PURIFICATION OF AN NADP-REDUCTASE AND OF FERREDOXIN DERIVED FROM THE FACULTATIVE PHOTOHETEROTROPH, RHODOPSEUDOMONAS PALUSTRIS

T. Yamanaka* and M. D. Kamen

From the Department of Chemistry, University of California, San Diego

La Jolla, California

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In green plant photosynthesis, it is known that the photoreduction of $NADP^+$ requires the cooperation of a flavoprotein (Keister et al. 1960, Shin et al. 1963) and a non-heme iron protein, ferredoxin (San Pietro and Lang 1958, Tagawa and Arnon, 1962). A similar enzymatic system may occur in bacterial photosynthesis. Indeed, from Chromatium and Rhodospirillum rubrum, ferredoxin (Arnon 1963) and a similar protein (Tagawa and Arnon 1962) have been isolated. However, it has not been determined whether the ferredoxin and a flavoprotein participate in the photoreduction of NAD^+. NAD^+ reduction with chromatophore fragments of the photosynthetic bacteria is not stimulated by ferredoxin, derived either from Chromatium or from R. rubrum (Vernon 1964).

We have purified the ferredoxin and a flavoprotein, NADP-reductase, found in the facultative photoheterotroph, Rhodopseudomonas palustris (#2137). In the present paper, we describe briefly properties of the two proteins.

Rps. palustris (#2137) cells were sonicated (20 kc, 500 W) for 3 min, allowed to stand 5 min in an ice-bath. This treatment was repeated twice. The sonicate was centrifuged at 35,000 x g for 20 min,

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and the supernatant was charged on a DEAE** -cellulose column at pH 8.0 (0.01 M Tris-HCl buffer) after overnight dialysis against the same buffer. The unadsorbed solution contained cytochrome c_2, RHP and cytochrome c-554 (de Klerk et al. 1964).

The column was washed with 0.1 M Tris-HCl buffer (pH 8.0), and a yellow eluate was obtained. The eluate contained a flavo(FMN)protein which exhibited diaphorase activity (i.e., catalysis of dye reduction and reduction of menadione and ferricyanide by NADH). This enzyme showed neither pyridine nucleotide reductase nor cytochrome reductase activities. The column was washed with 1.0 M KCl containing 0.1 M Tris-HCl buffer (pH 8.0), and a brown eluate was obtained. The eluate was dialysed 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 0.25 M Tris-HCl buffer (pH 8.0), a yellow eluate was obtained. Next, the column was treated with 0.35 M Tris-HCl buffer (pH 8.0). A brown eluate resulted.

The brown eluate showed an absorption spectrum (Fig. 1) more similar to that of plant ferredoxin (PPNR) (Gewitz and Volker 1962, Horio and Yamashita 1962, Tagawa and Arnon 1962, Bendal et al. 1963, Fry and San Pietro 1963, Katoh and Takamiya 1963) than to that of the bacterial ferredoxins obtained up to the present from various bacteria (Tagawa and Arnon 1962, Lovenberg et al. 1963). The absorption maxima were at 415 and 460mμ, with a shoulder at 330mμ. When it was reduced with $Na_2S_2O_4$, the brown color faded and the absorbancy between 400 and 500mμ was much decreased (Fig. 1). The reduced form was reoxidized by vigorous aeration. These properties together with the enzymatic

**In addition to the standard abbreviations, the following were used: Tris(hydroxymethyl)aminomethane = Tris, diethylaminoethyl = DEAE, benzyl viologen = BV, reduced form of BV = BVH, pyridine nucleotide = PN^+, reduced form of PN^+ = PNH, FMN = flavin mononucleotide, and Rps = Rhodopseudomonas.

properties described below, indicated that the brown substance was a ferredoxin.

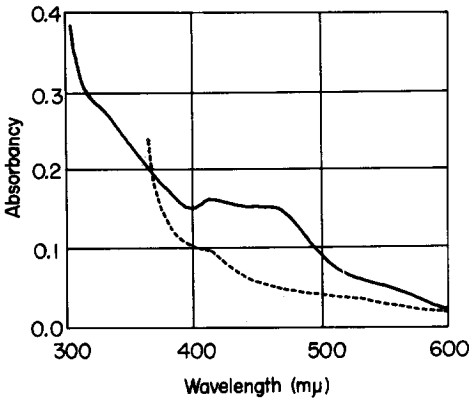

Fig. 1. The absorption spectrum of Rps. palustris #2137 ferredoxin. Solid line, oxidized form; broken line, reduced with $Na_2S_2O_4$.

The yellow eluate described above (eluted by 0.25 M Tris-HCl buffer, pH 8.0) was fractionated by $(NH_4)_2SO_4$, and the yellow precipitate obtained between 40 and 50% saturation was collected and dissolved in 0.1 M Tris-HCl buffer (pH 8.0). The solution obtained showed the absorption spectrum of a flavoprotein; there were absorption peaks at 277, 370 and 460mμ. The last peak was essentially removed by addition of a small amount of $Na_2S_2O_4$; after aeration, the original absorption spectrum reappeared. A trichloroacetic acid extract of the protein also showed an absorption spectrum of flavin.

As shown in Table I, the flavoprotein, in cooperation with the ferredoxin, reduced $NADP^+$ with BVH as the electron donor. For the reaction, the flavoprotein was essential. Although, as expected from its low redox potential, BVH functioned as a direct electron donor for the flavoprotein, nevertheless the ferredoxin accelerated significantly the reduction of $NADP^+$. It is very interesting that $NADP^+$ was a better electron acceptor than NAD^+, contrary to results noted with the R. rubrum system (Nozaki et al. 1963).

TABLE I

NADP reduction by the flavoprotein and ferredoxin.

Component	$-\Delta A_{550m\mu}$	$-\Delta BVH^*$ (μM)	$\Delta A_{340m\mu}$	ΔPNH^* (μM)
Complete	0.390	31.0	0.113	16.1
-Ferredoxin	0.270	21.4	0.091	12.5
-Flavoprotein	0.025	1.84	0.023	1.61
NAD^+ in place of $NADP^+$	0.121	9.52	0.073	9.51
$-PN^+$	0.002	0.00#	0.013	0.00#

*Concentration in the reaction mixture; the value in "$-PN^+$" was taken as 0.00.

The reaction mixture consisted of 2.0 ml of 0.1 M Tris-HCl (pH 7.0), 0.1 ml of 0.01 M BV, 0.05 ml of ferredoxin, 0.02 ml of the flavoprotein ($A_{460m\mu}$ = 0.209) and 0.25 ml of 3 mM $NADP^+$ in a total 2.5 ml. The reactions were carried out anaerobically at 20° using Thunberg-type cuvettes. The main chamber contained the buffer and BV, and the side arm contained the other reagents. BV was partially reduced with a small amount of $Na_2S_2O_4$ before evacuation. The reactions were measured by the decrease of the absorbancy at 550mμ (i.e. consumption of BVH). After 1 min-reaction, air was introduced to oxidize BVH, and the absorbancy at 340mμ read. Then, the reaction mixtures were allowed to stand under air for 5 hrs. During this treatment, PNH disappeared by cooperation of the flavoprotein and BV (the enzyme was added to the "-flavoprotein" cuvette after BVH was reoxidized). There was a technical difficulty in determining $\Delta A_{340m\mu}$ because PN^+ was further reduced during the air introduction procedures. However, with the reaction rates observed for the complete system, or in the absence of ferredoxin, note stoichiometry (2 BV to 1 PN) expected on the basis that BVH is a one-electron donor (Michaelis and Hill, 1933).

At pH 7.0 and 20°, about 1.3×10^{-2} moles of $NADP^+$ were reduced per min per $A_{460m\mu}$ of the best enzyme preparation so far obtained.

The flavoprotein functioned also as a PNH-cytochrome c_2 reductase as shown in Table II. NADPH was a better electron donor than NADH. The NADPH-cytochrome c_2 reduction was strongly inhibited by $NADP^+$ but only slightly by NAD^+. Thus, the reaction velocity with NADPH as the electron donor was affected considerably by the $NADP^+$

TABLE II

Reduction of cytochrome c_2 by the flavoprotein.

Electron donor \ Addition	$\Delta A_{552m\mu}$/min None	$NADP^+$	NAD^+
NADPH	0.368	0.098	0.341
NADH	0.123	0.029	0.088

The reaction mixture contained 0.2 ml of 0.1 M Tris-HCl buffer (pH 7.0), 0.7 ml of 48 μM cytochrome c_2 (Rps. palustris) dissolved in 0.01 M KCl, 0.1 ml of 3 mM NADPH or NADH, 0.02 ml of the flavoprotein ($A_{460m\mu}$ = 0.100), and 0.1 ml of 5 mM $NADP^+$ or NAD^+. The oxidized form of the cytochrome c_2 was prepared by addition of a small amount of $K_3Fe(CN)_6$ to the solution followed by chromatography on a Sephadex column to remove the oxidant. The reactions were carried out at pH 7.3 and 20° in air, and measured by the increase of the absorbancy at 550mμ (α-peak of the cytochrome c_2).

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present in the NADPH preparation. At pH 7.3 and 20°, about 3.1×10^{-2} moles of the cytochrome c_2 were reduced per min per $A_{460m\mu}$ of the best enzyme preparation so far obtained.

From the facts described above, it is expected that in bacterial photosynthesis both ferredoxin and a flavoprotein are necessary for the reduction of pyridine nucleotide, as well as in plant photosynthesis. It is very interesting that in the photosynthesis system of Rps. palustris $NADP^+$ appears to be the electron acceptor for the photoreductant.

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