Glyoxylate Carboligase of *Escherichia coli*: a Flavoprotein

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When *Escherichia coli* is adapted to grow on glycolate, the first step in the degradation of substrate involves its oxidation to glyoxylate (1-3). This substance is then converted to CO₂ and hydroxymalonic semialdehyde (Equation 1) by an adaptive enzyme, glyoxylate carboligase.

\[ 2 \text{CHO-COO}^- + \text{H}^+ \rightarrow \text{CO}_2 + \text{CHO-CHOH-COO}^- \] (1)

Krarow and Barkulis (1) first characterized this enzyme, and demonstrated its dependence on diphenolphthiamine and Mg⁺⁺. As part of a broader study of the bacterial metabolism of two carbon substrates, Kornberg and Gotto (4) have identified an analogous enzyme in some species of *Pseudomonas*. Reaction 1 has also been shown to occur in extracts of *Streptococcus pilosus* (5). The present study describes the purification of the carboligase from glycolate-adapted *Escherichia coli*, and presents evidence that this enzyme is an FAD flavoprotein.

**Experimental Procedure**

**Materials**—Sodium glyoxylate, FAD, FMN,1 protamine sulfate (salmine), and D-amino acid oxidase were purchased from Sigma Chemical Company.

**Assay of Carboligase**—The enzyme was assayed by a slight modification of the manometric procedure described by Krarow, Barkulis, and Hayashi (3). This involves a measurement of the rate of CO₂ released from added glyoxylate under anaerobic conditions. The determinations were made in double arm Warburg flasks at 30° under an atmosphere of argon gas. The reaction mixtures contained 500 μmoles of potassium phosphate buffer, pH 6.7, 300 μg of thiamine pyrophosphate, 600 μg of MgCl₂, and enzyme, in a volume of 1.8 ml. After gassing and equilibration, 30 μmoles of glyoxylate in 0.1 ml were added from one side arm. The reaction was allowed to proceed with shaking for 10 minutes, and stopped by addition of 0.4 ml of 2 N H₂SO₄ from the other side arm. The CO₂ released was calculated from the pressure increase read 5 minutes after tipping the acid. A correction for bicarbonate in the reagents was applied otherwise specified, all operations were performed at 0-4°. The FAD values given in Table I were determined by the fluorimetric method of Bessey, Lowry, and Love (8).

**Growth of Bacteria**—The bacterial strain was that used by Krarow et al. The conditions employed for growth were those previously described (3). Bacteria from 36 liters of a 24-hour culture were harvested by centrifugation in a Servall refrigerated centrifuge and stored in the frozen state. Such preparations were suitable for several months.

**Step 1. Crude Extract**—Of the frozen cells, 66 g (obtained from 36 liters of culture) were suspended in 200 ml of 1% KCl solution and ruptured by treatment in a Raytheon sonic oscillator (200-watt, 10-ke) for 9 minutes. The extract was clarified by centrifugation for 1 hour at 27,000 x g, and the clear supernatant was dialyzed overnight against 4 liters of 0.01 M phosphate buffer, pH 6.7.

**Step 2. Ammonium Sulfate Fractionation**—Solid ammonium sulfate was added slowly to the extract to bring it to 35% saturation. The suspension was stirred for 30 minutes and then centrifuged at 27,000 x g for 10 minutes. The precipitate so obtained was discarded. Solid ammonium sulfate was then added to bring the supernatant to 55% saturation. After 30 minutes of stirring, the precipitate, which contained most of the enzyme, was collected by centrifugation as described above, dissolved in 0.01 M phosphate buffer, pH 6.7, and dialyzed against 4 liters of the same buffer.

**Step 3. Heat Treatment**—The dialyzed solution was mixed with 0.25 volume of 1 M phosphate buffer, pH 0.0, and thiamine pyrophosphate and MgCl₂ were both added to a final concentration of 3 mg per ml. The solution was distributed among 25-ml thin walled test tubes in 15-ml portions. The tubes were placed in a water bath at 72° and incubated at this temperature for 5 minutes, then transferred to an ice bath and cooled for 5 minutes.

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1 The abbreviation used is: FMN, flavin mononucleotide.
**TABLE I**

<table>
<thead>
<tr>
<th>Fractionation of E. coli carboligase</th>
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<tbody>
<tr>
<td>Volume</td>
</tr>
<tr>
<td>ml</td>
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<tr>
<td>--------</td>
</tr>
<tr>
<td>1. Sonorated extract</td>
</tr>
<tr>
<td>2. First (NH₄)₂SO₄ precipitate</td>
</tr>
<tr>
<td>3. Heat-treated preparation</td>
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**TABLE II**

| Resolution and reactivation of carboligase |
| Conditions and procedure are described in the text. |

### RESULTS

**Flavin Content of Carboligase Preparations**—The absorption spectrum of a typical preparation of the enzyme, shown in Fig. 1, is that of a flavoprotein. The three major absorbance maxima were located at 275, 380, and 445 μm, with a distinct secondary maximum at 467 μm. The ratio of the absorbance at 275 μm to that at 445 μm ranged from 6.4 to 8.9 in different preparations. It may be assumed that some thiamine pyrophosphate is present and contributes to the absorbance at 275 μm. Although the crude enzyme has been reported to be readily dissociated to show an absolute requirement for thiamine pyrophosphate (3), the purified enzyme generally exhibited from 30 to 50% of its full activity when assayed in the absence of added thiamine pyrophosphate.

The average molar absorptivity, \( E \), at 445 μm for the four best preparations was 14.1 \( \times \) 10³ M⁻¹ cm⁻¹, with a range from 13.2 to 14.8 \( \times \) 10³ M⁻¹ cm⁻¹. This is rather high for a flavoprotein, but no evidence has yet been obtained for the presence of any chromogen other than FAD.

**FAD Dependence of Carboligase Reaction**—Application of the acid ammonium sulfate resolution procedure of Warburg and Christian (9) to the E. coli carboligase provided definitive evidence that the FAD of the protein was essential for enzyme ac-
tivity. On treatment with acid ammonium sulfate, the flavin was dissociated from the protein and remained in the supernatant, and a colorless apoenzyme was obtained as a precipitate. The inactive apoenzyme was specifically reactivated by FAD. The carboligase apoenzyme was quite unstable, and a variety of modifications of the resolution procedures were tested in an effort to maximize recovery of active enzyme. The best results were achieved with the procedure initially applied by Strittmatter (12) to the resolution of microsomal cytochrome b_5 reductase. One of the most successful experiments is summarized in Table II. The enzyme for this experiment had been carried through Step 3 of the fractionation. Enzyme solution, 1 ml containing 4 mg of protein per ml, was mixed with 1 ml of 3 M KBr and cooled in an ice bath. To this was added 1 ml of cold acid ammonium sulfate solution, prepared by adding 0.5 ml of 1 M H_2SO_4 to 7.5 ml of saturated ammonium sulfate. After the solution was mixed well, the suspension was centrifuged immediately for 5 minutes at 8000 x g in the cold. The yellow supernatant solution was poured off, and the liquid adhering to the inside of the centrifuge tube was removed with absorbent paper. The white precipitate was dissolved in 1 ml of 0.2 M phosphate buffer, pH 7.4, and aliquots were assayed immediately in the standard enzyme assay system with the various additions shown. The specific activity of the enzyme protein was reduced from 8.7 to 0.7 by the dissociation procedure and full recovery of specific activity (and of total enzyme units) was achieved by addition of 6.0 mmoles of FAD per 1.8 ml of assay mixture. The amount of apoenzyme employed per assay was equivalent to 0.7 mmole of FAD prior to resolution. Complete reconstitution and recovery was achieved in only about one-fourth of the many similar experiments performed to date, but all experiments consistently showed substantial reactivation of apoenzyme by FAD.

**DISCUSSION**

We are not yet able to estimate the purity of the carboligase enzyme preparation described here. The flavin content of 1 mole of FAD per 100,000 g of protein is rather low when compared with that of other flavoproteins. The enzyme product obtained is apparently a mixture of at least two proteins, one of which contains thiamine pyrophosphate and the other of which does not. The over-all purification is only about 50-fold, but this is perhaps not surprising in view of the fact that much of the substrate metabolized by the glycolate-adapted *E. coli* must presumably be handled by this enzyme.

The over-all recovery of 73% of total units is achieved mainly because the heat treatment of Step 3 causes an actual increase in enzyme activity. This increase has been observed consistently. The flavoprotein pyruvate oxidase of *E. coli* described by Williams and Hager (13) offers some analogies to the carboligase here described. Both enzymes are formed by the same bacterial species, both contain FAD, both require thiamine pyrophosphate, both are adaptive enzymes formed in response to the presence of an α-keto acid: pyruvate for the former enzyme, and glycolate for the latter. A major difference appears to lie in the function. The pyruvate oxidase enzyme is bleached by pyruvate, and although the reduced purified flavoprotein is not autoxidizable, the enzyme can be coupled with the particulate cytochrome b of the bacteria to give an oxidase system operating with molecular O_2_. The carboligase reaction, on the other hand, is not an oxidation-reduction reaction and occurs under completely anaerobic conditions. No bleaching of flavoprotein by glyoxylate has been observed. If the reaction is performed in the presence of O_2_, there is considerable O_2_ uptake, but only after an initial lag period. The reaction product, hydroxymalonialdehyde, is autoxidizable (5), and this complicates studies of the possible oxidase function of the carboligase. Such a function is not supported, however, by the metabolic studies which indicate that the hydroxymalonialdehyde is reduced physiologically to glycerate (3, 14, 15).

Evidence has been presented by Jaenicke and Koch (16) that hydroxymethyl thiamine pyrophosphate is an intermediate in the reaction catalyzed by the glyoxylic acid carboligase of *Pseudomonas*. It is not known whether this enzyme is a flavoprotein, and there is nothing in the proposed reaction mechanism to suggest the obligatory occurrence of an oxidation-reduction. In view of these facts, one is tempted to suggest an indirect function for the FAD of *E. coli* carboligase, involving perhaps the maintenance of the protein conformation necessary for activity. This question must be left open, pending further investigation.

**SUMMARY**

A procedure is described for purifying glyoxylate carboligase from glycolate-adapted *Escherichia coli*. Although the reaction catalyzed by this enzyme (the conversion of 2 molecules of glyoxylate to 1 molecule of hydroxymalonialdehyde) does not appear to be an oxidation-reduction reaction, the enzyme has been identified as a flavoprotein containing flavin adenine dinucleotide. The evidence rests on the demonstration of the presence of flavin adenine dinucleotide in the purified enzyme, and on the resolution of the active protein into a colorless inactive apoenzyme which is specifically reactivated by the dinucleotide.

**REFERENCES**

9. **Warrburg, O., and Christian, W., Biochem. Z., 296, 150 (1938).**
12. **Strittmatter, P., J. Biol. Chem., 236, 2329 (1961).**
15. **Krakow, G., Uda, S., and Vennesland, B., Biochemistry, 1, 254 (1962).**
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