Isolation, Purification, and Some Properties of Reduced Nicotinamide Adenine Dinucleotide Phosphate-Cytochrome c₂ Reductase from Rhodopseudomonas spheroides*

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SUMMARY

A method has been described for the isolation and purification of NADPH-cytochrome c₂ reductase from light-grown Rhodopseudomonas spheroides. The enzyme is a nonmetalloflavoprotein with flavin adenine dinucleotide as the prosthetic group, and it catalyzes the reduction of R. spheroides cytochrome c₂, 2,6-dichloroindophenol, and K₃Fe(CN)₆ with NADPH as the electron donor. It does not reduce R. spheroides cytochrome 5₅₃ but reduces Rhodospirillum rubrum cytochrome c₂ and mammalian cytochrome c very slowly. There is no NADH or NADPH oxidase activity, NADP⁺ reductase activity, or transhydrogenase activity associated with the enzyme.

The pH optimum was found to be 7.5; and Kₘ values of $3.7 \times 10^{-5}$ M, $1.25 \times 10^{-5}$ M, and $1.24 \times 10^{-4}$ M were calculated for cytochrome c₂, 2,6-dichloroindophenol, and K₃Fe(CN)₆ reduction, respectively. NADH functioned as a competitive inhibitor, and the $K_i$ was calculated to be $5.5 \times 10^{-5}$ M.

The enzyme was inhibited by p-chloromercuribenzoate, N-ethylmaleimide, iodoacetate, and thyroxine; and the inhibition by sulfhydryl reagents was reversed by reduced glutathione. Preincubation with NADPH protected the enzyme against the poisoning effects of sulfhydryl reagents but not thyroxine.

The behavior of the enzyme on Sephadex G-100 and in the analytical ultracentrifuge indicates the presence of several molecular forms of the enzyme.

EXPERIMENTAL PROCEDURE

Methods

Growth of Cells—Wild-type cells of R. spheroides were mass cultured photosynthetically in 32-ounce prescription bottles at 30°C in the synthetic medium described by Cohen-Bazire, Sistrom, and Stanier (15). The cells were harvested during the log phase.
of growth (48 hours) by centrifugation in a Sharples super-
centrifuge, then lyophilized and stored at −15°C.

Assays—All assays and absorption spectra were performed at
room temperature (25°C), in 1-ml cuvettes (1-cm light path),
with a Cary model 14 recording spectrophotometer. Cyto-
chrome reduction was measured by the increase in absorbance
at 550 nm. The reduction of ferricyanide was measured as the
decrease in absorbance at 600 nm and the reduction of DCI,
2.6-dichloroindophenol, as

\[ \text{DCI, } 2.6 \text{-dichloroindophenol.} \]

Materials

The following materials were purchased from Calbiochem:glucose 6-phosphate, glucose 6-phosphate dehydrogenase, re-
duced and oxidized forms of pyridine nucleotides, ATP, FMN,
FAD, Bio-Gel, and thyroxine. DEAE-cellulose was obtained
from the Brown Paper Company (Berlin, New Hampshire), and
Sephadex G 100 from Pharmacia. Reagents for the preparation
of polyacrylamide gel columns were purchased from the Canal
Industrial Corporation (Rockville, Maryland). Iodoscetate, p-
chloromercuribenzoate, and nonenzymatic protein molecular
weight markers (Kit 8109 A) were obtained from Mann, and

\[ \text{N-ethylmaleimide from K & K Laboratories. Horse heart} \]

cytochrome c was purchased from Mann, and

\[ \text{Rhodospirillum rubrum cytochrome c was available in the laboratory. All other} \]

reagents were standard laboratory reagent grade chemicals.

RESULTS

Purification of NADPH-Cytochrome c Reductase

Step 1. Extraction—One hundred grams of lyophilized cells
were mixed with 1 liter of cold 0.1 M Tris-HCl, pH 8.0, and
homogenized in a Waring Blender at 4°C. All subsequent opera-
tions were conducted in the cold at 2–4°C unless otherwise noted.
The suspension was extracted overnight with constant stirring
and then centrifuged for 2 hours at 39,000 × g. It was necessary
to centrifuge the reddish brown supernatant fraction at 80,000
× g for 2 hours to remove the bulk of the remaining chromato-
pores. Failure to include this step resulted in extremely slow
flow rates and distorted banding when the extract was chro-
matographed on DEAE-cellulose. The supernatant fraction
from the second centrifugation (80,000 × g) was then dialyzed
against 0.1 M Tris-HCl, pH 8.0, for 12 hours with at least three
changes of external buffer. The dialysate constituted the crude
extract and was designated Fraction 1.

Step 2. DEAE-cellulose Chromatography—Fraction 1 was
charged onto a DEAE-cellulose column (27 × 4.5 cm) which had
been equilibrated with 0.1 M Tris-HCl, pH 8.0. The enzyme
(yellow band) and pigmented material were adsorbed at the top
of the column while a broad, bright red band percolated slowly
through. When all the extract had been adsorbed, the column
was developed with 0.1 M Tris-HCl, pH 8.3, and 2-ml aliquots
were collected at a rate of 1 ml per min. The red band chro-
matographed under these conditions and was identified as
cytochrome c (16). When all the cytochrome c had been
eluted, the buffer was replaced with 0.2 M Tris-HCl, pH 8.0,
and the eluate, which consisted of nonenzymatic protein and a small
amount of reduced cytochrome c, was collected until the ab-
sorbance at 280 nm reached zero. Chromatography was con-

\[ \text{Step 3. Concentration on DEAE-cellulose—Fraction 2 was} \]

diluted 1:4 with ice-cold deionized water and applied to a DEAE-
cellulose column (10 × 3 cm) previously equilibrated with 0.1 M
Tris-HCl, pH 8.0. The enzyme was adsorbed and occupied
approximately three-fourths of the column bed, while about 1%
of the total DCI reductase activity passed through the column.
When all the enzyme had been adsorbed, the column was eluted
with 0.4 M Tris-HCl, pH 8.0, at a slow enough flow rate to pre-
vent spreading or distortion of the eluting band. The enzyme

\[ \text{Step 4. Chromatography on DEAE-cellulose—Fraction 3 was} \]

charged onto a DEAE-cellulose column (27 × 4.5 cm) which had
been equilibrated with 0.1 M Tris-HCl, pH 8.0. The enzyme
(yellow band) and pigmented material were adsorbed at the top
of the column while a broad, bright red band percolated slowly
through. When all the extract had been adsorbed, the column
was developed with 0.1 M Tris-HCl, pH 8.3, and 2-ml aliquots
were collected at a rate of 1 ml per min. The red band chro-
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eluted, the buffer was replaced with 0.2 M Tris-HCl, pH 8.0,
and the eluate, which consisted of nonenzymatic protein and a small
amount of reduced cytochrome c, was collected until the ab-
sorbance at 280 nm reached zero. Chromatography was con-

\[ \text{Step 5. Concentration on DEAE-cellulose—Fraction 4 was} \]

charged onto a DEAE-cellulose column (27 × 4.5 cm) which had
been equilibrated with 0.1 M Tris-HCl, pH 8.0. The enzyme
(yellow band) and pigmented material were adsorbed at the top
of the column while a broad, bright red band percolated slowly
through. When all the extract had been adsorbed, the column
was developed with 0.1 M Tris-HCl, pH 8.3, and 2-ml aliquots
were collected at a rate of 1 ml per min. The red band chro-
matographed under these conditions and was identified as
cytochrome c (16). When all the cytochrome c had been
eluted, the buffer was replaced with 0.2 M Tris-HCl, pH 8.0,
and the eluate, which consisted of nonenzymatic protein and a small
amount of reduced cytochrome c, was collected until the ab-
sorbance at 280 nm reached zero. Chromatography was con-

\[ \text{Step 6. Concentration on DEAE-cellulose—Fraction 5 was} \]

charged onto a DEAE-cellulose column (27 × 4.5 cm) which had
been equilibrated with 0.1 M Tris-HCl, pH 8.0. The enzyme
(yellow band) and pigmented material were adsorbed at the top
of the column while a broad, bright red band percolated slowly
through. When all the extract had been adsorbed, the column
was developed with 0.1 M Tris-HCl, pH 8.3, and 2-ml aliquots
were collected at a rate of 1 ml per min. The red band chro-
matographed under these conditions and was identified as
cytochrome c (16). When all the cytochrome c had been
eluted, the buffer was replaced with 0.2 M Tris-HCl, pH 8.0,
and the eluate, which consisted of nonenzymatic protein and a small
amount of reduced cytochrome c, was collected until the ab-
sorbance at 280 nm reached zero. Chromatography was con-

\[ \text{Step 7. Concentration on DEAE-cellulose—Fraction 6 was} \]

charged onto a DEAE-cellulose column (27 × 4.5 cm) which had
been equilibrated with 0.1 M Tris-HCl, pH 8.0. The enzyme
(yellow band) and pigmented material were adsorbed at the top
of the column while a broad, bright red band percolated slowly
through. When all the extract had been adsorbed, the column
was developed with 0.1 M Tris-HCl, pH 8.3, and 2-ml aliquots
were collected at a rate of 1 ml per min. The red band chro-
matographed under these conditions and was identified as
cytochrome c (16). When all the cytochrome c had been
eluted, the buffer was replaced with 0.2 M Tris-HCl, pH 8.0,
and the eluate, which consisted of nonenzymatic protein and a small
amount of reduced cytochrome c, was collected until the ab-
sorbance at 280 nm reached zero. Chromatography was con-

\[ \text{Step 8. Concentration on DEAE-cellulose—Fraction 7 was} \]

charged onto a DEAE-cellulose column (27 × 4.5 cm) which had
been equilibrated with 0.1 M Tris-HCl, pH 8.0. The enzyme
(yellow band) and pigmented material were adsorbed at the top
of the column while a broad, bright red band percolated slowly
through. When all the extract had been adsorbed, the column
was developed with 0.1 M Tris-HCl, pH 8.3, and 2-ml aliquots
were collected at a rate of 1 ml per min. The red band chro-
matographed under these conditions and was identified as
cytochrome c (16). When all the cytochrome c had been
eluted, the buffer was replaced with 0.2 M Tris-HCl, pH 8.0,
Fig. 1. Chromatographic separation of NADPH-cytochrome c reductase on DEAE-cellulose at pH 8.0.

Fig. 2 (left). Diagrammatic representation of the migration pattern of Fraction 3 on polyacrylamide gel. Direction of migration was from negative to positive.

Fig. 3 (right). Electrophoresis of Fraction 4 on polyacrylamide gel. Migrated as a dark yellow band and was recovered as a concentrated fraction with a 2-fold increase in specific activity (Fraction 3).

Step 4. Disc Gel Electrophoresis—Acrylamide gels were prepared as previously described (see “Experimental Procedure”), and Fraction 3 was subjected to electrophoresis. It was necessary to desalt the sample before application to the gel by passing it through a column (25 x 3 cm) of Bio-Gel P-2 that had been equilibrated with 0.1 M Tris-HCl, pH 8.0. This procedure was repeated if the conductivity of the eluate was not less than or equal to that of the buffer. Failure to desalt the enzyme sample resulted in distortion, spreading, and overlapping of the bands during electrophoresis. A similar result was obtained if the total protein applied to each gel exceeded 40 mg. The electrophoresis was stopped after 7 to 9 hours, and the condensing tubes were removed from the water jackets. A representative electrophoretic pattern is presented in Fig. 2. All of the enzyme activity was associated with the broad yellow band shown in Fig. 2, and this fraction was designated as Fraction 4. An aliquot of Fraction 4 was re-electrophoresed on a small trial gel, and a single band could be detected with Amido black staining (Fig. 3). This would suggest that the enzyme is of high purity.

The absorption spectrum presented in Fig. 4 (Curve A) is indicative of a flavoprotein in view of the shoulder at 470 nm and an absorption maximum at 380 nm. The presence of cytochrome 553 is suggested by the Soret maximum at 415 nm (22). The possibility that the enzyme is a heme flavoprotein seems remote since cytochrome 553 is not required for activity. Further, Mahler (23) has reported that even traces of the heme contaminant associated with NADH-cytochrome c reductase from pig heart completely obscured the flavin spectrum. Since characteristic flavin absorption maxima at 470 nm and 380 nm were detectable in these preparations (Fig. 4), we suggest that the amount of heme still associated with the enzyme represents...
an extremely small portion of the total protein and therefore is a contaminant.

Behavior of Fraction 4 on Sephadex G-100—The apparently homogenous Fraction 4 (8.0 mg of protein) was applied to a column of Sephadex G-100 (1.8 × 100 cm) that had been equilibrated with 0.01 M Tris-HCl buffer, pH 8.0. The enzyme was eluted with the same buffer, and the elution pattern presented in Fig. 5 was obtained. Three distinct protein peaks are evident and DCI reductase activity is coincident with each peak. An elution pattern similar to that presented in Fig. 5 was consistently obtained, and the specific activities of pooled Peak I fractions were found to be 50 to 63% of the pooled Peak II fractions.

![Fig. 5. Elution pattern of Fraction 4 from Sephadex G-100 chromatography. Eight milligrams of protein in 0.01 M Tris-HCl buffer, pH 8.0, were applied to the gel.](http://www.jbc.org/)

These differences in specific activity are predicted from the data of Fig. 5, where it is evident that the leading edge of the chromatogram is devoid of enzymatic activity. The absorption spectra presented in Fig. 4 indicate that Peak I (Curve B) contains a reduced c-type cytochrome with absorption maxima at approximately 550 μm and 415 to 418 μm. The flavoprotein nature of the enzyme is apparent from the absorption spectrum of Peak II (Curve C). A small amount of heme is still associated with the enzyme, as evidenced by the absorption maximum at 415 to 420 μm.

Chromatography on Sephadex G-100 resulted in considerable losses in enzyme protein. However, there is a significant increase in specific activity in the pooled eluate constituting Peak II, and this step was included as a final purification step. A summation of the purification is presented in Table I.

The gel pattern obtained after electrophoresis of Fraction 4 suggested that the preparation was homogeneous (Fig. 3). Thus, it was surprising to obtain the elution pattern for the gel filtration of Fraction 4 on Sephadex G-100 (Fig. 5). We have interpreted these data as being suggestive of a system which is heterogeneous with respect to molecular size and indicative of the presence of several molecular forms of the enzyme. This interpretation is further supported by ultracentrifugation data, and the sedimentation pattern presented in Fig. 6 shows that the enzyme does not sediment as a single peak, but rather three diffuse boundaries are in evidence. Sedimentation patterns similar to this have been observed for a number of enzymes which exhibit association-dissociation phenomena or polymerization (see Reference 24).

Molecular Weight Determination—The limited data presented in the preceding experiments may suggest an association-dissociation phenomenon, and the determination of molecular weight is of questionable value. However, gel filtration has been used to determine the molecular weight of the monomeric species in rapidly associating systems, and, while the method is subject to error from the quantitative point of view, weight average quantities can be calculated (25). Accordingly, we have calculated the weight average molecular weights of the molecular species appearing at Peaks I, II, and III with the aid of proteins of known molecular weights as markers. The procedure has been described by Andrews (26) and the results presented in Fig. 7 indicate average molecular weights of 67,000, 43,000, and 20,000 for Peaks I, II, and III, respectively.

Identification of Flavin Moiety—The pooled eluate constituting Peak II contained enzyme of the highest specific activity and stability and was used to determine the nature of the flavin prosthetic group. The flavin was liberated by treatment with

![Fig. 6. Sedimentation patterns of NADPH-cytochrome c reductase (Fraction 4). The sample cell contained 12 mg of protein in 0.1 M Tris-HCl, pH 8.0, and pictures were taken at 16-min intervals approximately 10 min after the centrifuge speed had reached 59,780 rpm.](http://www.jbc.org/)
FIG. 7. Calibration of Sephadex G-100 and estimation of weight average molecular weights of NADPH-cytochrome c$_2$ reductases. The column was equilibrated with 0.1 M Tris-HCl, pH 8.0 and standardized with 4 mg each of the following 2 ml of buffer: bovine serum albumin (mol wt, 68,000); ovalbumin (mol wt, 45,000); and myoglobin (mol wt, 17,500). Void volumes were estimated by the absorption at 280 nm. Then 8.6 mg of enzyme were applied in 1 ml of 0.1 M Tris-HCl, pH 8.0, and elution was in 3-ml fractions at a flow rate of 30 ml per hour with 0.01 M Tris-HCl, pH 8.0.

TABLE II

Acceptor specificity of NADPH-cytochrome c$_2$ reductase

The standard reaction mixture described in "Experimental Procedure" was employed. In addition, where indicated, the reaction mixture contained the following, in micromoles: DCl, 0.033; K$_3$Fe(CN)$_6$, 0.01; cytochrome, 0.005; and 7.0 pg of enzyme.

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Rates (mumoles reduced/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. c-type cytochrome</td>
<td></td>
</tr>
<tr>
<td>A. R. spheroides</td>
<td></td>
</tr>
<tr>
<td>1. c$_2$</td>
<td>2.40</td>
</tr>
<tr>
<td>2. c-553</td>
<td>0.43</td>
</tr>
<tr>
<td>B. R. rubrum</td>
<td>0.10</td>
</tr>
<tr>
<td>C. Horse heart</td>
<td>5.20</td>
</tr>
<tr>
<td>II. DCl</td>
<td>0.60</td>
</tr>
<tr>
<td>III K$_3$Fe(CN)$_6$</td>
<td>6.00</td>
</tr>
</tbody>
</table>

The purified enzyme was devoid of NADPH and NADH oxidase activity and transhydrogenase activity. The enzyme did not reduce NADP$^+$ with reduced benzyl viologen as the electron donor, which suggests that the enzyme is devoid of NADP$^+$ reductase activity (11).

pH Optimum—NADPH-cytochrome c$_2$ reductase had a pH optimum of 7.5 (Fig. 8). The increase in absorbance at 550 nm has been corrected for the nonenzymatic reduction which occurred at pH values below 6.8.

Stability—The purified concentrated enzyme (15 mg per ml) was stable for approximately 2 weeks at 4° in 0.4 M Tris-HCl, pH 8.0, and the gradual loss in activity which occurred beyond this time period could be restored by the addition of GSH to a final concentration of 10$^{-4}$ M. Higher concentrations were avoided since GSH promoted a nonenzymatic reduction of both DCl and cytochrome c$_2$. Activity was lost overnight if the enzyme was diluted 10-fold, and no protection was afforded in the presence of 10% bovine serum albumin. Partially purified preparations (Fractions 2 and 3) were stable at 4° for approxi-
mately 1 month and could be stored as lyophilized powders for at least 3 months at $-15^\circ$.

**Influence of Enzyme Concentration**—The relationship of NADPH cytochrome $c_2$ reductase concentration to the rate of cytochrome $c_2$ reduction is linear and typical of a system which involves a single enzyme (Fig. 9). These results provide additional support to the conclusion arrived at earlier that the small amount of $c$-type cytochrome associated with the purified enzyme is a contaminant and not necessary for enzymatic activity. Nonlinear kinetics would have been expected had the activity been dependent upon the presence of two soluble protein components, i.e. flavoprotein and cytochrome $553$.

**Michaelis Constant**—The effect of substrate concentration on enzyme activity was investigated with cytochrome $c_2$, DCI, and $K_3Fe(CN)_6$ as electron acceptors. The double reciprocal plots (27) were linear as a function of NADPH concentration, and $K_m$ values were calculated from these curves (Fig. 10, $A$ and $B$).

Statistical points for curve fitting were calculated by the least square method (28), and $K_m$ values of $3.7 \times 10^{-5}$ M, $1.25 \times 10^{-4}$ M, and $1.20 \times 10^{-4}$ M were calculated for cytochrome $c_2$, DCI, and $K_3Fe(CN)_6$, respectively.

The $K_m$ of $3.7 \times 10^{-5}$ M for cytochrome reduction is nearly identical with that of chloroplast cytochrome $f$ reductase ($3.3 \times 10^{-5}$ M) (6) but differs from the Rhodopseudomonas palustris preparation ($3.3 \times 10^{-4}$ M), where a 10-fold decrease in $K_m$ for cytochrome reduction was observed (12). The lower $K_m$ value observed with DCI can be explained in terms of its ability to accept 2 electrons directly, thereby being more efficient than cytochrome $c_2$ or $K_3Fe(CN)_6$, which are 1-electron acceptors.

**Inhibitors**—The effects of various inhibitors on the enzymatic reduction of cytochrome $c_2$, DCI, and $K_3Fe(CN)_6$ are presented in Table III. The enzyme is moderately sensitive to iodoacetate and N-ethylmaleimide (final concentration, 1 mM). Both reagents react slowly with free sulfhydryl groups, and the preincubation time of 5 min may not have been sufficient to promote full reactivity of the reagents. This could account for the low percentage of inhibition. In both experiments, full enzyme activity was restored by the addition of GSH to a final concentration of $10^{-3}$ M, and preincubation of the enzyme with NADPH resulted in complete protection against these reagents.

p-Chloromercuribenzoate (final concentration, $10^{-5}$ M) completely inhibits the enzyme when cytochrome $c_2$ is the electron acceptor. However, 75% and 70% inhibition was recorded when DCI and $K_3Fe(CN)_6$, respectively, were the electron acceptors. Inhibition by p-chloromercuribenzoate was completely reversed by GSH, and only partial protection was afforded by preincubation with $0.05 \mu$ mole of NADPH. The sensitivity of the enzyme to these inhibitors suggests that intact sulfhydryl groups are required for catalytic activity.

NADP$^+$ and NAD$^+$ had no effect on the reactivity of the enzyme. However, when NADH was added to a final concentration of $5 \times 10^{-4}$ M, the enzyme was inhibited 50%. The kinetic analysis presented in Fig. 10A clearly demonstrates that NADH is a competitive inhibitor, and a $K_i$ of $5.5 \times 10^{-4}$ M was calculated.

Thyroxine proved to be a very effective inhibitor and no protection was afforded by preincubation with NADPH. This hormone and a number of related halogenated phenols have been

![Fig. 9. Relationship between activity and enzyme concentration. In addition to the basic reaction mixture, the reaction systems contained 0.005 μmole of cytochrome $c_2$ and the amount of enzyme noted.](image)

![Fig. 10. Lineweaver-Burk plots for cytochrome $c_2$, DCI and $K_3Fe(CN)_6$ reduction, and NADH inhibition of cytochrome $c_2$ reduction. In addition to standard conditions, reaction mixtures contained, in micromoles: DCI, 0.033; $K_3Fe(CN)_6$, 0.01; cytochrome $c_2$, 0.005; and enzyme equal to 10 μg. O, statistical points calculated by the least squares method. The abscissa of $B$ should read $-1/K_m - 1/K_p$.](image)
reaction mixture and the reactions were started by the addition of NADPH. The final concentration of each of these reagents was 0.05 mule of NADPH and 0.005 pmole of cytochrome c2. Where NADPH is indicated in Column 1, both inhibitor and NADPH were preincubated with the enzyme in the standard reaction mixture and the reactions were started by the addition of R. spheroides cytochrome c2.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration</th>
<th>Cytochrome</th>
<th>DCI</th>
<th>Fe(CN)6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodoacetate</td>
<td>10⁻³</td>
<td>10</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>+GSH</td>
<td>10⁻³</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+NADPH</td>
<td>5 x 10⁻⁶</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>10⁻¹</td>
<td>25</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>+GSH</td>
<td>10⁻⁶</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+NADPH</td>
<td>5 x 10⁻⁶</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>10⁻⁵</td>
<td>98</td>
<td>74</td>
<td>70</td>
</tr>
<tr>
<td>+GSH</td>
<td>10⁻²</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>+NADPH</td>
<td>5 x 10⁻⁸</td>
<td>70</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>NADH</td>
<td>5 x 10⁻¹</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>10⁻²</td>
<td>100</td>
<td>96</td>
<td>40</td>
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<tr>
<td>+NADPH</td>
<td>5 x 10⁻⁵</td>
<td>100</td>
<td>96</td>
<td>40</td>
</tr>
</tbody>
</table>

shown to inhibit many pyridine nucleotide-dependent dehydrogenases (29). Studies with glutamic dehydrogenase have shown that the inhibitory effects of thyroxine are not due to competitive binding at the active site, but rather due to alterations in enzyme structure (30). Effects of this type could explain the lack of protection afforded by NADPH.

The enzyme retained full activity when preincubated for 5 min with each of the following metal-chelating agents: 8-hydroxyquinoline, o-phenanthroline, EDTA, ATP, sodium cyanide. The final concentration of each of these reagents was 10⁻² M. We have interpreted these results as being suggestive that NADPH-cytochrome c2 reductase is another example of a nonmetalloflavoprotein.

Preincubation of the enzyme with sodium arsenite (10⁻³ M final concentration) had no effect on enzymatic activity, which suggests that thiols are not involved in the catalysis.

**DISCUSSION**

From the extent of these studies, the major function of the enzyme isolated from R. spheroides appears to be that of mediating electron flow between NADPH and cytochrome c2. It appears to be similar to the green plant enzyme (1-9) and the enzyme isolated from R. palustris (11, 12), in that it is an FAD flavoprotein with a specificity toward NADPH as electron donor. The pH optimum, Kₘ, and specificity in cytochrome reduction are almost identical with those for the plant enzyme (30). The R. spheroides enzyme differs from the R. palustris enzyme in that the Kₘ for cytochrome reduction is increased by a factor of 10 and the R. palustris enzyme has no distinct pH optimum (12).

The behavior of the highly purified enzyme on Sephadex G-100 and in the analytical ultracentrifuge indicates heterogeneity with respect to molecular weight. This may be indicative of dissociation into subunits or suggest a chemically reacting system (24). However, such interpretations are restricted by the limited data presented. We have stated earlier that these phenomena preclude accurate determinations of molecular weight, but it is interesting to note that the fraction containing the highest specific activity (Peak II, Fig. 5) has a flavin to protein ratio of 1 and a molecular weight of 43,000. This molecular weight value is within the range of the molecular weight determined for the crystalline plant enzyme (40,000 to 46,000) (6).

The most striking differences between the R. spheroides enzyme and that of green plants and R. palustris is that the R. spheroides enzyme is devoid of transhydrogenase activity and NADP⁺ reductase activity and is not inhibited by NADP⁺.

The photoreduction mechanism in R. palustris appears to involve a ferredoxin-like nonheme iron protein and NADP⁺ reductase (12) and may be similar to that in green plants. Yamanaka and Kamen (12) have provided an explanation for the multiple activities of the NADP⁺ reductase preparation in terms of this mechanism. They have presented evidence which suggests that the enzyme is functional in the photoreduction reaction (i.e. ferredoxin-NADP⁺ reductase) as well as in the NADPH-mediated cytochrome 552 reduction. The latter activity is inhibited by NADP⁺, and this has been interpreted as indicating a role in biosynthetic regulation. Thus, when NADPH is formed in large amounts via photoreduction, the enzyme could function primarily as an NADP⁺-cytochrome 552 reductase. This activity would be inhibited when NADP⁺ is present, and thus this environment would favor photoreduction.

The role of the NADPH-cytochrome c₂ reductase of R. spheroides appears to be more consistent with a mechanism of photoreduction which differs from that of both green plants and other photosynthetic bacteria. In earlier reports (13, 14) we have suggested that the photoreduction of NADP⁺ in this organism occurs via an energy-linked transhydrogenase and not through ferredoxin and NADP⁺ reductase. Consequently, there would be no need for an enzyme with the multiple activities found in both green plants and R. palustris. Rather, there would be need for an enzyme the sole function of which is to oxidize NADPH formed via the light-dependent transhydrogenase. The enzyme may also function in biological control since NADH, a substrate for the transhydrogenase reaction, serves as a competitive inhibitor. Thus, when NADPH is being formed via the energy-linked transhydrogenase reaction, the enzyme functions in cytochrome c₂ reduction. Under conditions favoring photoreduction (an abundance of NADH and NADP⁺), the oxidation of NADPH is controlled by the competitive inhibitory effects of NADH.

In conclusion, this investigation has provided a method for the isolation and purification of NADPH-cytochrome c₂ reductase as an intact flavoprotein from light-grown R. spheroides. The enzyme, although similar to that found in green plants and in other photosynthetic bacteria, differs markedly with respect to the associated activities and specificity. It does not carry out the number of catalytic activities associated with the plant or the R. palustris preparations and does not exhibit the lack of specificity toward cytochrome acceptors observed with R.
pallidus. Its role in coupling hydrogen transfer to electron transport at the cytochrome c₅ level is consistent with the reductases studied in other photosynthetic bacteria (12), and the specificity toward NADPH as electron donor is consistent with the capacity of R. spheroides to photoreduce NADP.

REFERENCES

Isolation, Purification, and Some Properties of Reduced Nicotinamide Adenine Dinucleotide Phosphate-Cytochrome $c_2$ Reductase from *Rhodopseudomonas spheroides*

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