Evidence That Carboxyphosphate Is a Kinetically Competent Intermediate in the Carbamyl Phosphate Synthetase Reaction*

Mary J. Wimmer and Irwin A. Rose
From The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111
Sue G. Powers and Alton Meister
From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021

In the bicarbonate-dependent ATPase and carbamyl phosphate synthesis reactions catalyzed by Escherichia coli carbamyl phosphate synthetase, the Pi formed was found to contain one oxygen derived from bicarbonate. This indicates that the bicarbonate requirement for ATPase activity is due to the same direct involvement of HCO₃⁻ in the ATP cleavage reaction as occurs in the presence of ammonia or glutamine. Kinetic evidence for a carboxyphosphate intermediate in the ATPase reaction was obtained by studying reversible ATP cleavage using βγ bridge:β non-bridge positional oxygen exchange in βγ bridge-labeled [¹⁸O]ATP. The enzyme was found to catalyze the reversible cleavage of ATP to bound ADP in the presence of bicarbonate and absence of ammonia or glutamine, at a rate that is 1.4 to 1.7 times the rate of net ATP cleavage to free ADP and Pᵢ. These results lend support to earlier chemical evidence for such an intermediate. In the same experiment, bicarbonate oxygen was not incorporated to a measurable extent into the ATP γ-PO₃⁻. Therefore, the regeneration of ATP does not occur from a complex of the form E·ADP·Pᵢ·CO₂ or a form in rapid equilibrium with it. The bicarbonate requirement for the reversible cleavage of ATP suggests that this intermediate contains ADP and carboxyphosphate, O

HO—C—PO₃⁻².

The presence of glutamine decreases the rate of positional exchange, consistent with its reaction with carboxyphosphate on the main catalytic pathway of carbamyl phosphate synthesis, but does not cause its disappearance, as would have been expected for an ordered reaction sequence.

An Appendix is given which shows how an exchange rate may be corrected to include substrate that has disappeared during the period of incubation.

Carbamyl phosphate synthetase, the biotin-dependent carboxylases, and P-enolpyruvate carboxylase all catalyze the transfer of CO₂ from bicarbonate to an acceptor substrate at the expense of a high energy phosphate compound (ATP or P-enolpyruvate). The inherent lack of reactivity of bicarbonate toward nucleophilic substitution and the appearance of one bicarbonate oxygen in the Pi, formed during these reactions implies the direct participation of phosphoryl bond cleavage in a bicarbonate activation process (1-3). Mechanisms currently under consideration for activation are illustrated in Scheme 1 for the carbamyl phosphate synthetase reaction. It has been proposed that, as in the activation of glutamate during the glutamine synthetase reaction (4, 5), an acylphosphate intermediate (carboxyphosphate) is formed which reacts directly with the carboxyl acceptor (6-8). In an expansion of this, it is considered that, although bicarbonate is the true substrate, CO₂ is the actual carboxylating agent (9).

In this mechanism, carboxyphosphate is not attacked by the nucleophile but decomposes unimolecularly to bind CO₂ and Pᵢ. The role of ATP is then to provide a high, localized concentration of CO₂ for efficient carboxyl transfer. If there is an acyl phosphate intermediate, ATP:ADP exchange might be expected to occur in the presence of bicarbonate and the absence of acceptor. Such exchange, however, has been found to be insignificant whether the acceptor is biotin in the carboxylases (6) or NH₃ in (proposed) carboxylation of carbamyl phosphate synthetase (10, 11). A concerted reaction, therefore, has been seriously considered (12, 13).

The fact that carbamyl phosphate synthetase has a strong bicarbonate-dependent ATPase activity (14) can be accommodated to all mechanisms if water replaces NH₃ in the proposed mechanisms (Scheme 1) or if bound intermediates, such as CO₂ and Pᵢ, or carboxyl-P, dissociate from the enzyme in the absence of NH₃. The ATPase activity is important for studying exchange reactions, including those described below, in the absence of carboxyl acceptor, as it allows for regeneration of free enzyme for continued cycling with new ATP molecules. Similar studies cannot be done with biotin enzymes because formation of E-biotin-CO₂⁻, the decarboxylation of which is slow, terminates the reaction once ADP or Pᵢ dissociate.

Evidence consistent with a carboxyphosphate intermediate in the reaction catalyzed by Escherichia coli carbamyl phosphate synthetase has been reported from pulse-chase studies (8). Direct chemical evidence for carboxyphosphate formation was obtained recently by chemically trapping this compound during an incubation of enzyme with ATP and bicarbonate as the trimethyl ester with diazomethane, or as formate by reduction with borohydride (15, 16). These results, however, do not provide kinetic information that can be related to the rates of the catalytic reactions. The lack of partial exchange reactions in the absence of NH₃ suggests that ADP is a
component of the E-carboxyphosphate complex. This provides the possibility of measuring the rate of formation of bound ADP-carboxyphosphate by the ATP βγ bridge; β non-bridge 18O scrambling technique of Midelfort and Rose (6). This method, illustrated in Scheme 2, measures the reversible cleavage of ATP to bound ADP and depends on the occurrence of two steps that are not normal components of the synthetase reaction: establishment of torsional symmetry of the three β-PO3 oxygens of bound ADP and return to free ATP by reversal of the phosphoryl transfer. Because one of these “ancillary” steps could be rate-limiting for the scrambling reaction, the measurement provides a minimum rate of phosphoryl transfer from ATP to the acceptor required for intermediate formation. H2O is presumed to react in the place of NH3 or glutamine in the ATPase reaction.

The additional question, whether CO2 is formed on the enzyme at a significant rate, may also be approached with 18O techniques (Scheme 2) because its formation must be concomitant with formation of P. If the P contains an oxygen atom of bicarbonate, then return of bound P to ATP could lead to oxygen exchange between HCO3 and the ATP γ-PO3. Return of bound P would also be shown by the incorporation of more than one bicarbonate oxygen in the free P produced. Because no significant ATP-18O exchange is catalyzed by carbamyl phosphate synthetase (11, 13), ATP:HCO3- oxygen exchange can result only from the return of an E-ADP-P:CO3(HCO3-) intermediate to ATP and HCO3-.

The 18O molecular and positional exchange approach makes the assumption that bound P and the β-PO3 of bound ADP are torsionally symmetrical in their proposed intermediate states. Although each study makes this assumption anew, it can be noted that evidence for extensive mixing of oxygens has been obtained in all cases examined (5, 17). A limitation inherent in the use of carbamyl phosphate synthetase for this study is that the rate of P incorporation into ATP by this enzyme occurs at a negligible rate under all reaction conditions. Therefore, failure to show either positional exchange or ATP:HCO3- exchange does not negate participation of either pathway in the forward reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carbamyl phosphate synthetase (specific activity, 139 μmol/h/mg at 37°C) was purified from E. coli and assayed as previously described (8, 14). The preparation was made free of ammonium salts and glutamine by Sephadex gel filtration. [3H]K4HCO3 (37.2%; 18O-enriched) was prepared by equilibration of KHCO3 with [3H]H2O in a sealed tube followed by lyophilization. The 18O enrichment was kindly determined by Dr. C. K. Tu in the Department of Pharmacology and Therapeutics, University of Florida. All other materials were previously described (5, 17). The distribution in the two [γ-18O]ATP preparations used of species containing zero, one, two, and three 18O's in the γ-PO3 were: Preparation 1, 15.8, 3.6, 20.0, and 60.6%; Preparation 2, 4.8, 4.0, 23.1, and 68.1%. The 18O bridge oxygen contained 77.7 and 82.0% 18O, and the β non-bridge oxygens contained 2.3% 18O in both samples.

**Methods**—Bicarbonate-free 1 M Tris/HCl, pH 7.9, was prepared by adding 1.21 g of Trizma base to 9.5 ml of 0.65 N HCl through which nitrogen had been bubbled. A bicarbonate-free stock solution of 100 mM Tris/HCl, 5 mM γ-'*O]ATP, [3H]ATP (5.0 × 105 cpm/cell), 0.2 mM MgCl2, and [3H]ADP tracer (9.0 × 104 cpm/ml), pH 7.9, was prepared as follows: [γ-18O]ATP, triethylamine salt, was passed through a 0.6-mul column of Dowex 50-H+ and brought to pH 4.5 with a fresh solution of Trizma base. [3H]ADP, MgCl2, and [γ-3P]ATP were added and the volume was adjusted to 90% of that required for the final concentrations. The solution was degassed by shaking 5 min in vacuo, then bubbled with nitrogen, and diluted to the final volume with bicarbonate-free 1 M Tris/HCl, pH 7.9. This ATP solution was kept in ice during the procedure to prevent ATP hydrolysis and was stored under nitrogen until use on the same day.

Incubations of carbamyl phosphate synthetase with [γ-18O]ATP were carried out at 37°C using the bicarbonate-free stock solution with other additions as indicated in Tables II and III. [3H]ADP tracer was added to detect synthesis of ATP from the pool of free ADP. The reactions, assayed by sampling for ADP, were stopped by freezing after dilution, and the ATP and P1 samples were purified by DEAE-cellulose chromatography. Details of purification, sample work-up, analysis of ATP for β2 bridge/β non-bridge positional oxygen exchange and γ-PO3:HCO3- oxygen exchange have been described elsewhere (5, 17). The effect on the exchange rate calculation due to the changing size of the ATP pool is described under “Appendix.”

Incubations with [16O]HCO3- were carried out at 37°C as indicated in Table I, with the addition of solid [16O]HCO3- made last to prevent HCO3-/H2O oxygen exchange before initiation of the reaction. Reactions were terminated by adding 0.05 ml of 6 N KOH after cooling in ice. (This treatment will also lead to hydrolysis of the synthesized carbamyl phosphate exclusively by C-O bond cleavage and so will produce a second molecule of Pi (1).) After neutralization with perchloric acid and removal of KClO4, by centrifugation, the P1 from each incubation was isolated and the 18O content after methylation was determined by published procedures (5, 17). The half-time for HCO3-: H2O oxygen exchange at pH 7.9, 37°C, is 7.8 min.1

Corrections of the 18O content of P, formed in the reaction were necessary due to dilution by 18O1, added with the carbamyl phosphate synthetase solution. The extents of dilution were determined by the decreases in [3P] specific radioactivity of [3P], originating from

1 C. K. Tu, personal communication.
a) **Concerted Displacement Mechanism:**

\[
\text{AMP} \rightarrow \text{AMP} \cdot \text{P} \cdot \text{O} \cdot \text{HCO}_2
\]

Reisolated ATP Expected:

\[
\text{AMP} \rightarrow \text{AMP} \cdot \text{P} \cdot \text{O} \cdot \text{HCO}_2
\]

Considering the statistical corrections involved, \( \beta \) bridge: \( \beta \) nonbridge positional isotope exchange occurs at the same rate as \( \gamma\)-PO\(_3\):HCO\(_3\) oxygen exchange.

b) **Carboxy Phosphate Intermediate Mechanisms:**

\[
\text{AMP} \rightarrow \text{AMP} \cdot \text{P} \cdot \text{O} \cdot \text{HCO}_2
\]

Reisolated ATP Expected:

1. \[
\text{AMP} \rightarrow \text{AMP} \cdot \text{P} \cdot \text{O} \cdot \text{HCO}_2
\]
   Positional exchange occurs without \( \gamma\)-PO\(_3\):HCO\(_3\) exchange.

2. \[
\text{AMP} \rightarrow \text{AMP} \cdot \text{P} \cdot \text{O} \cdot \text{HCO}_2
\]
   and
   Positional exchange occurs faster than \( \gamma\)-PO\(_3\):HCO\(_3\) exchange.

**Scheme 2.** Elucidation of the mechanism of ATP-dependent bicarbonate activation: use of \( \beta \) bridge: \( \beta \) non-bridge positional oxygen exchange in \([\text{\textsuperscript{18}}\text{O}]\text{ATP}\).

\([\gamma\text{\textsuperscript{31}}\text{P}]\text{ATP}\). The P\(_i\) concentrations were determined by the method of Fiske and Subbarow (18) before methylation. Acetate was determined by the increase in absorbance at 233 nm upon conversion to acetyl-CoA with acetyl-CoA synthetase; \( \Delta\epsilon_{233} = 4.44 \).

**RESULTS**

Incorporation of HCO\(_3\)\(^-\) Oxygen into P\(_i\)—Jones and Spector were the first to observe that approximately one bicarbonate oxygen is incorporated into the P\(_i\) produced during carbamyl phosphate synthesis catalyzed by ammonia-dependent frog liver carbamyl phosphate synthetase (1). If the overall and the HCO\(_3\)\(^-\) dependent ATPase reactions catalyzed by the \( E. \text{coli} \) enzyme (14) are due to the same bicarbonate activation mechanism, a similar result might be expected. To establish the source of oxygen that goes to form P\(_i\) reactions were carried out in the presence and absence of glutamine using \([\text{\textsuperscript{18}}\text{O}]\text{HCO}_3\) and \([\text{\textsuperscript{18}}\text{O}]\text{ATP}\) in \([\text{\textsuperscript{18}}\text{O}]\text{H}_2\text{O}\) (Table I). The \( \text{\textsuperscript{18}}\text{O} \) content of the P\(_i\) formed is found to be the same in both cases and equal to (within 3%) the average \( \text{\textsuperscript{18}}\text{O} \) enrichment of bicarbonate oxygen during the incubations. The m/e distribution\(^2\) of the P\(_i\) analyzed shows no multiple oxygen incorporation (the m/e 145 seen represents only the natural abundance contribution). This establishes HCO\(_3\)\(^-\) as the source of one specific oxygen in P\(_i\) during both the ATPase and overall reactions. Thus, the ATPase reaction catalyzed by \( E. \text{coli} \)

\(^2\) The abbreviations used are: m/e, ratio of mass to charge of the molecular ion; Ap\(_5\)A, P\(_i\),P\(_i\)-di(adenosine 5')-pentaphosphate.
carbamyl phosphate synthetase seems to use the same ATP-dependent bicarbonate activation mechanism that is associated with overall carbamyl phosphate synthetase synthesis.

This result justifies a study of the mechanism of ATP participation in the absence of ammonia or glutamine. Carbamyl phosphate synthetase appears to have two binding sites for ATP with different functions, one for synthesis of a phosphorylated carbamate, and one for the kinase reaction to form carbamyl phosphate (10, 19). Complications that would be introduced by reversible reactions at the kinase site will be avoided by focusing on the ATPase reaction.

**ATP βγ Bridge: β Non-bridge Positional Oxygen Exchange**—When [γ-32P]ATP containing 32P in the βγ bridge was incubated with the enzyme and HCO₃⁻ in the absence of glutamine, it was found that βγ bridge β non-bridge positional oxygen exchange occurred in the reisolated ATP pool (Table II, complete systems). Because no ATP [3H]ADP exchange occurred in the same incubation, one can conclude that the positional exchange represents reversible ATP cleavage to bound ADP only. This exchange proceeds at a rate that is 1.4 to 1.7 times the rate of production of free ADP and P₃. This represents a minimum rate for phosphoryl transfer in the presence of bicarbonate because of the additional steps required for the exchange, and therefore implies that the process that is measured is part of the overall catalytic process. That the reversible formation of bound ADP is catalyzed by carbamyl phosphate synthetase itself is shown by the requirement for K⁺ and the inhibition by ApA (19) in a manner similar to the net forward reaction. Deletion of bicarbonate decreases the rate of reversible ATP cleavage by 90% when ATP positional oxygen exchange due to ATPase from residual HCO₃⁻ is subtracted (Table II, Experiments 1 and 2). The bicarbonate-independent positional exchange may be the result of alternate substrate acetate (20) added with reagents (0.1 to 0.3 mM). The effect of added acetate (1 mM) shows the sensitivity of the exchange to acetate contamination. In Experiment 3 of Table II, with 4 mM glutamine (~100 × Kₘ), P₃ is produced 1.6 times faster than without, and yet the rate of reversible ATP cleavage is decreased by 25%.

**ATP γ-P₃: HCO₃⁻ Oxygen Exchange**—To distinguish between the proposed mechanisms of bicarbonate activation during the ATPase reaction, it was of interest to determine whether the E-ADP complex that returned to free ATP had the potential γ-P₃ group in the form of bound P₃ (with CO₂ or HCO₃⁻), or in the form of carbamyl phosphate only (Scheme 2). Return from P₃ would introduce one 18O (75% of the time) into the γ-phosphate of reisolated ATP. The mass spectral analysis of trimethyl phosphate derived from this γ-phosphoryl would show a decrease in m/e 147 with statistical increases in m/e 145, 143, and 141. Analysis of the γ-P₃ of the ATP samples from Experiment 3, Table II, and of the P₃ produced in each of the two incubations, showed the samples to be very nearly identical to each other and to the original ATP γ-P₃ (Table III). No correlation was found with the amount of positional exchange observed at the β-phosphate of the same ATP (Table II). If 20% of the ATP pool that had been reversibly cleaved (or ~10% of the total ATP) had resulted from return of bound P₃, the effect would have been well above experimental error, as shown. A concerted mechanism requires that γ-P₃: HCO₃⁻ oxygen exchange and bridge-non-bridge positional exchange in ATP occur at similar rates, and this is clearly ruled out by the data. If bound CO₂ were an intermediate...

### Table I

Source of oxygen in P₃ from ATPase and carbamyl phosphate synthesis reactions

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>m/e of trimethyl phosphate</th>
<th>Atom % 18O in total P₃ pool</th>
<th>Atom % 18O in P₃ formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>143</td>
<td>145</td>
<td>147</td>
</tr>
<tr>
<td>+Glutamine</td>
<td>195</td>
<td>0.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>

- Includes contribution of [18O]P₃ added with the enzyme.
- Corrected for dilution of [18O]P₃ by added [18O]P₃ determined by 32P isotope dilution to be 56% and 18%, respectively. The average 18O enrichment of KHC₃O₃, considering HCO₃⁻:H₂O oxygen exchange as described under "Methods," was 24.4% for both incubations.

### Table II

ATP βγ bridge: β non-bridge positional oxygen exchange during the ATPase reaction of carbamyl phosphate synthetase

A aliquots of the bicarbonate-free [γ-32P]ATP stock solution (see "Methods") were brought, where indicated, to 100 mM in KCl or NaCl, 10 mM in NaHC₃O₃, 0.12 mM in ApA, 1 mM in sodium acetate, and 4 mM in glutamine. The final concentration of ATP was 3 mM. Reactions at 37°C were begun by addition of carbamyl phosphate synthetase. Half of each ATP sample was analyzed for 32P enrichment of KHC₃O₃, considering HCO₃⁻:H₂O oxygen exchange as described under "Methods," was 24.4% for both incubations.

**Note:** As 18O enrichment appearing in the β non-bridge oxygen of the final ATP from the βγ bridge was determined as previously described (5). At equilibrium, two-thirds of the original bridge enrichment will be in the β non-bridge oxygens.

- **Scheme:** The data are consistent with the hypothesis that the equilibrium is a result of alternate substrate acetate (20) added with reagents (0.1 to 0.3 mM). The effect of added acetate (1 mM) shows the sensitivity of the exchange to acetate contamination. In Experiment 3 of Table II, with 4 mM glutamine (~100 × Kₘ), P₃ is produced 1.6 times faster than without, and yet the rate of reversible ATP cleavage is decreased by 25%.

**ATP γ-P₃: HCO₃⁻ Oxygen Exchange**—To distinguish between the proposed mechanisms of bicarbonate activation during the ATPase reaction, it was of interest to determine whether the E-ADP complex that returned to free ATP had the potential γ-P₃ group in the form of bound P₃ (with CO₂ or HCO₃⁻), or in the form of carbamyl phosphate only (Scheme 2). Return from P₃ would introduce one 18O (75% of the time) into the γ-phosphate of reisolated ATP. The mass spectral analysis of trimethyl phosphate derived from this γ-phosphoryl would show a decrease in m/e 147 with statistical increases in m/e 145, 143, and 141. Analysis of the γ-P₃ of the ATP samples from Experiment 3, Table II, and of the P₃ produced in each of the two incubations, showed the samples to be very nearly identical to each other and to the original ATP γ-P₃ (Table III). No correlation was found with the amount of positional exchange observed at the β-phosphate of the same ATP (Table II). If 20% of the ATP pool that had been reversibly cleaved (or ~10% of the total ATP) had resulted from return of bound P₃, the effect would have been well above experimental error, as shown. A concerted mechanism requires that γ-P₃: HCO₃⁻ oxygen exchange and bridge-non-bridge positional exchange in ATP occur at similar rates, and this is clearly ruled out by the data. If bound CO₂ were an intermediate...

### Table I

| Source of oxygen in P₃ from ATPase and carbamyl phosphate synthesis reactions |
|-----------------------------------|-----------------|-----------------|-----------------|
| Each incubation contained 100 mM Tris/HCl (pH 7.9), 100 mM KCl, and 20 mM [18O]KHC₃O₃ (initial 18O enrichment, 37.2%) in a total volume of 10 mL. Incubations with or without 8 mM glutamine also contained, respectively, 6 or 10 mM [γ-32P]ATP (6.3 × 10⁵ or 3.5 × 10⁶ cpm/μmol), 6 or 11 mM MgCl₂, and 0.12 or 0.024 unit of carbamyl phosphate synthetase (measured as ATPase, where 1 unit = 1 pmol of ADP/min at 37°C). Reactions were terminated as described under "Methods" after 18 min at 37°C, during which time 2.16 and 1.91 pmol of P₃ were produced. The purified samples of P₃ were methylated and analyzed for 18O content as previously described (5, 17). m/e values are shown as percents of total signal area. |

### Table II

ATP βγ bridge: β non-bridge positional oxygen exchange during the ATPase reaction of carbamyl phosphate synthetase

**Note:** As 18O enrichment appearing in the β non-bridge oxygen of the final ATP from the βγ bridge was determined as previously described (5). At equilibrium, two-thirds of the original bridge enrichment will be in the β non-bridge oxygens.

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Carboxyphosphate in the Carbamyl Phosphate Synthetase Reaction

A: (-) Glutamine

\[ + \text{ATP} + \text{HCO}_3^- \rightarrow \text{ADP} \rightarrow \text{ADP} + P_i + \text{HCO}_3^- \]

Reversibly-cleaved ATP + \[ \text{H}_2\text{O} \]

B: (+) Glutamine

\[ + \text{ATP} + \text{HCO}_3^- \rightarrow \text{ADP} \rightarrow \text{ADP} + 2 \text{ADP} + P_i + \text{H}_2\text{NCO}_2\text{PO}_4^- + \text{Glu} \]

\[ \text{Reversibly-cleaved ATP} \]

Table III

Lack of ATP:HC\text{O}_3^- and P_i:HC\text{O}_3^- oxygen exchange during ATPase and carbamyl phosphate synthesis reactions

The y-phosphoryl of half of each ATP sample reisolated from Experiment 3, Table II, and the P_i produced during each incubation, were converted to trimethyl phosphate and analyzed for \(^{18}O\) as previously described (5, 17). Mass spectra were normalized to \(m/e\ 147 = 100.0\) for comparative purposes.

<table>
<thead>
<tr>
<th>Phosphate analyzed</th>
<th>Mass spectrum of trimethyl phosphate from (\gamma)-PO_3 or total P_i</th>
<th>(m/e\ 145/m/e\ 147)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original ATP (\gamma)-PO_3 (^a)</td>
<td>7.0 5.9 33.9 100.0</td>
<td>0.34</td>
</tr>
<tr>
<td>Table II, Experiment 3 (-glutamine) ATP (\gamma)-PO_3 (^b)</td>
<td>3.8 6.2 35.2 100.0</td>
<td>0.35</td>
</tr>
<tr>
<td>P_i (^b)</td>
<td>296.8 7.8 36.6 100.0</td>
<td>0.36</td>
</tr>
<tr>
<td>Table II, Experiment 3 (+glutamine) ATP (\gamma)-PO_3 (^b)</td>
<td>6.5 5.9 34.5 100.0</td>
<td>0.35</td>
</tr>
<tr>
<td>P_i (^b)</td>
<td>86.4 5.9 34.9 100.0</td>
<td>0.35</td>
</tr>
<tr>
<td>Expected (\gamma)-PO_3 for 10% return of bound P_i (^b)</td>
<td>7.7 8.1 42.9 100.0</td>
<td>0.43</td>
</tr>
</tbody>
</table>

\(^a\) Converted to trimethyl phosphate containing the \(\gamma\)-PO_3 and one \(^{18}O\) from dihydroxyacetone, as previously described (5). The original \(\gamma\)-PO_3 enrichment was 85.3%.

\(^b\) Includes contribution of \([^{16}O]P_i\), added with the enzyme.

\(^c\) Approximated as follows. If 10% of the ATP pool resulted from return of bound P_i, assuming torsional freedom of the P_i: 7.5% of \(m/e\ 147\) \(\rightarrow\) \(m/e\ 145\); 5.0% of \(m/e\ 145\) \(\rightarrow\) \(m/e\ 143\); 2.5% of \(m/e\ 143\) \(\rightarrow\) \(m/e\ 141\).

Discussion

The ATPase and overall reactions catalyzed by carbamyl phosphate synthetase have not been demonstrated to be reversible (13), and ATP:P_i and ATP:ADP partial exchange reactions associated with the bicarbonate activation step proceed at less than 1% the rate of ATPase (16). It has now been demonstrated, however, by ATP \(\gamma\) bridge:\(\beta\) non-bridge positional isotope exchange that the initial ATP cleavage is readily reversible, proceeding at a rate that is 1.4 to 1.7 times the ATPase rate in the absence of ammonia or glutamine; this finding suggests that a high energy intermediate is formed. The marked enhancement by bicarbonate of reversible ATP cleavage and the demonstration that one oxygen from bicarbonate is transferred to the P_i produced in the ATPase reaction support the conclusion that bicarbonate is covalently involved in the cleavage reaction. Prior phosphoryl transfer to a group on the enzyme is inconsistent with the requirement for bicarbonate in showing ATP positional exchange, unless HCO_3^- were to provide a necessary route for regeneration of free enzyme required for additional cycles of exchange. However, this explanation, which implies that ADP is released when E-P is formed, also requires that a significant ATP:ADP exchange rate be seen in the absence of HCO_3^- when in fact there is none.

\(^d\) M. J. Wimmer, unpublished observations.
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