Determination of Rate-Limiting Steps of *Escherichia coli* Carbamoyl-Phosphate Synthase. Rapid Quench and Isotope Partitioning Experiments†

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**Abstract:** The ATPase and carbamoyl phosphate synthesis reactions of *Escherichia coli* carbamoyl-phosphate synthase have been examined by using rapid quench and isotope partitioning techniques. The time course of the ATPase reaction is characterized by a "burst" of 1 enzyme equiv of acid-labile phosphate, followed by a slower steady-state rate (0.20 s⁻¹). The rate constant for the transient phase (4.5 s⁻¹) is close to the overall rate constant for the carbamoyl phosphate synthesis reaction, suggesting that the formation of carboxy phosphate is rate limiting for the overall reaction (3.1 s⁻¹). Binding of both molecules of ATP prior to the addition of glutamine could not be demonstrated by using isotope partitioning experiments. This suggests that either the ATP used in the reaction for the phosphorylation of carbamoyl does not bind significantly to the enzyme until after the addition of glutamine or its dissociation rate constant from the enzyme must be very fast compared with the rate constant for the overall reaction. In addition, carbamoyl-phosphate synthase was found not to catalyze an ¹⁸O exchange from [γ-¹⁸O]ATP during the ATPase reaction. When carbamoyl-phosphate synthase was incubated with ³²P, carbamoyl phosphate, and ADP, less than 1% of the ATP synthesized resulted from reaction with the P. This suggests that the complete forward reaction cannot be totally reversed and that some intermediate (presumably carbamate) must be dissociating irreversibly from the enzyme at least 100 times faster than its reaction with P. All of the above data are in accord with our previously published steady-state kinetic scheme showing sequential addition of substrates (Raushel, F. M., Anderson, P. M., & Villafranca, J. J. (1978) *Biochemistry* 17, 5587-5591).

Carbamoyl-phosphate synthase from *Escherichia coli* catalyzes the reaction shown in eq 1. It has been proposed

\[ 2\text{MgATP} + \text{HCO}_3^- + \text{l-Gln} \rightarrow 2\text{MgADP} + \text{carbamoyl-P} + \text{Pi} + \text{l-Glu} \] (1)

by Anderson & Meister (1965) that carbamoyl-phosphate synthase catalyzes this overall reaction via the series of reactions shown in eq 2-4. In support of this proposal it has been found that the enzyme catalyzes a bicarbonate-dependent ATPase reaction and that ATP can be synthesized from ADP and carbamoyl phosphate (Anderson & Meister, 1966). However, the complete forward reaction cannot be totally reversed since only 1 mol of ATP can be synthesized per mol of carbamoyl phosphate consumed, either in the presence or in the absence of Pi, (Anderson & Meister, 1966).

Evidence supporting the partial reactions catalyzed by carbamoyl-phosphate synthase of bacterial (Trotta et al., 1973) and mammalian (Jones, 1976) origin has been reviewed. Data from Meister's laboratory are consistent with the intermediate formed in a reaction pathway and not by an alternative pathway is that the formation and breakdown of the intermediate are equal to or faster than the slowest step in catalysis. Wimmer et al. (1978) showed formation of carboxy phosphate as a kinetically competent intermediate in the carbamylphosphate synthase reaction, and our present work demonstrates that the enzymatic reaction is characterized by "burst" kinetics. Thus a combination of the positional isotope exchange experiments (Wimmer et al., 1979) and our rapid kinetics experiments have defined the rate constants for the initial phase of the enzymatic reaction. The current results substantiate the steady-state kinetic scheme that we published last year (Raushel et al., 1978b) and suggest that formation of carboxy phosphate is rate limiting for the overall reaction.

Previous pulse-chase experiments of Powers & Meister (1978b) on the *E. coli* enzyme and of Rubio et al. (1979) on the enzyme from rat liver mitochondria demonstrated that the two ATP sites of this enzyme are functionally different. One ATP is involved in the formation of carboxy phosphate while the γ-phosphate of the other ATP is ultimately found in carbamoyl phosphate. Our studies on the stereospecificity of the enzymatic reactions with adenosine 5'- (2-thiothriphosphate) (Raushel et al., 1978a) show that the same diastereoisomer is used for both ATP reactions.

In this paper we report rapid-quench and isotope partitioning experiments that show that the ATP used in the reaction to synthesize carbamoyl phosphate does not bind significantly to the enzyme until after the addition of glutamine or that its dissociation rate constant from the enzyme must be fast compared to the rate constant for the overall reaction. These studies are in accord with our steady-state kinetic scheme (Raushel et al., 1978b) that shows sequential addition of ATP molecules interrupted by the chemical formation of carboxy phosphate.

Additional data utilizing ¹⁸O exchange techniques with [γ-¹⁸O]ATP and ³²P incorporation studies in the back-reaction...
have permitted calculation of partitioning of some of the intermediates in reactions 3 and 4.

Materials and Methods

Carbamoyl-phosphate synthase from E. coli was isolated by the procedure of Matthews & Anderson (1972). $[^{18}O]$P, was prepared by the method of Boyer & Bryan (1967). The $^{18}$O content was 78% as determined by mass spectrometric analysis (Midelfort & Rose, 1976) and by NMR (Cohn & Hu, 1978). $[^{18}O]$ATP was made by a procedure previously used to make $[^{18}O]$P (Mokrash et al., 1960). $[^{18}O]$P was obtained from ICN. All other biochemicals were obtained from Sigma.

Enzyme Assays. Carbamoyl-phosphate synthase activity in the forward and reverse directions was assayed spectrophotometrically as described previously (Rauschel et al., 1978a, b). At pH 7.5, 25 °C, the apparent Michaelis constants for MgATP in the ATPase and overall reaction in the presence of 10 mM ornithine were 15 and 200 μM, respectively.

Rapid Quench Experiments. A 0.3-mL solution containing 50 mM Hepes, pH 7.5, 25 mM MgCl₂, 16.7 mM HCO₃⁻, 10 mM ornithine, 0.33 mM $[^{32}P]$ATP (4.11 × 10⁶ cpm/μmol), and 100 mM KCl was added to rapid mixing to a 0.20-mL solution containing 37 μM carbamoyl-phosphate synthase, 50 mM Hepes, 10 mM ornithine, and 100 mM KCl. At various times the reaction was stopped by the addition of 0.5 mL of 0.1 N HCl containing 30 mg/mL acid-washed charcoal, with vigorous vortexing. The mixture was centrifuged, and an aliquot of the supernatant solution was counted for $^{32}$P activity. A blank contained everything but enzyme.

The ATPase reaction was also monitored at much faster times with a Durram Multi-Mixer apparatus as modified and described previously (Bennetovic et al., 1974). A solution containing 16 μM carbamoyl-phosphate synthase, 10 mM ornithine, 15 mM Mg²⁺, 50 mM Hepes, pH 7.5, and 100 mM KCl was added to an equal volume of 0.40 mM $[^{32}P]$ATP (7.08 × 10⁵ cpm/μmol, 15 mM Mg²⁺, 10 mM ornithine, 50 mM Hepes, pH 7.5, 20 mM HCO₃⁻, and 100 mM KCl. At varying times 2 volumes of 200 mM HCl were used to quench the reaction. A 0.30-mL aliquot of the quenched reaction solution was then mixed with 2.0 mL of 0.1 N HCl, containing 30 mg/mL charcoal. The mixture was centrifuged, and an aliquot of the supernatant solution was counted for $^{32}$P activity. A blank contained everything but enzyme.

Isotope Partitioning Experiments. Using the Durram Multi-Mixer apparatus, we added a solution containing carbamoyl-phosphate synthase (22-36 μM), 50 mM Hepes, pH 7.5, 100 mM KCl, 10 mM ornithine, and 10 mM Mg²⁺ to an equal volume of solution containing $[^{32}P]$ATP (0.4 or 4.0 mM), 50 mM Hepes, 20 mM HCO₃⁻, 10 mM ornithine, and 10 mM Mg²⁺. After a mixing time of 50 ms or 1.5 s, 2 volumes of a chase solution containing 20 mM MgATP, 50 mM Hepes, pH 7.5, 10 mM HCO₃⁻, 10 mM ornithine, 10 mM Mg²⁺, and 10 mM glutamate were added. The reaction was quenched at various times with 3.0 mL of 0.1 N HCl containing 30 mg/mL acid-washed charcoal.

Hydrolases of $[^{18}O]$ATP. In a volume of 1.0 mL, carbamoyl-phosphate synthase was incubated with 50 mM HEPES, pH 7.5, 100 mM KCl, 20 mM HCO₃⁻, 15 mM MgCl₂, 10 mM ornithine, and 2.0 mM $[^{18}O]$ATP until the hydrolysis of the ATP was nearly complete. The unreacted ATP and ADP were removed by the addition of 1.0 mL of 0.1 N HCl containing

![FIGURE 1: Time course for ATPase reaction catalyzed by carbamoyl-phosphate synthase. The reaction mixture contained 15 μM carbamoyl-phosphate synthase, 200 μM $[^{32}P]$ATP, 10 mM HCO₃⁻, and 15 mM MgCl₂. Additional details are described in the text.](image-url)

30 mg/mL acid-washed charcoal. The inorganic phosphate was isolated and the $^{18}$O content determined as described by Midelfort & Rose (1976).

$^{18}$O Exchange from $[^{18}O]$P, during the Back-Reaction. In a volume of 2.0 mL, 6 mg of carbamoyl-phosphate synthase was incubated with 5.0 mM ornithine, 100 mM Hepes, 100 mM KCl, 15 mM MgCl₂, 5 mM ADP, 5 mM EDTA, 50 mM carbamoyl phosphate, 50 mM $[^{18}O]$P, 75 mM glucose, 100 units of yeast hexokinase, and 20% D₂O. The $^{18}$O content and distribution in the P₇ were monitored approximately every 15 min by using the $^{31}$P NMR method of Cohn & Hu (1978) until all of the carbamoyl phosphate was exhausted. The $^{31}$P NMR measurements were made on a Bruker WH360 instrument operating at 145.7 MHz.

Reaction of $^{32}$P, during the Back-Reaction. In a volume of 1.5 mL, 2.4 mg of carbamoyl-phosphate synthase was incubated with 67 mM HEPES, pH 7.5, 134 mM KCl, 50 mM P₇ (6.02 × 10⁴ cpm/μmol), 10 mM MgCl₂, 50 mM carbamoyl phosphate, 100 mM glucose, 100 units of yeast hexokinase, and 3.33 mM ADP for 5 h. The reaction mixture was then diluted to 25 mL, titrated to pH 8.0 with KOH, and applied to a column (0.9 × 30 cm) of Dowex-1 (formate). The P₇ and glucose 6-phosphate were separated by using a 1.0-L gradient of 0.06 N formic acid (Bartlett, 1959). The fractions were counted for $^{32}$P activity and assayed for P₇ (Ames, 1966) and glucose 6-phosphate (Dreywood, 1946).

All of the above experiments were done at 22-25 °C.

Results

ATPase Reaction. The time course for the HCO₃⁻ dependent ATPase reaction catalyzed by carbamoyl-phosphate synthase at high enzyme concentrations is shown in Figures 1 and 2. Figure 1 shows the results when the reaction was quenched manually at times greater than 3 s. The reaction time course is characterized by a "burst" of acid-labile phosphate approximately equivalent to the concentration of enzyme sites. Shown in Figure 2 is the time course of the reaction with time points as low as 100 ms. The average steady-state rate from these experiments is 0.20 s⁻¹ as expected from earlier spectrophotometric studies. The average burst height corresponds to 86% of the total enzyme concentration. The transient rate constant was evaluated by subtracting the individual time points from the extrapolated steady-state rate and plotting the resulting data in a semilog plot as shown in the inset in Figure 2. This plot is characterized by a single exponential with a rate constant of 4.5 s⁻¹.

Isotope Partitioning Experiments. Shown in Figure 3A are the results from the isotope partitioning experiments when 15 μM carbamoyl-phosphate synthase, HCO₃⁻, and 0.20 mM radioactive MgATP are incubated for 50 ms, added to a

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1 Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.
indicates that 9.2 pM of ATP is trapped. This is exactly the
quenched at varying times. Since the plot extrapolates es-
sentially to zero at zero time, no amount of initially bound
ATPase reaction (see Figure 2).

100-fold excess of unlabeled MgATP and glutamine, and then
quenched at varying times. Since the plot extrapolates es-
entially to zero at zero time, no amount of initially bound
MgATP can be trapped as product (either as Pi or carbamoyl
phosphate). Figure 3 presents the results when the amount
of radioactive MgATP is increased to 2.0 mM. Again, none
of the initially bound MgATP could be trapped as product.

The results when 11 μM carbamoyl-phosphate synthase,
HCO₃⁻, and 0.2 mM radioactive MgATP are initially incu-
bated for 1.5 s before being added to a 100-fold excess of
unlabeled MgATP and glutamine are shown in Figure 3A.
The 1.5-s incubation is enough time for one turnover of the
ATPase reaction (see Figure 2). Extrapolation to zero time
indicates that 9.2 μM of ATP is trapped. This is exactly the

18O Exchange from [18O]P, during the Back-Reaction. Shown in Figure 4 is the 31P NMR spectrum of the reaction mixture
at various times when carbamoyl-phosphate synthase is added to 50 mM carbamoyl
phosphate, 5 mM ADP, and 50 mM [180]Pi. The peaks at 0 ppm correspond to the various Pi species containing zero to four atoms of
18O. The peak at 3.2 ppm upfield from [18O]Pi corresponds to
carbamoyl phosphate. Additional details are presented in the text.

Hydrolysis of [γ-18O]ATP. To determine if carbamoyl-
phosphate synthase catalyzed the 18O exchange from the
terminal phosphate of ATP in the ATPase reaction, the
enzyme was allowed to react with [γ-18O]ATP in the presence of
HCO₃⁻. When the reaction was nearly complete the nu-
cleotides were removed by the addition of charcoal, and the
phosphate was isolated and examined for 18O content and
distribution by mass spectrometry. Shown in Table I are the
results of the 18O analysis of the isolated phosphate along with
the analysis of 18O present initially in the γ-P of ATP. There is no detectable exchange of 18O catalyzed by car-
bamoyl-phosphate synthase. This experiment was also repeated at pH 6.5 and 8.5 with similar results. The inclusion of glutamine,
IMP, or UMP in the reaction mixture also did not change the
results. In addition, 2 mg/mL of carbamoyl-phosphate synthase was incubated with 5.0 mM MgADP, 50 mM
[18O]Pi, and 25 mM HCO₃⁻ for 68 h without any exchange of
18O.

Table I: Attempted Measurements of 18O Exchange Catalyzed by
Carbamoyl-Phosphate Synthase

<table>
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<tr>
<th>reaction</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<td>10.7</td>
<td>36.4</td>
<td>42.0</td>
<td>0.3</td>
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<td>10.8</td>
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</tr>
</tbody>
</table>

a Determined by the method of Midelfort & Rose (1976).
ferences in the various species of Pi containing 0-4 atoms of \(^{18}\text{O}\) (Cohn & Hu, 1978). The most downfield peak is due to \(\text{HP}^{18}\text{O}_2^-\). At 3.2 ppm is the singlet for carbamoyl phosphate. The extent of the chemical reaction was monitored by following the height of the carbamoyl phosphate peak. Any exchange of \(^{18}\text{O}\) from Pi was monitored by changes in the relative peak heights of the Pi peaks. Examination of the data showed a slight increase in the height of the peak due to \(\text{HP}^{18}\text{O}_2^-\), most likely due to some slow nonenzymatic hydrolysis of carbamoyl phosphate. The other peaks show no significant changes in their relative heights, indicating that there was no significant \(^{18}\text{O}\) exchange during the back-reaction.

**Reaction of \(^{32}\text{P}\) during the Back-Reaction**. When carbamoyl-phosphate synthase is incubated with MgADP, carbamoyl phosphate, and Pi, approximately 1 mol of ATP is produced per mol of carbamoyl phosphate consumed (Anderson & Meister, 1966). In addition, Pi does not affect the rate of the reaction, and thus the overall forward reaction cannot apparently be totally reversed (Anderson & Meister, 1966). To determine if a small fraction of the ATP produced came from reaction with Pi, carbamoyl-phosphate synthase was incubated with 5.0 mM MgADP, 50 mM carbamoyl phosphate, and 50 mM \(^{32}\text{P}\) in a volume of 1.5 mL. Glucose and yeast hexokinase were added to the reaction mixture to pull the reaction to completion. Any radioactivity found in the glucose 6-phosphate is thus a measure of the amount of reaction with Pi. After 5 h of incubation, the reaction products were separated on Dowex-1 (formate). The isolated glucose 6-phosphate totaled 68 amol and contained 34.300 cpm. From the specific activity of the radioactive Pi, the amount of the total ATP synthesized that results from reaction with Pi is 1.0%.

**Discussion**

**ATPase Reaction**. The minimal scheme for the ATPase reaction catalyzed by *E. coli* carbamoyl-phosphate synthase is

\[
E \overset{k_{5}}{\rightleftharpoons} ES \overset{k_{3}}{\rightleftharpoons} EP \overset{k_{1}}{\rightarrow} E + P
\]

In this scheme ES represents the enzyme-HCO\(_3^\cdot\)MgATP Michaelis complex and EP represents the enzyme-carboxy phosphate-MgADP complex. In the above scheme the relationships shown in eq 5-7 hold (Shafer et al., 1972).

\[
k_{\text{cat}} = \frac{k_{3}k_{S}}{k_{3} + k_{4} + k_{5}} \text{ (5)}
\]

transient burst amplitude \(= \frac{k_{3}(k_{3} + k_{4})}{(k_{3} + k_{4} + k_{5})^{2}} = \beta \text{ (6)}
\]

transient burst rate \(= k_{3} + k_{4} + k_{5} = \lambda \text{ (7)}
\]

following values were obtained from the pre-steady-state experiments: \(k_{\text{cat}} = 0.20 \text{ s}^{-1}, \beta = 0.86, \text{ and } \lambda = 4.5 \text{ s}^{-1}.
\]

In addition, Wimmer et al. (1979) have recently measured the \(\beta\) bridge/\(\beta\) nonbridge positional oxygen exchange in \(\beta\) bridge labeled \([\text{ATP}]\) by Midelfort & Rose (1976) with glutamine synthetase to demonstrate the participation of a \(\gamma\)-glutamyl phosphate intermediate (Rose, 1978). Wimmer et al. (1979) found this rate to be 1.4-1.7 times faster than the ATPase reaction rate.

In the above scheme this exchange rate \((k_{2})\), relative to the ATPase rate, has the relationship shown in eq 8. Since \(k_{2}\)

\[
k_{2} = \frac{k_{2}k_{4}}{k_{3}(l_{2} + k_{3})} \text{ (8)}
\]

\(> k_{3}\) (see below) this ratio reduces to \(k_{4}/k_{5}\). The values for \(k_{3}, k_{4}, k_{5}\) and \(k_{6}\) can now be computed. Using an average value of 1.5 for \(k_{3}/k_{\text{cat}}\), we obtained the values \(k_{3} = 3.9 \text{ s}^{-1}, k_{4} = 0.34 \text{ s}^{-1}, \text{ and } k_{5} = 0.23 \text{ s}^{-1}.
\]

Thus, \(k_{3}\) is predominate rate-determining for the ATPase reaction. This rate constant most likely represents the release of carboxy phosphate (or its hydrolysis products) or an enzyme conformational change that permits such product release. The value for \(k_{4}\) is very close to the \(k_{\text{cat}}\) for the overall synthase reaction (3.1 s\(^{-1}\)), and thus the formation of carboxy phosphate from ATP and bicarbonate is most likely rate limiting for the overall reaction in the formation of carbamoyl phosphate, ADP, and Pi from ATP, HCO\(_3^\cdot\), and glutamine (NH\(_3\)).

**Isotope Partitioning**. Since carbamoyl-phosphate synthase uses two molecules of ATP in the overall reaction, isotope partitioning experiments were initiated to determine if both ATP molecules can be on the enzyme at the same time. Our recent kinetic evidence indicates that the binding of the second ATP does not occur until after the release of Pi (Rauschel et al., 1978b). However, isotope partitioning experiments under somewhat different reaction conditions indicated that both molecules of ATP could be bound simultaneously (Powers & Meister, 1978).

When carbamoyl-phosphate synthase was incubated with radioactive ATP and HCO\(_3^\cdot\) for 50 ms and then mixed with a 100-fold excess of unlabeled ATP and glutamine, none of the initially bound ATP goes on to form product. The 50-ms incubation is enough time for the formation of the ternary complex (E-ATP-HCO\(_3^\cdot\)) but short enough so that there is no significant formation of acid-labile phosphate (see Figure 2). All of the enzyme should be in the ES complex in the above scheme. Since none of the initially bound ATP can be trapped as either Pi or carbamoyl phosphate, the ATP used in the formation of carboxy phosphate must be dissociating from the enzyme much faster than the \(k_{\text{cat}}\) for the overall synthase reaction. Therefore, \(k_{5}\) must be greater than 5-10 times faster than 3.1 s\(^{-1}\).

When the initial incubation period is increased so that there is enough time for one turnover (1.5 s; see Figure 2), the amount of Pi and carbamoyl phosphate trapped is approximately equal to 1 equiv of enzyme (Figure 3A). It is also equal to the amount of acid-labile phosphate formed when the
incubation mixture is immediately quenched after the 1.5-s incubation. Thus, the amount of ATP trapped corresponds to $P_i$ and a little or none corresponds to carbamoyl phosphate. Therefore, either the ATP used in the second half of the reaction does not bind to the enzyme until after the addition of glutamine or, if it is bound simultaneously with the first ATP, its release from the enzyme must be very fast compared with the overall turnover rate. If the dissociation constant for ATP at the second site, in the absence of glutamine, is significantly higher than the levels of ATP used in these experiments (0.2–2.0 mM), then ATP will appear not to bind. However, the levels used in our experiments are up to 10 times the $K_m$ for ATP in the overall synthase reaction (see Materials and Methods). This level of ATP should be high enough to detect a kinetically significant pathway that might differ from the one presented previously (Raushel et al., 1978b). The above results are fully consistent with the kinetic mechanism presented earlier that has an ordered addition of ATP, HCO$_3^-$, and NH$_3$, followed by $P_i$ release and the binding of the second ATP.

These results are in conflict with those obtained by Powers & Meister (1978). However, their experiments were conducted in phosphate (a product of the reaction) buffer at low Mg$^{2+}$ levels, while the experiments reported here were done in Heps buffer, 10 mM excess Mg$^{2+}$, and 10 mM l-ornithine. We have used excess Mg$^{2+}$ and the allosteric activator l-ornithine in our experiments because the double-reciprocal plot for ATP under these conditions is linear; this is an essential requirement for obtaining a meaningful kinetic scheme for an enzyme with so many substrates and products (Raushel et al., 1978b). The Michaelis constant for ATP is lowered by l-ornithine, and this allosteric effector also promotes oligomerization of the enzyme (Trotta et al., 1974). Another difference between the experimental conditions of Powers and Meister and ourselves is that they used up to 14 mM ATP while in our experiments 2.0 mM ATP was the highest concentration used. It should also be pointed out that the $K_m$ for ATP for the overall synthase reaction is 0.20 mM (Raushel et al., 1978b) under the conditions used in our experiments and 7.0 mM for half-maximal activity using their conditions (Anderson & Meister, 1966). Since both sets of experiments were conducted under different conditions that are known to affect the binding of ATP, it is quite possible for each pathway to predominate under different reaction conditions.

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$^1$ The amount of radioactive phosphate that is trapped with glutamine after 1.5 s corresponds to the amount that is trapped upon the addition of acid. For the calculation of the maximum amount of phosphate that can possibly be carbamoyl phosphate, the partitioning of the enzyme-carbamoyl phosphate (EP) complex has to be determined. (In the scheme the symbols have the same meaning as in the Discussion.)

\[ \frac{k_s}{k_d} = \frac{E + S}{E S} \times \frac{k_2}{k_4} = \frac{k_1}{k_3} \]

The rate constants $k_s$ and $k_d$ have the same value as before, but $k_1$ is now $>3.1 \text{s}^{-1}$, since this is the value of the enzyme turnover at saturating glutamine and thus no individual rate constant in the forward direction can be slower than this. If we assume that $k_2$ is fast, the partitioning of EP upon the addition of glutamine is $k_4/k_3$. This ratio is <0.11 and thus <11% of the isolated phosphate can be radioactive carbamoyl phosphate. The actual value is most likely much smaller than this since under our conditions the enzyme has been shown to operate with an ordered kinetic mechanism (Raushel et al., 1978b) and Wimmer et al. (1979) have shown that the positional isotope exchange ratio is reduced upon the addition of glutamine more than would be expected based on the increase in enzyme turnover only. Thus, the absolute maximum that can represent carbamoyl phosphate is <11%. This is within the experimental error of our data and thus the trapping of initially bound ATP as carbamoyl phosphate could not be accurately demonstrated by us.

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Rubio et al. (1979) have obtained evidence that the mammalian carbamoyl-phosphate synthase can catalyze the overall reaction by a different sequence of events than the enzyme from E. coli. They demonstrated a "burst" in $P_i$ production, but their pulse-chase experiments show that both ATP molecules can bind and react before they exchange with free ATP. They have therefore proposed an intermediate with the chemical composition of two ATP molecules and bicarbonate that subsequently reacts with ammonia (Rubio & Grisolia, 1977). Our data (this work and Raushel et al., 1978b) are consistent with an enzyme complex of chemical composition $E$-$ADP$-(carbamoyl or CO$_2$, NH$_3$)-ATP that reacts to form carbamoyl phosphate. Thus, the major difference between our results and those from Meister's and Grisolia's laboratories is the "timing" of the release of $P_i$ from the enzyme. The presence of excess Mg$^{2+}$ and the positive allosteric effector l-ornithine in our experiments (to promote full catalytic activity) may well accentuate this difference in the reaction mechanism.

**Hydrolysis of [$\gamma$-$^{18}$O]ATP.** Sauers et al. (1975) suggested that the activated form of bicarbonate that reacts with ammonia is not carboxy phosphate but enzyme-bound CO$_2$. This could arise from the breakdown of carboxy phosphate to CO$_2$ and $P_i$ as shown in Figure 5. The CO$_2$ would then react with NH$_3$ to form carbamate. In order to test this hypothesis, we synthesized [$\gamma$-$^{18}$O]ATP and allowed it to react with carbamoyl-phosphate synthase under the conditions of the ATPase reaction. If CO$_2$ is a reactive intermediate (Figure 5), it is formed from the breakdown of carboxy phosphate. Provided the enzyme-bound $P_i$ is free to rotate and recombine with CO$_2$, the bridge oxygen of carboxy phosphate is predicted to be labeled with $^{18}$O. Subsequent reaction with ADP on the enzyme would result in formation of ATP with one less $^{18}$O in the terminal phosphate.

When the ATP that has undergone $^{18}$O exchange is ultimately hydrolyzed, the resulting $P_i$ will have only two atoms of $^{18}$O instead of three. As shown in Table 1, carbamoyl-phosphate synthase does not catalyze an $^{18}$O exchange from the terminal phosphate of ATP. A negative result in this experiment does not disprove the existence of a CO$_2$ intermediate since the rotation of the bound $P_i$ might be hindered. A more likely explanation is that the rate constant for the resynthesis of carboxy phosphate from CO$_2$ and $P_i$ might be very slow compared with the release of carboxy phosphate from the enzyme ($k_s$) in the ATPase reaction.

The $^{18}$O exchange reported earlier in a preliminary abstract...
was found to be due to a contaminating enzyme (Raushel et al., 1978c).

Reaction of $^{32}$P during the Back-Reaction. When carbamoyl-phosphate synthase was incubated with MgADP, carbamoyl phosphate, and $^{32}$P, 1% of the ATP synthesized (after reaction with hexokinase and glucose to make glucose-6-phosphate) was found to have come from the P$_i$. Thus, the complete forward reaction catalyzed by carbamoyl-phosphate synthase can be reversed only to a very small extent. Since the reaction cannot be completely reversed (where 50% of the ATP would come from P$_i$), some intermediate in the reaction pathway must be dissociating irreversibly from the enzyme much faster than its reaction with P$_i$. The 1% conversion gives the partitioning ratio (1:100) for the reaction of the intermediate with P$_i$ vs. dissociation from the enzyme surface (Figure 6).

With the partial reactions proposed by Anderson & Meister (1965) (eq 2–4) and our overall kinetic scheme (Raushel et al., 1978a–c), the intermediate species under discussion is enzyme-bound carbamate (NH$_2$COO$^-$. In the back-reaction, P$_i$ will react with carbamate to form carboxy phosphate. This is most likely the slowest step, and thus carbamate must be dissociating at least 100 times faster from the enzyme than its reaction with P$_i$ to form carboxy phosphate. If the overall reaction goes through a CO$_2$ intermediate, then the P$_i$ would have to react with CO$_2$ to form carboxy phosphate after carbamate breaks down to form enzyme-bound CO$_2$ and NH$_3$.

The first-order rate constant for this reaction should be less than 0.003 s$^{-1}$. The small magnitude of this rate constant also explains why there is no observable $^{18}$O exchange for the ATPase reaction (if CO$_2$ is an intermediate) since this rate is almost 2 orders of magnitude slower than $k_{cat}$ for the ATPase reaction (0.20 s$^{-1}$).

Eliminated from consideration is the possibility of P$_i$ rapidly combining with CO$_2$ or carbamate to form carboxy phosphate which in turn reacts with ADP to form ATP with subsequent dissociation of ATP from the enzyme as the slowest step. This model is eliminated because in the isotope partitioning experiment it was determined that the release of ATP is very fast ($k_b$). Additionally, if this were the case, there would be no observable P$_i$ exchange from P$_i$ during the back-reaction and this is not observed in our 31P NMR experiment.

Acknowledgments

We thank Dr. P. M. Anderson for the preparation of the carbamoyl-phosphate synthase (GM-22434), Dr. T. R. Sharp for the preparation and analysis of the [$^{18}$O]P, Dr. S. J. Benkovic for the use of the Durran Multi-Mixer apparatus and for many helpful discussions, and Dr. I. A. Rose for sending us a preprint of his positional isotope exchange experiments. The 31P measurements were made at the Middle Atlantic NMR Facility, supported by National Institutes of Health Grant RR542 at the University of Pennsylvania.

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