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Malate and Dihydroxyacetone Phosphate-dependent Nitrate Reduction in Spinach Leaf Protoplasts[1]

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ABSTRACT

Isolated spinach (*Spinacia oleracea* L. var. Bloomsdale) leaf protoplasts reduced nitrate at rates of 9 micromoles per milligram chlorophyll per hour in light with a 3- to 4-fold stimulation in the presence of HCO_3^-. A similar stimulation of nitrate reduction in the absence of CO_2 fixation was obtained by the addition of malate, oxaloacetate (OAA), phospho-3-glyceric acid (PGA), or dihydroxyacetone phosphate (DHAP). Stimulation by malate and DHAP was light-independent, while the PGA and OAA effect was light-dependent. Nitrate reduction was found to be coupled to the cytoplasmic oxidation of DHAP or malate. The PGA/DHAP and OAA/malate shuttle across the chloroplast envelope has been demonstrated to support CO_2 fixation and/or nitrate reduction. The leaf protoplasts readily assimilated nitrate into amino-N in a stoichiometric relationship.

The first step in the assimilatory reduction of nitrate to ammonia is the reduction of nitrate to nitrite catalyzed by nitrate reductase and is the most rate-limiting enzyme in the over-all process (2, 3). The subcellular localization of nitrate reductase in leaves of higher plants has been the subject of considerable recent controversy (3, 14). As originally suggested by Beevers and Hageman (3), nitrate reductase is apparently associated with the outer membrane of the chloroplast envelope (14, 20). Other enzymes of subsequent nitrate assimilation and glutamate synthesis have been shown to be localized in the chloroplasts (3, 14, 20, 21).

Because of the reductive nature of both nitrate and nitrite assimilation, photosynthesis has been proposed to be the primary source of energy for these processes (3). Nitrite reduction by chloroplasts occurs in light and is directly dependent on the photosynthetic electron transport (17). However, isolated chloroplasts could not reduce nitrate (25; Rathnam, unpublished results). This was expected since the chloroplast inner envelope membrane is impermeable to the pyridine nucleotides (9, 10). Therefore, the nitrate reductase localized on the outer envelope membrane has no direct access for the intrachloroplastic reduced pyridine nucleotides, and has to depend on a cytoplasmic source for NADH. Efficient translocator systems, thought to be situated on the inner membranes, have been shown to facilitate an indirect transfer of reducing equivalents across the chloroplast envelope through malate/OAA[2] shuttle (dicarboxylate translocator) and PGA/DHAP shuttle (phosphate translocator) (9, 10, 24).

Some evidence for the generation of reductant from the oxidation of photosynthetic intermediates for nitrate reduction has beeen provided (3, 13, 19). Neyra (16) proposed a coupling between nitrite reduction and malate oxidation in leaves of corn and discussed the significance of such coupling in relation to the predominant formation of malate during C_4 photosynthesis in leaves of corn. Using leaf protoplasts of spinach, in which PGA but not malate is the primary product of photosynthesis, we provide evidence for an efficient interaction of the PGA/DHAP as well as the OAA/malate shuttle systems with nitrate reduction and CO_2 fixation.

MATERIALS AND METHODS

Plant Material. Spinach (*Spinacia oleracea* L. var. Bloomsdale) plants were grown in a naturally illuminated greenhouse on Vermiculite with full strength Hoagland nutrient solution. Young leaves harvested from 2- to 3-week-old spinach plants were used for the isolation of protoplasts.

Isolation of Protoplasts. Leaf sections stripped of their lower epidermis were digested in light for 1 hr in the incubation medium consisting of 0.6 M sorbitol, 20 mM MES, 5 mM $MgCl_2$, 2% (w/v) cellulase Onozuka 4S, and 0.1% (w/v) pectinase (both from Yakult Biochemicals Co., Nishinomiya, Japan) at pH 5.5. Protoplasts were obtained by sequential filtration followed by separation at the interface of an aqueous dextran-polyethylene glycol two-phase system (12). The protoplasts were suspended in a medium containing 0.3 M sorbitol, 50 mM HEPES at pH 7.6, 1 mM $MgCl_2$, 0.1 mM KH_2PO_4, and 3 mM Na-isoascorbate.

Assay Medium. The kinetic experiments for nitrate reduction, and for $^{14}CO_2$ fixation and reduction at the same time were carried out using a standard reaction mixture consisting of 0.3 M sorbitol, 50 mM Tricine-NaOH at pH 8.1, 0.75 mM $MgCl_2$, 0.1 mM KH_2PO_4, and 3 mM Na-isoascorbate in a final volume of 0.2 ml. Other additions were as indicated for individual experiments. The reactions were run at 25 C and the light intensity at the surface of the reaction vials, unless otherwise specified, was 6,000 ft-c.

$^{14}CO_2$ Fixation. Aliquots of spinach protoplasts (40 μl/vial to give 20–24 μg Chl/vial) were added to the vials and after 3 min of preillumination, the reactions were started by the addition of a solution of $NaH^{14}CO_3$ to give a final concentration of 6 mM. Periodically, 50-μl samples were removed from the vials and were immediately injected into scintillation vials containing 50 μl of 20% (w/v) trichloroacetic acid. The vials were flushed with an air stream to assure the complete removal of free $^{14}CO_2$ and the amount of ^{14}C incorporated into acid-stable products was determined by scintillation spectroscopy (22, 24).

Nitrate Reduction. The reaction vials containing the assay medium, protoplasts and additives were preilluminated for 3 min, and the reactions were initiated by the addition of K-nitrate solutions to give a final concentration of 1 mM. The rates of nitrate reduction were determined by measuring the disappearance of nitrate from the medium generally over a 5- to 10-min period. Samples (50 μl) were removed at 5-min intervals and analyzed immediately for nitrate. A sample was always removed from the reaction mixtures within 5 sec after the addition of nitrate and

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[2] Abbreviations: DHAP: dihydroxyacetone phosphate; Gld: D,L-glyceraldehyde; α-Kg: α-ketoglutarate; OAA: oxaloacetate; PGA: phospho-3-glyceric acid.

was considered as containing the initial concentration of nitrate in the reaction mixture.

Nitrate Determination. The amount of nitrate remaining in the reaction samples was estimated according to Cataldo *et al.* (4). The reaction samples were mixed thoroughly with 0.8 ml of 5% (w/v) salicyclic acid in concentrated H_2SO_4. After 20 min at room temperature, 19 ml of 2 N NaOH were added, cooled under running water, and the *A* was read at 410 nm.

Amino-N Determination. The measurements were made with the same reaction mixture that was used to follow nitrate reduction. At each sampling time, two aliquots (50 μl each) were removed simultaneously; one was used for nitrate determination (4), and the other for amino-N measurement. For the estimation of amino acid α-N, 50 μl of the reaction samples were mixed thoroughly with 1 ml of 10% (v/v) aqueous pyridine and 1 ml of 2% (w/v) aqueous ninhydrin. The test tubes were stoppered and placed in a boiling water bath for 20 min, cooled under running water, and the *A* was read at 570 nm (8). L-Isoleucine was used as a standard.

Chl. Samples from the original preparations of protoplasts were taken for Chl measurement. The protoplasts were incubated overnight with 96% ethanol at 4 C. Chl was determined using the extinction coefficients of Wintermans and De Mots (27).

Preliminary assays indicated linear responses with varying Chl concentration (up to 60 μg) and time over a 30-min period. Preliminary experiments (22) have also indicated that the plasmalemma of the isolated protoplasts is not a significant barrier for the uptake of added metabolites in that both isolated intact chloroplasts and protoplasts exhibited similar rates of substrate-dependent O_2 evolution in light. The rates of O_2 evolution (μmol/mg Chl·hr) were: 220 to 245 (HCO_3^--dependent), 210 to 240 (PGA-dependent), and 140 to 164 (OAA-dependent).

The data presented, unless otherwise stated, are from single experiments which generally were representative of several (4–6) such measurements. For the purpose of comparison, each set of experiments, constituting the data presented in each of the tables and figures, was carried with a single protoplast preparation, and repeated twice the same day.

RESULTS AND DISCUSSION

The spinach protoplasts used in the present study fixed $^{14}CO_2$ (in the absence of nitrate) with rates up to 243 μmol/mg Chl·hr. The protoplasts also reduced added nitrate in light with a 3-fold stimulation in the presence of HCO_3^- (Table I). The HCO_3^--stimulated rates of nitrate reduction were 28 to 32 μmol/mg Chl·hr. There was also a concomitant increase in the rate of CO_2 fixation in the presence of nitrate, the increased rate being almost equal to the sum of the rates of CO_2 fixation observed in the absence of nitrate and the rates of nitrate reduction in the presence of HCO_3^-.

The observed 3-fold increase in the rate of nitrate reduction by actively photosynthesizing protoplasts indicates the dependence of nitrate reductase activity on certain photosynthetic intermediates such as DHAP for reductive power. Even though the rate of nitrate reduction was only one-tenth of the observed rates of CO_2 fixation, a continued shuttling of DHAP/PGA across the chloroplasts to sustain the reduction of nitrate would result in a corresponding decrease in net photosynthesis. The Calvin cycle is an autocatalytic pathway (27), and a constant nonavailability of a portion of DHAP, an intermediate of the cycle, would either upset this autocatalysis or might delay attainment of steady-state by a short time interval. To offset this imbalance the chloroplasts would have to fix CO_2 at a higher rate, which was indeed the case (Table I); or some other cellular process should provide the NADH required for nitrate reduction. The ability of actively photosynthesizing protoplasts to support high rates of nitrate reduction, in the presence of malate and OAA, but without a net increase in the endogenous rates of CO_2 fixation (Table I) indicates a coupling between nitrate reduction and the oxidation of malate to OAA.

Figure 1 shows the effect of nitrate on $^{14}CO_2$ fixation by spinach protoplasts as a function of light intensity. In the absence of added nitrate, the rate of $^{14}CO_2$ fixation was linear up to 3,000 ft-c, and gradually leveled off with complete saturation around 5,000 ft-c. In the presence of nitrate, the rate of $^{14}CO_2$ fixation appeared to resemble an S-shaped hyperbola and reaching saturation at a relatively higher light intensity of 6,000 ft-c. Below 3,000 ft-c, the rate of $^{14}CO_2$ fixation in the presence of nitrate was lower than the control; but above 4,000 ft-c the rates were higher than the control. The exact nature of this bifunctional effect of nitrate on CO_2 fixation is not known at the present time, but it might be due to a competition for light energy at low light intensities, while stimulating the autocatalysis at high light intensity.

Interrelationships between CO_2 fixation, photosynthetic electron transport, DHAP/PGA shuttle, malate/OAA shuttle, and nitrate reduction were further examined by making use of DCMU and D,L-glyceraldehyde (1). Both glyceraldehyde and DCMU inhibited CO_2 fixation as well as the associated nitrate reduction (Tables II and III). In the glyceraldehyde-treated protoplasts exogenously added PGA and DHAP readily supported nitrate reduction in the light with the rates being comparable to those obtained with the actively CO_2-fixing protoplasts (Table II). The effect of PGA was light-dependent and that of DHAP was light-independent. DHAP also supported nitrate reduction by the DCMU-poisoned protoplasts in both light and dark, while PGA

Table I. Effect of Nitrate on $^{14}CO_2$ Fixation by Spinach Leaf Protoplasts in Light.
Two aliquots were taken simultaneously at each sampling time. One aliquot was treated for $^{14}CO_2$ fixation and the other for nitrate determination as described in MATERIALS AND METHODS. Rates were calculated from the linear phase of $^{14}CO_2$ fixation and nitrate reduction which lasted for more than 10 min. Substrate concentrations were: 6 mM $NaH^{14}CO_3$, 1 mM KNO_3, 10 mM L-malate and 10 mM OAA[1].

Conditions	$^{14}CO_2$ Fixation	Nitrate Reduction
	μmol/mg Chl. hr^{-1}	
NO_3^-	-	9±1.5[2]
$H^{14}CO_3^-$	221±3.1	-
$H^{14}CO_3^-$ + NO_3^-	259±2.4	29±2.4
$H^{14}CO_3^-$ + malate	222±2.5	-
$H^{14}CO_3^-$ + OAA	223±1.4	-
$H^{14}CO_3^-$ + NO_3^- + malate	224±1.9	27±1.8
$H^{14}CO_3^-$ + NO_3^- + OAA	222±2.2	28±2.1

[1] OAA solutions were prepared, just prior to use, as previously described (24).

[2] Results are means (± standard deviation) of eight assays with four preparations on four different days.

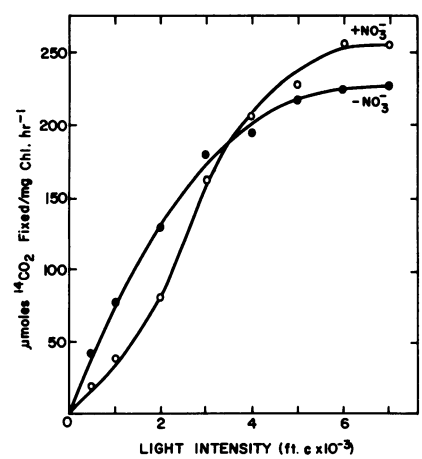

FIG. 1. Effect of nitrate on $^{14}CO_2$ fixation by spinach leaf protoplasts as a function of light intensity.

was ineffective (Table II). It is suggested that the cytoplasmic oxidation of DHAP is coupled to the reduction of nitrate with a stoichiometric balancing of pyridine nucleotide reduction-oxidation. As there is no net energy consumption this process would thus proceed in a light-independent manner. The inability of added PGA to support nitrate reduction by the protoplasts in the dark and in the presence of DCMU would be due to a lack of photochemically generated ATP and NADPH. These results (Table II) confirm that nitrate reduction is coupled to the cytoplasmic oxidation of DHAP, and the effect of PGA is through DHAP.

In isolated chloroplasts, Kaiser and Urbach (11) have shown that DHAP supports CO_2 fixation in the DCMU-treated chloroplasts. Recent evidence from protoplast studies (24) indicated that high concentration of OAA (~10 mM) prevents the entry of DHAP (when present at low concentrations, ~0.25 mM) into the chloroplasts from the medium. The present study with isolated protoplasts provides further evidence that DHAP (0.25 mM), in the presence of DCMU, would support cytoplasmic nitrate reduction but not chloroplastic CO_2 fixation (Table II). However, at a 10-fold higher concentration (2.5 mM), DHAP effectively supported both nitrate reduction and CO_2 fixation, *i.e.* a breakdown of the Calvin cycle at the PGA reduction step by DCMU will be overcome by the import of DHAP, when present in excess (24).

Like the DHAP/PGA shuttle, a shuttle involving malate and OAA has also been proposed for the indirect transfer of reducing equivalents across the chloroplasts (9, 10, 23, 24). Since the extrachloroplastic reduction of OAA (24) and nitrate (Table II) is dependent on cytoplasmic DHAP oxidation, experiments were designed to elucidate the dependence of nitrate reduction on malate/OAA shuttle and DHAP oxidation (Table III). In the glyceraldehyde-treated protoplasts, added malate and OAA readily supported nitrate reduction in the light. The effect of OAA was light-dependent and that of malate was light-independent. This indicates that the reduction of added OAA occurs in the chloroplasts and the malate thus formed is transported across the chloroplasts, and upon oxidation the NADH generated would support nitrate reduction. In this system cytoplasmic reduction of OAA is not proposed as it would compete with nitrate reductase for cytoplasmic NADH. The chloroplastic reduction of OAA to malate in the presence of DCMU is restricted due to a lack of photochemical generation of NADPH. In consequence, nitrate was not reduced (Table III). Therefore, it is proposed that the effect of OAA in supporting nitrate reduction is not direct but it is through malate.

Table II. Effect of PGA and DHAP on Nitrate Reduction by Spinach Leaf Protoplasts in the Presence of D,L-Glyceraldehyde and DCMU in Light.

Experimental conditions and procedures were the same as described for Table I. Substrate concentrations were: 6 mM $NaH^{14}CO_3$, 1 mM KNO_3, 2.5 mM PGA, 25 mM D,L-glyceraldehyde (Gld), and 1 μM DCMU and DHAP (as indicated)[1]. Data are means of four separate sets of experiments. Values in parentheses indicate the rate of nitrate reduction in the dark.

Conditions	$^{14}CO_2$ Fixation[2]	Nitrate Reduction
	μmol/mg Chl. hr^{-1}	
$H^{14}CO_3^- + NO_3^-$	275	32
$H^{14}CO_3^- + NO_3^-$ + Gld	0	0
$H^{14}CO_3^- + NO_3^-$ + Gld + PGA	0	26 (0)
$H^{14}CO_3^- + NO_3^-$ + Gld + DHAP (0.25 mM)	0	25 (27)
$H^{14}CO_3^- + NO_3^-$ + DCMU	0	0
$H^{14}CO_3^- + NO_3^-$ + DCMU + PGA	0	0 (0)
$H^{14}CO_3^- + NO_3^-$ + DCMU + DHAP (0.25 mM)	0	24 (27)
$H^{14}CO_3^- + NO_3^-$ + DCMU + DHAP (2.5 mM)	285	26 (29)

[1] DHAP was prepared from dihydroxyphosphate dimethylketal (Sigma Chemical Co.) according to the procedure advised by the manufacturer.

[2] The rate of $^{14}CO_2$ fixation, in the absence of nitrate, was 243 μmol/mg Chl hr^{-1}.

Table III. Effect of OAA, Malate and DHAP on $^{14}CO_2$ Fixation and Nitrate Reduction by Spinach Leaf Protoplasts in the Presence of D,L-Glyceraldehyde and DCMU in Light.

Experimental conditions and procedures were the same as described for Table I. Substrate concentrations used were: 6 mM $NaH^{14}CO_3$, 1 mM KNO_3, 25 mM D,L-glyceraldehyde (Gld), 1 μM DCMU, 10 mM OAA, 10 mM L-malate and 0.25 mM DHAP. Data are means of five separate sets of experiments.

Conditions	$^{14}CO_2$ Fixation	Nitrate Reduction
	μmol/mg Chl. hr^{-1}	
$H^{14}CO_3^- + NO_3^-$	256	31
$H^{14}CO_3^- + NO_3^-$ + Gld	0	0
$H^{14}CO_3^- + NO_3^-$ + Gld + OAA	0	22 (0)[1]
$H^{14}CO_3^- + NO_3^-$ + DCMU	0	0
$H^{14}CO_3^- + NO_3^-$ + DCMU + OAA	0	0
$H^{14}CO_3^- + NO_3^-$ + DCMU + OAA + DHAP	75	0
$H^{14}CO_3^- + NO_3^-$ + Gld + malate	0	28 (20)
$H^{14}CO_3^- + NO_3^-$ + DCMU + malate	76	0
$H^{14}CO_3^-$ + DCMU + malate + DHAP	76	-
$H^{14}CO_3^- + NO_3^-$ + DCMU + malate + DHAP	70	19

[1] Values in parentheses indicate the rate of nitrate reduction in the dark.

Although added DHAP (0.25 mM) has been shown to support nitrate reduction in the DCMU-treated protoplasts (Table II), the addition of OAA together with DHAP did not support nitrate reduction, but surprisingly supported CO_2 fixation (Table III). It is proposed that the site of OAA reduction, in the presence of DCMU and DHAP, shifts from inside of the chloroplasts to the cytoplasm and the resulting malate would support CO_2 fixation. The absence of nitrate reduction under these conditions needs further investigation.

Exogenously added malate partly restored DCMU-inhibited CO_2 fixation. This supports our earlier observation (23, 24) that under conditions of lack of photochemical generation of NADPH, malate oxidation occurs in the chloroplasts and the NADH generated would be utilized for 1,3-diPGA reduction. The absence of a cytoplasmic malate oxidation to support nitrate reduction is intriguing and needs additional experimentation.

In the absence of nitrate, added malate and DHAP effectively supported CO_2 fixation by the DCMU-treated protoplasts (Table III). In the presence of nitrate, however, malate and DHAP, when added together, readily supported both CO_2 fixation and nitrate reduction by the DCMU-treated protoplasts. Under these conditions, DHAP might support both nitrate and OAA reduction and/or CO_2 fixation; and malate might support both CO_2 fixation and/or nitrate reduction. These experiments, however, do not distinguish between the specific effects of DHAP and malate in supporting CO_2 fixation and nitrate reduction.

Although chloroplasts assimilate nitrite and synthesize amino-N (14, 15), any estimation of the *in vivo* potential for amino-N synthesis should come from a reaction sequence comprising nitrate reduction also, since nitrate reductase is the most rate limiting enzyme of the N-assimilation pathway (2, 3). It was of interest to study amino-N synthesis in our preparations as they exhibited highest rates of nitrate reduction recorded ever to date (2, 3, 13, 16, 19). Although Plaut *et al.* (18) have recorded nitrite reduction at rates up to 40 μmol/mg Chl·hr with chloroplast preparations, their system does not represent the *in vivo* potential as it was not coupled with nitrate reductase. Figure 2 shows a concomitant synthesis of amino-N during nitrate reduction by photosynthesizing spinach protoplasts in light. The protoplasts exhibited a slow but steady increase in amino-N in the absence of added nitrate. The addition of nitrate to the system caused a severalfold increase in the amino-N production over the control during the 30-min experimental period. The addition of α-Kg (1 mM) to the system had a remarkable effect on the rate of nitrate assimilation as a function of time. In the first 10 min of assay, both the loss of nitrate and amino-N synthesis proceeded in an almost linear fashion and were independent of added α-Kg. In the absence of α-Kg, these rates leveled off within 15 to 20 min. When α-Kg was present, a linear increase in the rate of nitrate loss and amino N-synthesis was recorded up to 30 min. Net losses of nitrate were 7.5

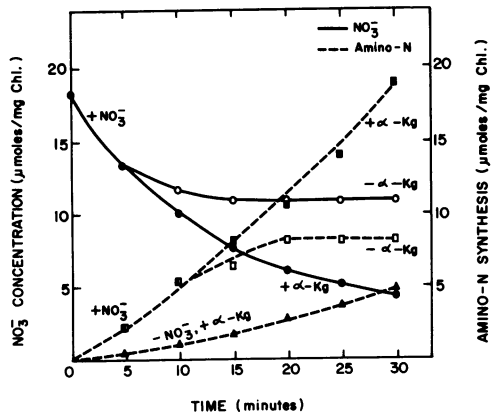

FIG. 2. Time course of nitrate loss from the medium and amino-N synthesis by spinach leaf protoplasts in light.

and 14 μmol/30 min·mg of Chl and net increases in amino-N were 8.25 and 19 μmol/30 min·mg of Chl, respectively, in the absence or presence of α-Kg, and the respective stoichiometric ratios between amino-N and nitrate loss, after subtracting the control values, were 0.43 and 1.0. This indicates that the reduction of nitrate ultimately resulted in the synthesis of amino-N in the presence of α-Kg in an almost stoichiometric fashion in photosynthesizing protoplasts.

The synthesis of amino-N, even in the absence of added α-Kg, could be attributed to the endogenous pools of α-Kg and other keto acids present in the chloroplasts that could be aminated. However, addition of α-Kg becomes necessary to maintain high rates of amino-N beyond 10 to 15 min as the endogenous keto acid pool gets completely utilized in that period. The presence of isocitric dehydrogenase in the chloroplast (5) suggests that the chloroplasts may generate at least part of the α-Kg required during the chloroplastic amino-N synthesis. We also believe that the chloroplasts are the major site of N-assimilation as they were shown earlier to contain the necessary enzyme compliment (3, 14, 20, 21). Isolated chloroplasts were also shown to photoreduce α-Kg to glutamate (7), and convert glutamate to glutamine (6). These data suggest that isolated protoplasts are capable of reducing nitrate and synthesizing amino-N *de novo*, when supplied with nitrate, α-Kg, bicarbonate, and light.

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