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Light-Dependent Oxygen Uptake, Glycolate, and Ammonia Release in L-Methionine Sulfoximine-Treated *Chlamydomonas*

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ABSTRACT

Glycolate and ammonia excretion plus oxygen exchanges were measured in the light in L-methionine-DL-sulfoximine treated air-grown *Chlamydomonas reinhardii*. At saturating CO_2 (between 600 and 700 microliters per liter CO_2) neither glycolate nor ammonia were excreted, whereas at the CO_2 compensation concentration (<10 microliters per liter CO_2) treated algae excreted both glycolate and ammonia at rates of 37 and 59 nanomoles per minute per milligram chlorophyll, respectively. From the excretion values we calculate the amount of O_2 consumed through the glycolate pathway. The calculated value was not significantly different from the component of O_2 uptake sensitive to CO_2 obtained from the difference between O_2 uptake of the CO_2 compensation point and at saturating CO_2. This component was about 40% of stationary O_2 uptake measured at the CO_2 compensation point. From these data we conclude that glyoxylate decarboxylation in air-grown *Chlamydomonas* represents a minor pathway of metabolism even in conditions where amino donors are deficient and that processes other than glycolate pathway are responsible for the O_2 uptake insensitive to CO_2.

Light-dependent oxygen uptake has been shown to occur both in higher plants (6, 13, 27) and algae (10, 12, 15, 16, 24). Enzymic studies on ribulose-1,5-bisphosphate carboxylase (4) and labeling studies using $^{18}O_2$ (1, 3, 10) led to the evidence that oxygenation of ribulose-1,5-bisphosphate and the associated photorespiratory metabolism of glycolate was involved in this process. Nevertheless other mechanisms, like direct photoreduction of oxygen (Mehler reaction) (21), chlororespiration (2), or mitochondrial respiration in the light (14) could also occur. In unicellular algae, part of the glycolate synthesized during photorespiration has been shown to be excreted (10). In higher plants, glycolate is metabolized through glyoxylate and glycine leading to the production of NH_3 and CO_2 during the mitochondrial conversion from glycine to serine (7). Photorespiratory ammonia was shown to be reassimilated via the GS-GOGAT[1] pathway (19). By using L-MSO, an inhibitor of GS (25), ammonia production was also shown to occur in *Chlamydomonas* (9, 23). From the effects of light (9), CO_2 (9, 23), O_2 and INH (23), this production was attributed to the photorespiratory metabolism of glycolate.

The present study was undertaken to determine the contribution of the glycolate pathway to the oxygen uptake in the light in air-grown *Chlamydomonas*. For this purpose, we measured oxygen uptake, glycolate, and ammonia release in L-MSO-treated algae both at the CO_2 compensation point and saturating CO_2 concentrations. From glycolate and ammonia excretion rates we calculate the amount of oxygen consumed through the glycolate pathway. Our results will be discussed in relation to the contribution of the different mechanisms for oxygen uptake in the light and to the existence of a CO_2-concentrating mechanism in air-grown algae.

MATERIALS AND METHODS

Chlamydomonas reinhardii (wild type 137c) was grown axenically and phototrophically as previously described (23). Air was bubbled through the culture at a flow rate of about 20 $l \cdot h^{-1}$. During the exponential phase, cells were harvested by centrifugation and resuspended in the same medium minus NH_4^+ · pH of the medium was 6.0. Chl concentration was between 20 and 30 $\mu g \cdot ml^{-1}$. Such high algal concentrations were required to measure O_2 uptake with a sufficient accuracy. We checked that during these conditions, the incident quantum flux (1000 $\mu E \cdot m^{-2} \cdot s^{-1}$, 400–700 nm) saturated photosynthetic CO_2 uptake at saturating CO_2.

The algal suspension (55 ml) was transferred into a thermostated (25°C) flask included in a gas circuit. CO_2-free air was bubbled through the suspension by means of a glass-frit at a flow rate of 30 $l \cdot h^{-1}$. After 2.5 h in the dark in the presence or in the absence of 0.5 mM L-MSO, the circuit was closed and 2 ml $^{18}O_2$ (98.1% ^{18}O from CEA Saclay, France) were injected. Light was switched on 30 min later. The circuit was connected to a mass-spectrometer (Mat Atlas CH_4) through a gas inlet valve (Balzers, UDV135) and included a metal bellows pump (Metal Bellows Corp, Sharon, MA), a two-way valve to open or close the circuit, a water trap (−12°C), and an IR gas analyzer (Hartmann & Braun, Uras 2). For the experiments carried out at saturating CO_2 concentrations, a peristaltic pump (Gilson, Minipuls 2) was used to inject CO_2. Oxygen uptake and evolution were measured by continuously recording $^{16}O_2$ (m/e = 32), $^{18}O_2$ (m/e = 36), Ar (m/e = 40). Argon contained in air was used as an internal standard to correct variations in gas concentration due to mass spectrometer consumption. Calculations of O_2 uptake and evolution were performed as published by Radmer and Kok (24).

GS activity, ammonia concentration in the medium, and Chl content were determined as in (23). Glycolate excreted in the medium was measured colorimetrically (5). After filtration, 1 ml of the culture medium was evapored to dryness and 1 ml of Calkins reagent (18.5 mg 2,7-dihydroxynaphthalene in 100 ml concentrated H_2SO_4) was added. After 20 min at 100°C glycolate was measured at 536 nm. We checked that NH_4^+ ions at the concentrations present in our experiments did not interfere with Calkins test. For internal ammonia determination, algae were harvested by filtration on a glass fiber filter (Whatman GFC). After extraction using 90% methanol, ammonia was determined by isotopic dilution. A known quantity of $^{15}NH_4Cl$ was added to

[1] Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; L-MSO, L-methionine-DL-sulfoximine; INH, isonicotinic acid hydrazide.

the sample and final ^{15}N content was measured as previously reported (23).

RESULTS

GS inactivation was followed in the darkened algal suspension treated by L-MSO (0.5 mM final concentration). GS activity was inhibited 3 h after the addition of the inhibitor (Fig. 1). During this period, internal ammonia concentration remained constant and no ammonia was excreted in the medium. After switching on the light, internal ammonia pool increased up to a stationary level which was reached after about 1 h. Ammonia excretion started only 40 min after the beginning of illumination.

Oxygen exchanges were measured at the CO_2 compensation point and at saturating CO_2 concentration (Fig. 2). L-MSO was found to have no effect on gas exchanges (data not shown). Previously, dark respiration was measured for 30 min after the gas circuit was closed and $^{18}O_2$ injected (Table I). CO_2 concentration in the circuit increased to about 600 $\mu l \cdot l^{-1}$

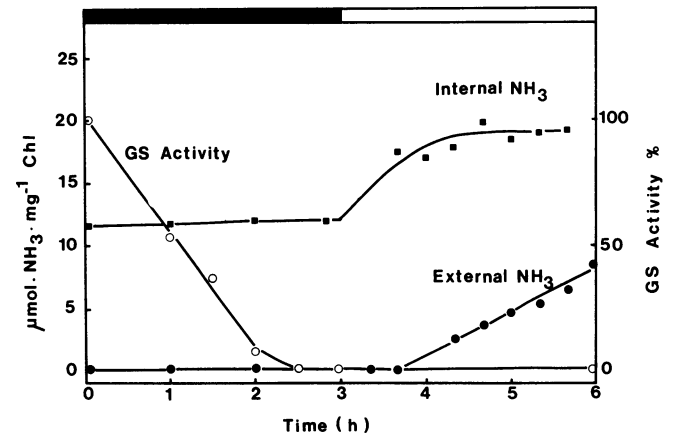

FIG. 1. GS activity, internal and external ammonia in L-MSO-treated *Chlamydomonas*. L-MSO (final concentration, 0.5 mM) was added at zero time in the dark. Algae were bubbled with CO_2-free air in the dark. At the beginning of the light period, the circuit was closed and the experiment was performed at the CO_2 compensation point. (○), GS relative activity; (■), internal ammonia concentration; (●), ammonia concentration in the medium.

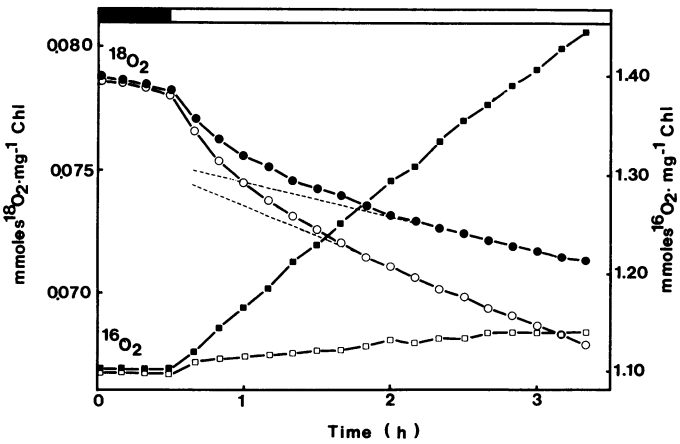

FIG. 2. Oxygen exchanges in air-grown *Chlamydomonas*. Light symbols: experiment carried out at the CO_2 compensation point (<10 $\mu l \cdot l^{-1}$ CO_2): (□) $^{16}O_2$ concentration; (○) $^{18}O_2$ concentration. Dark symbols: experiment carried out at saturating CO_2 (600–700 $\mu l \cdot l^{-1}$ CO_2): (■) $^{16}O_2$ concentration; (●) $^{18}O_2$ concentration. O_2 concentration was between 23 and 29% O_2. Gas exchanges were similar in the presence or in the absence of L-MSO.

Table I. *Ammonia and Glycolate Excretion, Oxygen Exchanges in L-MSO-treated Chlamydomonas*

Measurements were made at the CO_2 compensation point (<10 $\mu l \cdot l^{-1}$ CO_2) and at saturating CO_2 (600–700 $\mu l \cdot l^{-1}$ CO_2). Values for the light period were obtained between 1 and 3 h after switching on the light.

	CO_2 Compensation Point	Saturating CO_2
	$\mu mol \cdot min^{-1} \cdot mg^{-1}$ Chl ± SD	
Light O_2 uptake	0.72 ± 0.06 (5)[a]	0.42 ± 0.03 (5)[a]
Net O_2 evolution	0 (5)	1.99 ± 0.12 (5)
Ammonia excretion	0.059 ± 0.004 (3)	0 (2)
Glycolate excretion	0.037 ± 0.002 (3)	0 (2)
Dark O_2 uptake	0.22 ± 0.04 (5)	

[a] Number of experiments.

After turning on the light and when no CO_2 was injected, the CO_2 compensation concentration (<10 $\mu l \cdot l^{-1}$) was obtained after about 5 min. During this short period, net O_2 evolution was observed due to CO_2 uptake. After that, $^{16}O_2$ evolution was balanced by $^{18}O_2$ uptake and no net O_2 evolution was observed. O_2 uptake, calculated from mass spectrometer measurements, decreased from about 2.3 $\mu mol\ O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl at the beginning of the light-period to a constant rate of 0.7 $\mu mol\ O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl about 1 h later.

When CO_2 concentration was maintained in the light at a saturating level (between 600 and 700 $\mu l \cdot l^{-1}$) by continuous injection of CO_2, net O_2 evolution was 2.1 $\mu mol\ O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl. In this case, O_2 uptake decreased from an initial value of about 1.9 $\mu mol\ O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl to 0.4 $\mu mol\ O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl.

Consequently, increasing CO_2 concentrations partially inhibited O_2 uptake. The CO_2 sensitive O_2 uptake obtained by the difference between measurements at the CO_2 compensation point and at saturating CO_2 was constant during illumination (about 0.3 $\mu mol\ O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl). The remaining O_2 uptake, insensitive to CO_2, decreased during the 1st hour of illumination and kept a constant value afterward.

Figure 3 shows that L-MSO-treated algae excreted glycolate at the CO_2 compensation concentration. Ammonia excretion and O_2 exchanges were the same as described in Figures 1 and 2, respectively. At saturating CO_2, both glycolate and ammonia excretion stopped, while $^{18}O_2$ uptake decreased and $^{16}O_2$ evolution started. In the same conditions, untreated algae did not excrete a significant amount of glycolate either at the CO_2 compensation point or at saturating CO_2 (data not shown). Such an induction of glycolate excretion by L-MSO at subsaturating CO_2 levels was previously observed in *Ankistrodesmus* (20). It was interpreted as a protein turnover too slow to sustain the demand for amino groups for metabolizing all the glycolate formed when ammonia assimilation is inhibited by L-MSO.

Values of glycolate and ammonia excretion rates are summarized in Table I.

DISCUSSION

The purpose of this work was to determine the contribution of the photorespiratory carbon oxidation cycle to O_2 uptake in air-grown *Chlamydomonas*. Because *in vitro* studies showed that CO_2 can compete with O_2 on ribulose-1,5-bisphosphate carboxylase/oxygenase, attempts to identify O_2 uptake in higher plants were mainly based on the dependence of O_2 uptake with regard to CO_2 concentration (22). However, CO_2 could also possibly inhibit other O_2 uptake processes like Mehler reaction by draining reductive power from NADPH to reductive carbon cycle

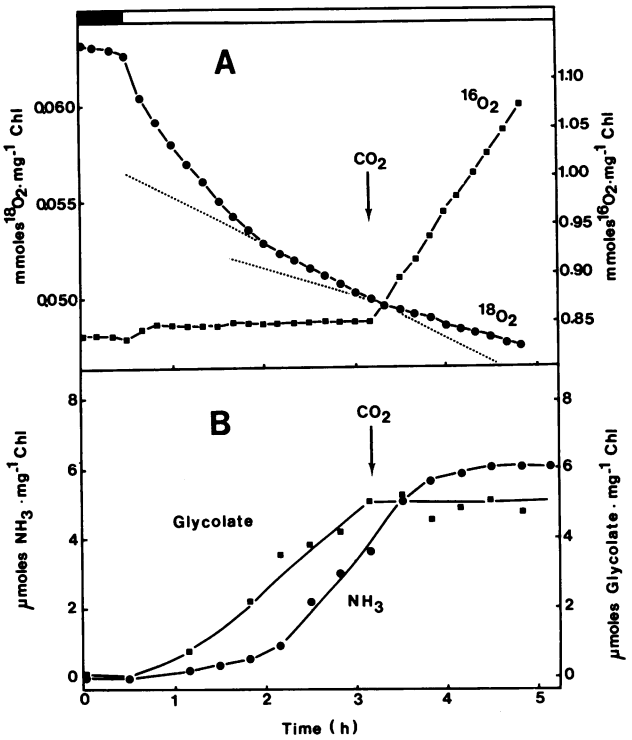

FIG. 3. Effect of CO_2 concentration on O_2 exchanges, ammonia and glycolate release in L-MSO-treated *Chlamydomonas*. CO_2 compensation concentration (<10 $\mu l \cdot l^{-1}$ CO_2) was reached about 5 min after switching on the light. After 2.5 h in the light CO_2 was injected and kept between 600 and 700 $\mu l \cdot l^{-1}$. A: (●), $^{18}O_2$ concentration; (■), $^{16}O_2$ concentration. B: (■), glycolate concentration; (●), ammonia concentration.

(13). A more suitable approach was to calculate metabolic flux through glycolate pathway from ^{18}O labeling kinetics. Such studies showed in C_3 plants that the O_2 flux through the glycolate pathway was close to the O_2 uptake rate in the light (3). In C_4 plants it was reported that less than 75% of O_2 taken up in the light was involved in this pathway (17).

High CO_2-grown unicellular algae have been shown to excrete part of the glycolate they produce during photorespiration (10). Furthermore, L-MSO-treated algae excrete photorespiratory ammonia (9, 23). Our results show that air-grown algae, when treated with L-MSO, excreted both glycolate and ammonia. Because the photorespiratory carbon oxidation cycle consumes 3.5 O_2 per ammonia and 1 O_2 per glycolate synthesized (3), the amount of O_2 consumed to produce excreted ammonia and glycolate is:

$$O_2 \text{ consumed} = 3.5\ NH_3 \text{ excreted} + 1 \text{ glycolate excreted}$$

Several conditions are required to make sure that this equation gives a valid measurement of the overall O_2 consumed through the glycolate pathway: (a) intermediary pools must have reached a stationary level during the measurement. We checked that ammonia internal concentration was constant during excretion measurements, but it must be the same for P-glycolate, glycolate, glyoxylate, and glycine pools. (b) Metabolic intermediates of the glycolate pathway must not be metabolized by another pathway. It was observed in a mutant of *Arabidopsis thaliana* lacking serine transhydroxymethylase activity that depletion of amino donors required for glyoxylate amination may lead to CO_2 release from direct decarboxylation of glyoxylate (26). (c) Ammonia assimilation must be completely inhibited to make sure that all of the ammonia produced will be excreted (23).

Oxygen consumption supported by the glycolate pathway and calculated from our results (Table I) is 0.24 ± 0.016 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl. This value is not significantly different from the CO_2-sensitive O_2 uptake in the light (0.30 ± 0.09 μmol $O_2 \cdot min^{-1}$ Chl). This implies that: (a)glyoxylate decarboxylation in *Chlamydomonas* represents, if it occurs, a minor pathway of metabolization even in conditions where amino donors are probably deficient (as indicated by glycolate excretion). (b) Mehler reaction and (or) other O_2 uptake processes occur at the same rate in the presence or in the absence of CO_2.

Consequently, the O_2 uptake observed during the light period can be divided into two components: the one which is inhibited by high CO_2 concentration is linked to the photorespiratory carbon oxidation cycle and leads to glycolate and ammonia excretion in the presence of L-MSO and the other one which does not depend on CO_2 concentration. The latter component is characterized by an O_2 uptake rate which decreased dramatically during the 1st h of illumination and stabilized at a constant value afterward (0.42 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl). The nature of the process responsible for this O_2 uptake remains unknown. Mehler reactions, chlororespiration, or mitochondrial respiration could be involved. It is interesting to note that the constant rate of O_2 uptake measured after 1 h of illumination (0.42 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl) is very close to the rate of dark respiration measured after 3 h of illumination (data not shown).

The CO_2-sensitive O_2 uptake measured in air-grown *Chlamydomonas* (about 15% of net O_2 evolution at saturating CO_2) is quite low compared to that observed in C_3 plants (about 75% of net O_2 evolution at saturating CO_2) (14). A previous report attributed this different behavior to the existence of a CO_2-concentrating mechanism in air-grown algae (12) which could increase the internal CO_2 concentration enough to inhibit the oxygenation of ribulose-1,5-bisphosphate. However, at the CO_2 compensation point, the ratio between oxygenation and carboxylation is two (11). So, for a given O_2 concentration, internal CO_2 at the enzyme level only depends on the kinetic properties of the ribulose-1,5-bisphosphate carboxylase/oxygenase (see equation in reference 18). Finally, the CO_2-concentrating mechanism present in air-grown algae results in lowering the measured external CO_2 compensation concentration without influence on internal CO_2 compensation concentration. A more suitable hypothesis to explain the different behavior observed between algae and C_3 plants would be to consider that *in vivo* kinetic properties of the ribulose-1,5-bisphosphate carboxylase/oxygenase are different from that observed *in vitro* with purified enzymes (18). For instance, it was recently reported that a large part of the carboxylase activity in blue-green algae was present in carboxysomes (8). Carboxylase activity in these particles, unlike soluble carboxylase activity, was shown to be insensitive to O_2 concentration. The existence of a large part of the carboxylase activity, under a form insensitive to the presence of O_2, could explain the low CO_2-sensitive O_2 uptake observed in air-grown *Chlamydomonas*.

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