ENZYMATIC ACTIVATION OF CARBON DIOXIDE

I. CRYSTALLINE CARBON DIOXIDE-ACTIVATING ENZYME

BY BIMAL K. BACHHAWAT AND MINOR J. COON

(From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor, Michigan)

(Received for publication, October 28, 1957)

Since the discovery of carbon dioxide fixation in heterotrophic organisms by Wood and Werkman (1) and in animal tissues by Evans and Slotin (2), many biological carboxylations have been described in which carbon dioxide (or bicarbonate ion) serves directly as the carboxylating agent. As recently proposed (3), however, an "activated carbon dioxide" is formed prior to the carboxylation of the methyl group of \( \beta \)-hydroxyisovaleryl coenzyme A, an intermediate in leucine metabolism (4). Further study of this system (5) has led to the conclusion that the reactive intermediate is adenylic carbonate (the mixed anhydride of adenylic and carbonic acids) and to the demonstration that the coupled action of two enzymes is required to effect the carboxylation, as follows:

1. \[ \text{CO}_2 + \text{ATP} \rightarrow \text{AMP-CO}_2 + \text{pyrophosphate} \]
2. \[ \text{AMP-CO}_2 + \beta\text{-hydroxyisovaleryl CoA} \rightarrow \text{AMP} + \beta\text{-hydroxy-\beta-methylglutaryl CoA} \]

The enzyme which catalyzes the bicarbonate-dependent cleavage of ATP according to Reaction 1 will be referred to as the carbon dioxide-activating enzyme. It is of wide occurrence in living organisms and has recently been isolated from pig heart. The reversibility of this reaction has been established by studies on the exchange of radioactive pyrophosphate with ATP, to be described in detail in a subsequent paper. Reaction 1 proceeds to the right when coupled with Reaction 2, which is catalyzed by hydroxyisovaleryl CoA carboxylase. Alternatively, Reaction 1 may be coupled with the non enzymatic decomposition of adenylic carbonate in the presence of hydroxylamine according to Reaction 3.

3. \[ \text{AMP-CO}_2 + \text{NH}_2\text{OH} \rightarrow \text{AMP} + \text{CO}_2 \]

* Supported by a grant from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service (Grant A-993).

The following abbreviations are used: adenosine triphosphate, ATP; adenylic acid, AMP; adenylic carbonate, the mixed anhydride of adenylic and carbonic acids, AMP-CO\(_2\); thiol ester of coenzyme A, acyl CoA; tris(hydroxymethyl)aminomethane, Tris.
The present paper is concerned with the purification and isolation of the carbon dioxide-activating enzyme in crystalline, homogeneous form from pig heart and with the assay and properties of the enzyme. The distribution of the activating enzyme in various tissues and organisms is also described. Subsequent papers will deal with the specificity of these enzymes, the identification of the reaction products, and the effect of biotin deficiency.

**EXPERIMENTAL**

**Enzyme Assay**—The carbon dioxide-activating enzyme is assayed by the bicarbonate-dependent cleavage of ATP in the presence of hydroxylamine, followed by deproteinization and estimation of the pyrophosphate formed. The following are added to a tube chilled in ice: potassium bicarbonate (freshly prepared solution), 500 μmoles; hydroxylamine hydrochloride, 200 μmoles; zinc acetate, 4 μmoles; and ATP (dipotassium salt), 10 μmoles. To this mixture the enzyme solution, diluted in 0.05 M Tris buffer, pH 8.1, is added, and the final volume is adjusted to 3.0 ml. The tube is immediately stoppered, incubated in a water bath at 38° for 30 minutes, and then placed in an ice bath prior to the addition of 1.0 ml of 40 per cent trichloroacetic acid. The mixture is centrifuged, and an aliquot of the supernatant solution is taken for the determination of pyrophosphate by the colorimetric method of Flynn, Jones, and Lipmann (6). A control tube with 500 μmoles of Tris buffer, pH 8.1, in place of bicarbonate is included, and the amount of pyrophosphate found is subtracted from that in the tube containing bicarbonate. The pH of both the complete reaction mixture and of the mixture containing Tris in place of bicarbonate is 6.5 as determined with the glass electrode. At the concentrations employed, the components of the assay system have no significant effect on the colorimetric determination of pyrophosphate. As shown in Fig. 1, pyrophosphate formation in this assay system bears a linear relation to enzyme concentration in the range employed.

In crude enzyme preparations containing pyrophosphatase, orthophosphate liberation serves as an assay for the carbon dioxide-activating enzyme. In assaying the enzyme fractions described below, orthophosphate liberation was therefore determined prior to the heating step in which pyrophosphatase is destroyed, and pyrophosphate liberation was determined with all subsequent fractions. 1 unit of activating enzyme is defined as that amount which will catalyze the formation of 1.0 μmole of pyrophosphate (or 2.0 μmoles of orthophosphate in the presence of pyrophosphatase) from ATP under the conditions described. The specific activity of the enzyme is expressed as the number of units per mg. of protein.
Isolation of Enzyme

The following operations are carried out at about 0°. The steps employed in isolating the activating enzyme from heart extracts are outlined in Table I.

Extraction—Pig hearts obtained immediately upon death of the animals are chilled in ice, trimmed of fat and connective tissue, cut into small pieces, and either homogenized at once or stored in the frozen state until needed. Each of four 500 gm. portions of the minced tissue is homogenized with 500 ml. of 0.05 M Tris buffer, pH 8.1, in a Waring blender for 3 minutes. The combined thick suspension from 2 kilos of heart tissue is diluted with 2 liters of 0.05 M Tris buffer, pH 8.1, and 20 ml. of 1 M Versene, pH 8.1, and the preparation is stirred mechanically for 30 minutes. 30 gm. of solid potassium bicarbonate are added, and stirring is continued for an additional 20 minutes. The resulting mixture is centrifuged at 13,000 r.p.m. for 15 minutes. The deep red supernatant solution contained 76 gm. of protein in a volume of 3.6 liters.

First Ammonium Sulfate Fractionation—The extract is brought to a final ammonium sulfate concentration of 0.29 gm. per ml. (0.55 saturation) by the slow addition with mechanical stirring of 1214 gm. of the salt, and the preparation is stirred for 30 minutes and then centrifuged for 20 minutes.

![Graph](http://www.jbc.org/) Fig. 1. Pyrophosphate formation from ATP as a function of activating enzyme concentration. The standard assay was employed with varying amounts of 280-fold purified activating enzyme.
at 13,000 r.p.m. The precipitate is taken up in 0.05 M Tris buffer, pH 8.1, to give 550 ml. of a turbid solution containing 37.3 gm. of protein.

Treatment with Calcium Phosphate Gel and Second Ammonium Sulfate Fractionation—To the preparation obtained in the above step an equal volume of calcium phosphate gel (7) (15.0 mg. per ml.) is rapidly added, and the mixture is stirred for 30 minutes and centrifuged at 13,000 r.p.m. for 15 minutes. The precipitate is discarded, and the supernatant solution (28.6 gm. of protein in a volume of 1 liter) is brought to a final ammonium sulfate concentration of approximately 0.21 gm. per ml. (0.4 saturation)

TABLE I

<table>
<thead>
<tr>
<th>Isolation of Carbon Dioxide-Activating Enzyme</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Units</th>
<th>Specific activity*</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract ..................................................................</td>
<td>75,600</td>
<td>4540</td>
<td>0.06</td>
<td>100</td>
</tr>
<tr>
<td>1st ammonium sulfate fractionation (0–0.55 saturation)</td>
<td>37,300</td>
<td>4100</td>
<td>0.11</td>
<td>90</td>
</tr>
<tr>
<td>Calcium phosphate gel supernatant solution .............</td>
<td>28,600</td>
<td>4000</td>
<td>0.14</td>
<td>88</td>
</tr>
<tr>
<td>2nd ammonium sulfate fractionation (0–0.40 saturation)</td>
<td>16,800</td>
<td>3360</td>
<td>0.20</td>
<td>74</td>
</tr>
<tr>
<td>Heating at 62° ..................................................</td>
<td>3,190</td>
<td>3190</td>
<td>1.0</td>
<td>70</td>
</tr>
<tr>
<td>3rd ammonium sulfate fractionation (0.30–0.41 saturation)</td>
<td>351</td>
<td>1020</td>
<td>2.9</td>
<td>22</td>
</tr>
<tr>
<td>Crystals from ammonium sulfate solution (0.45 saturation)</td>
<td>15</td>
<td>480</td>
<td>32</td>
<td>112</td>
</tr>
<tr>
<td>Recrystallization. ................................................</td>
<td>10</td>
<td>360</td>
<td>36</td>
<td>8</td>
</tr>
</tbody>
</table>

* Prior to the heating step, in which pyrophosphatase is removed completely, the assay is based on the liberation of orthophosphate (see the text).

by the addition of 235 gm. of the salt. The precipitate is collected by centrifugation and taken up in 0.05 M Tris buffer, pH 8.1, to give 350 ml. of a thick suspension containing 16.8 gm. of protein.

Heat Denaturation—50 ml. aliquots of the suspension are transferred to 250 ml. Erlenmeyer flasks and heated with constant swirling in a 70° bath until the temperature of the enzyme preparation reaches 62°. The temperature of the preparation is maintained at 62° for 1 minute, and the flask is then immediately chilled in an ice bath. The insoluble protein is removed by centrifugation at 13,000 r.p.m. to furnish 210 ml. of clear, reddish supernatant solution containing 3.2 gm. of protein. At this stage the enzyme preparation is free of pyrophosphatase and myokinase and also largely free of ATPase activity.

Third Ammonium Sulfate Fractionation—The solution is brought to a
final ammonium sulfate concentration of approximately 0.16 gm. per ml. (0.3 saturation) by the addition of 36 gm. of salt, and the supernatant solution obtained upon centrifugation (210 ml.) is then brought to a final ammonium sulfate concentration of approximately 0.22 gm. per ml. (0.41 saturation) by the addition of 14 gm. of salt. The resulting precipitate is collected by centrifugation and dissolved in 0.05 M Tris buffer, pH 8.1, to give 18 ml. of solution containing 351 mg. of protein.

Crystallization—The solution is brought to a final ammonium sulfate concentration of approximately 0.24 gm. per ml. (0.45 saturation) by the addition of 4.8 gm. of salt. The solution is stirred for 5 minutes and centrifuged to remove the slight precipitate, and the supernatant solution is allowed to stand for 48 hours in an open beaker in the refrigerator at about 4°. The crystals are harvested by centrifugation and dissolved in 0.05 M Tris buffer, pH 8.1, to give 3 ml. of solution containing 15 mg. of protein. Recrystallization is accomplished by the slow addition to incipient turbidity (about 0.45 saturation) of a saturated ammonium sulfate solution previously adjusted to pH 8.1 with concentrated ammonium hydroxide and by allowing the preparation to stand at 0° for 24 hours. 10 mg. of the recrystallized enzyme were harvested by centrifugation. The enzyme crystallizes as fine, slender needles which are easily visible in the presence of methylene blue (Fig. 2). The recrystallized enzyme had a specific activity of 36, representing 600-fold purification from the initial extract.

Purity—When recrystallized twice as described and three times from 0.3 M potassium succinate buffer, pH 5.9, by the addition of saturated ammonium sulfate solution, the enzyme had a specific activity of 39. This preparation was found to be homogeneous in the ultracentrifuge as shown in Fig. 3. It also appeared to be homogeneous on zone electrophoresis in starch gel in sodium borate buffer, pH 8.6, according to the procedure of Smithies (8). Under these conditions and a potential gradient of 6 volts per cm. a single narrow protein band moved 4.4 cm. during 6 hours. The enzyme has a typical protein absorption spectrum with end absorption in the ultraviolet region and a single maximum at about 280 μ. The absence of appreciable amounts of nucleotides is indicated by the $E_{280}/E_{260}$ ratio of 1.6. As further indication of the absence of bound nucleotides, the enzyme retains activity upon treatment with charcoal. The enzyme is stable to dialysis against dilute buffers overnight at 4° and to

2 Additional amounts of crystalline enzyme may be obtained from the protein fraction precipitating at 0-0.30 ammonium sulfate saturation in the preceding step. This fraction is diluted to a protein concentration of 18 mg. per ml., solid ammonium sulfate is added to 0.45 saturation, the precipitate is discarded, and the preparation is allowed to stand at 0° for 3 days. 30 mg. of protein, specific activity 20, were obtained upon centrifugation.

3 The authors are indebted to Dr. Walter D. Block for these determinations.
storage at 0° as a suspension in 50 per cent ammonium sulfate solution. Acetone powder extracts and alcohol-potassium chloride extracts of tissues contain less of the enzyme than does the buffer extract described above.
Optimal pH—The activating enzyme has maximal activity in the hydroxylamine assay at about pH 6.5, as shown in Fig. 4, with appreciable activity in the broad range pH 5.2 to 9.0. It should be noted that in the standard assay in which hydroxylamine hydrochloride is employed the pH of both the complete reaction mixture containing bicarbonate and the control reaction mixture containing Tris buffer is 6.5 as determined with the glass electrode. No significant change in pH occurs during the incubation.

Effect of Substrate Concentration on Enzyme Activity—The effect of the concentration of the components of the assay system on the activity of the enzyme is indicated in Fig. 5. The enzyme is most active in the presence of zinc ions. Manganous and magnesium ions are less effective, even at higher concentrations. ATP was found to be somewhat inhibitory in concentrations higher than that employed in the standard assay, whereas hydroxylamine shows no inhibitory effect at a concentration as high as 0.33 M. The $K_m$ values, determined by the method of Lineweaver and Burk (9), were found to be $1.6 \times 10^{-3}$ M for ATP (with total bicarbonate at 0.17 M) and $8.5 \times 10^{-3}$ M for bicarbonate (with ATP at 0.033 M). Since it is not known whether carbon dioxide, carbonic acid, or bicarbonate is the actual substrate in this or other enzymatic carboxylations, the $K_m$ has been calculated for total bicarbonate added. The $K_m$ would obviously be a much smaller value if calculated for carbon dioxide, for example. No special precautions need be taken to exclude dissolved carbon dioxide from
the reagents employed in the enzyme assay, since a relatively large amount of bicarbonate must be added to saturate the system.

Distribution of Enzyme—Quantitative determination of the carbon dioxide-activating enzyme in crude extracts of most organisms is difficult because of the presence of ATPase and pyrophosphatase. For this reason,

![Graph showing the effect of concentration of assay components on enzyme activity.](image)

**Fig. 5.** Effect of concentration of assay components on enzyme activity. The standard assay was employed with 30 γ of crystalline carbon dioxide-activating enzyme and with the individual components at varying concentrations as indicated in the figure, but with the other components at the concentrations specified in the standard assay. Neutralized hydroxylamine was employed in determining the effect of hydroxylamine concentration; the pH of the complete reaction mixtures was 8.1. In determining the effect of bicarbonate concentration, Tris buffer, pH 8.1, was added as necessary so that each reaction mixture had a total of 500 μmoles of bicarbonate and Tris combined, and the pH was maintained at 6.5 in each instance. In determining the effect of zinc and ATP concentrations, the pH of the complete reaction mixtures was 6.5, as in the standard assay.

most of the values recorded in Table II were determined after the extracts had been carried through the ammonium sulfate, gel, and heating steps known to remove these enzymes from the heart activating enzyme preparation. In the absence of information as to how the activating enzyme in many of these sources responds to such procedures, the results probably provide only a rough indication of its actual concentration. It is evident, however, that the enzyme is widespread in animal tissues and is also present in certain microorganisms as well as in a green plant (spinach leaf). Although *Rhodospirillum rubrum* and *Escherichia coli* extracts appear to be a rich
source of the enzyme, Neurospora crassa and Penicillium notatum extracts appear to contain little or none.

**Table II**

Distribution of Carbon Dioxide-Activating Enzyme

<table>
<thead>
<tr>
<th>Source</th>
<th>Extract</th>
<th>Partially purified preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig heart</td>
<td>0.06*</td>
<td>1.00</td>
</tr>
<tr>
<td>&quot; brain</td>
<td></td>
<td>0.70</td>
</tr>
<tr>
<td>&quot; kidney</td>
<td></td>
<td>0.46</td>
</tr>
<tr>
<td>&quot; liver</td>
<td></td>
<td>0.46</td>
</tr>
<tr>
<td>Chicken liver</td>
<td></td>
<td>0.64</td>
</tr>
<tr>
<td>Rat liver</td>
<td></td>
<td>0.40</td>
</tr>
<tr>
<td>Rabbit skeletal muscle</td>
<td></td>
<td>0.41</td>
</tr>
<tr>
<td>Spinach leaf</td>
<td></td>
<td>0.40</td>
</tr>
<tr>
<td>Bakers' yeast (Fleischmann)</td>
<td></td>
<td>0.40*†</td>
</tr>
<tr>
<td><em>R. rubrum</em></td>
<td>0.33*</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td><em>N. crassa</em></td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td><em>P. notatum</em></td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

The various animal tissues and spinach leaf were extracted with Tris buffer and bicarbonate according to the procedure described for pig heart. These extracts were then carried through the first ammonium sulfate fractionation, treatment with gel, second ammonium sulfate fractionation, and heating steps described in the text in order to remove pyrophosphatase, ATPase, and myokinase. These enzyme preparations were assayed by the bicarbonate-dependent liberation of pyrophosphate from ATP in the presence of hydroxylamine according to the standard procedure. Lyophilized preparations of *E. coli* B (grown in a medium containing glucosamine as the sole carbon source), *N. crassa*, and *P. notatum* were extracted by grinding with alumina in a mechanical mortar in the presence of 0.05 M Tris buffer, pH 8.1. In order to determine pyrophosphate liberation in these three extracts, 50 μmoles of fluoride were incorporated into the reaction mixture to inhibit pyrophosphatase. The activity of the *E. coli* preparation may be attributed to the carbon dioxide-activating enzyme because fluorokinase (10) is inhibited by hydroxylamine. For the preparation of *R. rubrum* and yeast extracts the harvested cells were suspended in 0.1 M Tris buffer, pH 8.1, containing 0.001 M glutathione, treated in a Nossal disintegrator for 90 seconds, and then centrifuged.

* The specific activity, based on phosphate liberation, is calculated as pyrophosphate.

† In assaying the yeast extract 20 μmoles of magnesium chloride were substituted for zinc acetate, and hydroxylamine was omitted from the reaction mixture (see the text).

The values recorded in Table II represent in all cases the bicarbonate-dependent cleavage of ATP in the hydroxylamine assay. As stated above,
this reaction in heart preparations is completely dependent upon the presence of hydroxylamine. On the other hand, extracts of pig brain, chicken liver, *R. rubrum*, and yeast have been found in some instances to catalyze the bicarbonate-dependent liberation of pyrophosphate (or phosphate in the presence of pyrophosphatase) when hydroxylamine is omitted from the reaction mixture. No satisfactory explanation has yet been obtained for this difference.

**Methods**

The concentration of the ammonium sulfate solutions is expressed in all cases as gm. per ml., the concentration at saturation at 0° being 0.526 gm. per ml. The protein concentration of the enzyme solutions was determined spectrophotometrically at 280 and 260 μ, with a correction for the nucleic acid content (11). Crystalline ATP was a commercial product.

A culture of *R. rubrum* was kindly furnished by Dr. A. S. Sussman, lyophilized preparations of *E. coli* and *P. notatum* by Dr. S. Roseman, and *N. crassa* by Dr. H. J. Blumenthal. Pig heart tissue was generously donated by the Peters Sausage Company of Ann Arbor.

The authors wish to acknowledge the technical assistance of Mrs. Mary Januszka.

**SUMMARY**

1. The isolation of the carbon dioxide-activating enzyme in crystalline and apparently homogeneous form from pig heart is described. The enzyme, which requires zinc ions for activity, is assayed by the carbon dioxide- and hydroxylamine-dependent liberation of pyrophosphate from adenosine triphosphate (ATP).

2. The enzyme is active in the range pH 5.2 to 9.0, with maximal activity at pH 6.5. The *Kₘ* values are 1.6 × 10⁻⁴ M for ATP and 8.5 × 10⁻² M for bicarbonate. The latter value is based on total bicarbonate added, in the absence of information as to whether the reactive species is carbon dioxide or bicarbonate ion.

3. The activating enzyme is also present in extracts of brain, kidney, liver, and skeletal muscle, as well as spinach leaf, *Escherichia coli*, *Rhodospirillum rubrum*, and bakers’ yeast.

**BIBLIOGRAPHY**

2. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, 136, 301 (1940); 141, 439 (1941).
ENZYMATIC ACTIVATION OF CARBON DIOXIDE: I. CRystalline Carbon Dioxide-activating Enzyme
Bimal K. Bachhawat and Minor J. Coon


Access the most updated version of this article at http://www.jbc.org/content/231/2/625.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/231/2/625.citation.full.html#ref-list-1