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Oxidation of Reduced Pyridine Nucleotides by Molecular Oxygen in Spinach Chloroplasts

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NADPH and NADH were oxidized by molecular oxygen via b-type cytochromes of chloroplasts. Reduction of cytochromes with NADPH was catalyzed by Fd-NADP reductase, whereas that with NADH was presumably due to the action of NAD-linked glyoxylate reductase.

We have recently reported [1] that photoreductive carboxylation of pyruvate and α-ketoglutarate in spinach chloroplasts is suppressed by molecular oxygen, and that this inhibition is partly due to the re-oxidation of photochemically reduced pyridine nucleotides by oxygen. However, the detailed mechanism of the oxidation has not been clarified. In the present study we investigated the stoichiometry of the reaction and absorbance change of cytochromes accompanying the reaction in chloroplasts and acetone-extract powder.

Chloroplasts were prepared from spinach leaves by the method of Whatley and Arnon [2]. Acetone-extract powder of chloroplasts was prepared as follows: seven volumes of cold acetone previously chilled at $-20°C$ was added slowly to the chloroplast suspension. The resulting precipitate was filtered on a Büchner funnel, washed thoroughly with cold acetone ($-20°C$) and dried in vacuo. The O_2-uptake and absorption spectra of chloroplast cytochromes were recorded with a Beckman oxygen analyzer, Model 777, and a Hitachi double beam spectrophotometer, Model 124, respectively. The anaerobic reactions were carried out in Thunberg-type quartz cells (1-cm light path). If necessary, glycerin was added to the reaction mixtures (40% in final concentration) to prevent the material from settling out while the recordings were being made.

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Abbreviation: cyt b_{563}, cytochrome b_{563}; cyt b_{559}, cytochrome b_{559}; cytochrome b, total b-type cytochromes (cyt b_{563}+cyt b_{559}): Fd-NADP reductase, ferredoxin-NADP reductase; NAD and NADH, nicotine adenine dinucleotide and its reduced form; NADP and NADPH, nicotine adenine dinucleotide phosphate and its reduced form.

In Table 1 are compared the amounts of excess O_2 consumed for the oxidation of 2.5 μmoles of NADPH or NADH by chloroplasts or acetone-extract powder. The reactions were continued until excess O_2-uptake due to the oxidation of substrate ceased. Simultaneous measurements of O_2- and NADPH- (NADH-) changes (Table 2) make it evident that the enzyme system concerned in the reaction was not impaired by acetone treatment. Furthermore, the ratio NADPH (NADH)/O_2=ca. 2 remained unchanged even in the presence of 10^{-4} M of KCN (a catalase inhibitor), suggesting that b-type cytochromes rather than some flavoprotein(s) might be involved in terminal oxidation (data not shown). The use of spectrophotometry proved this postulate correct (see later).

Table 1. O_2-uptake in the oxidation of reduced pyridine nucleotides.

Material	Reduced pyridine nucleotides* added (μmole)	Net amount of O_2-uptake** (μmole)	NADPH (NADH)/O_2
Chloroplasts (NADPH)	2.5	1.15	2.17
Chloroplasts (NADH)	2.5	1.26	1.99
Acetone-extract powder (NADPH)	2.5	1.26	1.99

All reaction mixtures contained 250 μmoles of Tris-HCl (pH 7.8), 2.5 μmoles of NADPH or NADH, chloroplasts (750 μg of chlorophyll***) or acetone-extract powder (50 mg) in a final volume of 10 ml. Temperature: 20°C.

* Reduced pyridine nucleotides were determined by absorbancy at 340 nm.

** Net amount of O_2-uptake was calculated by subtracting the amount of O_2-uptake in blank from that of total uptake.

*** Chlorophyll was determined by the method of Whatley and Arnon [2].

Table 2. Relationship between the amount of O_2 consumed and decrease of reduced pyridine nucleotides.

Experiments	NADPH (NADH) decreased (μmole)	O_2 consumed (μmole)	NADPH (NADH)/O_2
(1) NADPH	0.250	0.110	2.26
(2) NADH	0.410	0.215	1.90
(3) NADPH	0.665	0.330	2.10

All reaction mixtures contained 250 μmoles of Tris-HCl (pH 7.8), 1.0 μmole of NADPH or NADH and chloroplasts (750 μg of chlorophyll) in a final volume of 10 ml. Temperature: 20°C. O_2 and reduced pyridine nucleotides were determined as in Table 1.

As regards the reduction of cytochrome b, Cramer and Butler [3] reported that both components (cyt b_{563} and b_{559}) were reduced by NADPH. The enzyme concerned with the reaction was, however, not identified. In our experiments, the cytochromes in the acetone-extract powder were reduced by either NADPH or NADH (Fig. 1. trace a). Cytochrome f, on the other hand, was in the reduced state even in the

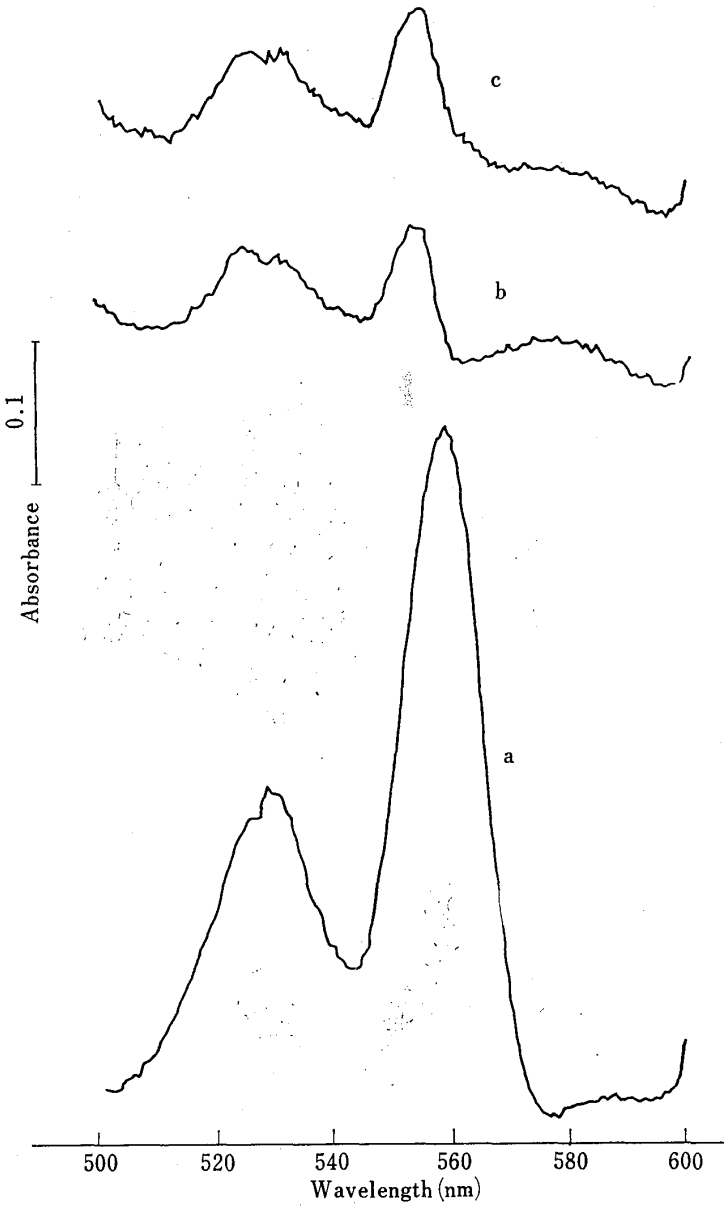

Fig. 1. Effect of NADPH and NADH on absorption spectra of cytochromes in acetone-extract powder.

Reaction mixtures contained 100 μmoles of Tris-HCl (pH 7.8), 400 μmoles of sorbitol, 2.5 μmoles of NADPH or NADH, 50 mg of acetone-extract powder in a final volume of 3.0 ml. Temperature: 20°C.

a: +NADPH (or +NADH), anaerobic. b: −NADPH (or −NADH), anaerobic.
c: +NADH, anaerobic.

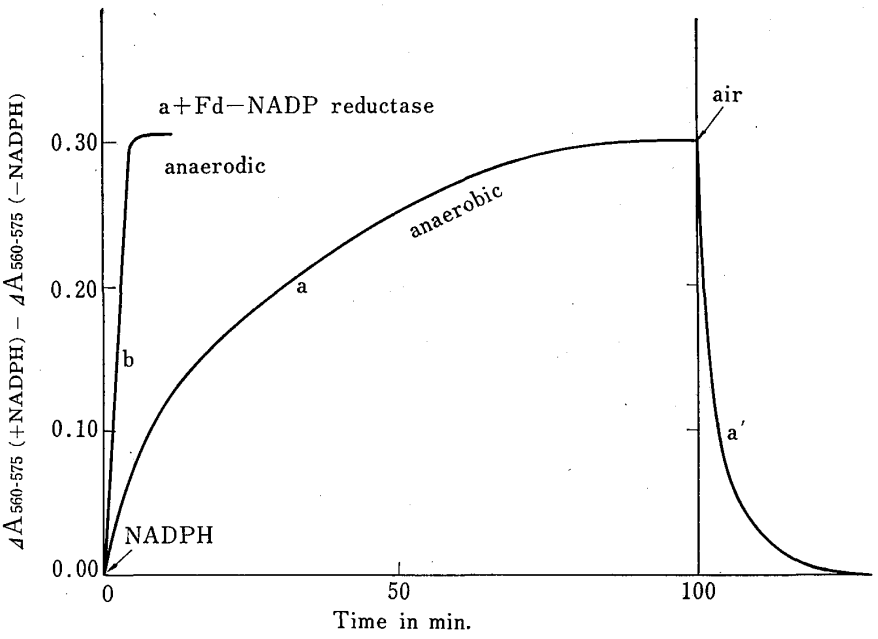

Fig. 2. NADPH-induced absorbance change at 560–575 nm (total b-type cytochromes) in washed acetone-extract powder and effect of Fd-NADP reductase.

Reaction mixtures contained 100 μmoles of Tris-HCl (pH 7.8), 400 μmoles of sorbitol, 2.5 μmoles of NADPH, 50 mg of acetone-extract powder washed four times with M/25 Tris-HCl (pH 7.8) and Fd-NADP reductase* in a final volume of 3.0 ml. Temperature: 20°C. At arrow, reaction mixture was exposed to air.

* Corresponding to the reduction of ca. 20 mμmoles of ferricyanide per minute.

presence of air without any reductant (Fig. 1, trace c) [5]. Thus the absorption spectrum with a peak around 560 nm represents the sum of three cytochromes (cytochrome f and two b-type cytochromes). Cytochrome b reduced by NADH anaerobically became oxidized under aerobic conditions (Fig. 1, trace b; Fig. 2, curve a′), while in the presence of NADPH it was oxidized more slowly. Fan and Cramer [4] reported that cytochrome b_{559} in the anaerobic chloroplast suspension is reducible by 0.1 mM NADH at a rate one-third that of NADPH. The results presented above and that of Fan and Cramer suggest that some diaphorase catalyzes the reduction of b-type cytochromes with either NADPH or NADH but its affinity for NADPH is much greater, or the NADH reaction is catalyzed by some other enzyme with low activity. The rate of cytochrome reduction with NADPH and NADH was greatly diminished by washing the acetone powder with M/25 Tris-HCl (pH 7.8) (Fig. 2, curve a; data for NADH not shown), indicating that the diaphorase(s) might be removed by this treatment. An affirmative result in this respect was obtained as shown in Fig. 2, cuave b; i.e., the activity of NADPH-dependent cytochrome reduction was restored by adding Fd-NADP reductase [6], which not only acts in this

function, but also behaves as a diaphorase, with respect to the washed acetone-extract powder. It can therefore be concluded that the sequence of NADPH-oxidation consists in the following two steps: (1) dehydrogenation of NADPH by cytochrome b via Fd-NADP reductase and (2) subsequent autooxidation of the reduced cytochrome.

Since the diaphorase activity of Fd-NADP reductase is highly specific for NADPH as the hydrogen donor [7], it will be reasonable to assume that the reduction of cytochrome b with NADH is catalyzed by some other enzyme. Recently Omata and Fukaya [8] found that, like Fd-NADP reductase, NAD-linked glyoxylate reductase of spinach leaves acts as an active diaphorase, catalyzing the reduction with NADH of various electron acceptors such as ferricyanide, cytochrome f, cytochrome c, 2, 6-dichlorophenol indophenol, methylene blue and menadion. Thus, in NADH-oxidation, cytochrome b would be reduced probably not via Fd-NADP reductase but more likely via NAD-linked glyoxylate reductase.

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