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AN NADP REDUCTASE, AN NADH-DYE REDUCTASE AND A NON-HAEM IRON PROTEIN ISOLATED FROM A FACULTATIVE PHOTOHETEROTROPH, *RHODOPSEUDOMONAS PALUSTRIS*

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SUMMARY

An NADP reductase, an NADH-dye reductase and a non-haem iron protein were isolated from a facultative photoheterotroph, *Rhodopseudomonas palustris*.

The NADP reductase is a flavoprotein; its absorption spectrum has peaks at 370 mμ and 460 mμ, and its prosthetic group is not FMN but, probably, FAD. The enzyme reversibly reduces $NAD(P)^+$ with reduced benzylviologen as the electron donor. It also catalyses reductions of *R. palustris* cytochrome *c*-552 and 2,6-dichlorophenolindophenol by NADPH. NADH is a less effective electron donor than NADPH. The NADPH-cytochrome *c*-552 reductase activity is stimulated by benzylviologen, menadione and FMN. The enzyme also exhibits a pyridine nucleotide transhydrogenase activity.

The NADH-dye reductase appears to be a flavoprotein with FMN as its prosthetic group. It reduces 2,6-dichlorophenolindophenol and ferricyanide with NADH as the electron donor, but does not reduce *R. palustris* cytochrome *c*-552. However, in the presence of 2,6-dichlorophenolindophenol, ferricyanide or menadione, the enzyme shows NADH-cytochrome *c*-552 reductase activity.

The non-haem iron protein preparation shows an absorption spectrum very similar to that of green plant ferredoxin; there are peaks at 330 mμ and 420 mμ. It contains inorganic sulfide as well as non-haem iron. The millimolar extinction coefficients at 420 mμ are 4.8 and 7.6, determined on the basis of non-haem iron and of inorganic sulfide, respectively. It is reduced rapidly by $Na_2S_2O_4$, and very slowly either by NADP reductase with NADPH as the electron donor, or by ascorbate. It is also reduced slightly by $K_4Fe(CN)_6$. The reduced form of the protein is very autoxidizable in all cases, but is not reoxidized by addition of $NADP^+$ and NADP reductase under anaerobic conditions.

INTRODUCTION

In green plant photosynthesis, it is known that photoreduction of $NADP^+$ requires the cooperation of a flavoprotein (NADP reductase), and a non-heam

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

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iron protein (ferredoxin)[1,2]. The spinach NADP reductase reduces $NADP^+$ with reduced ferredoxin as the electron donor. The same mechanisms have been found for $NADP^+$ reduction in the diatom, *Navicula pelliculosa*[3]. In *Chlorella pyrenoidosa*, it has been shown that ferredoxin and a flavoprotein are necessary for photosynthetic oxygen evolution[4].

A similar enzymatic system may occur in bacterial photosynthesis. Indeed, a ferredoxin has been isolated[5] from *Chromatium*, and a ferredoxin-like protein from *Rhodospirillum rubrum*[6]. However, reduction of NAD^+ with chromatophore fragments of the photosynthetic bacteria is not stimulated by ferredoxin, derived either from *Chromatium* or from *R. rubrum*[5], although photosynthetic reduction of pyridine nucleotide with *R. rubrum* chromatophore fragments is readily stimulated by the spinach photosynthetic pyridine nucleotide reductase preparation (perhaps a mixture of ferredoxin and NADP reductase)[7]. Recently, it has been shown also that the reduction of NAD^+ by H_2 in cell-free extracts of *Chromatium* and *Chlorobium thiosulfatophilum* is stimulated by addition of ferredoxin derived from *Clostridium pasteurianum*[8]. Therefore, it is not unlikely that, in bacteria, ferredoxin is necessary for photosynthetic pyridine nucleotide reduction[9]. However, the essential isolation of a pyridine nucleotide reductase from the photosynthetic bacteria has not been reported. Therefore, strictly speaking, it has not been established that the mechanisms of the photosynthetic pyridine nucleotide reduction in the bacteria are similar to those in green plants.

As has been reported in a preliminary paper[10], we have isolated and purified a non-haem iron protein (previously called ferredoxin), similar to ferredoxin, and a flavoprotein, NADP reductase*, from the photoheterotroph, *Rhodopseudomonas palustris*. This NADP reductase shows various enzymatic activities very similar to those of the green plant enzymes. In this paper we describe in greater detail purification of the NADP reductase and the non-haem iron protein and, in addition, of an NADH-dye reductase; some properties of these enzymes are also reported.

MATERIALS AND METHODS

Materials

Benzylviologen was purchased from British Drug Houses Ltd.; NAD^+, NADH, $NADP^+$, NADPH, glucose 6-phosphate and yeast glucose-6-phosphate dehydrogenase from Boehringer Mannheim Corporation; FAD, FMN, riboflavin and menadione from California Corporation for Biochemical Research; DEAE-cellulose (type 40) from Brown Company; and Amberlite CG-50 from Mallinckrodt Chemical Works. *R. palustris* (No. 2137) cytochrome *c*-552 and cytochrome *cc'* (RHP) were purified and crystallized by the methods of DE KLERK, BARTSCH AND KAMEN[11]. Horse-heart cytochrome *c* (type III) was purchased from Sigma Chemical Company. The oxidized form of the cytochrome *c*-552 was prepared by addition of a small amount of $K_3Fe(CN)_6$, followed by treatment with a DEAE-cellulose column to remove the oxidant and $K_4Fe(CN)_6$ formed during oxidation of the cytochrome *c* preparation. The reduced form of the cytochrome *c*-552 was prepared by addition of a small amount of $Na_2S_2O_4$ to the solution and subsequent dialysis against an appropriate buffer.

* This enzyme catalyses various reactions, as described later. However, as it seems to be a major characteristic property of the enzyme that it can reduce $NADP^+$ in the presence of an appropriate electron donor, it is called 'NADP reductase' in the present paper.

Purification of NADP reductase and non-haem iron protein

R. palustris (No. 2137) cells (about 500 g in wet weight), which were cultivated as previously[11], were suspended in 1 l of 0.01 M Tris–HCl buffer (pH 8.0), and the suspension was sonicated (20 kcycles, 500 W) for 3 min in the cold. The sonicate was centrifuged at 35000 × *g* for 20 min, and the supernatant was charged on a DEAE-cellulose column (5 cm × 10 cm) at pH 8.0 (0.01 M Tris–HCl buffer) after overnight dialysis against the same buffer as used for the chromatography. The column was washed with 0.1 M Tris–HCl buffer (pH 8.0). The eluate obtained contained in addition to all the original soluble haem proteins, an NADH-dye reductase activity, and was set aside for further purification of the enzyme.

A further wash with 0.1 M Tris–HCl buffer (pH 8.0) containing 1.0 M NaCl produced a brown eluate which was dialyzed overnight against 0.1 M Tris–HCl buffer (pH 8.0), then charged on the DEAE-cellulose column which had been equilibrated with the same buffer as used for dialysis. When the column was washed with 0.20 M Tris–HCl buffer (pH 8.0), a yellow eluate was obtained. Next, the column was treated with 0.2 M Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl. A brown eluate resulted.

The yellow eluate obtained above was dialysed against 0.05 M ammonium phosphate buffer (pH 7.0) overnight, and charged on the DEAE-cellulose column which had been equilibrated with the same buffer as used for dialysis. The column was washed with 0.1 M ammonium phosphate buffer. The eluate obtained was adjusted to pH 4.7 with dilute acetic acid, and $(NH_4)_2SO_4$ was added. The precipitate obtained between 40 % and 50 % satn. was collected and dissolved in 0.1 M Tris–HCl buffer (pH 8.0). The resultant solution was used as the *R. palustris* NADP reductase preparation.

The brown eluate, which was obtained when the column was washed with 0.2 M Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl, was dialysed against 0.1 M phosphate buffer (pH 7.0), and charged on the DEAE-cellulose column which had been equilibrated with the same buffer as used for dialysis. After the column had been washed with 0.1 M ammonium phosphate buffer (pH 7.0) the brown band was eluted with 0.15 M phosphate buffer (pH 7.0). To the eluate, $(NH_4)_2SO_4$ was added to 40 % satn. The precipitate which appeared was separated by centrifugation at 35000 × *g* for 10 min. To the supernatant, further $(NH_4)_2SO_4$ was added to 50 %, and the resultant precipitate was collected by centrifugation at 35000 × *g* for 10 min. The brown precipitate obtained was dissolved in 0.1 M Tris–HCl (pH 8.0). This solution was used as *R. palustris* non–haem iron protein.

Purification of NADH-dye reductase

The sonicate of the cells described above (or unadsorbed solution in the initial chromatography on the DEAE-cellulose column during the purification of NADP reductase and non-haem iron protein) was dialysed against 0.005 M ammonium phosphate buffer (pH 6.0) and charged on an Amberlite CG-50 column which had been equilibrated with the same buffer as used for dialysis. The column was washed with 0.25 M ammonium phosphate buffer (pH 7.0). The eluate was fractionated with $(NH_4)_2SO_4$, and the precipitate obtained between 50 % and 60 % satn. was collected. The precipitate was washed with 60 % satd. $(NH_4)_2SO_4$ solution containing 0.04 M ammonium phosphate buffer (pH 7.0) and suspended in about 2 ml of the same solution as used in washing. To the suspension, a few drops of water were added, until the precipitate was almost completely solubilized. The solution was centrifuged at 35000 ×

g for 10 min, and a small amount of $(NH_4)_2SO_4$ was added to the supernatant until the solution became a little turbid. Then the solution was kept in an ice bath for 1 h. The resultant crystalline precipitate was collected by centrifugation at 35000 $\times$ g for 10 min, then dissolved in 0.1 M Tris–HCl (pH 8.0). The solution so prepared was used as the *R. palustris* NADH-dye reductase preparation. The enzyme obtained by the above procedures required addition, specifically, of FMN for expression of activity. Therefore, the NADH-dye reductase preparation could be used to determine FMN (see below).

The enzyme was also purified by chromatography on a DEAE-cellulose column (see preceding section). When larger columns of DEAE-cellulose (5 cm $\times$ 30 cm) were used in the purification of NADP reductase and non-haem iron protein, the enzyme was adsorbed weakly on the lower part of the column as a yellow band. This band was eluted with many volumes of 0.01 M Tris–HCl (pH 8.0). The eluate was fractionated by $(NH_4)_2SO_4$, and the precipitate obtained between 30% and 60% satn. was collected. The precipitate was dissolved in 0.1 M Tris–HCl (pH 8.0) and the resultant solution was dialysed against 0.01 M Tris–HCl (pH 8.0). The dialysed solution was again subjected to chromatography under the same conditions as described above.

The resultant enzyme solution was active even without addition of FMN, but was still activated considerably by FMN. This enzyme solution was used as a control when the flavin of NADP reductase was determined by means of the apo-protein of NADH-dye reductase (see below).

Preparation of acid-treated chromatophore fragments

The cells which had been washed with 0.1 M Tris–HCl buffer (pH 8.0) were suspended in an appropriate volume of 0.1 M Tris–HCl buffer (pH 8.0) and the suspension was sonicated (10 kcycles/sec, 500 W) for 3 min in the cold. The sonicate was fractionated by centrifugation. The debris obtained between 10000 $\times$ g and 35000 $\times$ g centrifugations was suspended in 0.1 M Tris–HCl (pH 8.0). This suspension was used for the photosynthetic reduction of iron protein.

Next, the pH of the suspension was brought to 4.7 with dilute acetic acid, and the resultant precipitate was collected by centrifugation at 35000 $\times$ g for 20 min. The precipitate was washed 3 times with 0.1 M Tris–HCl (pH 8.0), and then suspended in the same buffer as used for the washing. This suspension was used as *R. palustris* acid-treated chromatophore fragments.

Determinations of enzymatic activities, non-haem iron, inorganic sulfide, and flavin

NADP reductase activity, NADPH-cytochrome *c*-552 reductase activity, NADPH-dichlorophenolindophenol (DCIP) reductase activity, pyridine nucleotide transhydrogenase activity, and flavin in NADP reductase were determined by the methods described previously[3]. Non-haem iron and inorganic sulfide in the non-haem iron protein were determined as described by LOVENBERG, BUCHANAN AND RABINOWITZ[12]. Absorption spectra were recorded with a Cary spectrophotometer, Model 14.

Photosynthetic reoxidation of reduced cytochrome c-552

The standard reaction mixture consisted of 1.5 ml of 0.1 M Tris–HCl buffer (pH 7.0), 0.5 ml of 87 μM *R. palustris* cytochrome *c*-552 (81% reduced), 0.25 ml of

5 mM $NADP^+$, 0.05 ml of non-haem iron protein ($A_{420m\mu}$, 0.198), 0.02 ml of NADP reductase (1.1 μM on the basis of flavin content), and 0.2 ml of acid-treated chromatophore fragments ($A_{868m\mu}$, 0.672). The chromatophore fragments were put in the side arm of Thunberg-type cuvettes; all the other reagents were placed in the main chamber. The reaction systems were made anaerobic by repeated evacuation and flushing with argon. The reaction was started by tipping the chromatophore fragments into the main chamber, carried out at 20° with about 1200 lux illumination, and monitored by the decrease in absorbance at 552 mμ.

RESULTS

NADH-dye reductase

As shown in Table I, the enzyme reduced DCIP and $K_3Fe(CN)_6$ with NADH as the electron donor, but did not reduce cytochrome *c*-552. However, a very high NADH-cytochrome *c*-552 reductase activity was observed in the presence of DCIP, $K_3Fe(CN)_6$ or menadione. The enzyme did not show any NADH-benzylviologen reductase activity, unlike NADP reductase (see below). The enzyme preparation was scarcely active without addition of flavins. It was strongly activated by FMN and FAD, but the latter was less effective than the former, especially at lower concentrations. Therefore, the enzyme preparation obtained seems to be the apo-protein

TABLE I

VARIOUS ENZYMATIC ACTIVITIES OF NADH-DYE REDUCTASE

Concentrations of reagents were as follows: NADH or NADPH, 0.23 mM; *R. palustris* cytochrome *c*-552, 31 μM; DCIP, 32 μM; $K_3Fe(CN)_6$, 0.78 mM; Tris–HCl buffer (pH 8.0), 77 mM; the enzyme, $A_{280m\mu}$, $3.8 \cdot 10^{-3}$. The total vol. of the reaction mixture was 1.3 ml. The reactions were carried out at 23°.

*Component**	*DCIP reduced per min (mμmoles)*	*$K_3Fe(CN)_6$ reduced per min (mμmoles)*	*Cytochrome c-552 reduced per min (mμmoles)*
NADH + enzyme	3.3	2.3	0.30
NADH + enzyme + 0.038 μM FMN	41	—	—
NADH + enzyme + 8 μM FMN	71	38	0.59
NADH + enzyme + 38 μM FMN	67	—	—
NADH + enzyme + 0.038 μM FAD	4.1	—	—
NADH + enzyme + 8 μM FAD	—	4.5	—
NADH + enzyme + 38 μM FAD	57	—	—
NADH + enzyme + 38 μM riboflavin	3.4	—	—
NADH + enzyme + 0.2 μM FMN + 0.77 mM menadione	—	—	44
NADH + enzyme + 0.77 mM menadione	—	—	2.9
NADH + 0.2 μM FMN + 0.77 mM menadione	—	—	0.30
NADH + enzyme + 0.2 μM FAD + 0.77 mM menadione	—	—	9.7
NADPH + enzyme + 8 μM FMN	4.2	0.89	—
NADPH + enzyme + 0.2 μM FMN + 0.77 mM menadione	—	—	3.1

* Other than electron acceptor and buffer.

of an FMN-enzyme. It may be noted that an enzyme preparation which was considerably active even without added FMN was obtained by chromatography on a DEAE-cellulose column instead of on an Amberlite CG-50 column. As electron donor, NADPH was almost completely ineffective. The enzyme also reduced cytochrome *cc'* very rapidly, when in the presence of menadione and FMN, with NADH as electron donor.

NADP reductase

The NADP reductase preparation was yellow and showed the absorption spectrum of a flavoprotein (Fig. 1); there were peaks at 370 mμ and 460 mμ, and a shoulder at 490 mμ. When $Na_2S_2O_4$ was added to the enzyme solution, the 460-mμ

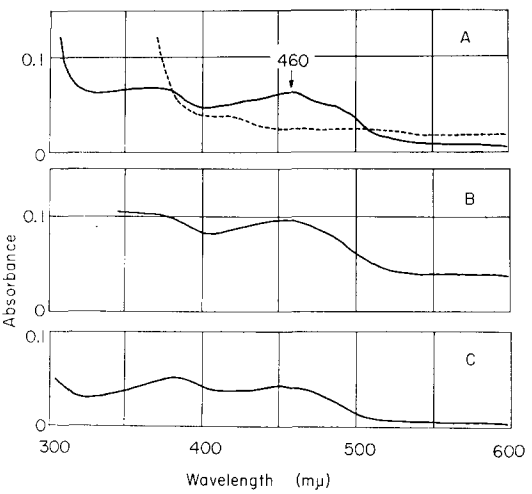

Fig. 1. Absorption spectra of NADP reductase. A. The enzyme dissolved in 0.1 M Tris–HCl buffer (pH 8.0). ——, oxidized form; – – – –, reduced with $Na_2S_2O_4$. B. The $Na_2S_2O_4$-reduced enzyme after reoxidation by aeration. C. Trichloroacetic acid extract of the enzyme.

peak disappeared. The reduced enzyme readily exhibited the original absorption spectrum upon aeration. The trichloroacetic acid extract of the enzyme showed the absorption spectrum of a typical flavin. The extract, after neutralization with NaOH, did not activate the NADH-dye reductase preparation which was highly activated by FMN, as described in the preceding section (Table II). Therefore, the prosthetic group of the NADP reductase was not FMN, but probably FAD. The various enzymatic activities of the enzyme preparation are described in later sections.

Non-haem iron protein

The non-haem iron protein preparation was brown; its absorption spectrum, as shown in Fig. 2, was very similar to those of the green plant ferredoxins (or photosynthetic pyridine nucleotide reductase)[3–5,13–17] rather than to those of the other bacterial ferredoxins[5,12]. There were peaks at 330 mμ and 420 mμ with shoulders at 460 mμ and 550 mμ. There was a slight shoulder at 380 mμ. The protein was reduced by $Na_2S_2O_4$, and the reduced form was very quickly reoxidized to the original state upon aeration. In a preliminary experiment, ε_{mM} at 420 mμ, based on non-haem iron,

TABLE II

ACTIVATION OF THE APO-ENZYME OF NADH-DYE REDUCTASE BY FLAVINS

The reaction mixture was 1.0 ml of 0.05 mM DCIP dissolved in 0.1 M Tris–HCl buffer (pH 8.0), 0.1 ml of 3 mM NADH, 0.01 ml of the apo-enzyme ($A_{280\,m\mu}$, 0.130), and 0.01–0.04 ml of flavin solution. Flavins from NADH-dye reductase and NADP reductase were extracted with 0.5 M trichloroacetic acid, and the extracts were used, after neutralization by 10 M NaOH. The NADH-dye reductase used for flavin extraction was purified by DEAE-cellulose column chromatography (see text). The reactions were carried out at 23°.

Flavin	*Flavin*		$\Delta A_{600\,m\mu}$ *per min*
	Concn. ($A_{600\,m\mu}$)	*Volume added* (*ml*)	
FMN	0.010	0.01	—0.160
FAD	0.008	0.01	—0.018
Flavin from NADP reductase	0.011	0.02	—0.023
Flavin from NADP reductase	0.036	0.02	—0.047
Flavin from NADP reductase	0.036	0.04	—0.047
Flavin from NADH-dye reductase	0.012	0.02	—0.129

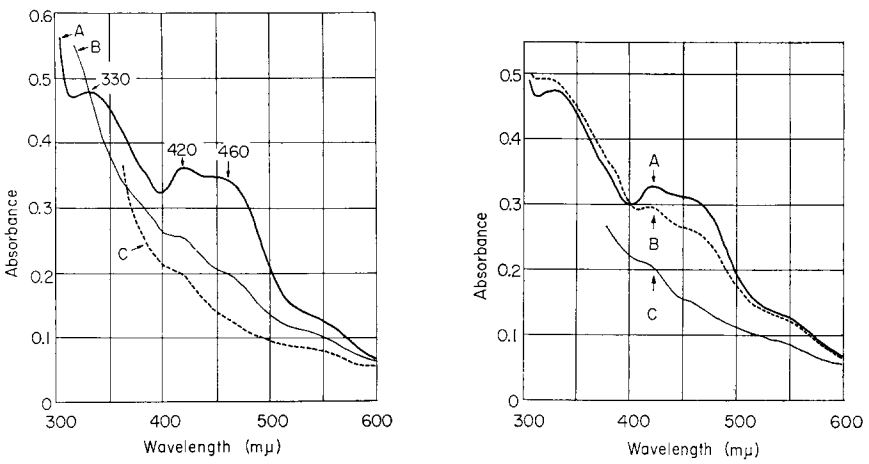

Fig. 2. Absorption spectrum of non-haem iron protein. The protein was dissolved in 0.1 M Tris–HCl buffer (pH 8.0). A, oxidized form; B, partially reduced with a very small amount of $Na_2S_2O_4$; C, fully reduced with $Na_2S_2O_4$.

Fig. 3. Reduction of non-haem iron protein by NADP reductase with NADPH as the electron donor. The reaction mixture consisted of 2.0 ml of the protein dissolved in 0.1 M Tris–HCl buffer (pH 8.0), 0.2 ml of NADP reductase (4.6 μM based on the flavin content), and 0.2 ml of 3 mM NADPH. A, 1 min after the protein, NADP reductase and NADPH were mixed; B, 20 min; C, 24 h.

was found to be 4.8; that based on inorganic sulfide, was 7.6. The non-haem iron protein preparation was approx. 90% reduced by overnight incubation with ascorbate under anaerobic conditions (the initial concentration of protein was 37 μM, based on non-haem iron, and of ascorbate, 4.8 mM). This reaction was very slow, and was not stimulated by addition of DCIP. The non-haem iron protein was also reduced by $K_4Fe(CN)_6$ very slowly. When 36 μM of the protein was incubated for 2 days with 2 mM $F_4Fe(CN)_6$ under anaerobic conditions, it was approx. 10% reduced.

The protein was reduced very slowly by the NADP reductase with NADPH as the electron donor under anaerobic conditions (Fig. 3), and fairly fast by the illuminated chromatophore fragments of the organism. The resultant reduced protein, obtained by any of the procedures described, was very autoxidizable, but was not reoxidized by anaerobic addition of $NADP^+$ and the NADP reductase.

Reduction of pyridine nucleotide by NADP reductase with reduced benzylviologen as the electron donor

The NADP reductase preparation reduced $NADP^+$ with reduced benzylviologen as the electron donor under anaerobic conditions. The result shown in Table III is the average of 9 experiments. The reaction proceeded to an insignificant extent without the NADP reductase. Although we reported in a preliminary paper[10] that non-haem iron protein (called 'ferredoxin') stimulated the reaction, the further purified protein

TABLE III

PYRIDINE NUCLEOTIDE REDUCTION BY NADP REDUCTASE WITH REDUCED BENZYLVIOLOGEN AS THE ELECTRON DONOR

The complete reaction mixture consisted of 2.0 ml of 0.1 M Tris–HCl buffer (pH 7.0), 0.1 ml of 0.01 M benzylviologen, 0.25 ml of 5 mM $NADP^+$, 0.1 ml of NADP reductase (6.3 μM on the basis of flavin content), and 0.05 ml of non-haem iron protein ($A_{420\,m\mu}$ = 0.200). The total vol. was 2.5 ml. Benzylviologen was carefully reduced by a small amount of $Na_2S_2O_4$ before the reactions were started. The reactions were carried out at 23°.

Component	$\Delta A_{550\,m\mu}$ *per min*	*Reduced benzylviologen oxidized per min* (μM^*)	$\Delta A_{340\,m\mu}$ *per min*	*Reduced pyridine nucleotide formed per min* (μM^*)
Complete	−0.525	58.3**	0.150	24.1
Minus non-haem iron protein	−0.450	50.0	0.127	20.4
Minus enzyme	−0.038	4.2	0.028	4.5
Minus pyridine nucleotide	−0.035	3.9	0.004	0.64
NAD^+ in place of $NADP^+$	−0.284	31.6	0.069	11.1

* Concn. in the reaction mixture (2.5 ml).
** ε_{mM} at 550 mμ of reduced benzylviologen was determined as 9.0.

described in this report did not show any appreciable stimulating effect on the $NADP^+$ reduction. NAD^+ functioned also as the electron acceptor in place of $NADP^+$, although less efficiently than $NADP^+$. The quantity of reduced pyridine nucleotide formed was less than that expected theoretically (*i.e.*, as reduced benzylviologen is a one-electron carrier, its 2 moles should reduce 1 mole of pyridine nucleotide). An explanation for this fact has been given previously[3]. The pH optimum in the $NADP^+$ reduction was at pH 7, and K_m's for $NADP^+$ and NAD^+ were 21 μM and 91 μM, respectively.

The enzyme reduced benzylviologen in the presence of NADPH under anaerobic conditions, and the reaction stopped when about 26 % of benzylviologen was reduced (benzylviologen, 0.47 mM and NADPH, 0.30 mM before the reaction was started).

NADPH–cytochrome c-552 reduction by NADP reductase

The NADP reductase preparation also showed NADPH-cytochrome *c*-552 re-

ductase activity. Although NADH was more efficient than NADPH as the electron donor in the pH range lower than 6.5, NADPH was better than NADH around pH 8. However, there was no distinct optimal pH with either electron donor. The NADPH-cytochrome *c*-552 reductase activity was strongly inhibited by $NADP^+$, but little inhibited by NAD^+ (Table IV). Therefore, it was very important for the determination of the enzyme activity to use an NADPH preparation which was not contaminated by $NADP^+$. Because commercial NADPH preparation usually contained some $NADP^+$, higher concentrations (about 0.3 mM) of NADPH were inhibitory to the enzyme. Therefore, the concentration of the reagent which was appropriate for measurement of the enzyme activity was about 0.03 mM. The inhibitory effect of $NADP^+$ was also observed in NADPH–DCIP reduction.

TABLE IV

NADPH (NADH)-CYTOCHROME *c*-552 AND -DCIP REDUCTASE ACTIVITIES OF NADP REDUCTASE

The concentrations of reagents were as follows: In the case of cytochrome *c*-552 reduction; NADPH, 28.6 μM; NADH, 286 μM; *R. palustris* cytochrome *c*-552, 55 μM; Tris–HCl buffer (pH 8.0), 96 mM; reductase, 88 mμM on the basis of flavin content. The total vol. of the reaction mixture was 1.05 ml, and the reactions were carried out at 20°. In the case of DCIP reduction; NADPH, 25.2 μM; NADH, 252 μM; DCIP, 39 μM; Tris–HCl buffer (pH 8.0), 84 mM; NADP reductase, 77 mμM on the basis of flavin content. The total vol. of the reaction mixture was 1.19 ml, and the reactions were carried out at 20°.

Addition	*Cytochrome c-552 reduction*			*DCIP reduction*		
	Concn. of reagent added (μM)	$\Delta A_{552\,m\mu}$ *per min*		*Concn. of reagent added* (μM)	$\Delta A_{600\,m\mu}$	
		NADH	*NADPH*		*NADH*	*NADPH*
None	—	0.118	0.501	—	−0.076	−1.63
$NADP^+$	47.6	—	0.089	41	—	−0.426
$NADP^+$	238	—	0.024	205	—	−0.110
$NADP^+$	476	0.008	0.012	410	−0.050	−0.060
NAD^+	476	0.090	0.453	410	−0.068	−1.47
Benzylviologen	95	0.111	1.40	84	—	−1.76
Menadione	95	0.162	1.42	84	—	−1.92
FMN	95	—	0.719	84	—	−1.68

Values for K_m in NADPH(NADH)–cytochrome *c*-552 reduction at pH 7.0 and 24° were 0.10 mM, 3.3 μM and 60 μM for cytochrome *c*-552, NADPH and NADH, respectively. The NADPH–cytochrome *c*-552 reduction was greatly stimulated by benzylviologen and menadione, whereas the NADH–cytochrome *c*-552 was scarcely affected by these reagents. Therefore, it seemed probable that the rate-limiting step in the NADPH–cytochrome *c*-552 reduction was the reduction of cytochrome *c*-552 by the enzyme, while that in the NADH–cytochrome *c*-552 reduction was the dehydrogenation of NADH. Horse cytochrome *c* was as efficient as cytochrome *c*-552 as electron acceptor. *R. palustris* cytochrome *cc'* was not reduced by the NADP reductase and NADPH under anaerobic conditions, but when benzylviologen or menadione were added to the reaction mixtures, it was reduced very rapidly.

Other enzyme activities of NADP reductase

The NADP reductase preparation reduced DCIP, benzylviologen and $K_3Fe(CN)_6$

TABLE V

COMPARISON OF VARIOUS ACTIVITIES OF NADP REDUCTASE

Reaction	*pH*	*Turnover number**	K_m** *at 24°* (*mM*)	
NADPH → cytochrome *c*-552	7.0	1170	0.0033	(NADPH)
			0.10	(cytochrome *c*-552)
NADH → cytochrome *c*-552	7.0	64	0.065	(NADH)
Reduced benzylviologen → $NADP^+$	7.0	600***	0.021	($NADP^+$)
Reduced benzylviologen → NAD^+	7.0	286***	0.091	(NAD^+)
NADPH → NAD^+	8.0	202***	2.2	(NAD^+)
NADPH → DCIP	8.0	993	—	
NADPH → $K_3Fe(CN)_6$	7.0	4600	—	

* The turnover number is expressed as moles of electron acceptor/mole of flavin of the enzyme per min) and calculated from v_{max} except for NADPH–DCIP reduction and NADPH–$K_3Fe(CN)_6$ reduction in which cases it was calculated on the basis of the best activities obtained.

** K_m's are for the compounds which are indicated in the parentheses.

*** Doubled for comparison with the case of one-electron carriers.

with NADPH as electron donor. NADH was much less efficient than NADPH as electron donor. The enzyme preparation showed also a pyridine nucleotide transhydrogenase activity, that is, NAD^+ was reduced by the enzyme with a continuous NADPH-forming system as the electron donor (NAD^+, 2.2 mM; NADPH, 78 μM). However, the reverse reaction was not catalysed by the enzyme at all, using lactate dehydrogenase and lactate as a continuous NADH-forming system. In Table V, we show turnover numbers and K_m's for the various reactions catalysed by the enzyme preparation.

Photosynthetic oxidation of reduced cytochrome c-552

Photosynthetic oxidation of reduced cytochrome *c*-552 by the acid-treated chromatophore fragments was observed under anaerobic conditions (Fig. 4). Addition

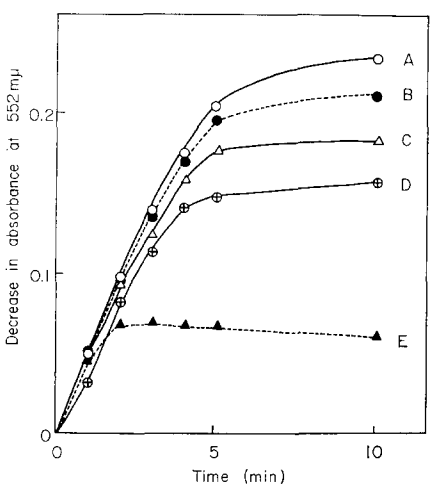

Fig. 4. Photosynthetic reoxidation of reduced cytochrome *c*-552 by acid-treated chromatophore fragments. A, complete; B, *minus* non-haem iron protein; C, *minus* $NADP^+$; D, NAD^+ in place of $NADP^+$; E, *minus* NADP reductase.

of the NADP reductase preparation markedly stimulated this oxidation, whereas addition of the non-haem protein was less noticeably effective. NAD^+ functioned less effectively than $NADP^+$, presumably as electron acceptor. However, the lesser photo-oxidation of the cytochrome could also be attributed to the lowered efficiency of NAD^+ as an inhibitor of the cytochrome reductase activity.

In the absence of added pyridine nucleotide, there was still considerable photo-oxidation of the cytochrome. This endogenous reaction varied in velocity from one preparation to another, probably because of variation in endogenous content of pyridine nucleotide. In all tests, with or without exogenous pyridine nucleotide, we observed that, when the reaction mixtures were kept overnight in the dark, the oxidized cytochrome *c*-552 reverted to its original level of reduction.

Attempts to observe photosynthetic reduction of pyridine nucleotide by the chromatophores of *R. palustris* were not successful. In some preliminary experiments, the illuminated cell-free extract (supernatant obtained by sonication and centrifugation at 35000 × *g*) appreciably reduced $NADP^+$ as well as NAD^+. Addition of the NADP reductase to the cell-free extract sometimes stimulated the photosynthetic $NADP^+$ reduction. However, this stimulating effect of the NADP reductase was not reproducible.

DISCUSSION

It has been stated that NAD^+ is the exclusive electron acceptor in 'non-cyclic photosynthesis' of bacteria[18]. However, the present investigation has made it clear that an NADP reductase exists in at least one photosynthetic bacterium. This NADP reductase, isolated and purified from *R. palustris*, is very similar to the green plant enzymes[1–3,19], in that it is a flavoprotein, the flavin of which is not FMN but probably FAD. The enzyme preparation reduces $NADP^+$ with reduced benzylviologen as the electron donor; NAD^+ is less efficient than $NADP^+$ as the electron acceptor in this reaction. It catalyses also NADPH–cytochrome *c*-552 reduction, NADPH–DCIP reduction, and shows a pyridine nucleotide transhydrogenase activity. NADH is a poorer electron donor than NADPH in these reactions, at about pH 8. Because the enzyme preparation used in the present investigation is not completely pure, these activities may be caused by more than one enzyme. However, the purified green plant enzymes have been known to show a similar variety of enzymatic activities[1–3,20]. Therefore, it seems very likely that the NADP reductase itself can act not only as an NADP reductase but also as an NADPH-cytochrome *c*-552 reductase, NADPH-DCIP reductase and pyridine nucleotide transhydrogenase.

A very striking property of the NADP reductase is that its dehydrogenase activity is strongly inhibited by $NADP^+$. The same is true of the NADP reductase isolated from the diatom, *Navicula pelliculosa*[3]. Therefore, it seems probable that, generally, dehydrogenation catalysed by the NADP reductase of photosynthetic organisms is strongly inhibited by $NADP^+$. This property of the enzyme may be important for regulation of biosynthesis in photosynthetic organisms. One may suppose the NADP reductase functions as NADPH-cytochrome *c*-552 reductase *in vivo* when a considerable amount of $NADP^+$ which is present in the chromatophores is changed into NADPH by a vigorous photosynthetic reduction of $NADP^+$. The inhibitory effect of $NADP^+$ on the dehydrogenase activity of the NADP reductase

cannot be attributed to existence of an equilibrium favorable to NADPH formation, because $NADP^+$ is not reduced at all by the enzyme when a continuous NADH-forming system is present as the electron donor.

The reoxidation of reduced cytochrome *c*-552 by the illuminated acid-treated chromatophore fragments is strongly stimulated by the NADP reductase. In the photosynthetic pyridine nucleotide reduction with a cell-free extract of *R. palustris*, $NADP^+$, as well as NAD^+, functions as the electron acceptor. These facts suggest that the NADP reductase participates in the photosynthetic formation of NADPH in the organism.

As is evident from our isolation of an NAD reductase from the organism, photosynthetic NAD^+ reduction in the organism may be catalysed in addition. NADH-dye reductase cannot reduce cytochrome *c*-552 without addition of menadione, or a dye such as DCIP. It has been reported that there is exceptionally little ubiquinone in *R. palustris* cells[21]. However, there may be present other compounds which mediate electron transfer from NADH-dye reductase to cytochrome *c*-552 in the organisms. Thus, cytochrome *cc'* may be a member of the electron-transfer system which is linked to the NADH-dye reductase.

The *R. palustris* non-haem iron protein preparation described in the present investigation resembles ferredoxins in many respects. It contains labile sulfide as well as non-haem iron, is very autoxidizable, is reduced by the NADP reductase, isolated from the same organism, with NADPH as the electron donor, and is reduced also by the illuminated chromatophore fragments of the same organism, with ascorbate as the electron donor. Its absorption spectrum is very similar to those of green plant ferredoxins[3–5,13–17] but different from those of bacterial ferredoxins[5,12].

These properties of the non-haem iron protein support the idea that it belongs to the class of ferredoxins. However, it is reduced by ascorbate to a large extent, and also by $K_4Fe(CN)_6$ although slightly. Therefore, its midpoint redox potential is much higher than those of well-known ferredoxins, although it seems to be not so high as those of *Chromatium*[22] and *Rhodopseudomonas gelatinosa*[23] high-potential iron proteins ($+0.35$ and $+0.33$ V, respectively). Roughly, its midpoint potential is estimated as between $+0.05$ and $+0.24$ V. The absorption spectra of the *Chromatium* and *R. gelatinosa* proteins are very different from that of the *R. palustris* protein. A non-haem iron protein isolated from *Azotobacter vinelandii*[24] shows an absorption spectrum very similar to that of the *R. palustris* protein. Therefore, it may be that the *R. palustris* and *A. vinelandii* proteins belong to a new group of non-haem iron proteins. In any case, further investigations are required to determine whether the present non-haem iron protein functions in the photosynthetic pyridine nucleotide reduction of *R. palustris* cells, that is, whether the protein is a ferredoxin.

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