The Influence of Oxygen on Nitrite Reduction in a Reconstituted System
Hartmut Spiller, Gerhard Bookjans, and Peter Böger
Department of Biology, University of Konstanz

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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·min⁻¹·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 system¹, the latter system presumably works only under strictly anaerobic conditions². Although NO₂⁻ reduction apparently is linked directly to non-cyclic photosynthetic electron transport³, a strict stoichiometry between oxygen evolved and NO₂⁻ reduced to ammonia was not observed⁴.⁵. Whereas photosynthesis in higher plants proceeds in an aerobic environment, the interaction of O₂ with NO₂⁻ 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 O₂ and NO₂⁻.

Materials and Methods

Fd and Fd-NADP reductase (EC 1.6.7.1, formerly 1.6.99.4) from autotrophically grown Bumilleriopsis⁶,⁷ and flavodoxin from Chlorella were isolated as described previously⁸,⁹. Isolation and some properties of nitrite reductase (EC 1.7.7.1) from Bumilleriopsis are reported elsewhere¹⁰.

All oxygen measurements were carried out with a Clark type electrode mounted in a thermostated water-jacketed cell⁷ 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 methosulfate¹¹.

Activity of nitrite reductase was determined as described elsewhere¹².

Protein determination was done according to Lowry et al.¹³. NH₃ was measured after alkalization with 0.1 N NaOH by means of an NH₃ 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.

Requests for reprints: To anyone of the authors, Lehrstuhl Physiologie und Biochemie der Pflanzen, Fachbereich Biologie der Universität Konstanz, Postfach 7733, D-7750 Konstanz.

* Abbreviations: Fd, ferredoxin; Fld, flavodoxin; IC/ICDH, isocitrate/isocitrate dehydrogenase; NiR, nitrite reductase; SOD, superoxide dismutase; Tris, tris(hydroxymethyl)-aminomethane (buffer, adjusted with HCl).
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 \( \text{Tris, pH 8.0, 100; Na-isocitrate 6; NADP 0.2; MgCl}_2 4; \text{KNO}_2 1; \) 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 \( \text{NO}_2^- \) 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 \( \text{NO}_2^- \) chemically and NADPH is maintained at a constant level, we chose this system as a convenient 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 \( \text{O}_2 \) to peroxide.\(^15\) Fig. 1 shows the influence of this reaction on \( \text{NO}_2^- \) reduction. Catalase was included to avoid secondary oxidations by peroxide. \( \text{NO}_2^- \) 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 \( \text{O}_2 \) within 15 min (dotted line). After \( \text{O}_2 \) depletion the rate of \( \text{NO}_2^- \) reduction was enhanced and attained a constant value of 4.63 \( \mu \text{mol} \) \( \text{NO}_2^- \) reduced per 10 min and mg NiR as compared to 2.1 \( \mu \text{mol} \) 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) \( \text{NO}_2^- \) is reduced in stoichiometric amounts to \( \text{NH}_4^+ \), which was found also with the NADPH-generating system (see legend of Fig. 1). Both systems have to be complete to achieve \( \text{NO}_2^- \) reduction. This indicates that in contrast to earlier reports\(^2\) catalytic amounts of NADPH, NiR, and Fd-NADP reductase are amply sufficient to maintain physiological rates of \( \text{NH}_4^+ \) production.

Table I. Nitrite reduction by NADPH in the presence of oxygen. The assay contained in a total volume of 1.8 ml in \( \mu \text{mol} \): Tris, pH 6.0, 100; MgCl\(_2\) 4; \text{KNO}_2 1; \text{Fd-NADP reductase 0.0025; Fd 0.018; NiR 3} \times 10^{-4}; \text{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.}

<table>
<thead>
<tr>
<th>NADPH at start [( \mu \text{mol} )]</th>
<th>( \text{NO}_2^- ) consumed [( \mu \text{mol} )]</th>
<th>Oxygen at start [( \mu \text{mol} )]</th>
<th>NADPH at the end of reaction [( \mu \text{mol} )]</th>
<th>( \text{NH}_4^+ ) produced [( \mu \text{mol} )]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>—</td>
<td>0.51</td>
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<td>—</td>
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<tr>
<td>2</td>
<td>0.11</td>
<td>0.51</td>
<td>0.61</td>
<td>—</td>
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<tr>
<td>5</td>
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<td>0.51</td>
<td>2.72</td>
<td>0.35</td>
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<tr>
<td>10</td>
<td>0.51</td>
<td>0.51</td>
<td>6.90</td>
<td>0.49</td>
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</table>

Apparently, there is no need for the complete exclusion of \( \text{O}_2 \) during nitrite reduction under physiological conditions. Hence we investigated the dependence on NADP concentration of enzymic reduction of \( \text{NO}_2^- \) in our reconstituted system with a distinct amount of oxygen present at start in the closed vessel (Table I). \( \text{NO}_2^- \) was not reduced at the lowest NADPH concentration used, whereas higher concentrations of NADPH resulted in an increased reduction of \( \text{NO}_2^- \). 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 O₂ 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 NO₂⁻ reduction was determined by taking aliquots with a gas-tight syringe, a) in air, b) anaerobic.

According to the equation

\[ a = 3b + 2c \]

where \( a \) denotes μmol NADPH oxidized, \( b \) and \( c \) μmol NO₂⁻ and O₂ 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 NO₂⁻ reduction by oxygen. Since reduced Fd can be regarded as substrate for the reduction of both O₂ and NO₂⁻, 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 O₂. The Fd concentration required for half maximum velocity remained unchanged at 2.5 μM, whereas the maximum rate was decreased by 21% v/v O₂ from 1.2 to 0.85 μmol NO₂⁻ per min and mg NiR. This effect of O₂ on NO₂⁻ reduction can be interpreted as decrease of the concentration of reduced ferredoxin available for nitrite reduction, rendering interaction of NO₂⁻ and O₂ at NiR rather unlikely.

These data, instead, point to Fd-NADP reductase as the key enzyme responsible for O₂ 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.

If both O₂ and NO₂⁻ draw on the pool of reduced low potential carriers through either the oxidase activity of Fd-NADP reductase or NiR, then NO₂⁻ reduction ought to have some influence also on O₂ 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 O₂⁻. Addition of 0.3 nmol NiR exhibited no change, but subsequent addition of NO₂⁻ lowered the rate of O₂ uptake (Fig. 3, tracing A). When the order of NiR and NO₂⁻ addition was reversed (tracing B), then again only further addition of NiR induced a pronounced decrease. This demonstrates that inhibition of O₂ uptake requires both NO₂⁻ and NiR of the NO₂⁻ reducing system. Inhibition of O₂ 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 O₂⁻ was also demonstrated when NADPH was produced by a glucose-6-phosphate/dehydrogenase system.

Evidence presented herewith, suggesting that NO₂⁻ and O₂ 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 NO₂⁻ with the
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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