ELECTRON TRANSPORT FUNCTION OF A HEAT-STABLE PROTEIN AND A FLAVOPROTEIN IN THE OXIDATIVE DECARBOXYLATION OF GLYCINE BY PEPTOCOCCUS GLYCINOPHILUS*

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The oxidative decarboxylation of glycine according to equation (1)

$$\text{glycine} + \text{tetrahydrofolate} + DPN^{+} \longrightarrow \text{5,10-methylene tetrahydrofolate} + CO_2 + NH_4^{+} + DPNH \qquad (1)$$

was described by Sagers and Gunsalus (1961) in cell-free extracts of Peptococcus glycinophilus and by Richert et al. (1962) in avian liver preparations. In both systems, there is a partial requirement for added pyridoxal phosphate and glyoxylate cannot replace glycine as the substrate. Sagers and Klein (1965) employed fractionation with ammonium sulfate, followed by chromatography on DEAE-Sephadex A-50, to separate the P. glycinophilus system into four protein fractions (P_1, P_2, P_3, and P_4), all of which were required to catalyze the overall reaction. Three of the fractions were shown to have special characteristics: P_1 contains bound pyridoxal phosphate (Klein and Sagers, 1965, 1966 and 1966a); P_2 is a heat- and acid-stable protein (Klein et al., 1964; Sagers and Klein, 1965); and P_3 is a flavoprotein (Sagers, private communication). The combination of P_1 and P_2 catalyzes the exchange of $^{14}CO_2$ with the carboxyl group of glycine (Klein et al., 1964; Klein and Sagers, 1966).

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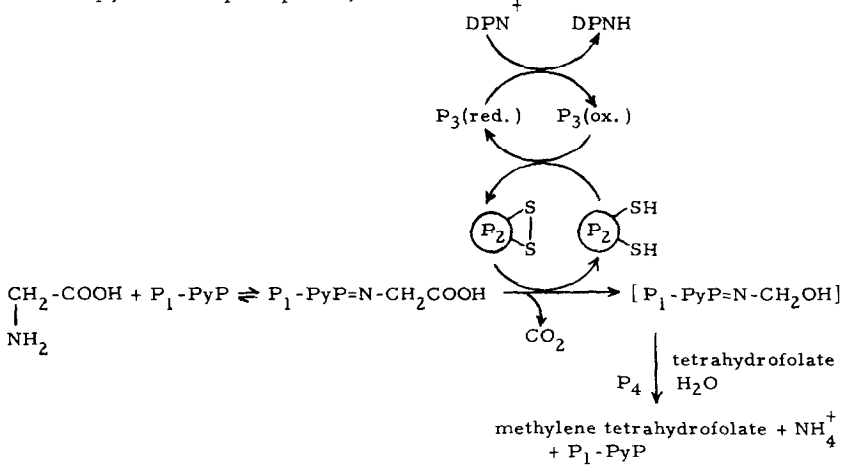
The present communication extends these observations by providing evidence that the heat-stable protein and the flavoprotein from P. glycinophilus are involved in the transfer of reducing power from glycine to DPN. These proteins, moreover, bear a close resemblance to thioredoxin (a low molecular weight, heat-stable protein) and thioredoxin reductase (a flavoprotein), which have been shown to be components of the ribonucleotide reductase system in Escherichia coli (Laurent et al., 1964). A tentative mechanism for reaction 1 is also proposed.

P. glycinophilus was grown anaerobically at 36° in 10-liter bottles containing the medium of Sagers and Gunsalus (1961) except that the concentration of phosphate buffer was decreased to 0.05 M, the amount of $MgSO_4 \cdot 7H_2O$ was increased to 200 mg per liter, and $MnSO_4 \cdot H_2O$ (0.34 mg per liter) was added. Cells were harvested by centrifugation after 60-72 hours of growth and were stored frozen until needed. Fifty ml aliquots of cell suspensions (1 part cells and 3 parts 0.02 M phosphate buffer, pH 7.2, which was 0.01% in Na_2S) were sonicated for 20 min in a Raytheon 10 KC. oscillator and centrifuged at 35,000 x g for 30 min to remove cell debris. Nucleic acids were precipitated with protamine sulfate and, after centrifugation, the supernatant solution was fractionated with ammonium sulfate. The fraction precipitating between 70 and 100% saturation (at 5°) was dissolved in a minimum volume of 0.02 M phosphate buffer, pH 7.0, and chromatographed on Sephadex G-50. The rapidly moving protein band contained P_1, P_3 and P_4 and a small amount of P_2; the latter followed the bulk of the protein but still moved well ahead of the colored bands of ferredoxin and rubredoxin. P_1, P_3 and P_4 were separated from each other by chromatography on DEAE-cellulose (phosphate buffer, pH 7.0; gradient 0.05 $\longrightarrow$ 0.4 M). P_1 and P_3 were purified further by chromatography on hydroxylapatite (phosphate buffer, pH 6.8; gradient 0.05 $\longrightarrow$ 0.4 M). Fractions from the Sephadex G-50 column that contained P_2 activity were pooled and lyophilized. The residue was dissolved in water, heated for 3 min in a boiling water bath, and the precipitate was removed by centrifugation (105,000 x g, 15 min). After dialysis, the supernatant solution was chromatographed on hydroxylapatite (phosphate buffer, pH 6.8; gradient 0.05 $\longrightarrow$ 0.2 M).

P_1 is a yellow-green protein ($\lambda_{max.}$ at 430 mμ) which is stable to

repeated freezing and thawing. P_2 is a colorless protein of low molecular weight, as judged by its behavior on Sephadex. It is strongly adsorbed on DEAE-cellulose and is stable to heating, to acid, and to repeated lyophilization. No labile sulfide groups were detected in this protein and the spectrum showed, in addition to the typical protein peak in the 280 mμ region, a shoulder at about 290 mμ and, in some preparations, a small peak at 325 mμ similar to that seen in oxidized lipoic acid (Calvin and Barltrop, 1952). P_3 is a flavoprotein with absorption maxima at about 280, 350 and 455 mμ and shoulders at about 370, 435 and 480 mμ; absorption minima occur at about 315 and 395 mμ. The flavin of P_3 is not detached when the protein is chromatographed on Florisil or repeatedly precipitated with acid ammonium sulfate. It can be released, however, by heat- or acid-denaturation of the protein. Paper chromatography using 5% Na_2HPO_4 or n-butanol:acetic acid: water (60:15:25) demonstrated that the flavin is FAD. Based upon the small amount (ca. 1 μgm) of this protein required for the overall reaction, P_3 appears to have a high turnover number. P_4 is a colorless protein that is poorly adsorbed on DEAE-cellulose. It loses activity upon repeated freezing and thawing.

Several properties of P_2 (heat stability, low molecular weight and strong adsorption on DEAE-cellulose) are similar to those of thioredoxin (Laurent et al., 1964). The latter protein contains a disulfide bridge which is reduced via a TPNH-dependent flavoprotein (thioredoxin reductase) to a dithiol structure. By analogy, P_2 and P_3 might be expected, therefore, to be involved in electron transport between glycine and DPN. A tentative mechanism for the oxidative decarboxylation of glycine is shown below (PyP represents pyridoxal phosphate):



Several observations support the assignment of P_2 and P_3 in the electron transfer portion of the overall reaction. First, chemically-reduced P_3 is readily reoxidized by DPN, but not by TPN; this is consistent with the pyridine nucleotide specificity of reaction 1. As shown in Fig. 1, addition of DPN to the dithionite-reduced flavoprotein partially restores the yellow color and gives rise to absorption maxima at about 445 and 475 mμ. It should be noted that the spectrum of the oxidized form of P_3 closely resembles that of lipoyl dehydrogenase from E. coli (Williams, 1965).

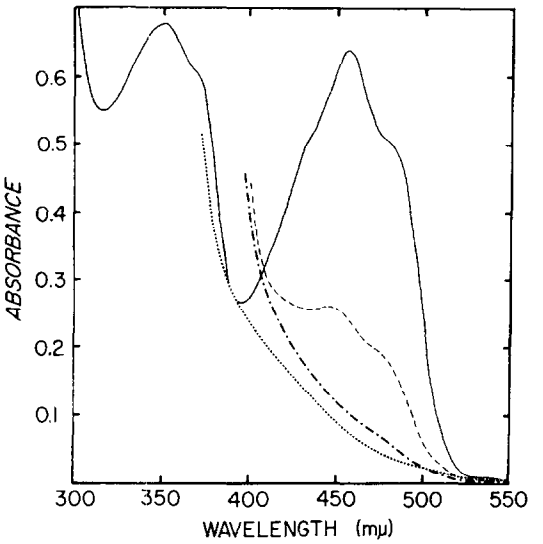


Fig. 1. Oxidation of reduced P_3 by DPN. Samples were examined under nitrogen in anaerobic cuvettes with side-arms, and spectra were recorded with a Cary Model 14 spectrophotometer. The experimental cuvette contained 24 mg of P_3 in 3.0 ml of 0.05 M potassium phosphate buffer, pH 7.0; the blank cuvette contained only buffer.(——) Initial spectrum. (· · · ·) After addition of ca. 0.5 mg of dithionite to the experimental cuvette . (·- ·-) After addition of 6 μmoles (in 0.1 ml) of TPN to the dithionite-reduced sample (- - - -) After addition of 6 μmoles of DPN (in 0.1 ml) to the dithionite-reduced and TPN-treated sample.

Second, DPN and P_3 may be replaced in the complete assay system (glycine-dependent reduction of DPN) by DTNB*, suggesting that the function of P_3 is to link reduced P_2 with DPN. Finally, reversed electron flow is also possible with these components. Thus, DPNH (but not TPNH)

* 5, 5' - Dithiobis - (2-nitrobenzoic acid)

will reduce DTNB in the presence of P_2 and P_3. A typical experiment illustrating this point is shown in Fig. 2.

Highly purified thioredoxin and thioredoxin reductase from E. coli cannot replace P_2 and P_3, respectively, either in glycine oxidation (equation 1) or in DPNH oxidation linked to DTNB. In turn, P_2 and P_3 are not able to catalyze the reduction of insulin by DPNH (or TPNH), a reaction known to occur with the thioredoxin system.

According to the mechanism proposed above, oxidation and decarboxylation of glycine would occur before the remnant of the substrate interacts with tetrahydrofolate. Thus, DPNH formation should be observed in the absence of tetrahydrofolate and P_4, provided that a sufficiently large amount of P_1 is present; preliminary experiments support this view. The mechanism also accounts for the observation by Klein and Sagers (1966) that only P_1 and P_2 are required for the exchange of $^{14}CO_2$ with the carboxyl group of glycine. The proposal by Richert et al. (1962) that glycine forms a Schiff base with

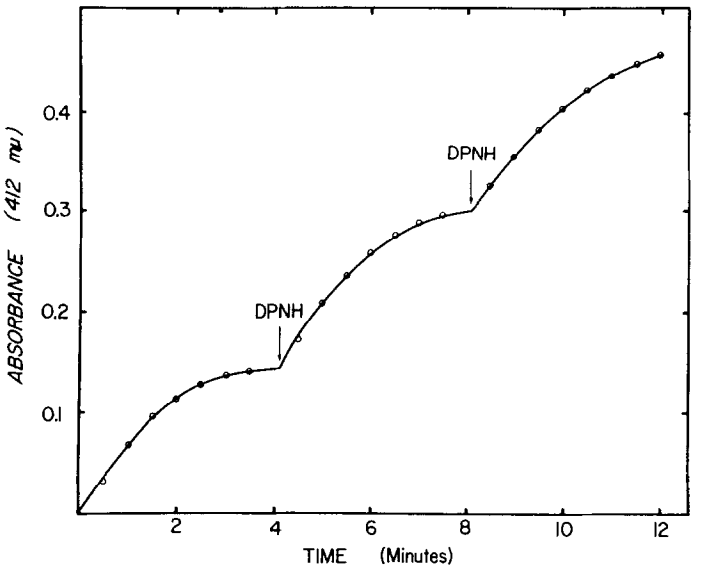
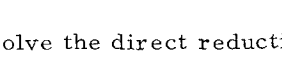
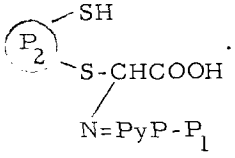
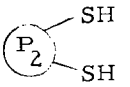


Fig. 2. Reduction of DTNB by DPNH. The experimental cuvette contained in a total volume of 3.0 ml: 50 μmoles of potassium phosphate buffer, pH 7.0, 30 μmoles of EDTA, 0.5 μmole of DTNB, 0.05 mg of P_3 and 0.12 mg of P_2. The blank cuvette contained all components except P_2. After pre-incubating the mixtures for 2 min at 37°, the reaction was started by adding 0.25 μmoles of DPNH (in 0.02 ml) to both cuvettes. The reaction was followed in a Beckman DU spectrophotometer by the increase in absorbance at 412 mμ. At the times indicated by arrows, further additions of DPNH (0.25 μmoles) were made.

pyridoxal phosphate is now susceptible to experimental proof following the isolation and detailed characterization of P_1 as a pyridoxal-containing protein (Klein and Sagers, 1966a). Oxidation of such a pyridoxal-glycine complex might involve the direct reduction of P_2 (), or it could proceed via expulsion of a proton from the α-carbon of glycine followed by attack of the carbanion upon the S-S bridge of P_2 to yield  . Decarboxylation of the latter complex, followed by hydrolysis, would yield CO_2,  and P_1-PyP=N-CH_2OH. A similar complex involving a linkage between a protein-bound thiol group and the cobalt of vitamin B_{12a} has been postulated as a step in the reduction of B_{12a} to B_{12s} (Walker, Schmidt and Huennekens, in preparation).

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