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Reduction of Nitrate via a Dicarboxylate Shuttle in a Reconstituted System of Supernatant and Mitochondria from Spinach Leaves[1]

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ABSTRACT

Substantial rates of nitrate reduction could be achieved with a reconstituted system from spinach leaves containing supernatant, mitochondria, NAD^+, oxaloacetate (OAA), and an oxidizable substrate. Appropriate substrates were glycine, pyruvate, citrate, isocitrate, fumarate, or glutamate. The reduction of NO_3^- with any of the substrates could be inhibited by *n*-butyl malonate, showing that the transfer of reducing power from the mitochondria to the supernatant involved the malate exchange carrier. The addition of ADP to the reconstituted system decreased NO_3^- reduction and this decrease could be reversed by the addition of rotenone or antimycin A. The operation of the OAA/malate shuttle was achieved most quickly in the system when low concentrations (≤0.1 millimolar) of OAA were added. A corresponding increase in the lag time for the operation of the OAA/malate shuttle was observed when the OAA concentration was increased. Concentrations for half-maximal activity of OAA, glycine, NAD^+, and NO_3^- in the reconstituted system were 42 micromolar, 0.5 millimolar, 0.25 millimolar, and 26 micromolar, respectively. The transfer of reducing power from the mitochondria to the soluble phase via the OAA/malate shuttle can not only provide NADH for cytoplasmic reduction but can also sustain oxidation of tricarboxylic cycle acids and the generation of α-ketoglutarate independently of the respiratory electron transport chain.

Nitrate reduction in leaves is light-dependent and ceases when the light is extinguished (6). Nitrate reduction in the dark, however, can be promoted by anaerobic conditions (1, 7) or by inhibiting the respiratory electron transport chain with CO (19) or antimycin A (7). The promotion of NO_3^- reduction in the dark has been attributed, in part, to the availability of cytoplasmic NADH when the external NADH dehydrogenase of the mitochondria is inhibited (7). The promotion of NO_3^- reduction in the light presumably also occurs because the respiratory electron transport chain is inhibited, but in this case the inhibition is thought to be due to the high ATP/ADP ratio that prevails in leaf cells in the light (11, 20).

The inhibition of the respiratory electron transport chain could be expected to result in an inhibition of tricarboxylic acid oxidation by the mitochondria unless there was an alternate mechanism for the reoxidation of the NADH that is produced. Woo and Osmond (23, 24) have proposed that with mitochondria oxidizing glycine, the mitochondrial NADH can be reoxidized using an OAA[3]/malate shuttle with a transfer of the malate to the external medium. The capacity of this shuttle system to provide NADH for NO_3^- reduction has been investigated using a soluble extract containing malate dehydrogenase and nitrate reductase combined with mitochondria capable of oxidizing organic acids or glycine. The results show that the OAA/malate shuttle is an effective means of reoxidizing NADH in the mitochondria, thereby allowing organic acid oxidation to proceed even though the respiratory electron transport chain may be inoperative.

MATERIALS AND METHODS

A supernatant extract was prepared from spinach leaves by grinding them in a medium containing 0.1 M Hepes-NaOH (pH 7.5), 5 mM DTT, at 0 C. The homogenate was filtered through cheesecloth and the filtrate centrifuged at 30,000*g* for 30 min. The supernatant was passed through a Sephadex G-25 column (1.5 × 11 cm). Nitrate reductase eluted in the void volume and this fraction was used for experiments.

Mitochondria were prepared from spinach leaves as previously described (23).

The standard assay for nitrate reduction in the reconstituted system contained 0.4 M sucrose, 0.1 M Hepes (pH 7.2), 5 mM K-phosphate, 5 mM $MgCl_2$, 1 mM DTT, 2 mM NAD^+, 5 mM glycine, 0.1 mM OAA supernatant, and mitochondria in a total volume of 1 ml. Alterations to the standard assay are as shown in the text. Assays were incubated at 25 C and NO_2^- was determined at 540 nm (12) after 40 min or at times indicated.

Protein was determined by Bio-Rad assay (2). Mitochondrial protein was corrected for thylakoid contamination (10). Activities are expressed on the protein content of the supernatant.

RESULTS

Mitochondrial addition alone, or the addition of oxidizable substrate without mitochondria to the supernatant fraction resulted in little production of NO_2^- (Table I) except for glutamate (Table I) or malate (235 nmol NO_2^- mg^{-1} protein h^{-1}). With mitochondria present, substantial NO_3^- reduction was observed on the addition of glycine, pyruvate, citrate, isocitrate, glutamate, or fumarate (Table I). After a lag of varying time the reduction of NO_3^- was linear for 50 min with the first five substrates mentioned above (Fig. 1). With all substrates, the reduction of NO_3^- was inhibited by the addition of *n*-butyl malonate (Table I), suggesting that the supply of reducing power for nitrate reduction involved the malate exchange carrier (17). Succinate α-ketoglutarate and aspartate were unable to support NO_3^- reduction (Table I).

The addition of ADP to the reconstituted system decreased the reduction of NO_3^-, presumably by stimulating NADH reoxidation

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[3] Abbreviation: OAA: oxaloacetate.

through the respiratory electron transport chain (Table II). The inhibition of the electron transport chain by rotenone or antimycin A partially or fully restored NO_3^- reduction (Table II).

Fluorocitrate, an inhibitor of aconitase (5) inhibited citrate supported NO_3^- reduction (Table III), and α-cyano 4-hydroxycinnamic acid, an inhibitor of the pyruvate exchange carrier (9), inhibited the NO_3^- reduction observed from the oxidation of pyruvate and fumarate (Table III).

The reduction of NO_3^- in the soluble phase by the transfer of reducing power from the mitochondria via the OAA/malate shuttle was extremely dependent on the OAA concentration (Fig. 2). Low concentrations of OAA stimulated NO_3^- reduction within 15 min (Fig. 2) but higher concentrations of OAA did not support NO_3^- reduction until after a lag period (Fig. 2) that was linearly related to the initial OAA concentration (Fig. 3). The longer lag period at higher OAA concentrations is presumably related to the longer time required for malate to accumulate sufficiently to drive the equilibrium toward NADH production and consequently leads to NO_3^- reduction.

The relationship between initial OAA concentration and the operation of the OAA/malate shuttle is shown (Fig. 4) where the rate of reduction of OAA has been varied by varying the glycine concentration. Without glycine or at 0.2 and 0.5 mM glycine, increased OAA concentrations inhibited the reduction of NO_3^-. As the concentration of glycine and presumably its rate of oxidation was increased, a balance between OAA and malate was more quickly achieved and the OAA/malate shuttle could then operate. Only small concentrations of OAA were required for the effective operation of the OAA/malate shuttle (Fig. 5). The apparent K_m for OAA was 42 μM with a V_{max} of 250 nmol mg^{-1} protein h^{-1}.

The apparent K_m for glycine was 0.5 mM with a V_{max} of 263 nmol mg^{-1} protein h^{-1} (Fig. 6). The apparent K_m for NAD^+ was

Table I. *Effect of Metabolites on NO_3^- Reduction in the Presence of OAA*

All metabolite additions were 2 mM; OAA and butyl malonate were 0.1 mM and 10 mM, respectively. Supernatant and mitochondria containing 198 and 109 μg protein, respectively, were used in assays.

Metabolite	NO_2^- Formed				
	− mito (A)	mito (B)	mito + butyl malonate (C)	(B − A)	(C − A)
	nmol/h·mg protein				
None	4.7	13.9	11.9	9.2	7.2
Glycine	3.3	204.2	69.6	200.9	66.3
Pyruvate	3.6	72.3	25.9	68.7	22.3
Citrate	10.6	149.2	31.3	138.6	20.7
Isocitrate	15.9	296.1	171.1	280.2	155.2
α-Ketoglutarate	5.0	23.8	15.9	18.8	10.9
Succinate	4.0	13.9	10.6	9.9	6.6
Fumarate	12.6	72.6	35.8	60.0	23.2
Glutamate	59.7	131.3	83.5	71.6	23.8
Aspartate	3.2	13.3	8.6	10.0	5.3

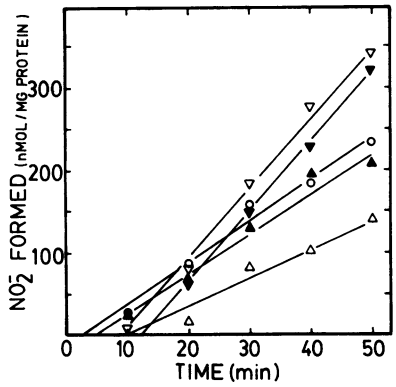

FIG. 1. Time course of NO_3^- reduction in a reconstituted system from spinach leaves in the presence of 0.1 mM OAA with various metabolites. (○): Glycine; (△): pyruvate; (▲): glutamate; (▽): isocitrate; (▼): citrate. Additions of metabolites were 2 mM. Supernatant and mitochondria used contained 183 and 97 μg protein, respectively.

Table II. *Effect of ADP and Respiratory Inhibitors on NO_3^- Reduction in the Reconstituted System with OAA in the Presence of Organic Acids*

Additions were 5 mM for pyruvate, citrate, and isocitrate, 2 mM for ADP, 1 μM for rotenone and antimycin A, and 0.1 mM OAA. Supernatant and mitochondria contained 185 and 59 μg protein, respectively.

Treatment	NO_2^- Formed			
	No addition	+ ADP	+ ADP rotenone	+ ADP Antimycin A
	nmol/h·mg protein			
Control[a]	21	11	9	17
+ Pyruvate	147	125	149	—
+ Citrate	306	228	292	278
+ Isocitrate	500	366	394	432

[a] No glycine was present in the control assay, otherwise conditions were as described.

Table III. *Effect of α-Cyano 4-Hydroxycinnamic Acid (CHCA) and Fluorocitrate on NO_3^- Reduction in the Reconstituted System in the Presence of OAA*

All additions were 2 mM. The concentrations of CHCA, fluorocitrate, and OAA were 10, 5, μM and 0.1 mM, respectively. Supernatant and mitochondria used contained 180 and 105 μg protein, respectively.

Addition	NO_2^- Formed			
	− Mito	Mito	Mito + CHCA	Mito + fluorocitrate
	nmol/h·mg protein			
None	4	15	10	32
Glycine	4	189	197	155
Isocitrate	10	217	233	228
Citrate	8	181	153	95
Pyruvate	4	130	60	119
Fumarate	8	64	38	67
Glutamate	31	174	163	151

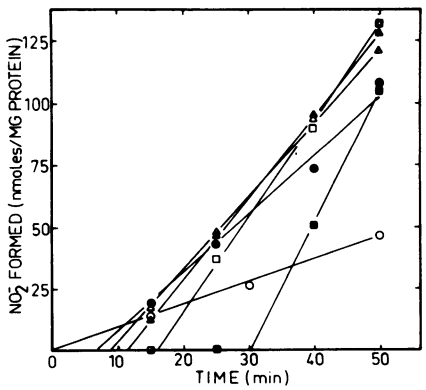

FIG. 2. Time course of NO_3^- reduction in the reconstituted system at various OAA concentrations. (○): 0 μM OAA; (●): 20 μM OAA; (△): 50 μM OAA; (▲): 100 μM OAA; (□): 200 μM OAA; (■): 500 μM OAA. Supernatant and mitochondria used contained 188 and 119 μg protein, respectively.

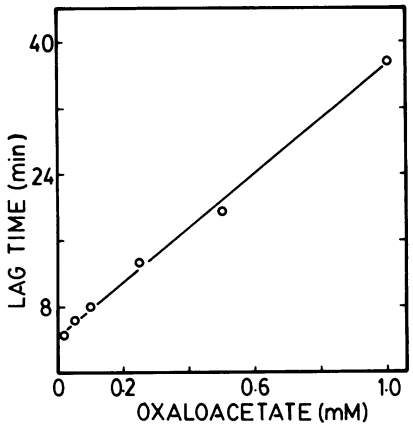

FIG. 3. Relationship between lag time and OAA concentration during NO_3^- reduction in the reconstituted system. Assays were carried out at 0, 15, 30, 40, and 50 min for each OAA concentration. Supernatant and mitochondria used contained 190 and 122 μg protein, respectively.

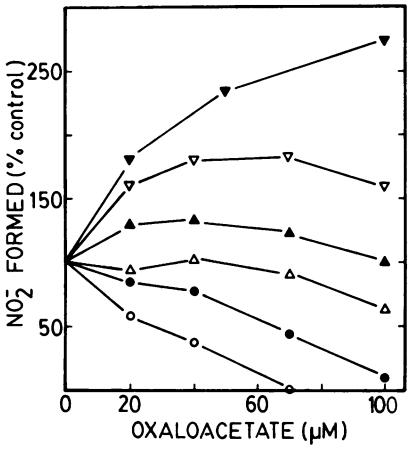

FIG. 4. Relationship between OAA and glycine concentrations on NO_3^- reduction in the reconstituted system. (○): No glycine; (●): 0.2 mM glycine; (△): 0.5 mM glycine; (▲): 1 mM glycine; (▽): 2 mM glycine; (▼): 5 mM glycine. Control rate in the absence of glycine and OAA was 115 nmol NO_2^- formed μg supernatant protein·h.

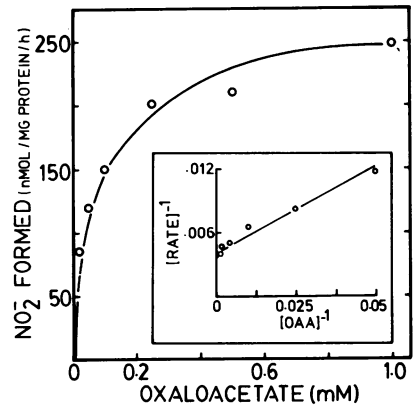

FIG. 5. Effect of OAA on NO_3^- reduction in the reconstituted system. Assays were carried out as in Figure 3.

0.25 mM with a V_{max} of 343 nmol mg^{-1} protein h^{-1} (Fig. 7). The apparent K_m for NO_3^- in the reconstituted system was 26 μM and the V_{max} was 192 nmol mg^{-1} protein h^{-1} (Fig. 8). These kinetic constants indicate that the system operates with reasonable concentrations of metabolites.

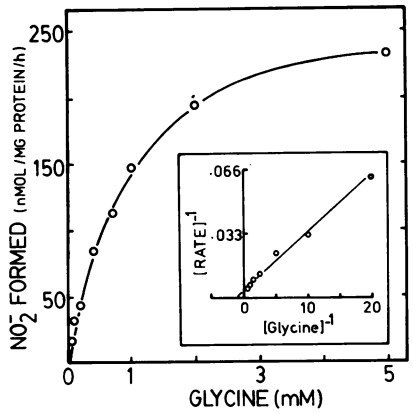

FIG. 6. Effect of glycine on NO_3^- reduction in the reconstituted system. Supernatant and mitochondria used contained 207 and 108 μg protein, respectively.

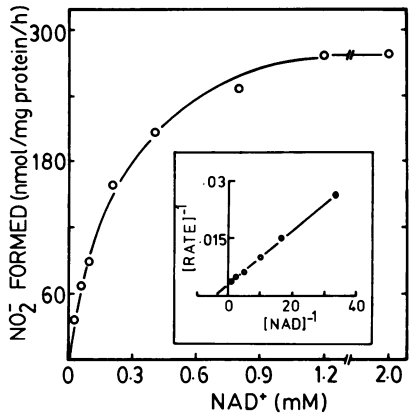

FIG. 7. Effect of NAD^+ on NO_3^- reduction in the reconstituted system. Supernatant and mitochondria used contained 207 and 108 μg protein, respectively.

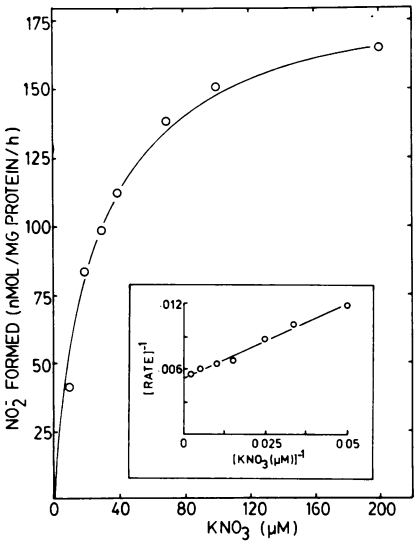

FIG. 8. Effect of NO_3^- on NO_3^- reduction in the reconstituted system. Supernatant and mitochondria used contained 168 and 97 μg protein, respectively.

DISCUSSION

Several papers (8, 14) suggest that carbon traffic through the tricarboxylic acid pathway continues in the light, whereas other

evidence on O_2 transients (16) and ATP/ADP ratios (11, 13) suggests that the respiratory electron transport chain is inhibited. The continued oxidation of organic acids in the mitochondria without the ability to reoxidize the NADH through the electron transport chain would be possible only if an alternate method of regenerating NAD^+ were operative. An alternate method of regenerating NAD^+ in the mitochondria has been described by Woo and Osmond (23, 24). OAA reduction to malate in the mitochondria regenerates NAD^+, the malate is transferred to the cytoplasm via the dicarboxylate exchange carriers and there oxidized to OAA with the generation of NADH in the cytoplasm, the return of OAA to the mitochondria completes the cycle.

The continued operation of this method of regenerating NAD^+ in the mitochondria requires malate dehydrogenase in both the mitochondria and cytoplasm (18) and a means of reoxidizing the NADH produced in the cytoplasm. One method of reoxidizing the NADH *in vivo* would be the reduction of NO_3^- (12) and we have used this method in the reconstituted system to determine the degree to which the OAA/malate shuttle would operate in such a system.

Our data suggest that when conditions favorable to NADH production had been attained in the reconstituted system, presumably mediated at optimal NAD^+/NADH and/or OAA/malate ratios, the OAA/malate shuttle was an effective mechanism for the transfer of reducing power from the mitochondria to the soluble phase. At 0.5 mM OAA the rate of NO_3^- reduction by the OAA/malate shuttle in the reconstituted system was 63% of the *in vitro* nitrate reductase activity in the supernatant (unpublished data). In the presence of an oxidizable substrate for the mitochondria and a reducible substrate for the supernatant, the optimal condition for NADH production and NO_3^- reduction was achieved by the system itself, showing that in a closed system such as a cell the system could be self-regulating. The transfer of the reducing power via the shuttle could supply NADH for the use of cytoplasmic reactions such as NO_3^- reduction and also would allow the oxidation of acids to proceed in the mitochondria when the respiratory electron transport chain was inhibited in the light by a high ATP/ADP ratio (11, 13). Under these circumstances only NAD^+-linked oxidations would proceed and the tricarboxylic acid cycle would presumably be blocked, in the absence of electron flow, at succinate whose oxidation is linked directly to the electron transport chain. Succinate was not an effective substrate in the reconstituted system (Table I), nor was α-ketoglutarate whose oxidation was presumably blocked by the lack of CoA or GDP. The partial operation of the cycle, however, would presumably allow for the production of α-ketoglutarate (which would be required for the assimilation of the ammonia produced from NO_3^- reduction) directly from organic acids in a modified tricarboxylic acid cycle (15) and/or from OAA generated by dark CO_2 fixation (22).

In the present studies glycine was routinely used as an oxidizable substrate in the reconstituted system as the glycine-decarboxylating system is located in the mitochondria (21, 24). In this system the regeneration of NAD^+ for the oxidation of glycine was linked to NO_3^- reduction but *in vivo* it could be entirely linked to the reduction of hydroxypyruvate to glycerate (23) in the peroxisome involving a dicarboxylate shuttle and the malate dehydrogenase isozyme present in the peroxisome (18). The glycine decarboxylation that occurs in mitochondria in the light is probably linked to the dicarboxylate shuttle (23, 24) and not to the respiratory electron transport chain. It can, of course, be linked to the electron transport chain with concurrent phosphorylation in isolated mitochondria (3, 4) where the energy charge inhibition of the electron transport chain is removed.

If the NADH from glycine decarboxylation is utilized via dicarboxylate shuttles for the reduction of hydroxypyruvate (23), the oxidation of glycine in the light would not interfere with the oxidation of other tricarboxylic cycle acids, nor would it supply any net NADH production for other cytoplasmic reactions. Thus, if NO_3^- reduction utilized NADH from the mitochondria via the dicarboxylate shuttle, the reduction of NO_3^- would, to some extent, be linked to the oxidation of organic acids and the generation of α-ketoglutarate in a modified tricarboxylic acid cycle (15).

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