The Influence of Oxygen on Nitrite Reduction in a Reconstituted System

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Data regarding the role of oxygen in nitrite reduction are presented. In an NADPH-generating system including homogeneously purified ferredoxin-NADP reductase, ferredoxin (or flavodoxin) and nitrite reductase from the alga *Bumilleriopsis filiformis*, oxygen and nitrite can be reduced simultaneously. In air, rates of 1.2 μmol nitrite reduced$\cdot$min$^{-1}\cdot$mg^{-1} nitrite reductase are obtained, which are physiologically feasible. Ferredoxin is inhibited non-competitively by oxygen during nitrite reduction. Oxygen uptake due to the oxidase reaction of ferredoxin-NADP reductase mediated by flavodoxin from *Chlorella fusca* and ferredoxin from *Bumilleriopsis* involves superoxide and is inhibited by the nitrite reducing system.

Introduction

Nitrite reduction in plants is closely connected to the photosynthetic electron transport but can also proceed as an enzymatic dark reaction using NADPH, Fd-NADP reductase, nitrite reductase and ferredoxin *. Whereas the former reaction requires at least a chloroplast lamellar system [1], the latter system presumably works only under strictly anaerobic conditions [2]. Although NO_2^- reduction apparently is linked directly to non-cyclic photosynthetic electron transport [3], a strict stoichiometry between oxygen evolved and NO_2^- reduced to ammonia was not observed [4, 5]. Whereas photosynthesis in higher plants proceeds in an aerobic environment, the interaction of O_2 with NO_2^- reduction has not received proper attention and remained unclear. This investigation, therefore, presents some data concerning the direct role of oxygen in an NADPH-generating system used for nitrite reduction with components isolated from the alga *Bumilleriopsis* in a homogeneous form. Our findings suggest a close interrelationship between the reduction of O_2 and NO_2^-.

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* *Abbreviations:* Fd, ferredoxin; Fld, flavodoxin; IC/ICDH, isocitrate/isocitrate dehydrogenase; NiR, nitrite reductase; SOD, superoxide dismutase; Tris, tris(hydroxymethyl)-aminomethane (buffer, adjusted with HCl).

Materials and Methods

Fd and Fd-NADP reductase (EC 1.6.7.1, formerly 1.6.99.4) from autotrophically grown *Bumilleriopsis* [6, 7] and flavodoxin from *Chlorella* were isolated as described previously [8, 9]. Isolation and some properties of nitrite reductase (EC 1.7.7.1) from *Bumilleriopsis* are reported elsewhere [10].

All oxygen measurements were carried out with a Clark type electrode mounted in a thermostated water-jacketed cell [7] of 2 — 2.7 ml volume with adjustments for anaerobic incubation. Reaction temperature was 24 °C unless stated otherwise. NADPH oxidation was performed in elongated 1 ml cuvettes flushed with argon purified by passing through Fieser's solution, 5% KOH and dried Silica gel and was followed by the 340 nm absorbance change with a Hitachi double-beam spectrophotometer (mod. 124). Alternatively, the oxidation of NADPH by oxygen and by nitrite was carried out in 1.9 ml conical flasks closed by a rubber stopper and equipped with a syringe outlet. For nitrite determination, aliquots from samples were removed with a gas-tight Hamilton syringe (50 μl). Nitrite was determined after dilution and oxidation of surplus NADPH with phenazine methosulfate [11].

Activity of nitrite reductase was determined as described elsewhere [12].

Protein determination was done according to Lowry *et al.* [13]. NH_3 was measured after alkalization with 0.1 N NaOH by means of an NH_3 electrode (Orion, type 95 — 100) using a calibration curve. ICDH, catalase and pyridine nucleotides were purchased from Boehringer, SOD (from bovine blood; E 1.15.1.1) from Sigma. All other reagents of analytical grade were obtained from Merck.
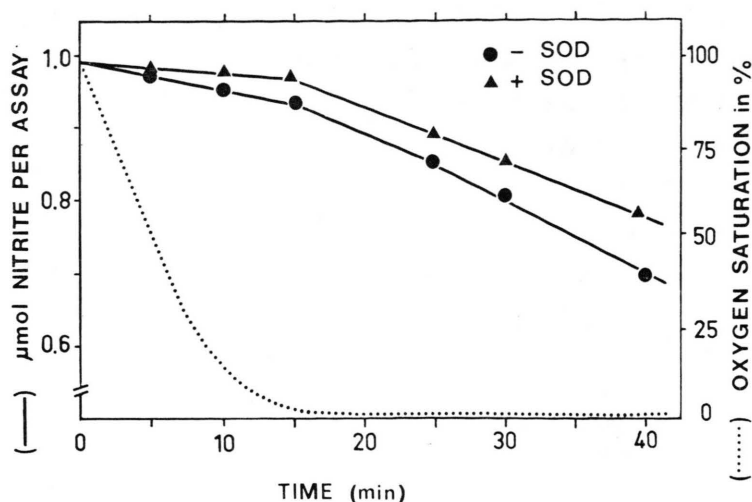
Fig. 1. Kinetics of oxygen consumption and nitrite reduction in a reconstituted system. The reaction mixture contained in a final volume of 2 ml in μmol: **Tris, pH 8.0**, 100; Na-isocitrate 6; NADP 0.2; MgCl$_2$ 4; KNO$_2$ 1; and in nmol: Fd 15; Fd-NADP reductase 3; NiR 0.3. Furthermore, the assay included catalase 200 units; SOD (Sigma) 170 units, and 0.2 mg ICDH. Reaction was carried out in an air-saturated mixture with stoppered tubes and in a closed oxygen-electrode vessel (dotted line). Aliquots for NO$_2^-$ determination were withdrawn at intervals as indicated. With air (21% oxygen v/v), inhibition of nitrite reduction was 55% *vs* anaerobic control.

Results and Discussion

Reduced ferredoxin is regenerated *via* the primary acceptor of photosystem I in photosynthetic electron transport and is considered also to act as physiological donor for nitrite reductase [1]. Regeneration of reduced Fd is also possible by coupling the NADPH-producing system of IC/ICDH to Fd-NADP reductase [14]. Since NADPH or IC will not reduce NO$_2^-$ chemically and NADPH is maintained at a constant level, we chose this system as a convient method to study nitrite reduction in the presence of oxygen (Fig. 1).

It is known that NADPH in the presence of Fd-NADP reductase and Fd reduces O$_2$ to peroxide [15]. Fig. 1 shows the influence of this reaction on NO$_2^-$ reduction. Catalase was included to avoid secondary oxidations by peroxide. NO$_2^-$ is reduced both in the presence and absence of SOD at constant rates for over 45 min. Due to the oxidase reaction the assay sample was depleted of O$_2$ within 15 min (dotted line). After O$_2$ depletion the rate of NO$_2^-$ reduction was enhanced and attained a constant value of 4.63 μmol NO$_2^-$ reduced per 10 min and mg NiR as compared to 2.1 μmol during the first 15 min. Addition of SOD decreased the rate by approx. 20%, which cannot be explained at present. As demonstrated further (Table I, last column) NO$_2^-$ is reduced in stoichiometric amounts to NH$_4^+$, which was found also with the NADPH-generating system (see legend of Fig. 1). Both systems have to be complete to achieve NO$_2^-$ reduction. This indicates that in contrast to earlier reports [2] catalytic amounts of NADPH, NiR, and Fd-NADP reductase

Table I. Nitrite reduction by NADPH in the presence of oxygen. The assay contained in a total volume of 1.8 ml in μmol: Tris, pH 8,0, 100; MgCl$_2$ 4; KNO$_2$ 1; Fd-NADP reductase 0.0025; Fd 0.018; NiR 3×10^{-4}; catalase 200 units and NADPH as indicated. Reaction was carried out in stoppered vessels for 90 min at 20 °C until equilibrium was attained. Nitrite was determined with aliquots treated with phenazine methosulfate before addition of NiR at start and at the end of the incubation period. NADPH was measured spectrophotometrically with an appropriate dilution of samples.

NADPH at start [μmol]	NO$_2^-$ consumed [μmol]	Oxygen at start [μmol]	NADPH at the end of reaction [μmol]	NH$_4^+$ produced [μmol]
0.5	—	0.51	—	—
2	0.11	0.51	0.61	—
5	0.38	0.51	2.72	0.35
10	0.51	0.51	6.90	0.49

are amply sufficient to maintain physiological rates of NH$_4^+$ production.

Apparently, there is no need for the complete exclusion of O$_2$ during nitrite reduction under physiological conditions. Hence we investigated the dependence on NADP concentration of enzymic reduction of NO$_2^-$ in our reconstituted system with a distinct amount of oxygen present at start in the closed vessel (Table I). NO$_2^-$ was not reduced at the lowest NADPH concentration used, whereas higher concentrations of NADPH resulted in an increased reduction of NO$_2^-$. Since the presence of catalase warranted the disappearance of peroxide, the amount of NADPH consumed ought to show a simple stoichiometric relationship with respect to the concurrent reduction of nitrite and oxygen ac-
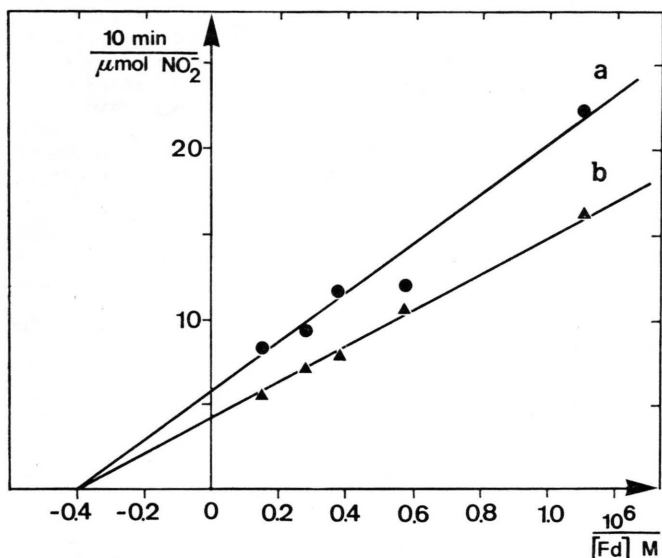
Fig. 2. Double reciprocal plot of the effect of ferredoxin concentration on nitrite reduction under aerobic and anaerobic conditions. Composition of the reaction medium was the same as in Fig. 1, except for NADP 0.4 μmol, minus SOD, and varying Fd concentration. Aerobic reaction was carried out in an open oxygen-electrode vessel (2.4 ml) gassed with air. Anaerobic conditions were obtained by gassing with purified argon for 3 min and consumption of the remaining O_2 by the inherent oxidase properties of the mixture (comp. Fig. 1) prior to adding NiR. Furthermore, glucose/glucose oxidase (20 mg/1.4 units) was included to ensure anaerobiosis. The initial rate of NO_2^- reduction was determined by taking aliquots with a gas-tight syringe, a) in air, b) anaerobic.

cording to the equation

$$a = 3\,b + 2\,c$$

where a denotes μmol NADPH oxidized, b and c μmol NO_2^- and O_2 reduced, or fractions thereof, to ammonia and to water. This balance rests on the assumption that 6 or 4 electrons are required to completely reduce 1 molecule of nitrite or oxygen, respectively. The experimental data of Table I support this stoichiometry.

Fig. 1 demonstrates a partial inhibition of NO_2^- reduction by oxygen. Since reduced Fd can be regarded as substrate for the reduction of both O_2 [14, 16] and NO_2^- [1], the mode of inhibition was checked by varying the concentration of Fd (Fig. 2). The double reciprocal plot exhibits non-competitive inhibition of nitrite reduction by O_2. The Fd concentration required for half maximum velocity remained unchanged at 2.5 μM, wheres the maximum rate was decreased by 21% v/v O_2 from 1.2 to 0.85 μmol NO_2^- per min and mg NiR. This effect of O_2 on NO_2^- reduction can be interpreted as decrease of the concentration of reduced ferredoxin available for nitrite reduction, rendering interaction of NO_2^- and O_2 at NiR rather unlikely.

These data, instead, point to Fd-NADP reductase as the key enzyme responsible for O_2 uptake, diverting electrons to either NiR or to oxygen. The oxidase activity of the flavoprotein itself is quite low (Bookjans, to be published), but can be greatly stimulated by ferredoxin [14, 15].

If both O_2 and NO_2^- draw on the pool of reduced low potential carriers through either the oxidase activity of Fd-NADP reductase or NiR, then NO_2^- reduction ought to have some influence also on O_2 uptake. Evidence for this proposition is presented in Fig. 3 using flavodoxin.

Addition of 1.5 nmol Fld increased the native activity more than 3-fold, whereas addition of SOD (from bovine blood or *Bumilleriopsis*) diminished the rate significantly, suggesting the involvement of superoxide. Decreasing the maximum rate to half by catalase indicates that oxygen reduction mediated by Fld (or Fd) is carried on to O_2^{2-}. Addition of 0.3 nmol NiR exhibited no change, but subsequent addition of NO_2^- lowered the rate of O_2 uptake (Fig. 3, tracing A). When the order of NiR and NO_2^- addition was reversed (tracing B), then again only further addition of NiR induced a pronounced decrease. This demonstrates that inhibition of O_2 uptake requires both NO_2^- and NiR of the NO_2^- reducing system. Inhibition of O_2 uptake by NiR can be increased by varying the amount of Fd-NADP reductase or NiR (not shown); Fd produces similar effects in this system. Generation of O_2^- was also demonstrated when NADPH was produced by a glucose-6-phosphate/dehydrogenase system.

Evidence presented herewith, suggesting that NO_2^- and O_2 reduction occur concurrently, with Fd or Fld as electron carriers, is corroborated by several observations reported: a) intact and broken chloroplasts are capable of reducing NO_2^- with the
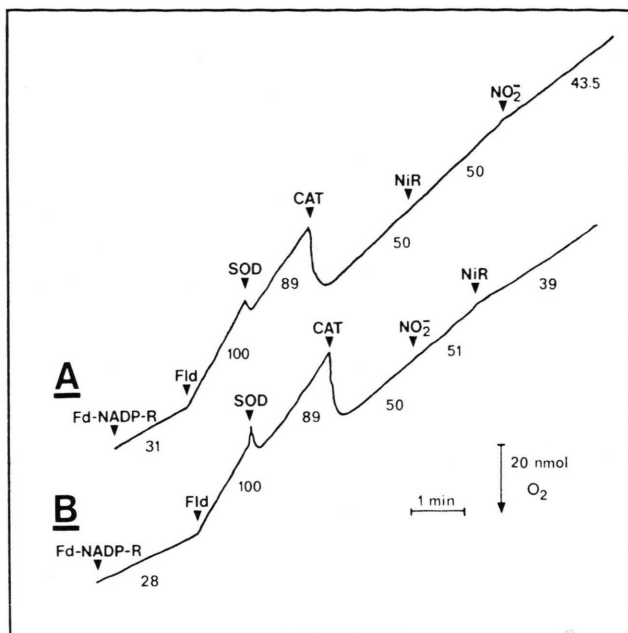
Fig. 3. The effect of Fld (2.4 nmol), SOD (170 units) and NiR (0.3 nmol) on oxygen uptake in an NADPH-regenerating system in the presence of Fd-NADP reductase (=Fd-NADPH-R, 0.75 nmol). Rates of O_2 uptake are indicated as percentage of the maximum rate (100%=3.64 μmol O_2 per 100 nmol Fd-NADP reductase and min). Other components and conditions as in Fig. 1. CAT = catalase.

oxygen-evolving apparatus still intact [1, 4], b) oxygen appears to be a prerequisite for the proper control of the NADPH/ATP ratio [17], c) during nitrite reduction in chloroplasts most of the NADP is in the reduced state [18], d) the decrease of the primary fluorescence peak by NO_2^- in algae occurs also in air, indicating electron transport partially due to NO_2^- reduction. The physiological significance of our findings may well be another regulatory function of Fd-NADP reductase in connection with photosynthetic nitrite reduction, in addition to reduction of NADP and O_2 as indicated by Allen [16], Heber and Kirk [17], maintaining a balance between nitrogen and carbon metabolism by directing reducing power towards oxidized nitrogen when NADPH and carbohydrates are in excess.

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