A PYRIDINE NUCLEOTIDE-CYTOCHROME C REDUCTASE
ISOLATED FROM CHLOROPLASTS

KEN TAKAMATSU, MITSUO NISHIMURA AND HIROSHI TAMIYA
DEPARTMENT OF BIOPHYSICS AND BIOCHEMISTRY, FACULTY OF SCIENCE, UNIVERSITY OF TOKYO, AND THE TOKUGAWA INSTITUTE FOR BIOLOGICAL RESEARCH, TOKYO

In 1957 Avron et al (1) extracted from spinach leaves a TPNH-diaphorase which transfers electrons from TPNH to various oxidoreductive dyes but not to cytochrome c. Recently Marré et al (5, 6) isolated from chloroplasts of pea seedling a pyridine nucleotide-cytochrome c reductase which utilizes TPNH, markedly preferentially to DPNH, as an electron donor. The existence of these enzymes is of interest in view of the current concept among the investigators of photosynthesis that some cytochrome(s) as well as pyridine nucleotide(s) are involved in the mechanism of photosynthesis. Independently of these studies, we have been pursuing investigations on the oxidoreductive enzymes contained in chloroplasts, and from the chloroplasts of spinach and parsley an enzyme was isolated, which is similar to, but not identical with, that studied by Marré et al. The methods of isolation and purification as well as the properties of this enzyme form the subjects of this paper.

MATERIALS AND METHODS

The enzyme could be extracted from green leaves of spinach and parsley or chloroplasts isolated therefrom. To obtain chloroplasts, fresh leaves were homogenized in a Waring blender for 3 minutes with the addition of 0.35 M NaCl solution. The homogenate was squeezed through cloth and centrifuged at 400 G for 5 minutes to remove the cell debris. The supernatant was centrifuged at 1700 G for 20 minutes, and the precipitate was collected, washed twice with 0.35 M NaCl solution by centrifugation, and used for further experiments. Two extraction procedures were employed. These led to the isolation of almost identical crude enzyme preparations.

PROCEDURE A: Fresh leaves or isolated chloroplasts (spinach) were ground in a mortar with quartz sand with the addition of chilled ammoniacal acetone-water (containing in volume percent: 98.5 acetone, 1.1 water and 0.4 ammonia) in a proportion of 85 ml per 100 g of fresh leaves or chloroplast suspension. The resulting mash was centrifuged at 1700 G for 15
minutes and the coarse precipitate was discarded. To the greenish-brown supernatant was added an equal volume of chilled ammoniacal acetone-water and the mixture was kept in an ice bath for 15 minutes. The precipitate formed was collected by filtration on a Buchner funnel and washed with weakly ammoniacal acetone-water (containing in volume percent: 74.5 acetone, 25 water and 0.15 ammonia) and then with 70% saturated ammonium sulfate solution (pH 8.0). The filter cake obtained was repeatedly extracted with small volumes of 0.02 M disodium phosphate solution, and the brownish-orange supernatant obtained on the final centrifugation was used as a crude enzyme preparation. The orange tint of this solution was due to contamination by the carotene protein complex previously reported from our laboratory (8).

Procedure B: Fresh leaves (250 g fresh weight) or chloroplasts (50 g fresh weight) were ground in a mortar with quartz sand and 100 ml of 0.02 M ammonium buffer (pH 8.0), to which polyethylene sorbitan monooleate (Tween 80) was added to a final concentration of 2%. The resulting mash was centrifuged at 1700 G for 20 minutes and the green supernatant was brought to 30% saturation with ammonium sulfate. The solution was kept in an ice bath for 15 minutes and then centrifuged at 1700 G for 25 minutes. The supernatant was filtered on a Buchner funnel using talc as a filter aid. The brown filtrate containing the enzyme was dialyzed in a refrigerator against 0.02 M disodium phosphate solution.

The crude enzyme preparations obtained by these 2 methods showed almost similar specific activity, but the yield was superior with Procedure B.

Purification of the enzyme: The crude enzyme solution obtained by Procedure B was brought to 35% saturation with ammonium sulfate (pH 8.0) and was centrifuged to remove the brownish precipitate formed. The supernatant was made to 75% saturation with ammonium sulfate, and the precipitate collected by centrifugation was dissolved in 0.02 M disodium phosphate solution. The fractionation with ammonium sulfate was repeated two more times. The enzyme precipitate obtained was dissolved in a minimum volume of pure water and dialyzed in a refrigerator against 0.005 M phosphate buffer (pH 7.0). The dialysate was centrifuged at 7000 G for 15 minutes, and calcium phosphate gel was added to the clear supernatant to adsorb the enzyme. After 15 minutes, the gel was collected by centrifugation. This procedure of enzyme adsorption was repeated until the supernatant became almost colorless. The combined calcium phosphate precipitate was washed twice with pure water, and the enzyme was eluted from the gel by repeated extraction with 0.2 M disodium phosphate solution. The eluate thus obtained was dialyzed overnight in a refrigerator against 0.02 M ammonium buffer at pH 8.0 and used as the purified enzyme in the following experiments. By this purification procedure an 8- to 9-fold increase in the specific activity of the enzyme could be attained.

Activity Measurement: The enzyme activity in reducing cytochrome c in the presence of TPNH or DPNH was measured by following the increase in absorption at 550 mμ using a recording spectrophotometer. The reaction mixture used for the measurement contained: 0.02 M ammonium buffer (pH 8.0), 2.5 ml; solution of oxidized cytochrome c, 0.1 ml (final concentration: 1.7 x 10⁻³ M); solution of reduced pyridine nucleotide, 0.1 ml (final concentration: 1.6 x 10⁻³ M); enzyme preparation, 0.1 ml; and water to make a total volume of 3.0 ml. The reaction was started by adding the enzyme (or pyridine nucleotide) to the reaction mixture.

Enzymic reduction of dyes was measured by the decrease in optical density at 340 mμ (p-benzoquinone) or at 600 mμ (DPIP). The composition of the reaction mixture was the same as that given above except that the dye was added instead of cytochrome c. In some experiments the oxidation of pyridine nucleotide was followed by measuring the decrease in absorption at 340 mμ. All reactions were run at room temperature (around 23° C).

TPNH was prepared from TPN by reduction with glucose-6-phosphate dehydrogenase purified from the yeast (3). DPNH was prepared by the enzymic reduction of DPN with yeast alcohol dehydrogenase (7) or by sodium dithionite reduction according to LePage (4). Both TPNH and DPNH were prepared immediately before each experiment and their concentrations were determined spectrophotometrically at 340 mμ. The preparation of cytochrome c used was that from equine heart muscle, purified by the method of Keilin and Hartree (2).

Results

Stability and Susceptibility to Hydrogen Ion Concentration: The enzyme was found to be rela-
tively stable in weakly alkaline solution, no decrease in enzyme activity being detected after several days of storage in a refrigerator under such conditions. The optimum for the reduction of cytochrome c (with TPNH as the electron donor) was found to lie at pH 8.5 to 9.0 (fig 1). All the following experiments were therefore performed at pH 8.5.

**Specificity:** The reduction of cytochrome c in the presence of TPNH and the enzyme as measured by the change in transmittance at 550 μm is shown in figure 2. Under the experimental conditions adopted, the added amount of cytochrome c was completely reduced within 40 seconds. No significant change in optical density occurred when 1 of the components of the reaction was omitted, or when the enzyme had been heated at 100° C for 3 minutes. The reactions with TPNH and DPNH are compared in figure 3. As will be seen from the figure, the activity with TPNH was about 3 to 4 times as high as that with DPNH. This situation existed at different stages of purification of the enzyme (see table I).

Among the electron acceptors tested, p-benzoquinone and DPIP, besides cytochrome c, were found to be utilized by the enzyme, indicating a diaphorase activity of the enzyme. Also in this case the relative utilizability of TPNH and DPNH as electron donors was 3 to 4 : 1. The enzyme had no activity of catalyzing the oxidation of pyridine nucleotides by molecular oxygen.

**Cofactor Requirements:** Prolonged dialysis of crude enzyme preparation, e.g., for 3 days at 3° C against 0.005 M phosphate buffer (pH 8.0) caused a slight decrease of enzyme activity. The decrease became more marked—reducing the original activity by about 50%—when EDTA or cyanide (10^-3 M) was added to the dialyzing medium. In both cases the lost activity was completely recovered on the addition of molybdate (10^-4 M) to the dialyzed enzyme (table II), indicating the role of this substance as a cofactor of the enzyme under investigation. Tungstate was also effective in this respect but with weaker efficiency. In the concentration range investigated, Ni++, Ca++, Zn++, Mg++ and Fe++ were without any effect; other metal ions were more or less inhibitory to the enzyme activity (table II). Neither FAD nor FMN showed any restoring action upon the dialyzed enzyme in the concentration range studied (10^-4 to 10^-2 M) (table III). The results were also negative when FAD or FMN was added to the acid-treated preparation of the enzyme (precipitated at pH 6.0 with ammonium sulfate and redissolved in ammonium buffer of pH 8.0).

**Effect of Inhibitors:** PCMB (0.4 × 10^-4 M) and potassium cyanide (3 × 10^-4 M) did not affect the activity of the enzyme as estimated by the reduction of cytochrome c in the presence of TPNH and molybdate. The enzyme was inhibited by EDTA, but the activity was recovered by the addition of molybdate (or tungstate) to the reaction mixture.

**Discussion**

Considering the still insufficient purity of the enzyme preparations obtained in the present study, it may be premature to conclude decisively whether the oxidations of TPNH and DPNH were catalyzed by one and the same enzyme, or whether 2 separate enzymes, one specific towards TPNH and the other towards DPNH, were operative. However, the fairly constant ratio of 3 to 4 : 1 with respect to the (diaphorase- and cytochrome c reductase-) activities towards TPNH and DPNH at different steps of enzyme purification seems to support the former view. The above-mentioned ratio is in striking contrast to that found by Marré et al (5, 6), whose enzyme acted with TPNH.

![Fig. 2. Reduction of cytochrome c by the enzyme in the presence of TPNH.](https://www.plantphysiol.org/)

### Table I

<table>
<thead>
<tr>
<th>ENZYME PREPARATION</th>
<th>ACCEPTOR USED</th>
<th>RATE OF REDUCTION* OF ACCEPTOR IN THE PRESENCE OF TPNH</th>
<th>DPNH</th>
<th>RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>Cytochr. c.</td>
<td>91.0</td>
<td>26.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Partially purified</td>
<td>Cytochr. c.</td>
<td>245.3</td>
<td>81.7</td>
<td>3.0</td>
</tr>
<tr>
<td>Purified</td>
<td>DPIP</td>
<td>316.0</td>
<td>90.3</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Cytochr. c.</td>
<td>839.0</td>
<td>227.1</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* Rate of reduction of cytochrome c was measured in terms of ΔE_{550}/E_{230}, where ΔE_{230} represents the change of optical density (× 10^4) at 550 μm occurring in 60 seconds, and E_{230} the optical density at 230 μm, which was taken as a reference. The values given for DPIP are those recalculated from the rate of decrease (occurring in 60 seconds) of the optical density at 600 μm, so as to make the values correspond—i.e., relative to the extent of electron transfer—to ΔE_{230}/E_{230}.
in marked contrast to the findings of the previous investigators. These discrepancies in the results obtained with similar materials may partly be due to the different conditions adopted in extracting the enzymes: Avron et al. extracted chloroplasts with a TRIS buffer (pH 8.0) for 1 hour at room temperature, and Marré et al. started the preparation with acetone powder of chloroplasts, from which the enzyme was extracted with a TRIS buffer (pH 7.3) at a lower temperature. The question as to whether or not these enzymes and ours are essentially different from each other is left open to further investigations.

**Table III**

**Effects of Flavines and Molybdate on the Activity of Dialyzed (EDTA-treated) Enzyme**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>MoO₄⁻⁻ (3.3 x 10⁻⁴ M)</td>
<td>173</td>
</tr>
<tr>
<td>FMN (2.7 x 10⁻⁴ M)</td>
<td>97</td>
</tr>
<tr>
<td>FMN + MoO₄⁻⁻</td>
<td>151</td>
</tr>
<tr>
<td>FAD (3.0 x 10⁻⁴ M)</td>
<td>100</td>
</tr>
<tr>
<td>FAD + MoO₄⁻⁻</td>
<td>173</td>
</tr>
</tbody>
</table>

* Compared by the initial velocities of reduction of cytochrome c in the presence of TPNH.

**Summary**

1. From green leaves or chloroplasts of spinach and parsley a pyridine nucleotide-cytochrome c reductase was extracted either with ammoniacal acetone-water and disodium phosphate solution or with ammonium buffer of pH 8.0 containing a small amount of Tween 80. The enzyme was further purified to 8- to 9-fold increase in specific activity by fractionation with ammonium sulfate followed by adsorption on tricalcium phosphate gel.

2. The enzyme utilizes as electron donors both TPNH and DPNH and as electron acceptors cytochrome c as well as benzoquinone and 2,6-dichlorophenol-indophenol. Irrespective of the kind of electron acceptors applied, TPNH was dehydrogenated 3 to 4 times faster than DPNH. Molecular oxygen was not used as an acceptor.

3. Molybdate was found to be a cofactor of the enzyme. Flavine compounds (FMN and FAD) had no marked effect on the activity of the enzyme.

4. The results obtained were compared with those of previous investigators working with similar enzymes.

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THE MECHANISM OF THE PHOTOCHEMICAL ACTIVITY OF ISOLATED CHLOROPLASTS. III. DEPENDENCE OF VELOCITY ON LIGHT INTENSITY 1, 2

JOHN S. RIESKE, 3 RUFUS LUMRY AND JOHN D. SPIKES

DEPARTMENT OF EXPERIMENTAL BIOLOGY, UNIVERSITY OF UTAH, SALT LAKE CITY, UTAH, AND THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF MINNESOTA, MINNEAPOLIS, MINNESOTA

Chloroplast fragments when illuminated in suspension media containing an oxidant such as p-benzoquinone, potassium ferricyanide or TPNH support the photoproduction of oxygen from water (1). In previous papers we have shown that the actual free-energy requirement of this type of Hill reaction is approximately independent of oxidant despite the fact that the formal requirement varies from oxidant to oxidant and is in general less than that of the complete photosynthetic process (2, 3). As a consequence, it may be concluded from free energy considerations that such Hill-reaction systems retain the remarkable efficiency of the natural process of photosynthesis. Since we have also shown that such systems yield reproducible quantitative results in rate measurements, reactions with inhibitors and the like (4), they provide a relatively simple and attractive point of attack on the photosynthetic process. Recent work, particularly by Jagendorf and his co-workers (5), has intensified interest in the Hill reaction through demonstrations of the relationship between this reaction and photophosphorylation. The later work has been carried out on chloroplast preparations which are undoubtedly more complex in chemical constitution and perhaps in structural interactions than fragments. The distinction between whole chloroplasts and fragments has not always been made in the past and, indeed, preparations have not usually been carefully characterized as to the presence or absence of intact chloroplasts. Nevertheless, it appears that chloroplast fragments, combined with necessary water-soluble cofactors and reactants, do carry out photophosphorylation just as well as intact chloroplasts. Thus, fragment preparations also provide a direct and much simplified system for investigation of this important reaction (6).

This paper confines itself to the Hill reaction of well-washed chloroplast fragments, our intention being to provide further fundamental information leading to a detailed characterization of the process. Eventually, the collection of biochemical, chemical and physical facts about photosynthesis will have to be organized on a framework of chemical kinetics if the process is to be understood in any really fundamental sense. It seems obvious that this undertaking will be very much facilitated if attention is first given to the Hill reaction, which has been shown consistently to yield simple kinetics entirely reminiscent of results obtained with chemical and enzymic systems. As will be shown in this paper, not the least fortunate appearance of simplicity is in the rate law relating velocity and light intensity. Under a very wide range of conditions, this rate law assumes a single, simple form in contradiction to the behavior of the rate law of complete photosynthesis, which is complicated by many dependencies on extrinsic and intrinsic variables, some of which are difficult, if not impossible, to control (6). In this paper we shall establish the rate law for light intensity and provide quantitative data relative to temperature and wave length dependencies of the reaction parameters. This will be followed in the subsequent paper by a consideration of the consequences of the remarkably simple form of this law. In our treatment, we shall assume that the Hill reaction should be studied as an independent entity quite free of implications derived from observations on the total photosynthetic process in intact cells. Thus, we shall work to explain photosynthesis in terms of the Hill reaction, rather than the reverse.

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3 Present address: C. W. Operations Division, U.S. Army Chemical Corps, Proving Ground, Dugway, Utah.

LITERATURE CITED


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