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Communication

Nitrite Photoreduction *in Vivo* Is Inhibited by Oxygen

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ABSTRACT

It was hypothesized previously that an O_2 inhibition of NO_2^- photoreduction would reflect a competition between O_2 and NO_2^- for electrons from ferredoxin at the site of plastid nitrite reductase. In order to test this *in vivo*, intact spinach (*Spinacia oleracea* L.) leaf chloroplast and mesophyll cell isolates held in high light were aerated with streams of 20% O_2/80% N_2 (250 micromolar O_2 in aqueous solution) or, alternatively, streams of 100% N_2. Bicarbonate plus CO_2 and NO_2^- were supplied to reaction mixtures at levels just sufficient to promote maximal assimilations of CO_2 and NO_2^-. In chloroplast isolates, there was a 9 to 30% O_2 inhibition of NO_2^- reduction while there were high rates of CO_2 fixation. In spinach and soybean (*Glycine max*) leaf cell isolates, NO_2^- photoreduction rates were 10 to 55% inhibited by O_2 at near ambient levels. It is possible that O_2 may compete, albeit weakly, with NO_2^- (nitrite reductase) for equivalents derived from reduced ferredoxin. Also, O_2 may oxidize sulfhydryl groups on nitrite reductase which are involved in substrate binding and/or activation.

The site of the O_2 inhibition (the Warburg effect) of carbon dioxide photoassimilation in green plants possessing the pentose phosphate reductive cycle generally is accepted to be the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (6). However, molecular oxygen is an alternative Hill oxidant, and it is able to compete with other Hill oxidants (*e.g.* NADP) for electrons derived from Fdr[1] (3, 9, 11, 13). This competition, as well the resulting products of O_2 reduction (*e.g.* H_2O_2), have been attributed to be causal factors of the O_2 inhibition of photosynthesis (10, 13). Additionally, O_2 or H_2O_2 also may oxidize —SH groups on the chloroplast carbon assimilatory enzymes (*e.g.* GAPase) (1, 8, 13) and protein reductants (*e.g.* reduced thioredoxin) (1, 4, 5) resulting in inhibitions of carbon assimilation (10, 13). For example, recent reports indicate that in isolated, reconstituted chloroplast systems, the Fd-thioredoxin mediated activation of maize chloroplast MDHase (4) as well as the thioredoxin driven activation of the spinach chloroplast MDHase and FBPase (5) were inhibited by O_2 apparently via oxidations of functional protein sulfhydryl groups on the reduced thioredoxin.

The question remained whether, *in vivo*, O_2 could inhibit NO_2^- photoreduction in chloroplasts (another photogenerated Fdr dependent event). Behrens *et al.* (2) demonstrated that NO_3^- reduction (and presumably subsequent NO_2^- reduction) repressed O_2 photoreduction in isolates of intact soybean leaf mesophyll cells. Those observations suggested that O_2 and inorganic nitrogen, *i.e.* NO_2^-, were alternative Hill oxidants in weak competition for photogenerated Fdr (2, 13).

This report presents the first evidence that O_2, equilibrated in reaction mixtures at air levels (0.25 mM in solution), appears to mediate a mild inhibition of NiPR ongoing in the presence of maximal PPRC activity in isolated intact chloroplasts as well as leaf mesophyll cells. The implications of this O_2 inhibition of NiPR are discussed in relation to the controversy (10) concerning the number of chloroplast enzyme sites associated with the Warburg effect, *i.e.* the O_2 inhibition of CO_2 photoassimilation (7).

MATERIALS AND METHODS

Plant Material

Isolates of intact leaf mesophyll cells and chloroplasts were prepared from mature "source" leaves of 30 to 40 d old, growth chamber propagated *Spinacia oleracea* cv Wisconsin Dark Green. Isolates of soybean leaf mesophyll cells were prepared from 26 to 30 d old *Glycine max* cv Williams; leaves were selected from the fifth and sixth trifoliolates (acropetally numbered). Spinach and soybean plants were propagated in the growth chamber with a 12 h day-12 h night cycle at 25°C as previously described (12, 14).

Leaf Chloroplast and Mesophyll Cell Isolation

Isolates of intact spinach leaf chloroplasts or mesophyll cells were prepared, Chl in the isolates was quantitated, and plastid and cell intactness was estimated as previously described (10, 12, 14). Soybean leaf cell isolates were prepared following the protocols described for spinach (14) except that leaf cells were separated from the Calbiochem[2] Mascerase Digest (14) using

[1] Abbreviations: Fdr, reduced ferredoxin; CO_2, CO_2 gas dissolved in solution; DIC, dissolved inorganic carbon, *i.e.* CO_2 plus HCO_3^-; FBPase, fructose-1,6-bisphosphate (C-1) phosphatase (chloroplast isozyme); Fdo, oxidized ferredoxin; FNRase, ferredoxin-NADP reductase; FTRase, ferredoxin-thioredoxin reductase; GAPase, glyceraldehyde-3-phosphate dehydrogenase (NADPH) (chloroplast isozyme); HCO_3^-, bicarbonate; MDHase, malate dehydrogenase-NADP, chloroplastic isoform; NiRase, nitrite reductase (reduced ferredoxin); NiPR, nitrite photoreduction; PPRC, pentose phosphate reductive cycle.

[2] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

a 153 μm pore size nylon filter; cell Chl was measured as reported previously (12). Chl reported in all reaction mixtures (figure legends) was that associated with only the intact plastids or cell isolates.

Simultaneous CO_2 Photofixation and NO_2^- Photoreduction in Isolates

Reaction mixture compositions that supported maximal intact plastid CO_2 and NO_2^- assimilation, contained in 2.0 mL, 50 mM Tricine (pH 8.1), 0.33 M sorbitol, 2 mM DiK EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.25 mM Pi, 5 mM [^{12}C] DIC or [^{14}C]DIC, 75 μM NO_2^-, and 1000 Sigma units of catalase (Sigma C-40, Thymol Free).

Reaction mixture compositions which supported maximal rates of CO_2 and NO_2^- assimilation in spinach and soybean leaf mesophyll cell isolates contained, in 2.0 mL, 50 mM Tricine-NaOH (pH 8.1), 0.5 M sorbitol, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM Pi, 5.0 mM [^{12}C]DIC or [^{14}C]DIC (20 μCi ^{14}C), 1 mM $NaNO_2$, and 1000 units catalase (Sigma Chem. Co. C-40) in a final volume of 2.0 mL.

During experiments, cell and plastid isolates were incubated in reaction mixtures within glass tubes mounted in a constant temperature, illuminated water bath. The tubes were illuminated from one side with white light supplied by General Electric reflector flood bulbs which provided incident quantum flux densities of approximately 800 μE/s·m^2 which were saturating with respect to both CO_2 and NO_2^- assimilations (14).

In the light, it has been observed repeatedly that NO_2^- disappearance in plastid isolates was dependent upon the photolytic cleavage of H_2O. The ratio of O_2 photoevolved to NO_2^- photoreduced has been observed repeatedly to be 1.5 (see ref. 12, p. 679). This reflects the transfer of 6 mol-equivalents of electrons from 3 mol H_2O to reduce 1 mol NO_2^- to 1 mol NH_4^+. For this reason, the y axes of the figures reflect both the quantitative, light dependent loss of NO_2^- or the reciprocal gain of NH_4^+. It is the light dependent disappearance of NO_2^- which is monitored for this study, but it is clear from the O_2 stoichiometry that the first product must be NH_4^+ (13, 15).

Estimations of an O_2 Mediated Inhibition of NiPR

It was hypothesized that a reflection of competition for Fdr electrons between O_2 and NO_2^- at the site of plastid NiRase would be an O_2 inhibition of NiPR (13). To test this, NiPR was monitored in intact spinach leaf chloroplast isolates or spinach and soybean leaf mesophyll cell isolates which were aerated with streams of either 20% O_2/80% N_2 (where indicated), 40% O_2/60% N_2 (as described) or 100% N_2 (where indicated) (see refs. 7 and 10 for apparatus and refs. 12 and 14 for NiPr monitoring). Total CO_2 fixation rates in plastid isolates and net carbon assimilation rates in leaf cell isolates was monitored as CO_2 dependent, O_2 evolution with a Clark-type electrode (10, 12, 14).

Plastid and cell isolates were fed [^{12}C]DIC and NO_2^- at levels sufficient to promote simultaneous maximal CO_2 and NO_2^- photoassimilations. The presence of an O_2 inhibition of NiPR always was tested in the presence of saturating levels of DIC in high light. This assured that there was rapid recycling of NADP(H) and Fdo/Fdr *in vivo* to support PPRC activity, and it facilitated the maximal demands for reductant and ATP by simultaneous carbon and nitrogen photoassimilatory processes. This prevented an excess of Fdr from accumulating so that potential competition between NO_2^- and O_2 for electrons might not be apparent. Also, maximal CO_2 fixation eliminated the opportunity for the onset of a "photoinhibition" (13) which could confuse the interpetation of the data.

Catalase was employed in the reaction mixtures to prevent H_2O_2, synthesized by broken plastids (in both intact plastid as well as cell isolates), from accumulating at levels sufficient to mediate inhibition of the PPRC enzymes within the intact plastids' stromal compartments (10).

RESULTS

Intact Plastid Isolates

In 9 out of the 12 consecutive experiments, it was observed that O_2 mediated an apparent inhibition of NiPR in intact spinach leaf isolates. With approximately 250 μM O_2 in aqueous solution, the severity of this inhibition was in the range of approximately 9 to 30% in the first period (≈6.5 min) and 20 to 55% in the subsequent 10 min in the light (*e.g.* Fig. 1). For example, in the presence of maximal total CO_2 fixation, NO_2^- photoreduction rates were, at null and 250 μM O_2, respectively, 8.3 and 6.2 μmol/h·mg Chl or 24.7%

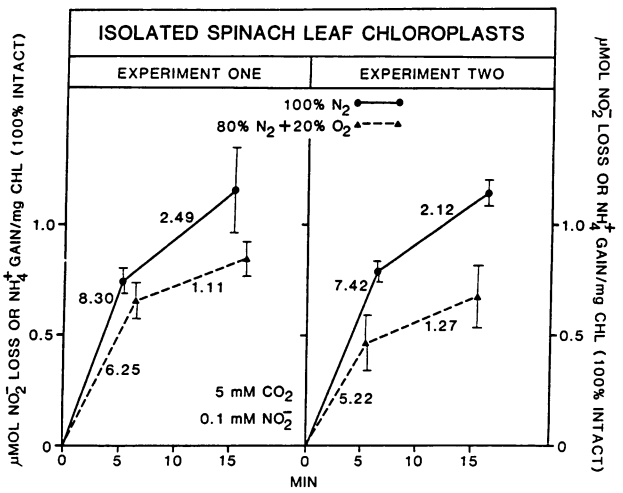

Figure 1. Inhibition by O_2 of nitrite photoreduction in isolated, intact spinach leaf chloroplast preparations. Reaction mixture components are described in the "Materials and Methods." In experiments 1 and 2, intact chloroplast Chl was, respectively, 37.0 and 35.2 μg. The numerical values displayed on the traces are the rates of NO_2^- reduction in the units μmol/h·mg Chl, and the bar associated with each time point reflects the standard deviation about the mean for that point. Each experiment was conducted with an isolate prepared from a different selection of spinach plants. In these studies, when the initial O_2 level in solution was 250 μM, the estimated rates of maximal total CO_2 fixation in experiments 1 and 2 were, respectively, 91.2 ± 0.7 and 83.2 ± 6.4 μmol CO_2 fixed/h·mg Chl (assessed with the O_2 electrode).

O_2 inhibition (Fig. 1, experiment 1). Regardless of the presence of O_2, rates of plastid NiPR usually diminished after approximately 6.5 min in the light (Fig. 1). However, the O_2 mediated inhibition appeared to be most severe during the 6.5 to 16.5 min time period. For example, the results displayed in Figure 1 indicate that the O_2 directed inhibition, during the last 10 min of the light period, increased to as much as 55% in experiment 1 and 40% in experiment 2.

The O_2 inhibition of NiPR observed in the presence of 40% O_2/60% N_2 aeration (≈500 μM O_2 in aqueous solution) was no more severe than the O_2 inhibition observed when 20% O_2/80% N_2 was employed for solution aeration (data not shown). Also, it should be noted that from experiment to experiment there was considerable variability in the severity of the O_2 mediated inhibition of NiPR. Indeed, in 3 other separate experiments, out of the 12 studies, no O_2 inhibition of NiPR was observed (data not shown).

There was no O_2 inhibition of CO_2 fixation when DIC was supplied in amounts saturating with respect to CO_2 assimilation and when NO_2^- reduction was functioning concurrently. It was observed that the rates of intact spinach plastid $^{14}CO_2$ photoassimilation (with 75 μM NO_2^- present), in the presence of 100% O_2 aeration compared with 100% N_2 aeration, were, respectively, 101.3 and 101.2 μmol/mg Chl·h (no O_2 inhibition). In this same experiment, the simultaneous rates of NO_2^- photoreduction, in the initial 4 min light period, and in the presence of 100% O_2 aeration compared with 100% N_2 aeration, were, respectively, 5.3 compared with 6.2 μmol/h·mg Chl (14.5% O_2 inhibition) (data not shown).

Intact Spinach and Soybean Leaf Cell Isolates

In the presence of a maximal rate of net CO_2 assimilation, NO_2^- photoreduction rates were, at null and 250 μM O_2, respectively, 23.3 and 21.0 or 10% apparent inhibition by O_2 in the 0 to 6.5 min illumination period. Oxygen inhibition rose to only 13.2% in the subsequent 10 min period (Fig. 2). However, this weak O_2 inhibition of NiPR in spinach leaf cells was variable in three other experiments (10–15% inhibition). Also, in three other studies, there was no difference between the rate of NiPR when assayed anaerobically as compared to the rate derived with 250 μM O_2 present (data not shown).

The O_2 mediated inhibition of NiPR in soybean cell isolates was often much more pronounced, especially during the initial minutes of illumination. For example, in one study, during 7 min of illumination, the rate of NiPR with 100% N_2 aeration was 9.9 ± 2.5 μmol NO_2^- reduced/h·mg Chl, but was 4.4 ± 1.4 in the presence of 21% O_2 (measurements were made simultaneously with maximal net CO_2 fixation). There was approximately 55% inhibition mediated by O_2 (data not shown). In another study with soybean leaf cells, the O_2 mediated inhibition was 60 to 76% over a 16 min illumination period (data not shown).

DISCUSSION

Molecular oxygen, equilibrated at 250 μM in the plastid and cell isolate reaction mixtures, was found to mediate inhibition of NiPR, often by as much 30 to 55% (Figs. 1 and 2 and

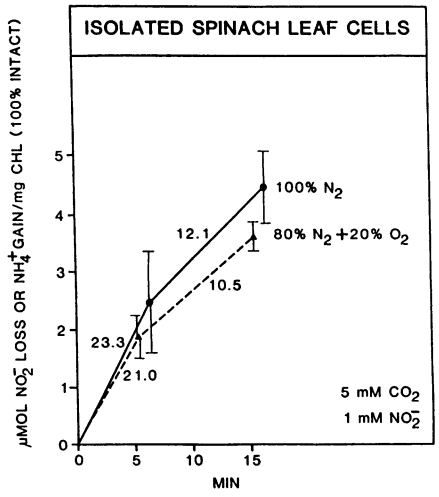

Figure 2. Inhibition by O_2 of nitrite photoreduction in isolates of intact spinach leaf mesophyll cells. Reaction mixture components are described in the "Materials and Methods." Intact cell Chl was 36.0 μg. The numerical values displayed on the traces are the rates of NO_2^- reduction in the units μmol/h·mg Chl, and the bar associated with each time point reflects the standard deviation about the mean for that point. In this experiment, when the initial O_2 level in solution was 250 μM, the estimated rate of maximal net CO_2 fixation was 71.4 ± 4.3 μmol CO_2 fixed/h·mg Chl (assessed with the O_2 electrode).

"Results"). Since this inhibition, apparently at the site of NiRase, occurred in the presence of maximal CO_2 assimilation, it was concluded that this was not a reflection of "photoinhibition" of some electron transport component (13).

In higher plant source leaf chloroplasts, NiRase does not compete with FNRase nor FTRase for electrons from Fdr, because it appears likely that these sites are not served by common pools of Fdr/Fdo (12, 14). It is conceivable that the terminal reductase of each linear, photosynthetic electron transport chain is served by its own pool of ferredoxin, thus negating the need for competition for electrons between sites (12–14). Without question, Fdr may be oxidized by O_2 at any site where Fdr functions as an electron donor, and this appears to be the basis for competition for electrons between O_2 and other physiological Hill oxidants (13). Indeed, it is becoming increasingly clear that there is a weak competition for electrons from Fdr that occurs between O_2 and the physiological Hill oxidant NADP (3, 9, 11, 13) as well as between O_2 and oxidized thioredoxin (4, 5). Further, Behrens *et al.* (2) found that when NO_3^- was supplied to isolates of soybean leaf cells, the ongoing rate of O_2 photoreduction (the Mehler reaction) was diminished by ≈40% (see Fig. 5, A and B, of ref. 2). Their work indicated that NO_2^-, which resulted from the reduction of the supplied NO_3^-, served as a successful competitor with O_2 for electrons derived from Fdr (see ref. 13 for a discussion of this point). However, the competition between O_2 and NO_2^- must be weak since the inhibition of NiPR did not appear to be more severe at 40% O_2 aeration (≈500 μM O_2 in solution) compared with that observed with 20% O_2 aeration ("Results"). Since the K_m O_2 for O_2 reduction (to H_2O_2 with Fdr as reductant) is rather low (≈22 μM O_2) (3, 9), and the level of O_2 that fully saturates Fdr driven O_2 photoreduction

is as low as 100 μM O_2 (3, 9), then it is likely that O_2 levels much above 250 μM O_2 would not be effective in enhancing the severity of the competition by O_2.

Further work is underway to examine the reversibility and the precise O_2 concentration properties of this apparent "Warburg effect" upon NiRase *in vivo*. However, at this point, it has been difficult to assess accurately the reversibility of the O_2 inhibition of NiPR, because after approximately 5 to 7 min, the rate of spinach chloroplast nitrite reduction often diminishes by approximately 50 to as much as 90% (Figs. 1 and 2 and data not shown). Under conditions of N_2 aeration or, alternatively, O_2 aeration, there has been repeated difficulty in assessing the nitrite reduction after the change over of aeration (O_2 to N_2 or the reverse), because of the severe diminution in linearity of the NiPR rate after even 5 to 7 min of illumination. Further attempts are being made to solve this problem.

The magnitude of severity of O_2 inhibition of NiPR was greater in the intact spinach plastid isolates and soybean leaf cell isolates than it was in the spinach leaf mesophyll cell isolates (Figs. 1 and 2, and "Results"). Depending on the plant species, the level of organization of the leaf isolate also could be a factor in the magnitude of severity of O_2 inhibition of NiPR. For example, purified PPRC enzymes and PPRC enzyme systems in reconstituted chloroplast systems are more vulnerable to oxidation by O_2 of functional sulfhydryl groups than are those same enzymes *in situ* (1, 4, 5). For example, DTT is always needed to provide sulfhydryl protection for purified chloroplast FBPase (1). In reconstituted chloroplast systems, DTT is needed to provide a sufficient sulfhydryl reducing environment to support complete activation and sustain activities of PPRC enzymes such as GAPase and FBPase (1, 8). In contrast, DTT is not needed to sustain the highest activities of CO_2 photoassimilation in intact spinach leaf plastid and cell isolates (10, 14). However, the NiRase within an isolated, intact plastid may be less well protected from sulfhydryl inactivation than is NiRase within intact plastids embedded in the whole cell cytoplasmic matrix. This could have been an additional causal factor for the more severe O_2 inhibition of NiPR visible in spinach plastid isolates compared with the lesser inhibition observed in spinach leaf cell isolates. Since it is known that the NiRase protein possesses functional sulfhydryl groups and iron-sulfur centers necessary for maximal catalytic activity (15), then it is feasible that O_2 could disrupt the sulfhydryl status as well as the iron sulfur center status of NiRase. Certainly, this could be another site of the O_2 mediated inhibition of NiPR (NiRase).

Based upon recent research in several laboratories (2–5, 9, 11, 13), as well as the results of this study, it is suggested that an additional increment of the O_2 inhibition of photosynthesis could be expressed because of competition between O_2 and other physiological Hill oxidants for Fdr at enzyme sites which are dependent upon Fdr for electrons. Also, another potential site for mediation of O_2 inhibition are those enzymes whose maximal catalytic activities depend upon the protein sulfhydryl status.

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