Glycine Metabolism

III. A FLAVIN-LINKED DEHYDROGENASE ASSOCIATED WITH THE GLYCINE CLEAVAGE SYSTEM IN PEPTOCOCCUS GLYCINOPHILUS

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SUMMARY

One of the four proteins involved in the cleavage of glycine to 1-carbon units and the transfer of electrons from glycine to diphosphopyridine nucleotide is a flavoprotein.

Precipitation of the protein with acid ammonium sulfate in the presence of 1 M potassium bromide separated the co-enzyme from the apoenzyme. The latter was fully reactivated by incubation with flavin adenine dinucleotide, but showed no activity with flavin mononucleotide.

The flavoprotein could be reduced either by glycine or by reduced diphosphopyridine nucleotide. When reduced by the latter, no protein other than the flavoprotein was required for the electron transfer, but to achieve a measurable rate of reduction by glycine it was necessary to add the three other proteins plus tetrahydrofolate. Dihydrofolate was entirely inactive in the system.

In an earlier report (1) the over-all pathway of glycine catabolism in Peptococcus glycophilus was outlined, and in Papers I and II of the present series (2, 3) some properties of the enzyme system catalyzing the labilization of the glycine carboxyl group were described. Preliminary reports of portions of the present work, describing the separation of the glycine cleavage system into four protein fractions, have appeared (4, 5). The summary of the activities of these four proteins is shown in Fig. 1. Labilization of the glycine carboxyl group, measured as the exchange of bicarbonate with glycine-1-14C, is catalyzed in the presence of two proteins, one of which is a pyridoxal phosphate-containing enzyme with a molecular weight of approximately 125,000 and previously designated P1 (2). The other is a strongly acidic protein, stable to boiling in 5% ammonium sulfate for 10 min, with a molecular weight of approximately 10,000 and previously designated P2 (2). Upon labilization of the glycine carboxyl group, the α carbon is transferred to tetrahydrofolate under the mediation of the protein designated P4. Coupled to this sequence is the transfer of electrons from the glycine molecule to enzyme-bound flavin adenine dinucleotide, as mediated by the dehydrogenase designated P3 (5), which finally transfers the electrons to DPN. The present report describes the properties of P3, the FAD-containing flavoprotein, particularly the optical properties of the holo- and apoenzymes, and the function of these proteins in the series of reactions associated with glycine catabolism in P. glycophilus.

EXPERIMENTAL PROCEDURE

Preparation of Cells, Cell Extracts, and Protein Fractions—Methods for the preparation of cells of P. glycophilus were described previously (2), as were the methods for the preparation of cell extracts and ammonium sulfate fractions.

Preparation of Flavoprotein P3—The protein fraction (3.15 g) precipitating between 55 and 75% ammonium sulfate saturation was placed on an Ecteola1 column (4 x 50 cm) equilibrated with 0.02 M potassium phosphate buffer, pH 7.1. Proteins were eluted from the column by stepwise increases in the concentration of potassium chloride contained in the above buffer. The flow rate of the column was 38 ml per hour. Effluent from the column was collected in 5-ml portions, and the protein peak containing P3 (248 mg) was subjected to a temperature of 70° for 5 min, which resulted in the precipitation of a large portion of the protein, but did not result in the loss of any of the P3 activity. The precipitated protein was separated by centrifugation and the supernatant fraction (containing 60 mg of protein) was placed on a diethylaminoethyl Sephadex A-50 column (2 x 27 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.1. The column was eluted with increasing concentrations of potassium chloride in 0.05 M phosphate buffer, pH 7.1. The effluent was collected in 5-ml volumes at a flow rate of 30 ml per hour, and the P3-containing protein peak, eluted with 0.25 M potassium chloride, was used in the present experiments.

Preparation of Apoenzyme of P3—The flavin prosthetic group of P3 was removed by precipitation of the protein in acid ammonium sulfate in the presence of 1 M potassium bromide (6). The precipitated protein was separated by centrifugation, washed in saturated ammonium sulfate, and was redissolved in 0.2 M potassium phosphate buffer, pH 7.1. It was then dialyzed against the same buffer for 2 hours.

Determination of P3 Activity—Of the reactions shown in Fig. 1, the exchange of bicarbonate with glycine-1-14C could be assayed independently of the others and required only proteins P1 and P3. The reduction of the flavoprotein P3 could be measured with

1 The abbreviation used is: Ecteola, epichlorohydrin triethanolamine cellulose.
either glycine or DPNH as electron donor. When DPNH was used, no protein other than P₁ was required to catalyze the reduction of the flavin, which could be measured either by the decrease in the FAD absorption band at 450 nm, decrease in the DPNH absorption at 340 nm, or by the transfer of electrons from DPNH to benzyl viologen with concomitant increase in the violet band at 555 nm. When glycine served as the electron donor, however, it was necessary to couple P₁ to P₂, P₃, and P₄ plus tetrahydrofolate in order to achieve a measurable rate of electron transfer. In the latter case activity could be measured as an increase in absorption at 340 nm when DPN was present as an acceptor, or at 555 nm when benzyl viologen was substituted for DPN, or by following the direct reduction of the flavin band at 450 nm when neither DPN nor benzyl viologen was present in the reaction mixtures.

Optical Determinations—Optical measurements were made with a Cary model 15 recording spectrophotometer or with a Beckman model DU spectrophotometer.

Tetrahydrofolate and Dihydrofolate—Tetrahydrofolate was prepared by catalytic hydrogenation of folic acid in an aqueous bicarbonate solution as suggested by Blakley (7). The dihydrofolate used in these experiments was kindly supplied by Dr. F. M. Huennekens.

RESULTS

A convenient method of obtaining three of the four proteins involved in the over-all cleavage of glycine was by chromatography of a 55 to 75% ammonium sulfate fraction on an Ecteola column as shown in Fig. 2. The P₁, P₄, and P₃ proteins were completely separated from each other and from several other protein peaks by this method. Protein P₃, because of its highly acidic nature, was tightly bound to the Ecteola column and was not eluted by this procedure, but was prepared by chromatography on Sephadex G-25 and G-100, and by heat treatment as described previously (2). The protein P₁ used in these experiments was obtained from the Ecteola column in the fractions collected between 540 and 700 ml as shown in Fig. 2, and was used without further purification. Properties of this protein will be described in another report.

The P₃ peak shown in Fig. 2 contained a mixture of proteins as shown by the fact that the activity curve was not entirely superimposable on the protein curve. Inasmuch as the P₃ protein was stable to heating to 70° for 5 min, the fractions from the Ecteola column containing P₃ were subjected to this treatment, and the precipitated proteins were removed by centrifugation. The bright yellow supernatant solution containing P₃ was placed on a DEAE-Sephadex A-50 column, and in this case the activity curve of the effluent fractions was entirely superimposable upon the protein curve.

Because of the presence of other DPNH oxidizing flavoproteins present in crude extracts and in ammonium sulfate fractions precipitating below 55% saturation, it was difficult to determine accurately the level of P₃ activity in these fractions when assayed by a procedure in which the flavoprotein was reduced by DPNH, or in which the latter was generated from DPN. However, the ammonium sulfate fractions precipitating above 55% saturation were free of DPNH oxidases (except for P₁). Thus, by knowing that chromatography on Ecteola, heat treatment, and chromatography on DEAE-Sephadex resulted in 6-, 5-, and a-fold increases, respectively, in specific activity compared with the 55 to 75% fraction, and by assuming that the ammonium sulfate fractionation resulted in a 2- to 5-fold increase in activity (as we have frequently noted for other enzymes), it was estimated that the P₃ activity was increased at least 120-fold.

Requirements for DPN Reduction by Glycine—As mentioned earlier, transfer of electrons from glycine to DPN required all four proteins: P₁, P₃, P₄, and P₅ plus tetrahydrofolate. This is shown in Fig. 3, wherein it is shown that any combination other than the one containing the four proteins was inactive in catalyzing the electron transfer. When all four proteins plus tetrahydrofolate were present, the reduction of DPN proceeded rapidly.

Requirement of P₃ Reduction—Although the P₃ flavoprotein was easily reduced either by DPNH or by glycine, the requirements for additional proteins or cofactors differed, depending upon the direction in which the electrons were flowing. When P₃ was reduced by DPNH, no protein or cofactor other than the FAD-containing P₃ was necessary. However, as shown in Fig. 4, in order for P₄ to be reduced by glycine, all four proteins, P₁,
P₃, P₄, and P₅ as well as tetrahydrofolate were required, similar to the requirements for DPN reduction by glycine. If any one of the proteins or tetrahydrofolate was omitted, no change in the absorption spectrum of P₅ was observed after 15 min of incubation. Upon addition of tetrahydrofolate to the reaction, however, the reduction of P₃ became evident within 30 sec. Increasing the P₁ and P₂ protein levels from 0.036 and 0.85 to 1.8 and 1.7 mg, respectively, increased the rate of P₃ reduction, but still there was no detectable change in the P₅ spectrum in the absence of tetrahydrofolate. Addition of DPN to the reduced enzyme oxidized the flavin to give the original spectrum of P₅.

**FAD as Prosthetic Group**—The absorption spectrum of the purified P₅ holoenzyme measured independently was the same as the spectrum represented by the solid line in Fig. 4. The enzyme exhibited typical properties of a flavoprotein with maxima at 457 and 340 nm, and shoulders at 485, 435, and 370 nm. When the flavin was separated from the holoenzyme by treatment with acid ammonium sulfate and potassium bromide (6), all absorptivity due to the flavin was removed. The supernatant solution from the apoenzyme precipitation contained the flavin coenzyme and exhibited an absorption curve superimposable upon that of an FAD solution, with a typical A₅₅₀/A₃₄₀ of 3.5 (8). Reactivation of the P₅ apoenzyme by combination with FAD is shown in Fig. 5.

**Fig. 3. Rate of DPN reduction by glycine with various combinations of the four proteins.** A 1.5-ml portion of reaction mixture, in a cuvette with a 1.0-cm light path, contained 0.1 M K₂HPO₄, pH 7.1; 75 nm glycine; 10⁻² M 2-mercaptoethanol; 10⁻⁸ M EDTA; 1.5 X 10⁻⁴ M tetrahydrofolate; 10⁻⁴ M DPN; and where added, 0.036 mg of P₁; 0.36 mg of P₂; 0.006 mg of P₃; and 0.016 mg of P₄. Measurements were made with a Beckman DU spectrophotometer. Control cuvette was minus DPN.

**Fig. 4. Reduction of the flavoprotein P₅ by glycine.** The reaction mixture was the same as in Fig. 3 except that DPN was omitted, the concentration of P₂ was increased to 3.6 mg, and P₄ was decreased to 0.066 mg. The curves were traced from the data obtained with a Cary model 15 recording spectrophotometer.

**Fig. 5. Reactivation of P₅ apoenzyme by FAD.** P₅ apoenzyme (3 mg in 1.0 ml of K₂HPO₄, pH 7.1) was incubated either with 0.05 μM FAD or 0.05 μM FMN for 2 hours at 37°. The preparations were then tested for their ability to transfer electrons from DPNH to benzyl viologen in a reaction mixture containing 2 mM DPNH; 10⁻⁴ M benzyl viologen (Mann); 0.1 M K₂HPO₄, pH 7.1; 10⁻³ M 2-mercaptoethanol; 10⁻⁸ M EDTA; 0.003 mg of reactivated apoenzyme or 0.3 mg of apoenzyme. The reaction was initiated by the addition of DPNH. Readings were made with a Beckman DU spectrophotometer.
In this case, the enzyme was assayed by its ability to catalyze the transfer of electrons from DPNH to benzyl viologen. The apo-enzyme alone showed no catalytic activity, and incubation of the apo-enzyme with FMN did not re-activate the system. However, when the apo-enzyme was incubated with FAD, the original activity of the holo-enzyme was regained. With the use of an estimated molecular weight of 120,000 for the P₃ protein, which value was obtained by determining the relative migratory rate of PB on Sephadex G-100 and G-200 columns as outlined by Whitaker (9), it was determined that 1 mole of FAD was bound to 1 mole of the P₃ protein to constitute the holo-enzyme.

Inactivity of Dihydrofolic—Although tetrahydrofolate had been used in all of the experiments in which a folate cofactor was required, the possibility existed that dihydrofolic could also function in the system. A tetrahydrofolate dehydrogenase, if present, could oxidize the tetrahydrofolic to dihydrofolic and thus allow the interconversion of these two forms of the cofactor. However, as shown in Fig. 6, the system showed a definite requirement for tetrahydrofolate as cofactor and was completely inactive in the presence of dihydrofolic.

**DISCUSSION**

A feature held in common by the glycine-metabolizing system from *Escherichia coli*, described by Pitts and Crosbie (10), the avian liver system described by Richert, Amberg, and Wilson (11), and the *P. glycinophilus* system is the transfer of electrons from glycine to DPN and the involvement of pyridoxal phosphate and tetrahydrofolic in the over-all scheme. It has now been established that one of the proteins involved in the electron transfer is an FAD-containing protein described in this communication as P₃. Although the separation and identification of a similar flavoprotein from *Escherichia coli* and liver systems has not yet been accomplished, it is possible that such a flavoprotein is involved in these systems as well as in *P. glycinophilus*. It has been established (12) that the heat-stable protein P₁, required for the activation of the glycine cleavage system in *P. glycinophilus*, is also present in *E. coli* and in various animal liver systems.

In considering the sequence in which the four proteins function during the cleavage of glycine to 1-carbon units and transfer of electrons to DPN, it is apparent from the experimental data that electron transfer from P₁ to DPN is the terminal step, as suggested in Fig. 1. It is further apparent that labilization of the carboxyl group in the presence of the pyridoxal phosphate-requiring enzyme P₁ and the heat-stable protein P₂ precedes the reduction of the flavoprotein P₃, since the latter enzyme is not required for exchange of bicarbonate with the glycine carboxyl group (2, 3). The requirement for proteins P₁, P₂, and P₃ to achieve the reduction of the flavoprotein P₂ by glycine would suggest the interaction of these proteins prior to reduction of the flavin, but the precise role of P₁ and its possible association with tetrahydrofolic, and of P₂ and its possible function in the release of the glycine carboxyl group and transfer of electrons to the flavoprotein has not yet been adequately established by experimental evidence.

Following the appearance of the preliminary reports (4, 5) of the present communication, Baginsky and Huennekens (13) also described the preparation of the electron-carrying flavoprotein P₃ from *P. glycinophilus*, with properties similar to those described in this report, and identified the prosthetic group as FAD by paper chromatography following heat or acid denaturation of the protein. They further postulated an interaction of P₂ and the heat-stable protein P₃ in the transfer of electrons from glycine to DPN. Studies related to the structure and function of P₂ as observed in our laboratory will be described in a separate report.

**REFERENCES**

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