Glyoxylate Carboligase of *Pseudomonas oxalaticus*. A Possible Structural Role for Flavine–Adenine Dinucleotide*

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ABSTRACT: Glyoxylate carboligase was purified to homogeneity from *Pseudomonas oxalaticus*. The enzyme had a molecular weight of 230,000 in the sucrose gradient centrifugation and each mole of enzyme contained 2 moles of FAD. When FAD was removed by acid ammonium sulfate treatment, the molecular weight of enzyme decreased to 115,000. With the addition of FAD the molecular weight increased to the original level regaining the catalytic activity. This reversible change in molecular weight was confirmed also in the change in the s_{20} value from 8.6 to 6.0 S. Electrophoresis of the enzyme in polyacrylamide gel with sodium dodecyl sulfate gave a subunit molecular weight of 61,000. It was concluded that the active form of glyoxylate carboligase is a tetramer and FAD is required for the formation of tetramer from inactive dimers.

No evidence was obtained from a stopped-flow spectrophotometric study for the reduction of flavin during the reaction.

Glyoxylate carboligase (EC 4.1.1.b) catalyzes the condensation of 2 moles of glyoxylate to tartronate semialdehyde and carbon dioxide:

$$2CHOCOO^- + H^+ \longrightarrow CHOCHOHCOO^- + CO_2$$

Krakow *et al.* (1956, 1961) characterized the enzyme from glycollate-grown *Escherichia coli* as thiamine diphosphate and Mg^{2+}-dependent, analogous to the classical pyruvate carboligase of *Aerobacter aerogenes*. Gupta and Vennesland (1964) purified the enzyme and demonstrated conclusively the presence of FAD which is essential for the activity of the enzyme. Although FAD is not reduced by addition of glyoxylate, the enzyme becomes inactive when the FAD component is chemically reduced (Gupta and Vennesland, 1966).

Pseudomonads produce glyoxylate carboligase when grown on glycollate (Kornberg and Gotto, 1959) or oxalate (Quayle *et al.*, 1961). We have purified the enzyme from *Pseudomonas oxalaticus* and found it to be a flavoprotein similar to the *E. coli* enzyme. This paper reports the role of FAD in associating enzyme subunits into an active tetramer of glyoxylate carboligase.

Materials and Methods

DEAE-cellulose (medium), protamine sulfate (Grade I), sodium glyoxylate, Na_2NADH, thiamine diphosphate chloride, Na_2FAD, NaFMN, catalase (bovine liver), aldolase (rabbit muscle), and glutamate dehydrogenase (bovine liver) were purchased from Sigma Chemical Co. Dithiothreitol was a product of Calbiochem. Bovine serum albumin (crystallized) was obtained from Mann Research Laboratories. Sodium docecyl sulfate was purchased from Fisher Scientific Co. Phosphorylase *b* was kindly donated by Dr. J. H. Wang. Deamino FAD was prepared from FAD according to Chung and Aida (1967). Protein was determined by the method of Lowry *et al.* (1951).

Assay of Glyoxylate Carboligase. The principle of assay is to couple the formation of tartronate semialdehyde to the oxidation of NADH in the presence of excess tartronate semialdehyde reductase (EC 1.1.1.60). The standard reaction mixture (3 ml) contained: 0.1 M potassium phosphate (pH 7.5), 1 mM $MgCl_2$, 0.1 mM thiamine diphosphate, 0.15 mM NADH, tartronate semialdehyde reductase (partially purified as described later and used in excess), and carboligase. The mixture was incubated for 15 min at room temperature before initiation of the reaction with 10 μl of 1 M sodium glyoxylate. The decrease in optical density at 340 mμ was followed spectrophotometrically using 1-cm light path.

One unit of enzyme was defined as the amount of enzyme which oxidized 1 μmole of NADH/min under the standard conditions.

When the preincubation of glyoxylate carboligase with tartronate semialdehyde reductase and NADH was omitted an initial lag was observed before the rate of NADH oxidation reached the maximum level corresponding to the level obtained with the standard preincubation assay method. The reason for this lag period is at present unknown.

Purification of Glyoxylate Carboligase. Pseudomonas oxalaticus ATCC 11883 was grown on oxalate as described by Quayle *et al.* (1961). Cells were collected by Sharples centrifugation, washed in 0.05 M potassium phosphate buffer (pH 7.5) with 0.1 mM EDTA, and stored frozen at −50°. All the following procedures were carried out at 0–4°.

The cells were suspended in 0.05 M potassium phosphate (pH 7.5) buffer (120-g cells in 360 ml of buffer) and were disrupted in a Raytheon 10KC sonic oscillator for 12 min. The crude extract was obtained after centrifugation for 1 hr at 48,000*g*.

A solution of protamine sulfate (2%, pH 5.5) was added to the crude extract (0.06 mg of protamine sulfate/mg of protein) and the mixture was stirred for 15 min. After centrifugation at 12,000*g* for 10 min the precipitate was eluted with 0.1 M potassium phosphate (pH 7.5) and the eluate was saved for the purification of tartronate semialdehyde reductase. To the supernatant was added 0.06 mg of protamine

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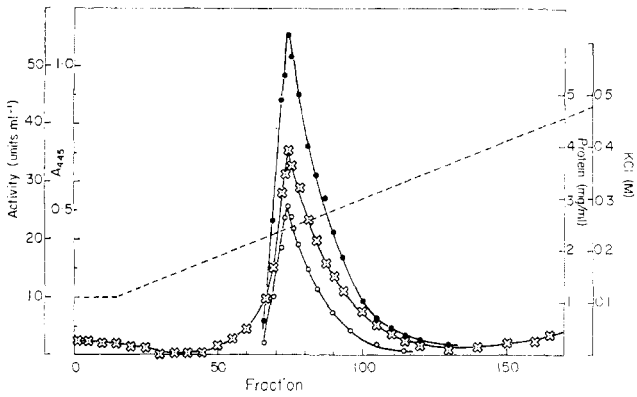

FIGURE 1: DEAE-cellulose chromatography of glyoxylate carboligase. Fractions of 5.8 ml were collected. Glyoxylate carboligase activity (●), protein concentration (⊠), and flavin concentration as absorption at 445 mμ (○). The dotted line shows the concentration of KCl.

TABLE I: Purification of Glyoxylate Carboligase from *P. oxalaticus*.

Fraction	Vol (ml)	Protein (mg/ml)	Sp Act. (Units/mg of Protein)	Recov (%)
Crude extract (from 360-g cells)	1170	34	0.26	100
Protamine sulfate	210	25	1.35	68.5
$(NH_4)_2SO_4$, 25–50%	50	40	3.06	59.2
DEAE	155	2.2	15.4	50.8

sulfate/mg of protein and after stirring the mixture was centrifuged. The precipitate was suspended in 70 ml of 0.2 M potassium phosphate buffer with 1 mM $MgCl_2$, 0.1 mM thiamine diphosphate, and 1 mM dithiothreitol. The insoluble material was removed by centrifugation to obtain a clear solution of carboligase. This protamine sulfate fraction was stable for several months at −20°. Three batches of this fraction (corresponding to 360-g cells) were combined before further purification.

Solid ammonium sulfate was added slowly to the protamine sulfate fraction to 25% saturation. After 30-min stirring the precipitate was removed by centrifugation at 27,000*g* for 15 min. Solid ammonium sulfate was then added to the supernatant to bring it to 50% saturation. After 30-min stirring the precipitate containing carboligase was collected by centrifugation as described above. The precipitate was dissolved in 50 ml of 0.05 M potassium phosphate (pH 7.5) buffer containing 1 mM $MgCl_2$, 0.1 mM thiamine diphosphate, and 1 mM dithiothreitol.

The ammonium sulfate fraction was desalted by passing through a Sephadex G-25 column (2.5 × 40 cm) in 0.05 M potassium phosphate (pH 7.5) buffer containing 1 mM $MgCl_2$, 0.1 mM thiamine diphosphate, and 1 mM dithiothreitol. The desalted enzyme was placed on a DEAE-cellulose column (2.5 × 40 cm) equilibrated with the same buffer. The column was washed with 500 ml of the above buffer and then with 500 ml of 0.1 M potassium phosphate buffer of the same pH containing the same protective compounds. The enzyme was finally eluted with a linear gradient of KCl (0–0.5 M) in 1 l. of the above 0.1 M potassium phosphate buffer.

As shown in Figure 1 the enzyme activity peak was eluted between 0.25 and 0.30 M KCl coinciding with the protein and flavin peaks. The tubes 72–84 were combined and used as the purified enzyme preparation in this work.

The results of purification of glyoxylate carboligase described above are shown in Table I. The final DEAE-cellulose eluate had a specific activity of 15.4 units/mg (60-fold increase over crude extract) with a recovery of 50%.

Preparation of Tartronate Semialdehyde Reductase. The precipitate of the first protamine sulfate treatment during the purification of glyoxylate carboligase was eluted with 0.1 M potassium phosphate buffer. The reductase was precipitated with solid ammonium sulfate (50–75% saturation fraction). After desalting with a column of Sephadex G-25 the enzyme was placed on a column of DEAE-cellulose and the column was washed with a linear gradient of KCl (0–0.5 M) in 0.05 M potassium phosphate buffer (pH 7.5). The reductase was eluted between 0.1 and 0.15 M KCl. The enzyme was precipitated with solid ammonium sulfate (50–75% saturation) and was dissolved in 0.05 M potassium phosphate (pH 7.5). The enzyme preparation thus prepared was stored at −20° in small portions. The enzyme was stable when stored frozen, but lost activity after repeated freezing and thawing. The final preparation was free of glyoxylate carboligase and showed a single major peak in an analytical ultracentrifuge with a minor faster moving peak.

The reductase solution contained 8 mg of protein/ml and 0.1 ml was used for the assay of glyoxylate carboligase activity.

Sucrose Gradient Centrifugation. Sucrose density gradient centrifugation was performed according to Martin and Ames (1961) in order to determine the molecular weight of enzyme. Sucrose density gradient (5–20%) was prepared in 0.05 M potassium phosphate (pH 7.5) containing 1 mM $MgCl_2$, 0.1 mM thiamine diphosphate, and 1 mM dithiothreitol. Samples were centrifuged for 14.5 hr at 37,000 rpm in a Spinco Model L2-65B ultracentrifuge equipped with a swinging-bucket rotor, SW 50.1. Catalase used as standard was detected by the method of Beers and Sizer (1952).

Analytical Ultracentrifugation. Sedimentation velocities of holo enzyme and apo enzyme were measured with a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. The rotor temperature was maintained at 20° and the rotor speed was 59,780 rpm. After the rotor reached full speed, photographs were taken at 8-min intervals with the schlieren diaphragm angle of 70°.

Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis. The determination of subunit molecular weight was carried out according to Weber and Osborne (1969) with minor modifications. For the 10% acrylamide solution, 22 g of acrylamide and 0.3 g of methylenebisacrylamide were dissolved in water to give 100 ml of solution. Staining of protein bands was performed with 0.02% coomassie brilliant blue in a mixture of 50% methanol and glacial acetic acid (9:1) at room temperature for 2–4 hr.

Preparation of Glyoxylate Carboligase Apo Enzyme. Resolution of glyoxylate carboligase holo enzyme into apo enzyme and FAD was carried out by the acid ammonium sulfate procedure of Warburg and Christian (1938) as de-

TABLE II: Inhibition of Carboligase Activity by FMN, AMP, and ATP.

Inhibitor	Act. ($\Delta A_{340\ m\mu}$ min^{-1})[a]	Inhibn (%)
None	0.195	
FMN, 3.3 μM	0.132	32
AMP, 1 mM	0.140	28
ATP, 1 mM	0.140	28
FMN, 3.3 μM; AMP, 1 mM	0.043	78
FMN, 3.3 μM; ATP, 1 mM	0.005	97
Atebrin, 30 μM	0.132	32

[a] Glyoxylate carboligase (20 μg) purified in the absence of dithiothreitol was preincubated with inhibitors for 15 min in 0.05 M potassium phosphate (pH 7.5) containing 1 mM $MgCl_2$ and 0.1 mM thiamine diphosphate before the assay of carboligase activity as described in Materials and Methods.

scribed for the *E. coli* enzyme by Gupta and Vennesland (1966). The colorless apo enzyme was dissolved in 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM $MgCl_2$, 0.1 mM thiamine diphosphate, and 1 mM dithiothreitol.

Results

Glyoxylate carboligase of *P. oxalaticus* was intensely yellow and showed a flavoprotein spectrum similar to the enzyme from *E. coli* (Gupta and Vennesland, 1964). The molecular weight determined by the sucrose gradient centrifugation method was 230,000. Since the optical density of an enzyme solution (5 mg of protein/ml) at the 445-mμ peak was 0.6 with a 1-cm light path the FAD content of the enzyme was calculated to be 2 moles of FAD/mole of enzyme using an E value of 14×10^3 M^{-1} cm^{-1} (Gupta and Vennesland, 1964). The *E. coli* enzyme contained 1 mole of FAD/100,000 g of protein.

Specific activity of the enzyme, 15.4 units/mg, also agrees closely with that of *E. coli* enzyme (14–15 units/mg).

The double-reciprocal plot of enzyme activity *vs.* glyoxylate concentration (0.1–5 mM) was linear and the K_m value was calculated as 0.25 mM under the standard conditions.

Absence of FAD Reduction During the Reaction. In agreement with Gupta and Vennesland (1964) there was no bleaching of flavoprotein color when glyoxylate was added to the enzyme. It was thought that possibly a temporary reduction and reoxidation of flavin might occur during the reaction. Rapid kinetic experiments were performed in a Durrum stopped-flow spectrophotometer at 450 mμ with 20-mm light path. No evidence was obtained for flavin reduction when a solution of 5 μM carboligase in 0.1 M potassium phosphate (pH 7.5) buffer containing 1 mM $MgCl_2$ and 0.1 mM thiamine diphosphate was mixed with a solution of 10 mM sodium glyoxylate in the same buffer. Under the conditions of experiments the reduction of only 1% of total flavin occurring slower than 2 msec should have been detected.

Inhibition of Activity by FMN and AMP or ATP. Because of the lack of evidence for FAD reduction during the carboligase reaction the structural role of FAD was investigated. As shown in Table II FMN and AMP or ATP inhibited the enzyme activity individually, but when FMN was combined with ATP or AMP the degree of inhibition was greatly increased. The presence of thiamine diphosphate and $MgCl_2$

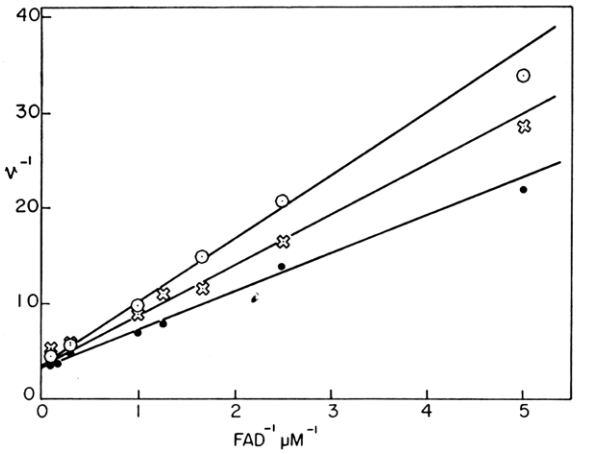

FIGURE 2: Inhibition of carboligase reconstitution with FAD by FMN and AMP. Glyoxylate carboligase purified in the absence of dithiothreitol (2 mg of protein/ml) was resolved of FAD by the acid ammonium sulfate procedure and the colorless apo enzyme was dissolved in the original volume of 0.05 M potassium phosphate buffer containing 1 mM $MgCl_2$ and 0.1 mM thiamine diphosphate. Aft 30-min preincubation of 10 μl of the apo enzyme solution witn FAD with and without FMN or AMP in the same buffer (total volume 3 ml) the assay of carboligase activity was carried out as described in Materials and Methods. v, $\Delta A_{340m\mu}$ min^{-1}; FAD alone (●), FAD plus 1 mM AMP (⊠), and FAD plus 1 μM FMN (○).

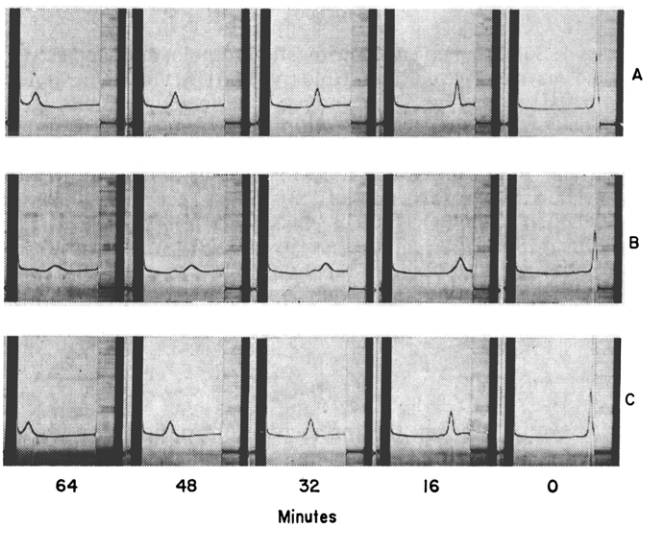

FIGURE 3: Sedimentation behavior of holo, apo, and reconstituted enzyme of carboligase. Glyoxylate carboligase holo enzyme (A) was prepared by ammonium sulfate (50% saturation) precipitation of enzyme and by dissolving the precipitate in 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM $MgCl_2$, 0.1 mM thiamine diphosphate and 1 mM dithiothreitol. Apo enzyme (B) was prepared as described in Materials and Methods and was dissolved in the same buffer. Reconstituted enzyme (C) was prepared by incubation of the apo enzyme with 50 μM FAD for 1 hr at 4°. All the three samples were dialyzed overnight at 4° against the same buffer used above before the sedimentation studies in a Spinco Model E analytical ultracentrifuge as described in Materials and Methods. Protein concentration was 5 mg/ml.

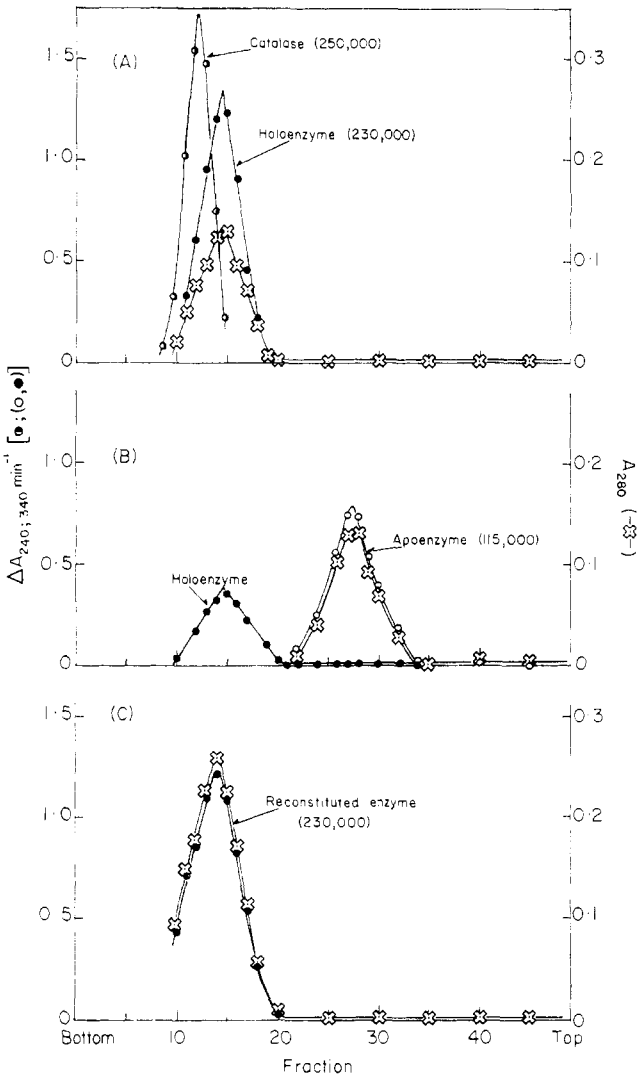

FIGURE 4: Sucrose gradient centrifugation of holo, apo, and reconstituted enzyme of carboligase. Holo (A), apo (B), and reconstituted enzyme (C) preparations were prepared as in Figure 3 and used after dialysis. Sucrose gradient centrifugation was carried out as described in Materials and Methods. Fractions of 0.1 ml were collected and 10-μl sample was used for enzyme assay. Activity of glyoxylate carboligase determined by the standard assay procedure. $\Delta A_{340m\mu}$/min (●); protein concentration measured as absorption at 280 mμ (⊗); catalase activity in $\Delta A_{340m\mu}$/min (centrifugation performed in a separate tube) (◑); and apo enzyme activity determined after preincubation with 5 μM FAD for 20 min. $\Delta A_{340m\mu}$/min (○).

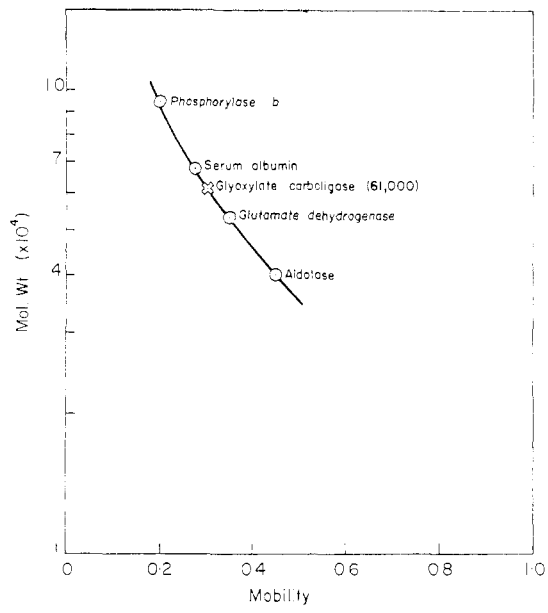

FIGURE 5: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of carboligase subunit. Procedures were as described in Materials and Methods.

was essential during the preincubation period for FMN and adenine nucleotide inhibition of carboligase. If the enzyme was preincubated with 3.3 μM FMN and 1 mM ATP there was only 20% inhibition of activity when measured under the standard method.

Reconstitution of Holo Enzyme from Apo Enzyme and FAD. Apo enzyme freed of FAD as described in Materials and Methods had no carboligase activity, but regained the activity when reconstituted with FAD to holo enzyme. As shown in Figure 2 FMN and AMP inhibited the reconstitution process competitively with respect to FAD concentration. FMN and deamino FAD were ineffective in replacing FAD for the reconstitution of active holo enzyme.

Sedimentation Velocities of Holo Enzyme and Apo Enzyme. Glyoxylate carboligase holo enzyme showed a single peak in a Spinco Model E analytical ultracentrifuge with the observed sedimentation coefficient (s_{20}) value of 8.6 S as shown in Figure 3A. The apo enzyme preparation showed a major protein peak which moved much slower (s_{20} value 6.0 S) with a smaller protein peak moving ahead indicating a residual amount of unresolved holo enzyme (B). When the apo enzyme was reconstituted with FAD the original holo enzyme centrifugation pattern was recovered (C).

The analytical ultracentrifugation results were confirmed and extended with the sucrose gradient centrifugation studies. As shown in Figure 4 the molecular weight of holo enzyme was 230,000 ± 10,000 (A). When the enzyme was resolved of FAD the molecular weight decreased to 115,000 ± 5000 (B). The reconstituted enzyme had a molecular weight identical with that of holo enzyme (C).

Molecular Weight of Enzyme Subunit. The molecular weight of carboligase subunit was determined by the polyacrylamide gel electrophoresis with sodium dodecyl sulfate as shown in Figure 5. The molecular weight was found to be 61,000 ± 3000. It is concluded therefore that the holo enzyme consists of four subunits and the apo enzyme consists of two subunits.

Discussion

From the present studies it is concluded that glyoxylate carboligase of *P. oxalaticus* consists of four protein subunits each with a molecular weight around 60,000 and of two FAD. When FAD is removed from the holo enzyme the enzyme dissociates to inactive dimers with a molecular weight of 115,000. With the reconstitution of apo enzyme with FAD the molecular weight increases to 230,000, indicative of association of dimers to the original tetramer.

A tentative model that emerges from these studies is an enzyme molecule consisting of two dimers joined together by two FAD molecules. The inhibition and reconstitution studies with FMN and adenine nucleotides suggest that both flavin and adenine moieties of FAD are involved in keeping the tetramer in an active form and in the formation of an active tetramer from dimers. Flavin and adenine moieties

may bind to the two dimer molecules joining them together. The presence of two FAD molecules in the holo enzyme molecule suggests that two such FAD bridges are formed between two pairs of subunit monomers. Clearly a more detailed study is necessary before the mechanism of tetramer formation is elucidated. For example, the experimental results may alternatively be explained by a conformational change of protein induced by FAD binding to a dimer leading to the association of dimers to tetramers. At the present time this alternative explanation cannot be excluded.

The reversible inactivation of the *E. coli* enzyme by reduction of FAD with dithionite (Gupta and Vennesland, 1966) as well as the inability of deamino FAD to replace FAD in our reconstitution experiments suggest a specific structural requirement for the molecule of FAD. It should be interesting to prepare the semiquinone form of the FAD enzyme and study its reaction with glyoxylate.

The lack of evidence for flavin reduction during the reaction of glyoxylate carboligase in our studies as well as in the *E. coli* enzyme studies (Gupta and Vennesland, 1964, 1966) seems to favor the structural role of FAD in this enzyme, although it does not rule out entirely the direct FAD participation in the condensation of glyoxylate. It is still possible that FAD might participate in the carboligase reaction in such a way that the reduction and oxidation are over within 2 msec, the dead time of the stopped-flow instrument used. Other types of rapid kinetic studies should be attempted before the catalytic role of FAD is ruled out.

Inhibition of carboligase activity by FMN and adenine nucleotides as well as that of reconstitution of the active carboligase by FMN and AMP was maximal when $MgCl_2$ and thiamine diphosphate were present and dithiothreitol was absent. The holo enzyme was protected from inhibition also by the presence of tartronate semialdehyde reductase and even bovine serum albumin during the preincubation with these nucleotides. The concentration of enzyme was also an important factor in the inhibition. A higher enzyme concentration during preincubation produced a lower degree of inhibition. In fact, in ultracentrifugation studies where the enzyme concentration was high and dithiothreitol was present FMN at the same concentration as FAD showed no inhibition of the reconstitution process. Although these results suggest that conformational changes induced under various conditions affect the affinity of FAD to the enzyme further work is necessary to elucidate this problem. It is, however, interesting to note that Gupta and Vennesland (1966) obtained a K_m for FAD of 0.2 μM in their reconstitution experiment in the presence of cysteine while the K_m calculated from Figure 2 was 1.3 μM. In our experiment of Figure 2 no sulfhydryl reducing agent was present.

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