Enzymatic Synthesis of Acetyl Phosphate and Formyl Phosphate by Carbamyl Phosphate Synthetase*

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The utilization of carbamyl phosphate and acetyl phosphate for synthetic purposes has been studied in this laboratory (1-4). Frog liver carbamyl phosphate synthetase catalyzes the synthesis of adenosine triphosphate from acetyl phosphate and adenosine diphosphate (4); it has also been briefly reported that the enzyme could synthesize ATP from formyl-P and ADP (5). The reverse reactions, the syntheses of acetyl-P and formyl-P from acetate and formate and from ATP (Equations 1 and 2), remained to be demonstrated.

\[
\begin{align*}
\text{Acetyl-P} + \text{ADP} &\quad \xrightarrow{\text{Mn}^{++}, \text{Mg}^{++}} \text{acetyl + ATP} \\
\text{Formyl-P} + \text{ADP} &\quad \xrightarrow{\text{Mn}^{++}, \text{Mg}^{++}} \text{formate + ATP}
\end{align*}
\]

Equations 1 and 2

This paper is concerned with the study of the synthesis of acetyl-P and formyl-P by carbamyl-P synthetase.

EXPERIMENTAL PROCEDURE

Frog liver carbamyl-P synthetase was prepared according to Marshall, Metzenberg, and Cohen (6). The cetyltrimethylammonium bromide extracts were made 0.05 m in Tris-HCl, at pH 7.4, and the enzyme was precipitated with acetone (15°C; final concentration, 25% by volume) or with ammonium sulfate (pH 7.4, 40 to 50% saturation). Their specific activities were about 11 and 16, respectively. The 85 to 90% pure enzyme (6) would approximate a specific activity of 22 under our assay conditions. Purified rat liver carbamyl-P synthetase (7) varied in specific activity from 10 to 15.

Acetyl-P and carbamyl-P were purchased from the Sigma Chemical Company. Formyl-P was a gift from Dr. E. Stadtman.

Acetyl-P and formyl-P were measured by the method of Lipmann and Tuttle (8).

ATP was measured in an assay system containing glucose, TPN, hexokinase, and glucose 6-phosphate dehydrogenase. Citrulline, PPi, Pi, ornithine, and protein were determined by standard procedures (9-13).

The first extraction with cetyltrimethylammonium bromide yields most of the enzyme; we have therefore eliminated the second extraction. The enzyme is often denatured during the acetone precipitation step unless salt is added; the fraction precipitating between 25 and 40% contains a low percentage of the synthetase, of very low specific activity.

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Reagents were neutralized with KOH.

Carbamyl-P synthesis was determined in an assay system containing the following (in micromoles): Tris-HCl, pH 7.4, 100; ATP, 10; acetylglutamate, 10; MgCl2, 20; KHCO3, 100; NH4Cl, 50; ornithine, 10. Twenty units of ornithine transcarbamylase (14) were added to each tube. The final volume was 2 ml; incubation was performed at 37°C for 15 minutes. The reaction was stopped by the addition of 2 ml of 1 M perchloric acid. Specific activity is defined as micromoles of carbamyl-P synthesized per mg of protein, under these conditions.

Acetyl-P and formyl-P synthesis was routinely assayed in a system containing the following (in micromoles): Tris-HCl, pH 7.4, 100; acetate, pH 7.5, 1000 (or formate, pH 7.4, 1100); ATP, 10; acetylglutamate, 10; MnCl2, 20; hydroxylamine, pH 7.4, 200. The final volume was 2 ml; incubation was performed at 37°C for 30 minutes. The reaction was terminated by addition of 1 ml of 2 m hydroxylamine at pH 7.4, followed by a FeCl3 reagent.

When acetyl-P formation was coupled to acetylornithine synthetase (3), no hydroxylamine was added, but the system was supplemented with 10 μmoles of ornithine and 50 units of rat liver ornithine transcarbamylase (14).

RESULTS

Effect of pH—Fig. 1 shows that frog liver carbamyl-P synthetase is active in acetyl-P and formyl-P synthesis within a broad pH range. It is noteworthy that synthesis proceeds at pH 10, even though the pH optima are 6.5 and 6.8, respectively. This may be related to the activation of the synthetase by acetylglutamate, a phenomenon which begins at pH 5.6 and is still close to maximal at pH 10 (5).

To facilitate comparison with carbamyl-P synthetase, most experiments were conducted at pH 7.4.

Effect of Metals—A divalent cation is necessary and, as shown in Table I, acetyl-P and formyl-P synthesis proceeds much more readily in the presence of Mn++ than of Mg++; this is in contrast to carbamyl-P synthesis, in which Mg++ is more effective than Mn++ (14). Ca++ cannot replace Mg++ or Mn++. Also, Mn++ is more stimulatory than Mg++ in several partial reactions catalyzed by the synthetase: (a) the release of P1 in the absence of ammonia (15, 16); (b) the release of P1 when hydroxylamine replaces ammonia (14, 16); and (c) the synthesis of ATP from carbamyl-P and ADP (14).
Effect of pH—The addition of coenzyme A or passage of the enzyme through a Dowex 2 column to insure the removal of possible traces of coenzyme A (21) does not affect the acetate-activating capacity of the synthetase.

Utilization of Formyl-P—Carbamyl-P synthetase catalyzes

Effect of Mg++ and of Mn++ on synthesis of formylhydroxamate and of acetylhydroxamate by rat and frog liver carbamyl-P synthetase

For assay with formate or with acetate, 0.6 or 0.7 mg of carbamyl-P synthetase from rat liver and 1.6 or 6 mg of the enzyme from frog liver were used, respectively. The conditions were as described in the text. Mg++ or Mn++ was added as indicated.

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>Metal added</th>
<th>Rat enzyme</th>
<th>Frog enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg++</td>
<td>Mn++</td>
<td>Mg++</td>
</tr>
<tr>
<td>Formate</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>1.9</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.2</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>1.1</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>1.1</td>
<td>2.4</td>
</tr>
</tbody>
</table>

TABLE II

Effect of acetylglutamate on synthesis of acetyl- and formylhydroxamate by frog and rat liver carbamyl-P synthetase

Frog liver carbamyl-P synthetase, 1.8 mg (3.5 for the values in parentheses), was incubated with the following (in micromoles): MnCl₂, 20; ATP, 16; hydroxylamine, 200; acetate or formate, 1000. Final volume, 2 ml. Incubation was performed at 37° for 30 minutes.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Acetylglutamate added</th>
<th>Hydroxamate synthesized from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>Acetate</td>
</tr>
<tr>
<td>Frog liver</td>
<td>0.0</td>
<td>0.3 (0.2)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.3 (0.8)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.5 (1.3)</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.7 (1.9)</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.7 (2.2)</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.8 (2.3)</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>0.8 (2.3)</td>
</tr>
<tr>
<td>Rat liver</td>
<td>0.0</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Specificity—Under our experimental conditions, propionate and β-hydroxybutyrate do not appear to be substrates for the enzyme.
Enzymatic Synthesis of Acetyl and Formyl Phosphates

Vol. 239, No. 4

1274

the synthesis of ATP from acetyl-P and carbamyl-P (4, 15). Table III shows that ATP is also synthesized from formyl-P.

Stoichiometry—Many experiments were carried out with the rat liver enzyme. The ratios of ATP utilization to both formyl-P and acetyl-P formation varied from 0.7 to 1.3. Although the

TABLE III
Effect of formyl-P concentration on synthesis of ATP

Frog liver carbamyl-P synthetase, 1.6 mg, was incubated with the following (in micromoles): ATP, 10; MgCl₂, 50; Tris-HCl, pH 7.4, 1000 (Experiment I), or formate, pH 7.5, 1100 (Experiments II and III); acetylglutamate, 5; formyl-P, as indicated. Final volume, 1 ml. Incubation was performed at 37° for 15 minutes. In a control tube containing 5 pmoles of carbamyl-P in place of formyl-P, 1.5 pmoles of ATP were synthesized.

<table>
<thead>
<tr>
<th>Formyl-P added</th>
<th>ATP synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>1.8</td>
<td>1.3</td>
</tr>
<tr>
<td>3.5</td>
<td>2.0</td>
</tr>
<tr>
<td>7.0</td>
<td>2.4</td>
</tr>
</tbody>
</table>

TABLE IV
Stoichiometry of acetyl- and formylhydroxamate synthesis with frog liver carbamyl-P synthetase

In Experiment I, 7.2 mg of frog liver carbamyl-P synthetase per tube were used, and in Experiments II and III, 3.6 mg. The enzyme was incubated with the following (in micromoles): ATP, 10; MnCl₂, 20; hydroxyamine, 200; acetate, pH 7.6, 1000 (Experiment I), or formate, pH 7.5, 1100 (Experiments II and III); acetylglutamate, 10, when indicated. Final volume, 2 ml. Incubation proceeded at 37° for 30 minutes for Experiments I and II, and 60 minutes for Experiment III.

Experiment and additions | Hydroxamate formed | ATP utilized | P₁ liberated |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>µmoles</td>
<td>µmoles</td>
<td>µmoles</td>
<td></td>
</tr>
<tr>
<td>I. None</td>
<td>1.4</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Acetylglutamate</td>
<td>9.6</td>
<td>11.5</td>
<td>10.8</td>
</tr>
<tr>
<td>II. None</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Acetylglutamate</td>
<td>7.6</td>
<td>8.6</td>
<td>8.6</td>
</tr>
<tr>
<td>III. None</td>
<td>1.2</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Acetylglutamate</td>
<td>12.4</td>
<td>14.4</td>
<td>14.4</td>
</tr>
</tbody>
</table>

TABLE V
RF values of acetyl- and formylhydroxamate

Acetyl- and formylhydroxamate were isolated from standard incubation mixtures. The samples were heated at 100° for 2 minutes, then centrifuged; the supernatant fluids were extracted with 1-butanol, and the extracts were evaporated to dryness. Controls of chemically synthesized acetyl- and formylhydroxamate were treated identically. Solvent A contains isobutyl alcohol-formic acid-water (75:15:10); Solvent B, butanol-acetic acid-water (40:10:60); Solvent C, ethanol-water (80:20). Ascending chromatography was carried out on Whatman No. 1 paper, developments with FeCl₃ (22).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Acetylhydroxamate</th>
<th>Formylhydroxamate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental</td>
<td>Control</td>
</tr>
<tr>
<td>A</td>
<td>0.60</td>
<td>0.58</td>
</tr>
<tr>
<td>B</td>
<td>0.64</td>
<td>0.60</td>
</tr>
<tr>
<td>C</td>
<td>0.80</td>
<td>0.78</td>
</tr>
</tbody>
</table>

enzyme had been purified some 50-fold (the specific activity of liver extracts is about 0.3), it often contained enough adenylate kinase to account for high control values. These controls, however, were not entirely adequate, since carbamyl-P synthetase and adenylate kinase competed for ATP. Within the limits of accuracy imposed by the enzyme preparations, the data indicated that 1 mole of acetyl- or formylhydroxamate was synthesized per mole of ATP utilized.

With frog liver carbamyl-P synthetase, the stoichiometry is clear. Adenylate kinase was not detectable in the preparations used in these experiments. Further, there was no synthesis when ATP was replaced by ADP. No PP₁ was liberated in the course of the reaction. It is apparent that 1 mole of acetyl- or formylhydroxamate was synthesized per mole of ATP utilized.

Identity of End Products—The RF values obtained with the enzymatically synthesized products correspond to those of authentic acetyl-P and formyl-P (see Table V). The only spots detected were those characterized in the table.

The synthesis of acetyl-P was coupled to δ-acetylornithine synthesis by ornithine transcarbamylase; the acetylornithine was
identified by paper chromatography (3). It is important to add that ornithine transcarbamylase does not catalyze the synthesis of δ-ornithinorhine from acetyl coenzyme A and ornithine.

Identity of Enzyme Catalyzing Activation of Formate and Acetate—The ratio of carbamyl-P and acetyl-P synthesis, followed throughout the enzyme purification procedures, remained constant.

The rat liver enzyme is very unstable when in solution. The ratios of activity with the different substrates were determined at several levels of enzyme inactivation (see Table VI). As noted above, the rat liver enzyme has appreciable activity in the absence of acetylglutamate; other experiments revealed that the extent of enzyme inactivation is the same whether activity is tested with or without acetylglutamate and with either formate or acetate. This indicates that the decrease in activity is not due to the loss of either a non-acetylglutamate-dependent or an acetylglutamate-dependent enzyme with the retention of a second activity.

The frog liver enzyme is inactivated if stored in the cold and in the presence of acetylglutamate (18). This is a specific effect; we know of no other enzyme bearing the same property. The effect of acetylglutamate-induced cold inactivation on carbamyl-P and on formyl-P synthesis is illustrated in Table VII. The data presented in the tables strongly support the conclusion that the same enzyme catalyzes the activation of acetate and formate, and of bicarbonate, in carbamyl-P synthesis.

Discussion

The acetylglutamate requirement of the rat liver enzyme is not absolute. This appears to eliminate, as far as acetel-P and formyl-P synthesis, nontransient intermediates involving this cofactor, and strengthens the concept that a role of acetylglutamate, if not the only one, may be in changing the conformation of the enzyme (5). It was suggested, in an extension of earlier findings (23), that “possibly, the activation and the denaturation processes with acetylglutamate are directly related” (14). Since 2-acetoxyglutarate (24) can replace acetylglutamate, at least one of the few remaining possible structures postulated for an acetylglutamate derivative in carbamyl-P synthesis can be eliminated (25).

Since 1 mole of ATP is used per mole of acetate-P synthesized, and since CoA is not needed, the enzyme does not resemble acetyl-CoA kinase (26). The activation of formate resembles that studied by Sly and Stadtman (27). It appears that Reaction 1 and 2 do not proceed stepwise, as postulated for carbamyl-P synthesis (15). Acetate, formate, and bicarbonate may be activated in an analogous manner, resulting, in the latter case, in the formation of an unidentified compound, perhaps the postulated carbonyl-P (5, 25).

The higher stimulation by Mn++ than by Mg++ of acetate-P and formyl-P synthesis suggests that the synthesis of ATP from carbamyl-P and ADP (also more stimulated by Mn++) may represent a reversal of the initial partial reaction, which postulates an ‘active CO₂’ (15).

Additional work is needed to assess the biological significance of Reactions 1 and 2. Although the affinity of the synthetase for acetate and formate is low, often the requirements determined under conditions in vitro seem unphysiological, e.g., methionine activation (28). On the other hand, the existence of two hexokinases (29) in liver, with widely different affinities for a substrate, is known, and may be of much biological interest.

The synthesis and utilization of acetyl-P by animal and bacterial carbamyl-P enzymes occur in many instances, beyond what seems a priori to be coincidental group specificity. Indeed, to our knowledge, δ-ornithinorhine, found in many plants (30), can only be synthesized enzymatically via acetyl-P.

Summary

Preparations of carbamyl phosphate synthetase from rat and frog liver catalyze the synthesis of acetyl phosphate and formyl phosphate from acetate or formate and from adenosine triphosphate. The pH optimum is 6.5 for the synthesis of acetyl phosphate, and 6.8 for the synthesis of formyl phosphate. Mn++ is more effective than Mg++ as a cofactor.

Acetylgutamate stimulates activation of acetate and formate with frog liver carbamyl phosphate synthetase; the stimulation by acetylgutamate is less marked with the rat liver enzyme.

The stoichiometry of the reactions differs from that of carbamyl phosphate synthesis; 1 mole of adenosine triphosphate is utilized per mole of acetyl- or formylhydroxamate formed.

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References

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