of CPS in a total volume of 1.0 mL at a temperature of 25 °C. After the addition of CPS and [^{18}O]H_2O, the reaction was allowed to proceed for 30 min until it was quenched by the addition of 150 μmol of KOH. Following the addition of 100 μL of D_2O, the sample was centrifuged at 15 000 rpm for 30 s to remove any particulate material. A control sample was prepared under identical reaction conditions except that the CPS was omitted from the incubation solution.

Scheme 3



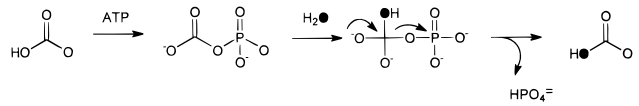
The exchange of ^{18}O from labeled bicarbonate to the solvent during glutamine-dependent carbamoyl-P synthesis was measured at pH 8.0. The sample contained 100 mM HEPES buffer (pH 8.0), 5.0 mM ATP, 10 mM $MgCl_2$, 15 mM [^{18}O,^{13}C]HCO_3^-, 100 mM KCl, 25 mM glutamine, 25 mM ornithine, 25 mM PEP, 50 units of ornithine transcarbamoylase, 50 units of pyruvate kinase, and 2 mg of CPS in a volume of 1.0 mL. The reaction was stopped after 5 min with the addition of KOH which raised the pH above 12. A total of 100 μL of D_2O was added, and then the sample was centrifuged as described above. A control reaction was conducted in the absence of added CPS.

Quantitative Analysis of Exchange Reaction Rates. The overall rate for solvent water−bicarbonate exchange catalyzed by CPS in the presence and absence of a nitrogen source was monitored by measuring the relative amounts of bicarbonate containing zero to three atoms of ^{18}O. Time courses were simulated using the model presented in Scheme 3, where x is the mole fraction of ^{18}O in the solvent water and k is the first-order rate constant for the exchange reaction. The simulations were conducted with the program KINSIM (*15*) to obtain the rate constant that best fit the experimental data. In the presence of glutamine, an additional step was added from each bicarbonate species in the mechanism presented in Scheme 3 to account for the net reduction in the total bicarbonate concentration during the synthesis of carbamoyl-P.

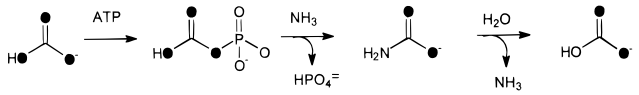
RESULTS AND DISCUSSION

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

Scheme 4



Scheme 5



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 (*4*). 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 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 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 source, whereas the parallel mechanism requires that an exchange of oxygen from water into the bicarbonate pool 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 ^{18}O from the solvent into the bicarbonate. The bicarbonate was labeled with ^{13}C, and NMR spectroscopy was used to follow the exchange reaction by measuring the small chemical shift difference when an ^{18}O is directly bonded to the carbon relative to when an ^{16}O is bonded to the carbon. The ^{13}C NMR signal for bicarbonate in unlabeled water at pH 8.5 is shown in Figure 1A. There is a single resonance centered at approximately 161 ppm. When 30 at. % ^{18}O-labeled water is added to this sample, three new resonances gradually appear upfield from the original signal as illustrated in Figure 1B. These three new resonances are due to the

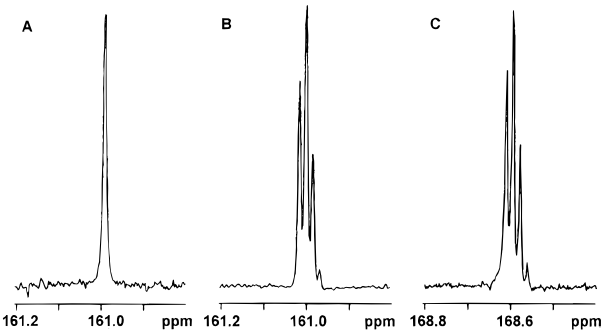


FIGURE 1: ^{13}C NMR spectra for bicarbonate and carbonate. (A) ^{13}C NMR spectrum for [^{13}C]bicarbonate (10 mM) at pH 8.5. The approximate chemical shift for the single resonance is 161 ppm. (B) Same as in part A except that the ^{18}O content of the solvent water was increased to 30 at. %. The difference in chemical shift for the species of bicarbonate containing zero to three atoms of ^{18}O is 0.017 ppm. (C) Same as in part B except the pH was raised from 8.5 to 12. The chemical shift is approximately 168 ppm. Additional details are given in the text.

appearance of labeled bicarbonate with either one, two, or three atoms of ^{18}O. The difference in chemical shift between each of these resonances is 0.017 ppm. Similar differences in chemical shift have been noted on other carboxylates (*16*). The relative ratio of the peak heights for these four resonances after equilibration is 32:44:21:3. These values are very consistent with the values expected from a simple statistical distribution of ^{18}O and ^{16}O within the three oxygens bonded directly to carbon (34:44:19:3) when the mole fraction of ^{18}O is 0.30. The chemical shift for these four resonances moves to approximately 168 ppm when the pH is adjusted to 12 and the bicarbonate is deprotonated to carbonate. No significant difference is observed in the relative peak intensities (Figure 1C).

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 the label from the solvent water to 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 nonenzymatic exchange of solvent water with bicarbonate. To effectively stop the exchange reaction after a fixed period of time, the incubation reaction 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 at. % [^{18}O]H_2O) in the presence of 9.2 mg CPS for 30 min, there is a significant amount of oxygen exchange between the solvent water and the bicarbonate pool. The relative ratio of bicarbonate containing zero to two atoms of ^{18}O is 42:46:12 (23 ± 1 at. % ^{18}O), while in the control reaction in the absence of enzyme, the ratio is 69:27:4 (11 ± 1 at. % ^{18}O). The NMR spectra are shown in Figure 2.

The combined enzymatic and nonenzymatic first-order rate constant for the water−bicarbonate exchange during the bicarbonate-dependent ATPase reaction of CPS was found to be 0.12 ± 0.01 min^{-1}. This value was determined by direct comparison of the experimental data with the simulated time courses generated with the model in Scheme 3 and the

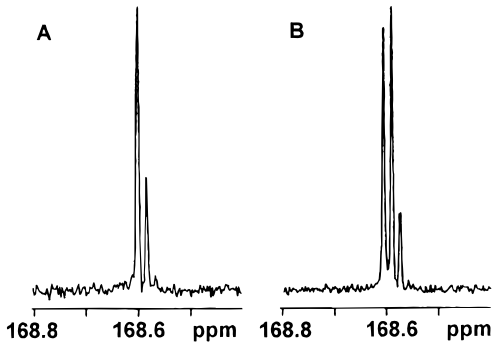


FIGURE 2: ^{13}C NMR spectra for bicarbonate. (A) [^{13}C]Bicarbonate (6.7 mM) was incubated with 20 mM MgATP in the presence of 33 at. % ^{18}O water at pH 8.5 for 30 min, and then the reaction was quenched by raising the pH to 12. (B) Same as in part A except that 9.2 mg of carbamoyl phosphate synthetase was included in the reaction mixture. Additional details are given in the text.

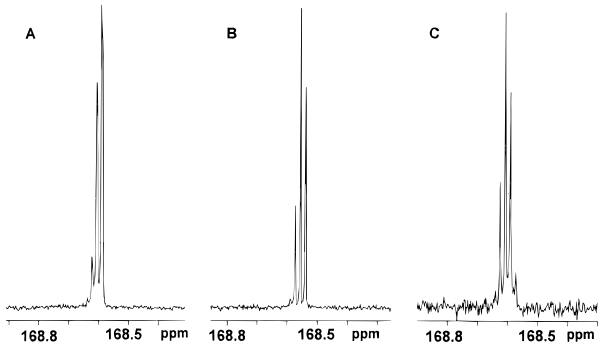


FIGURE 3: ^{13}C NMR spectra for bicarbonate. (A) [^{18}O,^{13}C]-Bicarbonate (79 at. % ^{18}O) prepared by incubation of [^{13}C]-bicarbonate in ^{18}O-labled water. (B) [^{18}O,^{13}C]Bicarbonate (15 mM) was incubated with 20 mM MgATP and 20 mM glutamine in the presence of unlabeled water for 5 min, and then the reaction was quenched by raising the pH to 12. (C) Same as in part A except that 2 mg of carbamoyl phosphate synthetase was included in the reaction mixture. Additional details are given in the text.

KINSIM program. The observed rate of oxygen exchange in the nonenzymatic control was 0.044 ± 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 ± 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 min reaction period showed that 13.2 ± 0.6 μmol of ADP was formed. Thus, the average rate for the enzymatic production of ADP during the 30 min incubation period was 0.44 ± 0.02 μmol min^{-1}. This reaction rate conforms reasonably well with the initial rate for the CPS-catalyzed water−bicarbonate exchange of 0.51 ± 0.07 μmol min^{-1} (6.7 μmol × 0.076 min^{-1}). This result thus confirms that an exchange of an oxygen atom from solvent water to 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 ^{18}O from labeled bicarbonate to the solvent water pool. The initial concentrations of ATP and bicarbonate were adjusted to ensure that measurable amounts of bicarbonate would remain at the end of the incubation period. Ornithine transcarbamoylase and ornithine were added to the reaction mixture 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 NMR spectrum of the initial [^{18}O,^{13}C]bicarbonate (at pH 12) is shown in Figure 3A. The ratio of species containing zero to three atoms of ^{18}O is 2:9:38:51 (79 ± 3 at. % ^{18}O). When 15 mM labeled bicarbonate was incubated with MgATP and glutamine in the presence or absence of CPS in a volume of 1.0 mL for 5 min, there was no significant difference in the final ratios of the bicarbonate species containing variable amounts of ^{18}O. The NMR spectra of the labeled bicarbonate after incubation in the absence and presence of CPS are shown in parts B and C of Figure 3, respectively. The net turnover of ATP in the presence of CPS was determined to be 13.0 ± 0.6 μmol on the basis of 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 ± 0.3 μmol 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 ratio of the various species of bicarbonate containing zero to three atoms of ^{18}O was found to be 2:16:47:35 (72 ± 3 at. % ^{18}O). These values correspond to a rate constant of 0.061 ± 0.011 min^{-1} for the nonenzymatic exchange reaction. In the presence of CPS, the ratio of the various species of bicarbonate containing zero to three atoms of ^{18}O was found to be 2:19:46:33 (70 ± 3 at. % ^{18}O). These values correspond to a rate constant for water−bicarbonate exchange of 0.075 ± 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 ± 0.012 min^{-1} (0.075−0.061 min^{-1}). The upper limit for the initial rate of exchange would be 0.21 μmol min^{-1} (0.014 min^{-1} × 15 μmol of bicarbonate). The nucleotide switch mechanism requires that a water−bicarbonate exchange reaction occur in concert with carbamoyl-P synthesis. However, in the presence of glutamine, the rate of carbamoyl-P formation (1.3 μmol min^{-1}) is greater than 6 times faster than the rate of water−bicarbonate exchange.

The NMR 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 imposed by the parallel mechanism (*13*) as originally formulated. If the nucleotide switch mechanism was fully functional, then the expected ratio of labeled bicarbonate at the end of the incubation period would be 6:26:44:24 (62 at. % ^{18}O). This ratio was calculated with the model for isotopic exchange that appears in Scheme 3 and the measured values for the rates of nonenzymatic exchange and the formation of carbamoyl-P. The predicted values for the 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.

In the original proposal for the parallel reaction mechanism for CPS, specific reference was made to seven experimental

observations that were interpreted to be consistent with the nucleotide switch mechanism but inconsistent with the sequential mechanism (*13*). However, none of the points raised in the original presentation of the nucleotide switch mechanism can be used to support one mechanistic proposal compared to the other. The experimental observations are summarized below.

Positional Isotope Exchange (PIX). Positional isotope exchange experiments have been used by a number of laboratories to demonstrate the kinetic competence of carboxy phosphate formation in the absence of a nitrogen source (*4*, *5*, *17*, *18*). In some, but not all, of these investigations, the PIX reaction was not eliminated upon the addition of either ammonia or glutamine to the assay mixture. Kothe et al. (*13*) have interpreted this observation to suggest that there is an alternative pathway for the formation of carboxy phosphate from a second site. A more likely alternative to this interpretation is the occurrence of a PIX reaction from either the enzyme·ADP·carboxy phosphate·NH_3 or the enzyme·ADP·carbamoyl-P·ADP complex. There is thus no requirement for the occurrence of a PIX reaction from a second site.

Binding of Both Molecules of ATP before Ammonia. There is some disagreement in the literature with regard to whether one or both molecules of ATP are able to bind to the enzyme prior to the binding of the nitrogen source (*5*, *19*, *20*). However, as formulated in the reaction mechanisms depicted in Schemes 1 and 2, it makes no difference whatsoever whether the binding of NH_3 and both molecules of ATP is ordered, sequential, or random since the chemical events outlined in either of these schemes do not dictate a particular kinetic mechanism.

Lability of Carbamate through a Tunnel. The sequential mechanism requires that the carbamate intermediate migrate from its site of synthesis within the N-terminal half of CPS to the site of phosphorylation within the C-terminal half of the protein. Examination of the electron density obtained for the X-ray crystal structure of CPS from *E. coli* clearly indicates the existence of a molecular tunnel connecting the two active sites (*7*). There is no a priori reason to believe that carbamate cannot migrate fast enough between these sites without decomposition to bicarbonate and ammonia. Carbamate is a known chemical entity that is quite stable at elevated pH. The measured half-life for carbamate at pH 8.0 in aqueous solutions is approximately 3×10^{-1} s (*21*). A rough estimate for the mean diffusion time (τ) for a one-dimensional walk of 50 Å through the molecular tunnel can be calculated from eq 4

$$\tau = L^2/3D \qquad (4)$$

where L is the distance traveled and D is the one-dimensional diffusion constant (*22*). There is no way of knowing precisely the diffusion constant for carbamate within the interior of CPS, but if a value of 10^{-5} cm^2 s^{-1} is assumed, then $\tau = \sim 10^{-7}$ s (*22*). It would thus appear that the mean residence time of carbamate in the tunnel of CPS is not a significant factor for either the overall stability of this intermediate or the turnover of the protein, even if the diffusion constant is inaccurate by many orders of magnitude.

Sequence Identity with Carbamate Kinase. Kothe et al. (*13*) have taken note of the fact that CPS proteins from most organisms do not have much sequence in common with carbamate kinase (*23*). The original formulation of the sequential reaction mechanism for CPS suggested that the modern-day enzyme arose through the mutation of a protein with catalytic properties similar to those of carbamate kinase, since the last step in the mechanism depicted in Scheme 1 is identical to the reaction catalyzed by carbamate kinase. However, CPS has been shown to be homologous to a number of other proteins such as biotin carboxylase (*24*) and D-alanyl-D-alanine ligase (*25*) that are known to catalyze very similar chemical reactions. The chemical events depicted in Scheme 1 are in no way dependent on whether CPS is related to carbamate kinase or any other protein.

Structural "Equivalence" of N- and C-Terminal Domains of CPS. The X-ray structure of the *E. coli* protein has clearly demonstrated that the two major domains in the N- and C-terminal halves of the large subunit of CPS are topologically similar but not identical (*7*). Sequence alignment studies have shown that approximately 40% of the amino acid residues between these two halves of the protein are identical (*8*). Comparison of CPS proteins from many different sources has identified a significant number of "differentially conserved" residues. These are residues that are fully conserved in all organisms within one domain but are not conserved with the identical amino acid at the homologous site in the other domain.[2] Kothe et al. (*13*) have argued that the sequential mechanism, as written in Scheme 1, does not require a "full N-ligase" type of mechanism for the chemical events occurring at the C-terminal domain since there is no formal requirement for amide formation at this site. However, the molecular switch mechanism for any CPS that utilizes glutamine as a nitrogen source cannot require a phosphorylation event and subsequent amide formation at both sites since only 1 equiv of ammonia is available from the hydrolysis of glutamine during each turnover. Therefore, the structural and sequence similarities of the two catalytic domains in CPS provide the same experimental support for either mechanism.

Catalytic Properties of Selected Mutants. It has been repeatedly demonstrated that site-directed mutations constructed within the N-terminal half of the large subunit of the CPS from *E. coli* predominantly affect the turnover number for the bicarbonate-dependent ATPase reaction, while the analogous mutants made within the C-terminal half of the same subunit predominantly affect the ATP synthesis reaction (*9*−*12*). These results have been used to assign the functional properties to each of these structural domains (*9*). Kothe et al. (*13*) have noted a slightly bigger increase in the K_m values for the nucleotide in the ATPase reaction with mutants constructed within the C-terminal domain than in the K_m values for the nucleotide in the ATP synthesis reaction when mutants were constructed in the N-terminal domain of CPS. However, these effects on K_m are in general much smaller than the effects on V_{max}. Moreover, the Michaelis constants are a complex function of many individual rate constants and should not be equated with general effects on binding alone.

Functional Equivalence of the Two Catalytic Domains. In the intact protein, the two homologous catalytic domains

[2] J. E. Raushel, unpublished results.

of CPS are neither structurally nor functionally equivalent (*7*). The parallel mechanism does not predict that the two domains must be functionally equivalent since only one of these domains can utilize ammonia in enzymes that require glutamine as a nitrogen source. Moreover, reports of catalytic equivalence for each of these structural domains in some of the truncated versions of the *E. coli* CPS are incompatible with the known X-ray structure (*26*).

Summary. Isotopic oxygen exchange between solvent water and bicarbonate was measured during the bicarbonate-dependent ATPase reaction and the overall biosynthetic reaction catalyzed by carbamoyl phosphate synthetase. An oxygen atom from bicarbonate was found to exchange with solvent water during every turnover of the bicarbonate-dependent ATPase reaction in the absence of added glutamine. This exchange reaction was fully suppressed upon the addition of glutamine and the subsequent formation of carbamoyl phosphate. These results are incompatible with the nucleotide switch mechanism, but they are fully consistent with a mechanism that utilizes the physical migration of carbamate between the two phosphorylation domains via a molecular tunnel.

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