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LIGHT DEPENDENT REDUCTION OF NITRATE BY PEA CHLOROPLASTS IN THE PRESENCE OF NITRATE REDUCTASE AND C_4-DICARBOXYLIC ACIDS

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Key Word Index—*Pisum sativum*; Leguminosae; peas; *Triticum aestivum*; Gramineae; wheat; nitrate reduction; nitrite reduction; malate shuttle; O_2 evolution; nitrate reductase; NAD-specific-malate dehydrogenase; NADP-specific-malate dehydrogenase; light dependent; chloroplasts.

Abstract—In the presence of purified nitrate reductase (NR) and 1 mM NADH, illuminated pea chloroplasts catalysed reduction of NO_3^- to NH_3 with the concomitant evolution of O_2. The rates were slightly less than those for reduction of NO_2^- to NH_3 and O_2 evolution by chloroplasts in the absence of NR and NADH (*ca* 6 μg atoms N/mg Chl/hr). Illuminated chloroplasts quantitatively reduced 0.2 mM oxaloacetate (OAA) to malate. In the presence of an extra-chloroplast malate-oxidizing system comprised of NAD-specific malate dehydrogenase (NAD-MDH), NAD, NR and NO_3^-, illuminated chloroplasts supported OAA-dependent reduction of NO_3^- to NH_3 with the evolution of O_2. The reaction did not proceed in the absence of any of these supplements or in the dark but malate could replace OAA. The results are consistent with the reduction of NO_3^- by reducing equivalents from H_2O involving a malate/OAA shuttle. The ratios for O_2 evolved: C_4-acid supplied and N reduced: C_4-acid supplied in certain experiments imply recycling of the C_4-acids.

INTRODUCTION

Illuminated pea chloroplasts catalyse the reaction sequence: $NO_2^- \rightarrow NH_3 \rightarrow$ glutamine $\rightarrow$ glutamate. Incorporation of N from NO_2^-, NH_3 and the amide group of glutamine into glutamate in chloroplasts and protoplasts is light-dependent, accompanied by O_2 evolution and inhibited by DCMU [1–8] thus demonstrating that the reducing equivalents and ATP required for the reaction sequence are supplied by the light reactions. The reaction sequence shown above can therefore be viewed as the dark reaction of NO_2^- assimilation, analogous in many aspects to the reductive assimilation of CO_2. However, illuminated chloroplasts fail to catalyse the reduction of NO_3^- [5, 8], the form of inorganic-N normally available to plants in the physical environment. NR is localized outside the chloroplast, possibly in association with the outer membrane of the chloroplast itself [9, 10]. The reduction of NO_3^- in leaf tissue, however, is enhanced by light and malate [11–15]. To account for these observations it has been proposed [11, 15] that light-generated reducing equivalents effect the reduction of OAA to malate in the chloroplast [16, 17] which is exported to the cytoplasm via the C_4-dicarboxylic acid translocator [18] and serves as a source of NADH for NO_3^- reduction through the action of NAD-MDH (Fig. 1, component C). OAA produced by the oxidation of malate could be recycled while NO_2^- would be subject to further reduction in the chloroplasts [1, 2, 5]. Similar mechanisms involving other carriers [e.g. dihydroxyacetone phosphate (DHAP)/phosphoglycerate (PGA)] have also been proposed [15, 19], but sustained cycling of the carriers for any of these mechanisms has not been demonstrated.

This paper describes studies of the hypothesis shown in Fig. 1 using isolated pea chloroplasts. The various components of the scheme shown in Fig. 1 were investigated independently. Component A represents light-coupled reduction of NO_2^- to NH_3 by chloroplasts with the concomitant evolution of O_2. Component B entails reduction of NO_3^- by purified NR (from wheat) in the presence of exogenous NADH, and subsequent reduction of the NO_2^- so formed by component A Component C involves light dependent reduction of OAA by chloroplastic NADP-specific-malate dehydrogenase (NADP-MDH) with the evolution of O_2 [16]. The complete scheme was examined in the presence of NR, NAD, NAD-MDH and catalytic quantities of C_4-dicarboxylic acids.

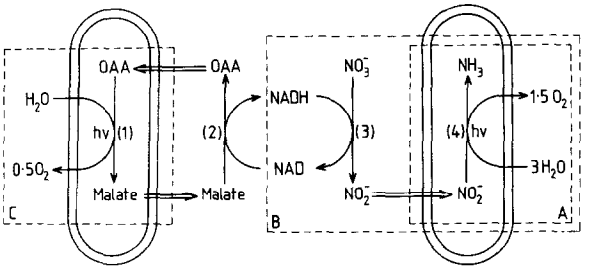

Fig. 1. Scheme for examining the utilization of light-generated reducing equivalents for the reduction of NO_3^- to NH_3. The regions enclosed within double lines represent chloroplasts. Reactions (1)–(4) are catalysed by light-coupled NADP-MDH, exogenous NAD-MDH, exogenous NR and light-coupled NO_2^- reductase. Portions of the scheme (A–C) enclosed within dotted lines were investigated independently of the complete scheme.

RESULTS

Light dependent NO_2^- consumption, O_2 evolution and NH_3 production (component A)

Table 1 summarises the results from a number of experiments in which NO_2^- (0.3–0.4 mM) was incubated with illuminated chloroplasts in the presence of 0.5 mM DL-methionine-DL-sulphoximine (MSO), an inhibitor of glutamine synthetase [20]. MSO was added to minimise loss of NH_3 by incorporation into amino acids. It had no effect on NO_2^--dependent O_2 evolution or NO_2^- reduction. The ratios of O_2 evolved to NO_2^- consumed and O_2 evolved to NH_3 produced are in approximate agreement with the theoretical stoichiometry (1.5). The reaction as determined by all three indices was dependent on light and NO_2^-.

Light coupled NO_3^- consumption, O_2 evolution and NH_3 production (component B)

Component B was examined by incubating chloroplasts in the light with NR (0.028 units/ml), 0.4 mM NO_3^- and 1 mM NADH. Under these conditions O_2 and NH_3 were produced at slightly slower rates than component A and some NO_2^- accumulated indicating that reaction 3 (Fig. 1) was not rate limiting. NO_2^-, but not NH_3, accumulated in the dark (Table 2).

OAA-dependent O_2 evolution and malate production (component C)

This reaction has been studied previously in pea chloroplasts [16]. The mean rate of O_2 evolution was 10.9 μmol/mg Chl/hr. It involves light-coupled reduction of OAA to malate by chloroplast NADP-MDH.

NO_3^- reduction, O_2 evolution and NH_3 production in the absence of exogenous NADH (coupling of components B and C)

Component C was examined as a source of reductant for NO_3^- reduction by supplying NAD, NAD-MDH and catalytic concentrations of OAA/malate in addition to NR and NO_3^-. Figs. 2A and 2D show respectively that illuminated chloroplasts in the presence of NO_3^-, NAD and NR quantitatively reduce 200 and 60 μM OAA to malate as judged by O_2 evolution (0.5 mol O_2 evolved/mol OAA supplied). Subsequent addition of NAD-MDH caused immediate resumption of O_2 evolution (range for 9 experiments using 200 μM OAA: 3.0–6.25 μmol/mg Chl/hr). Resumption of O_2 evolution did not occur in the absence of any one of NO_3^- (Fig. 2B), NAD-MDH (Fig. 2C), NR or NAD or in the dark (results not shown). However, O_2 evolution proceeded at slow rates in the absence of OAA (Fig. 2E) presumably due to endogenous C_4-dicarboxylic acids, estimated (as malate) at

Table 1. NO_2^- reduction, NH_3 production and O_2 evolution by illuminated pea chloroplasts (component A)

Experiment	Chloroplast intactness (%)	Chl concn (μg/ml)	NO_2^- concn (mM)	Treatment	O_2 evolution	NO_2^- consumption	NH_3 production	O_2/NO_2^-	O_2/NH_3
					(μmol/mg Chl/hr)				
1	90	200	0.3	Complete*	7.04	4.75	n.d.†	1.48	
2	83	100	0.3	Complete	9.79	n.d.	7.23		1.35
3	92	200	0.4	Complete	6.30	5.05	5.59	1.25	1.13
				Dark	0	0.84	0.13		
				Minus NO_2^-	0	0	0.05		

* Incubations were as described for component A and contained 0.5 mM MSO. All rates were calculated from differences between zero time samples (i.e. at time of illumination) and samples removed after 11.5–14 min.

† n.d.: Not determined.

Table 2. Consumption of NO_3^- and associated reactions by illuminated chloroplasts in the presence of NR, substrate amounts of NADH and MSO (component B)

Treatment	NO_3^- consumption	NO_2^- production	NH_3 production	O_2 evolution	O_2/NH_3
	(μmol/mg Chl/hr)				
Complete*	7.19	3.81	3.41	5.90	1.73
Dark	7.35	8.75	−1.04	0	
Minus NADH	0.33	0	0.04	0	
Minus NR	−1.42	−0.03	−0.15	0	

* Chloroplasts (93% intact and 118 μg Chl/ml) were incubated with 0.4 mM NO_3^-, 1 mM NADH and 0.5 mM MSO. All rates were calculated from differences between zero time samples (i.e. at time of illumination) and samples removed after 20 min.

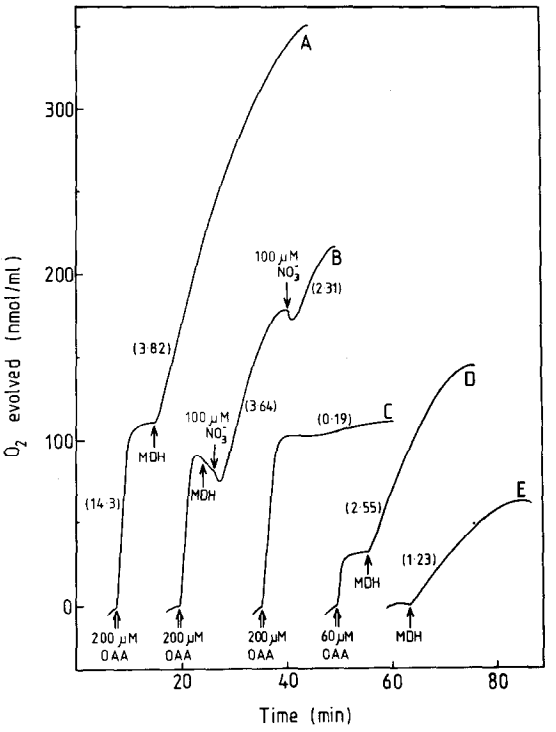

Fig. 2. Effect of NAD-MDH, OAA and NO_3^- on O_2 evolution by illuminated pea chloroplasts in the presence of NR and NAD. Incubation mixtures A–E initially contained chloroplasts (200 μg Chl/ml, 69% intact), NR (0.015 units/ml), 1 mM NAD and 10 mM DL-glyceraldehyde in chloroplast incubating medium. In addition, all incubation mixtures except B initially contained 1 mM $NaNO_3$. Other additions were made as shown. Where added, NAD-MDH was supplied after cessation of the initial stoichiometric OAA-dependent O_2 evolution. Values in parentheses beside the curves denote the rate of O_2 evolution in μmol/mg Chl/hr.

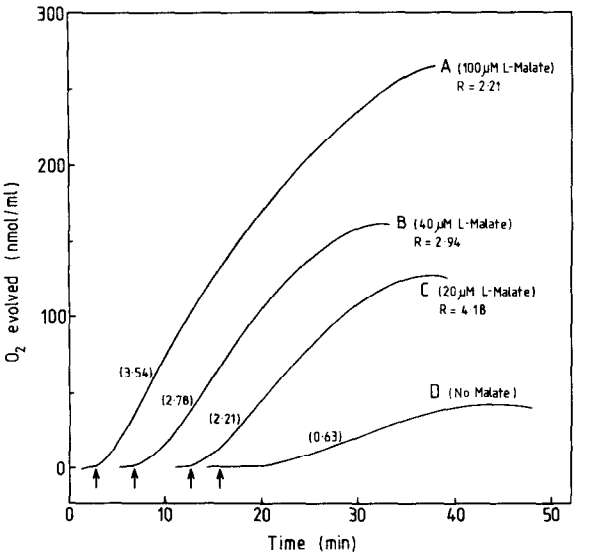

Fig. 3. Effect of malate on O_2 evolution by illuminated pea chloroplasts for condition (B plus C). Reaction mixtures (A–D) initially contained chloroplasts (200 μg Chl/ml, 63% intact), and other additions as described for components (B plus C). Malate was used as C_4-acid at the concentrations specified and NAD-MDH was added as indicated by the arrows. Values in parentheses beside the curves denote the rate of O_2 evolution in μmol/mg Chl/hr; R denotes the O_2: L-malate ratio corrected for O_2 evolution in the absence of L-malate.

0.15 μmol/mg Chl or 0.03 μmol/ml of reaction mixture. Conditions which supported reinitiated O_2 evolution also supported the production of NH_3 (Table 3). When low concentrations of NO_3^- were supplied, O_2 evolution ceased after the production of 0.91–1.02 mol O_2/mol NO_3^- supplied (Fig. 2B). Addition of more NO_3^- reinitiated O_2 evolution (Fig. 2B) implying that NO_3^- was the eventual electron acceptor. Typically, about 75% of the NO_3^- consumed was recovered as NH_3 and NO_2^- (Table 3).

Chloroplasts did not catalyse malate-dependent O_2 evolution either in the presence or absence of NR, NAD and NO_3^-. However, in the presence of NAD, NO_3^-, NAD-MDH and NR, malate (0.02–0.1 mM) supported O_2 evolution (Fig. 3); the rate of O_2 evolution increased with the concentration of malate up to 0.1 mM. Negligible O_2 evolution occurred in the absence of any one of these additions though some O_2 was evolved in the absence of malate (Fig. 3D). O_2 evolution under the conditions shown

Table 3. Production of O_2 and NH_3 and consumption of NO_3^- by illuminated chloroplasts in the presence of NR, NAD-MDH and NAD (components B plus C)

Experiment	Chloroplast intactness (%)	C_4-acid (μM)	Reaction conditions	O_2 evolved	NH_3 produced	NO_3^- consumed
				(μmol/mg Chl/hr)		
1	74	OAA (200)	Complete*	2.79	1.71	n.d.†
			−Light	0	0.59	n.d.
2	79	OAA (200)	Complete	6.20	2.03	3.54
			−MDH	1.62	0.57	1.05
3	84	Malate (30)	Complete	2.54	1.65	2.12
			−Malate	0.79	0.68	1.22

* Reaction mixtures were as described for components (B plus C). All rates were calculated from differences between zero time samples (i.e. at time of illumination) and samples removed after 16–20 min.

† n.d.: Not determined.

Table 4. N-flux and O_2 evolution by illuminated chloroplasts in the presence of NR, NAD-MDH and NAD (components B plus C)

Experiment	Chloroplast intactness (%)	C_4-acid (nmol/ml)	Conditions	N-flux (nmol/ml)	O_2 evolved (nmol/ml)	N-flux / C_4-acid	O_2 evolved / C_4-acid
1	83	OAA (80)	Complete*	202	234		
			OAA dependent†	116	155	1.45	1.94
2	84	Malate (30)	Complete	114	165		
			Malate-dependent†	71	116	2.37	3.87

* Chloroplasts (200 μg Chl/ml) were incubated as described for components (B plus C) in the presence of 0.5 mM MSO. All values were calculated from differences between zero time samples (i.e. at time of illumination) and samples removed when O_2 evolution ceased (see the text). N-flux refers to the net increase in NH_3 and NO_2^- formed from NO_3^-.

† OAA-dependent and malate-dependent values were corrected for controls lacking these C_4-acids.

in Fig. 3A was accompanied by the reduction of NO_3^- to NH_3 though, as for experiments using OAA as the C_4-acid, some NO_2^- accumulated. The rates observed with malate were similar to the reinitiated rates of O_2 evolution (after addition of NAD-MDH) for equivalent concentrations of OAA.

Regardless of the experimental conditions, O_2 evolution with NO_3^- as eventual electron acceptor invariably ceased after 20–30 min when either OAA or malate was used as the C_4-acid (Figs. 2A and 3A–C). Further additions of any one of the initial components at this stage did not reinitiate further O_2 evolution (except for an almost stoichiometric evolution of O_2 on addition of OAA). Some ratios for O_2 evolution and N-flux relative to the amount of C_4-acid supplied, determined on cessation of O_2 evolution, are shown in Table 4. Although the total amount of O_2 evolution and N-flux increased with the concentration of C_4-acid, in general the highest ratios were observed at low concentrations of exogenous C_4-acid (e.g. 30 μM L-malate, Table 4; 20 μM L-malate, Fig. 3).

DISCUSSION

NO_2^--dependent O_2 evolution and production of NH_3 by illuminated chloroplasts (Table 1) are consistent with light-coupled NO_2^- reductase activity using reducing equivalents from water as reductant (Fig. 1, component A). The rates of NO_2^- reduction (5 μmol/mg Chl/hr) and NH_3 production (6 μmol/mg Chl/hr) were slower than the nitrite reduction rates reported previously for pea (13 μmol/mg Chl/hr [5]) and spinach chloroplasts (9 μmol/mg Chl/hr [21] and 6–12 μmol/mg Chl/hr [22]) but are consistent with the lower rates for most light-coupled reactions observed in pea cv Massey Gem [16, 23]. The reduction of NO_3^- to NH_3 with the concomitant evolution of O_2 by illuminated chloroplasts in the presence of NR and substrate amounts of NADH (Table 2) implies that chloroplasts reduce the NO_2^- produced from NO_3^- by NR outside the chloroplasts (Fig. 1, component B); accumulation of NO_2^- in the dark is consistent with the requirement for light for NO_2^- reduction [1, 2, 5].

Illuminated pea chloroplasts reduce OAA to malate with the concomitant production of O_2 [16] demonstrating that H_2O serves as the reductant. In the absence of substrate amounts of NADH, the requirement for NAD, NAD-MDH and catalytic amounts of OAA for reduction of NO_3^- to NH_3 implies that malate, formed from OAA by chloroplasts in the light, serves as an intermediate reductant for NO_3^- reduction outside the chloroplast (Fig. 1). In this event, malate in the presence of NAD and NAD-MDH should also support reduction of NO_3^- to NH_3 with the concomitant evolution of O_2. In the dark, NO_3^- would be reduced to NO_2^-, the amount reduced being dependent on the amount of malate supplied. The data in Table 3 and Fig. 3 are consistent with these predictions.

An essential feature of chloroplast/cytoplasm shuttle mechanisms for the transport of reducing equivalents across the chloroplast envelope is that the reduced and oxidized forms of the carrier molecule can recycle indefinitely. Evidence for cycling of the exogenous C_4-acids is provided by the ratios of O_2 evolved to C_4-acid supplied and N-flux to C_4-acid supplied (Table 4); for a single turnover of C_4-acid the theoretical values predicted from Fig. 1 are 2 and 1 respectively. At concentrations of C_4-acid <100 μM, values in excess of these ratios were observed after *ca* 20–30 min when O_2 evolution ceased. For example, in the presence of 30 μM L-malate, the O_2 : malate and N-flux : malate ratios were 5.50 and 3.80 respectively. After correcting for the production of O_2 and NH_3 produced in the absence of malate the corresponding values were 3.86 and 2.36 implying that the added malate was cycling, or about to cycle, for the third time. Further O_2 : malate ratios are shown in Fig. 3.

The N-flux (NH_3 production) in the coupled system (components B plus C) was within the range 1.6–2.5 μg atoms N/mg Chl/hr. These were slower than those observed for NO_2^- reduction (component A) of 5.6 to 7.2 μg atoms N/mg Chl/hr (Table 1), and for the reduction of NO_3^- in the presence of substrate amounts of NADH and NR (component B, 3.4 μg atoms N/mg Chl/hr). These results suggest that the rate limiting step involves some step(s) in the supply of light-generated reducing equivalents for NO_3^- reduction. This is attributed to the oxidation of malate to OAA catalysed by NAD-MDH [24]. However, other factors could serve to enhance the rate of this reaction *in vivo* (e.g. high cytoplasmic concentrations of C_4-acids, maintenance of high NAD/NADH ratios by competing cytoplasmic reactions etc.).

The requirement for light and catalytic concentrations of OAA for the reduction of NO_3^- under the conditions described demonstrates that light generated reducing equivalents from H_2O can serve as the reductant. Our results therefore provide experimental evidence to support the mechanism proposed by Hageman and co-workers on the role of light and malate on NO_3^- reduction *in vivo*

[11–13] and in isolated protoplasts by Rathnam [15]. Since the reduction of OAA to malate in the chloroplast involves NADP-MDH [16] which is light-activated [25, 26] it follows that the effect of light on NO_3^- reduction by the C_4-shuttle could be regulated by this means in addition to the availability of light generated NADPH.

Techniques similar to those described for the study of the C_4-shuttle could also be used to investigate the role of the C_3-shuttle in light coupled reduction of NO_3^- [15, 19, 13]. Like the C_4-shuttle, it could also be regulated by light since glyceraldehyde 3-phosphate dehydrogenase is light activated [27]. Rathnam's experiments [15] demonstrate that the C_3- and C_4-shuttles are equally satisfactory mechanisms for light-dependent reduction of NO_3^- in protoplasts provided CO_2 assimilation is inhibited by glyceraldehyde. However, in the absence of added PGA, DHAP or C_4-acids, glyceraldehyde inhibited both CO_2 fixation and NO_3^- reduction [15], suggesting that the C_3-shuttle is the functional mechanism in spinach protoplasts.

EXPERIMENTAL

Plant material. Pea seedlings (*Pisum sativum* cv Massey Gem) were grown as before [23] and harvested 13–15 days after imbibition. Wheat (*Triticum aestivum* cv Olympic) was grown in a mixture of sand: pearlite: peat moss (2:2:1) in a growth room on a 12/12 day/night cycle at 25/25°. The plants were irrigated daily with a complete nutrient soln (essentially as in ref. [30] but with 0.1 mM Pi and without NH_4^+) and harvested after 8–10 days. Light intensity was 100 μE/m^2/sec at plant height.

Chemicals. NAD-MDH (in 50% glycerol) was obtained from Boehringer-Mannheim (West Germany), glutamate dehydrogenase (for NH_3 determinations) in 50% glycerol [$(NH_4)_2SO_4$ free] from Calbiochem (La Jolla, CA, U.S.A.), MSO from Sigma (St Louis, MO, U.S.A.). The concn of OAA solns was determined immediately prior to use [28]. Blue dextran-Sepharose 4B was prepared as per ref. [29].

Chloroplasts were prepared as described previously [16]. Chl was measured in EtOH, O_2 evolution by polarography and chloroplast intactness by the ratio of the uncoupled rates of O_2 evolution for osmotically shocked and intact chloroplasts using $Fe(CN)_6^{3-}$ as electron acceptor [16].

Purification and assay of NR. NR was prepared as in [29] with the following modifications: the extract, without $(NH_4)_2SO_4$ fractionation, was absorbed directly onto blue dextran-Sepharose 4B; NR was eluted with 10 μM NADH in 4 ml fractions into tubes containing 1 ml of 5% BSA in extracting medium (i.e. 1% final concn) to stabilize the enzyme [31]; the active fractions were concentrated by treatment with 1 g of DEAE-cellulose (equilibrated in the extracting medium of [29]) and NR displaced with 250 mM KCl. NR activity was determined as per ref. [32] and activity is expressed as μmol NO_2^- produced/min (NR units). Specific activities were not determined due to the presence of 1% BSA.

Reaction mixtures for estimating the activities of components A, B, C and (B plus C). All incubations were conducted at 25°C in medium containing 0.33 M sorbitol, 50 mM HEPES, 0.1% BSA, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and adjusted to pH 7.6 with KOH [16]. These conditions were maintained by addition of double strength incubating medium where necessary. In addition, all incubations were conducted in the presence of 10 mM DL-glyceraldehyde to inhibit CO_2 assimilation [33]. Some reaction mixtures also contained 0.5 mM MSO but this is specified for each experiment. For component A (100–200 μg Chl/ml), reactions were initiated with 0.3–0.4 mM $NaNO_2$ [5]. For component B (*ca* 100 μg Chl/ml), incubations contained NR (*ca* 0.025 units/ml), 1 mM NADH and reactions were initiated with 0.4 mM $NaNO_3$. For component C (100 μg Chl/ml), reactions were initiated with 0.8 mM OAA [16]. Components (B plus C) were determined with either OAA or malate as the C_4-acid. When OAA was used, reaction mixtures contained Chl (200 μg/ml), NR (0.028 units/ml), 1 mM NAD, 0.4 mM $NaNO_3$ and reactions were initiated with 60–200 μM OAA. After cessation of the initial OAA-dependent O_2 evolution (denoting reduction of OAA to malate, see Fig. 2A), reactions were reinitiated with NAD-MDH (21 units/ml). When malate was used as the C_4-acid the same conditions were employed except that 20–200 μM malate replaced OAA and reactions were initiated with NAD-MDH (see Fig. 3).

Determination of inorganic-N. NO_3^-, NO_2^- and NH_3 were determined by the procedures of refs. [34], [32] and [35] respectively. The results of all determinations of inorganic-N (including NR activity) are expressed as the means of duplicate estimations. The coefficient of variation was typically $<5\%$; for example the s.e. for the determinations of NO_3^-, NO_2^- and NH_3 in Table 2 were 0.29, 0.05 and 0.18 μmol/mg Chl/hr respectively.

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