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Metabolic Interactions between Spinach Leaf Nitrite Reductase and Ferredoxin-NADP Reductase

COMPETITION FOR REDUCED FERREDOXIN

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ABSTRACT

Steady state rates of NADP reduction decline upon commencement of nitrite reduction in reconstituted chloroplast preparations. Similarly, steady state rates of nitrite reduction are lower, but not zero, during concurrent NADP reduction. These results imply that competition for substrate occurs and suggest that nitrite reduction can successfully compete for reduced ferredoxin, even at high rates of NADP reduction.

In the chloroplast, reduced ferredoxin is utilized by nitrite reductase for the reduction of nitrite to ammonia (22) and by ferredoxin-NADP reductase for the reduction of NADP to NADPH (3). The use of a common substrate has led to the suggestion that a competition between the two enzymes may occur (21, 22). Under conditions of reduced electron transport such competition, if present, could severely affect the relative rates of CO_2 and nitrate assimilation.

Evidence for competition would be a decline in the rate of nitrite reduction during active CO_2 fixation or NADP reduction. Conversely, the rate of CO_2 fixation or NADP reduction should decline during nitrite reduction. Results from the first approach have been mixed. Both reductions (19, 20) and enhancements (10, 13) in the rate of nitrite reduction have been reported. In experiments measuring the effect of nitrite on CO_2 fixation or NADP reduction, reductions in the rate of CO_2 fixation or NADP reduction are generally found upon the addition of nitrite (4, 9–12, 14, 21). Although these results would seem to be a good indication of competition, this interpretation is complicated by the problem of toxicity. Nitrite toxicity, which may result from an inhibition of fructose bisphosphatase due to lowering of the stromal pH (11, 14), an inhibition of carbonic anhydrase (4, 8), or an inhibition of PSII (20), is thought to account for much of the inhibition observed in these experiments. Thus, although competition between NIR[1] and FNR for reduced ferredoxin appears likely, a rigorous demonstration is lacking.

In this study we used a reconstituted spinach leaf chloroplast preparation to monitor the change from steady state in the rate of nitrite reduction upon the addition of NADP, and of NADP reduction upon the addition of nitrite. This approach has the advantage of eliminating two of the proposed sites of nitrite toxicity, fructose bisphosphatase and carbonic anhydrase, and so permits a more precise evaluation of competition.

[1] Abbreviations: NIR, nitrite reductase (EC 1.7.7.1); FNR, ferredoxin NADP reductase (EC 1.6.99.4); GRD, glutathione reductase (EC 1.6.4.2).

MATERIALS AND METHODS

Plant Material. Spinach (*Spinacea oleracea* L. cv Wisconsin Dark Green) plants were grown as previously described (15). The photosynthetic period in these experiments was 12 h.

Stroma and Thylakoid Preparation. Chloroplasts from 7 g of deveined leaves were isolated following the methods of Walker (23). Stroma and thylakoid fractions were prepared by osmotic shock in a 1/25 dilution of reconstitution media containing 0.33 M sorbitol, 1 mM EDTA, 10 mM $MgCl_2$, 4 mM KH_2PO_4, 10 mM KCl, and 50 mM Hepes-KOH (pH 7.9). In some experiments 5 mM DTT was included in this media. Following centrifugation at 6000*g*, the supernatant (0.75 ml, 2.7–3.5 mg protein) was desalted on a 0.5 × 20 cm Sephadex[2] G-25 column using reconstitution media for equilibration and elution. The thylakoid fraction was resuspended in reconstitution media, centrifuged, and the pellet (0.57–0.71 mg Chl) resuspended in 0.75 ml of fresh reconstitution media.

NADP and Nitrite Reduction. Reactions were conducted in 1.2 ml of reconstitution media containing 1 mM ADP, 1 mM GSH, 3 mM GSSG, 10 μM ferredoxin, 500 units catalase, and equal volumes of the stroma and thylakoid fractions. Thus, the stroma/thylakoid ratio in the reaction mixture was equivalent to that of the intact chloroplast. The experiments were carried out with a Clark type O_2 electrode at 25°C with a radiant flux density of 3000 $\mu E\ m^{-2}\ s^{-1}$. Nitrite reduction was initiated by the addition of 0.4 mM KNO_2, which was saturating with respect to NIR activity. NADP reduction was initiated, unless otherwise noted, by the addition of 0.4 mM NADP. At various times thereafter aliquots were removed for analysis. Chlorophyll was estimated according to Arnon (2). Spinach ferredoxin was prepared as in Buchanan and Arnon (7). Protein concentration in the stroma and thylakoid fractions was determined by the method of Bradford (6) using ovalbumin as a standard.

Metabolite Assays. Nitrite in 10 μl of sample was mixed with 0.4 ml H_2O, followed by the addition of 0.2 ml of 1% sulfanilamide in 3 M HCl and 0.2 ml of 0.02% *N*-(1-naphthyl)ethylenediamine hydrochloride. Absorbance at 540 nm was read after 10 min. Under these conditions NADPH and GSH present in the sample did not interfere with color formation.

NADP and NADPH were measured in 25 μl aliquots from the reaction mixture as in Robinson and Gibbs (17). GSSG was measured as the decrease in absorbance at 340 nm upon addition of a 25-μl sample to 0.1 mmol NADPH and 3 units glutathione

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

reductase in 1 ml Hepes-KOH (pH 7.6).

Chemicals. Bovine liver catalase (thymol-free), yeast glutathione reductase, and all other biochemicals were obtained from Sigma.

RESULTS AND DISCUSSION

To determine the effect of NADP on nitrite reduction, nitrite reduction by the reconstituted system was allowed to reach steady state, NADP was then added, and the effect of NADP reduction on the rate of nitrite reduction measured. The results from one of several repetitive experiments are displayed in Figure 1. In the absence of NADP, the rate of nitrite reduction was 9.0 μmol mg^{-1} Chl h^{-1}; during NADP reduction this rate dropped to 3.9 μmol mg^{-1} Chl h^{-1}. Following the completion of NADP reduction, nitrite reduction increased to 9.6 μmol mg^{-1} Chl h^{-1}, approximately its initial rate. In seven experiments the average decline in nitrite reduction during concurrent NADP reduction was 60 + 11%. Similar values are observed when glutathione is replaced by DTT in the reconstitution media. When the ferredoxin concentration was increased from 10 to 30 μM, the rate of nitrite reduction declined by 45% upon the addition of NADP (data not shown). These results suggest that the observed inhibition is due to competition for reduced ferredoxin rather than a sequestering of ferredoxin by FNR.

Previous work has shown that the presence of NADPH in the reaction mixture significantly enhances nitrite dependent O_2 evolution (1). Although we found a 40% enhancement of nitrite-dependent O_2 evolution in the presence of NADPH (data not shown), nitrite reduction itself was not significantly enhanced (Fig. 1). The reason for this discrepancy is not clear.

To determine the effect of nitrite on NADP reduction, a steady state rate of NADP reduction was obtained, the system was then perturbed by the addition of nitrite, and the effect on NADP reduction measured. To maintain a constant rate of NADP reduction, NADPH needs to be recycled. To do so we employed the reaction catalyzed by GRD, the formation of reduced glutathione and NADP from NADPH and oxidized glutathione. As the K_m of yeast glutathione reductase for NADPH is low, 8 μm (5), the rate of reaction is proportional to the concentration of

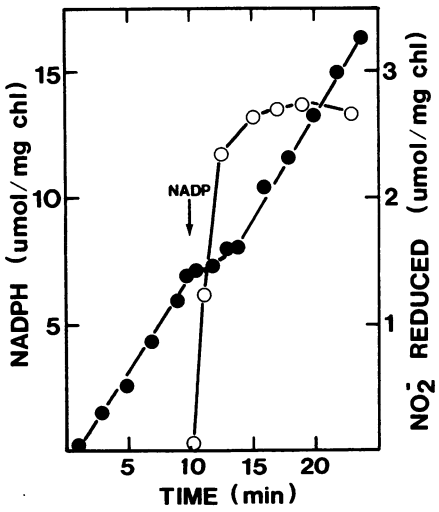

FIG. 1. Effect of NADP reduction (O) on the steady state reduction of NO_2^- (●). Reaction mixtures contained 1 mM ADP, 1 mM GSH, 3 mM GSSG, 3 mM P_i, 0.4 mM NO_2^-, 10 μM ferredoxin, 175 μg stromal protein, and 38 μg Chl. Arrow indicates addition of NADP to a final concentration of 0.4 mM. Rates of NO_2^- reduction before, during, and after the addition of NADP were 9.0, 3.9, and 9.6 μmol mg^{-1} Chl h^{-1}, respectively. The initial rate of NADP reduction (10–13 min) was 260 μmol mg^{-1} Chl h^{-1}.

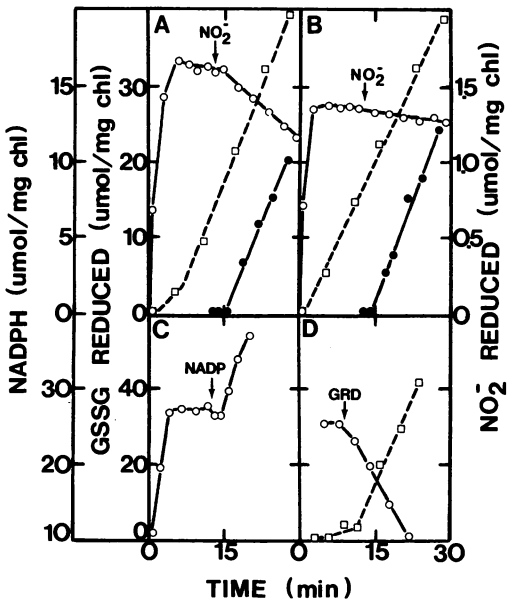

FIG. 2. Effect of NO_2^- reduction (●) on the level of NADPH (O). NADPH was recycled through the addition of (A) 2.4 units mg^{-1} Chl, (B) 2.0 units mg^{-1} Chl, or (D) 4.8 units mg^{-1} Chl glutathione reductase. NADPH utilization was measured as the reduction of GSSG (□). In (C), the rate of NADP reduction was measured after 13 min of illumination with no glutathione reductase present. In (A), rates of nitrite and GSSG reduction were 4.5 and 102 μmol mg^{-1} Chl h^{-1}. The initial rate of NADP reduction was 280 μmol mg^{-1} Chl h^{-1}. Following the addition of NO_2^-, the level of NADPH declined at a rate of 16.5 μmol mg^{-1} Chl h^{-1}. In (B), NO_2^- and GSSG were reduced at rates of 5.2 and 84 μmol mg^{-1} Chl h^{-1}, respectively, while the initial rate of NADP reduction was 270 μmol mg^{-1} Chl h^{-1}. In (C), the initial rate of NADP reduction was 325 μmol mg^{-1} Chl h^{-1} and the rate subsequent to the second addition of NADP was 96 μmol mg^{-1} Chl h^{-1}. In (D), GSSG was reduced at a rate of 190 μmol mg^{-1} Chl h^{-1}, while the level of NADPH declined at a rate of 72 μmol mg^{-1} Chl h^{-1}. In (A) through (D), reaction mixtures contained 1 mM ADP, 1 mM GSH, 3 mM GSSG, 3 mM P_i, 1 mM NADP, and 10 μM ferredoxin. (A) and (B) contained 320 μg stromal protein and 76 μg Chl. (C) and (D) contained 160 μg stromal protein and 38 μg Chl. In (A) and (B), the arrow indicates addition of NO_2^- to a final concentration of 0.4 mM; in (C), the addition of mM NADP to a final concentration of 1.0 mM; and in (D), the addition of GRD.

the enzyme at the levels of NADPH found in our system. By varying the amount of added GRD, the rate of NADPH utilization can be adjusted to match that of NADP reduction and a steady state level of NADPH maintained. For example, in Figure 2C the rate of NADP reduction after 13 min of illumination is 96 μmol mg^{-1} Chl h^{-1}. When additional NADP is added after 40 min of illumination, the rate is 90 μmol mg^{-1} Chl h^{-1} (data not shown). If excess GRD is added at the point at which all NADP is reduced (Fig. 2D), the level of NADPH declines. The rate of NADPH decline (72 μmol mg^{-1} Chl h^{-1}) being approximately equal to the rate of utilization by GRD (190 μmol mg^{-1} Chl h^{-1}) minus the rate of production (96 μmol mg^{-1} Chl h^{-1}). If the amount of added GRD is reduced to match the rate of NADP reduction (Fig. 2A, GRD activity of 102 μmol mg^{-1} Chl h^{-1}), no decline in the level of NADPH is observed prior to the addition of nitrite. When nitrite is added to the poised system (reduction balancing utilization) any competition between FNR and NIR for ferredoxin should be reflected in a decline in the steady state level of NADPH.

The results of typical experiment are shown in Figure 2A. Prior to the addition of nitrite, NADP and NADPH are reduced and oxidized at approximately equivalent rates (100 μmol mg^{-1}

Chl h^{-1}) and so the concentration of NADPH remained constant. Nitrite was then added and, following a lag period of about 3 min, nitrite reduction commenced. At this time a linear decline in NADPH levels occurs. The rate of this decline (16.5 μmol mg^{-1} Chl h^{-1}) is 3.5 times that of nitrite reduction (4.5 μmol mg^{-1} Chl h^{-1}). As nitrite reduction to NH_3 consumes six reducing equivalents and NADP reduction consumes two reducing equivalents, these results suggest that nitrite reduction alone is responsible for the decline in NADPH levels. This was confirmed in other experiments where less glutathione reductase was added, giving the system a slight excess of NADP reduction capacity, sufficient to match that required for nitrite reduction. Here, when nitrite was added, no decline in NADPH levels was observed (Fig. 2B). These data suggest that nitrite toxicity affecting PSII (20) is not a factor in these experiments.

The preparations used in these experiments reduced nitrite at rates of 8 to 14 μmol mg^{-1} Chl h^{-1} in the absence of NADP. Although this rate is lower during concurrent NADP reduction, it is not zero (Figs. 1, 2). Apparently, nitrite reduction can successfully compete for reduced ferredoxin, even at high rates of NADP reduction.

Our results are similar to those of Spiller and Boger (20) and Shin and Oda (19) in that in a reconstituted system, nitrite reduction is partially inhibited by concurrent NADP reduction. In contrast, Hattori (10) using unicellular algae, and Plaut *et al.* (13) working with whole chloroplasts, found that nitrite reduction is enhanced by concurrent CO_2 assimilation. Similarly, in intact plants nitrate fertilization can enhance (16, 18) the rate of photosynthesis. Thus, although competition for ferredoxin by NIR and FNR can be demonstrated in a reconstituted system limited to the reactions catalyzed by these two enzymes, the considerably more complex metabolic environment in the intact chloroplast may introduce additional control mechanisms not evident in a simple system. Excess electron transport capacity, for example, may permit an increased rate of nitrite reduction without a decline in CO_2 assimilation. This possibility is currently under investigation.

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