Cytochrome and Alternative Pathway Respiration during Transient Ammonium Assimilation by N-Limited Chlamydomonas reinhardtii

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ABSTRACT

Mass spectrometric analysis of gas exchange in light and dark by N-limited cells of Chlamydomonas reinhardtii indicated that ammonium assimilation was accompanied by an increase in respiratory carbon flow to provide carbon skeletons for amino acid synthesis. Tricarboxylic acid (TCA) cycle carbon flow was maintained by the oxidation of TCA cycle reductant via the mitochondrial electron transport chain. In wild-type cells, inhibitor studies and 18O2 discrimination experiments indicated that respiratory electron flow was mediated entirely via the cytochrome pathway in both the light and dark, despite a large capacity for the alternative pathway. In a cytochrome oxidase deficient mutant, or in wild-type cells in the presence of cyanide, the alternative pathway could support the increase in TCA cycle carbon flow. These different mechanisms of oxidation of TCA cycle reductant were reflected by the much greater SHAM sensitivity of ammonium assimilation by cytochrome oxidase-deficient cells as compared to wild type.

Ammonium assimilation by higher plants and microalgae occurs predominately via the glutamine synthetase/glutamate-2-oxo-glutarate amino-transferase pathway (1, 9). Operation of this pathway for net glutamate production requires the provision of αKG2, while the subsequent biosynthesis of other amino acids by transamination requires TCA cycle and glycolytic intermediates (9). Nitrogen assimilation also requires ATP and reducing power. In darkness, the requirements for carbon skeletons, ATP, and reducing power, can be met by mitochondrial respiration, whereas in the light ATP and reducing power may also be supplied by the light reactions of photosynthesis.

Ammonium assimilation results in increased TCA cycle carbon flow to provide the carbon skeletons necessary for amino acid synthesis (10, 16). Maintenance of TCA cycle carbon flow is dependent upon the continued oxidation of TCA cycle reductant (NADH, FADH2). One possible mechanism for this oxidation is via the mitochondrial electron transport chain (17, 18), utilizing either the phosphorylating Cyt pathway or the nonphosphorylating alternative pathway. While the capacity for alternative pathway respiration is widespread among plants and algae, the in vivo role is poorly understood. One hypothesis is that operation of the alternative pathway allows oxidation of TCA cycle reductant during adenylate restriction of Cyt pathway activity, thus maintaining TCA cycle carbon flow for provision of biosynthetic intermediates (2, 12, 14). This may be especially important in the light, when photophosphorylation may meet cellular ATP demands.

In the present study, we examine the role of the Cyt and alternative electron transport chains in support of respiratory carbon flow to ammonium assimilation by N-limited cells of a green alga, Chlamydomonas reinhardtii. This organism displays substantial capacities for both Cyt and alternative pathway activity (19). Use of inhibitors of the alternative and Cyt pathways, as well as examination of the discrimination against 18O2, permits the conclusion that enhanced rates of respiratory carbon flow during amino acid synthesis are supported by increased activity of the Cyt pathway. Under conditions where Cyt pathway activity is absent (i.e. in a Cyt oxidase-deficient mutant, or in the presence of cyanide), the alternative pathway may support biosynthetic carbon flow.

MATERIALS AND METHODS

Algal Strains and Culture Methods

Wild type Chlamydomonas reinhardtii R34 and Cyt ox− C. reinhardtii R4 were obtained via crosses using existing strains (19). Respiratory capabilities of both strains have been assessed using both inhibitor titrations and discrimination against 18O2 (19). C. reinhardtii R34 was shown to possess a substantial capacity for both Cyt and alternative pathway respiration, while in C. reinhardtii R4 Cyt pathway capacity was undetectable (19).

Cells were grown in N-limited chemostats (4), using a modification of Surzycki’s medium (20). Cultures were bubbled with air enriched with 2.5% CO2. Dissolved inorganic carbon in the culture was approximately 2.5 mm. Nitrogen was supplied as 1 mm NH4Cl at a dilution rate of 0.3 d−1. Temperature was maintained at 30°C.
Use of Isotope Discrimination

Discrimination against $^{18}$O$_2$ by mitochondrial respiration was calculated from changes in the stable oxygen isotope composition of the medium with time (7, 19). Samples were analyzed for stable oxygen isotope ratios via a VG Isotech (Middlewich, England) Prism triple-collecting mass spectrometer. Due to the production of O$_2$ in the light from water photolysis, the isotopic discrimination method of measuring respiratory electron partitioning is not appropriate under photosynthetic conditions. Consequently, this technique was only used to evaluate partitioning of respiratory electron flow between Cyt and alternative pathways in the dark.

Inhibitor Effects on Gas Exchange

Respiratory electron flow through the alternative pathway (in both light and dark) was also evaluated by the SHAM inhibition of O$_2$ consumption. Concomitant TCA cycle CO$_2$ release was also recorded. On-line gas exchange was measured using a membrane inlet-equipped VG Gas Analysis (Middlewich, England) MM14-805C mass spectrometer (17). Inorganic carbon was added as 99% [14C]Na$_2$CO$_3$ to a concentration of 2 mM, sufficient to inhibit photorespiration. $^{18}$O$_2$ was added as a bubble to the algal suspension; the bubble was removed prior to the experiment. Total [O$_2$] ($^{18}$O$_2$ + $^{16}$O$_2$) was never allowed to reach more than 110% air saturation. Illumination, when provided, was at 250 $\mu$E m$^{-2}$ s$^{-1}$ from a 300 W projector lamp (General Electric). Gas exchange rates were calculated as previously described (16, 18). Isotopes were obtained from Merck, Sharpe & Dohme (Canada).

One potential problem with the use of SHAM in assessing alternative pathway engagement in the light is that high SHAM concentrations may result in a partial inhibition of photosynthesis, possibly due to effects on CO$_2$ transport (6). Thus, SHAM concentrations used with each strain were optimized to yield maximal inhibition of alternative pathway activity (measured in the presence of KCN) with a minimal effect on gross O$_2$ evolution (measured by mass spectrometry in the absence of KCN). Based on these preliminary experiments SHAM was added to a concentration of 1 mM to Cyt ox$^-$ cells, and 2 mM to wild-type cells. The SHAM solution was prepared fresh daily as a 1 M stock in methoxyethanol. KCN was added to a concentration of 1 mM, which was found to be saturating for the inhibition of Cyt pathway activity (19). Since KCN is a potent inhibitor of photosynthesis, its use in the light was restricted to the end of experiments, allowing assessment of residual respiration.

Other Measurements

Ammonium assimilation was measured as disappearance from a cell suspension in a water-jacketed cuvette (18). Unfiltered samples (2.5 mL) were combined with 25 $\mu$L of 10 N NaOH and mixed vigorously to convert NH$_4^+$ to NH$_3$. The total NH$_3$ concentration was then measured with an NH$_3$ electrode. As this treatment releases internal NH$_4^+$, the rate of NH$_4^+$ disappearance represents the rate of NH$_4^+$ assimilation.

Chl was measured spectrophotometrically following extraction in 90% acetone (8).

RESULTS

Oxygen Isotope Discrimination

Previous work has established values for the $^{18}$O$_2$ discrimination associated with Cyt and alternative pathway-mediated O$_2$ consumption in wild type Chlamydomonas reinhardtii cells (Table I). These values were determined by measuring the $^{18}$O$_2$ discrimination in the presence of KCN for alternative pathway activity, and in the presence of SHAM for Cyt pathway activity (19). Under control conditions in the dark (i.e. absence of inhibitors or ammonium) the $^{18}$O$_2$ discrimination associated with respiratory O$_2$ consumption was completely consistent with operation of only the Cyt pathway in wild-type cells, and only the alternative pathway in Cyt ox$^-$ cells (19). These results agreed with the inhibitor data presented in the same study.

In the present study we measured the $^{18}$O$_2$ discrimination associated with respiratory O$_2$ consumption during dark ammonium assimilation by N-limited wild-type cells (Table I), which was accompanied by a marked stimulation of mitochondrial O$_2$ consumption (Fig. 1). The $^{18}$O$_2$ discrimination associated with this increased respiratory O$_2$ consumption was the same as that previously reported for the Cyt pathway (Table I), indicating that O$_2$ consumption was mediated entirely via that pathway during dark ammonium assimilation.

Effects of Inhibitors on Mitochondrial Respiration and Photosynthesis

SHAM concentrations used for both algal strains were optimized to produce maximal inhibition of alternative pathway activity while minimizing inhibition of photosynthetic O$_2$ evolution. Mitochondrial O$_2$ consumption by wild-type cells was only slightly affected by 2 mM SHAM in the absence of KCN in light or dark (Table II). This SHAM concentration resulted in approximately a 20% decrease in gross photosynthetic O$_2$ evolution, and a similar decrease in CO$_2$ fixation (Table III). Subsequent addition of KCN resulted in a 70% decrease in mitochondrial O$_2$ consumption (Table II). Residual respiration (O$_2$ consumption) under these conditions was 30% of control. This could be reduced to less than 10% by using higher SHAM concentrations (up to 40 mM; see ref. 19); however, gross photosynthetic O$_2$ evolution was also greatly reduced. Addition of KCN alone resulted in a 30% stimulation of O$_2$ consumption in the dark (Table II).

Table I. Discrimination Against $^{18}$O$_2$ by Dark Respiration in Wild-Type Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (R34)</td>
<td></td>
</tr>
<tr>
<td>+KCN*</td>
<td>25.46 ± 0.18 (6)</td>
</tr>
<tr>
<td>+SHAM*</td>
<td>20.02 ± 0.24 (7)</td>
</tr>
<tr>
<td>+NH$_4^+$</td>
<td>20.70 ± 0.54 (7)</td>
</tr>
</tbody>
</table>

* From Weger et al. (19).

From the table, it can be seen that the discrimination against $^{18}$O$_2$ by dark respiration in wild-type Chlamydomonas is similar to that observed in the presence of KCN, indicating that the alternative pathway is involved. The addition of SHAM further reduces the discrimination, consistent with the inhibition of the Cyt pathway.
Addition of 1 mM SHAM (in the absence of KCN) to Cyt ox− (R4) cells resulted in a 70% inhibition of mitochondrial O2 consumption (Table II). This SHAM concentration resulted in a 22% decrease in gross photosynthetic O2 evolution and a slightly larger decrease in photosynthetic CO2 fixation (Table II). A very small additional inhibition of mitochondrial O2 consumption was observed upon subsequent addition of KCN. Residual respiration under these conditions was approximately 30% of control. This could be reduced to 5% at 40 mM SHAM (19), but gross photosynthetic O2 evolution also decreased substantially. Addition of KCN alone did not affect O2 consumption by Cyt ox− cells, but resulted in approximately a 75% decrease in gross O2 evolution by both strains when added in the light (Table III).

**Effects of Ammonium Assimilation on Mitochondrial Respiration**

Addition of 500 μM NH4Cl to both wild-type and Cyt ox− cells resulted in a large stimulation of mitochondrial respiration in the dark, measured both as increased respiratory O2 consumption and increased TCA cycle CO2 release (Fig. 1, A and B). However, the SHAM sensitivity of this increased respiration differed between the two strains. Addition of SHAM to wild-type cells had little effect on gas exchange (Fig. 1A), while in Cyt ox− cells SHAM addition resulted in a large decrease in both O2 consumption and CO2 release (Fig. 1B). Subsequent addition of KCN resulted in a large decrease in mitochondrial respiration by wild type cells, but had little effect on Cyt ox− cells.

The addition of 1 mM KCN to wild-type cells in the dark stimulated O2 consumption (Table I; Fig. 2). Dark O2 consumption in the presence of KCN (i.e. via the alternative pathway) could be further increased by the addition of ammonium, and inhibited by the subsequent addition of SHAM (Fig. 2). Patterns of TCA cycle CO2 release were consistent with the effects on O2 consumption (Fig. 2).

Results similar to those in the dark were observed during photosynthesis (Fig. 3, A and B). Increased mitochondrial respiration resulting from NH4+ assimilation was largely SHAM insensitive in wild-type cells and SHAM sensitive in Cyt ox− cells. Conversely, the wild type exhibited substantial KCN-sensitivity, while Cyt ox− cells did not. It should be noted that rates of CO2 release observed during photosynthesis probably underestimate actual TCA cycle activity due to photosynthetic refixation of respired CO2 (18). On the other hand, measurement of respiratory O2 consumption as deter-

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**Table II. Cyt and Alternative Pathway Respiration by Wild-Type and Cyt ox− Cells as a Percentage of Control**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Control O2 Consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type (R34)</td>
</tr>
<tr>
<td>Dark</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>+SHAM</td>
<td>109 ± 1 (6)</td>
</tr>
<tr>
<td>+SHAM + KCN</td>
<td>30 ± 2 (6)</td>
</tr>
<tr>
<td>+KCN</td>
<td>131 ± 4 (6)</td>
</tr>
<tr>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>+SHAM</td>
<td>96 ± 2 (3)</td>
</tr>
<tr>
<td>+SHAM + KCN</td>
<td>21 ± 5 (3)</td>
</tr>
</tbody>
</table>

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**Table III. Effect of NH4+ and Inhibitors of Mitochondrial Respiration on Photosynthetic Gas Exchange by C. reinhardtii**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>O2 Evolution</th>
<th>CO2 Fixation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% ± SE (n)</td>
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</tr>
</tbody>
</table>

| Wild type (R34)     |              |              |
| +SHAM (2 μM)        | 81 ± 2 (7)   | 72 ± 9 (7)   |
| +KCN                | 16 ± 1 (3)   | 18 ± 18 (3)  |
| Cyt ox− (R4)        |              |              |
| +SHAM (1 μM)        | 78 ± 3 (5)   | 83 ± 3 (3)   |
| +KCN                | 22 ± 4 (5)   | 7 ± 5 (3)    |

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**Figure 1. Mitochondrial O2 consumption (C) and TCA cycle CO2 release (●) in the dark by (A) C. reinhardtii R34 (wild-type) and (B) C. reinhardtii R4 (Cyt ox−) cells. The data have been corrected for the effects of changing isotopic composition and mass spectrometer consumption of gases (16). Rates are expressed as μmol·mg−1 Chl·h−1. Scale bars represent 100 μM O2 or CO2. Representative experiments are shown.**

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**Figure 2.**
Ammonium Assimilation

The ammonium assimilation rate was maximal in the light for both strains (Table IV); however, assimilation by Cyt ox^- cells was much more sensitive to the presence of SHAM. Darkness only slightly decreased the rate of ammonium assimilation by wild type, but resulted in a 75% decrease in Cyt ox^- cells. Addition of SHAM in the dark resulted in approximately a 30% decrease in ammonium assimilation by wild type, and completely inhibited assimilation by the Cyt ox^- strain.

DISCUSSION

The oxidation of mitochondrial reductant (NADH, FADH2) necessary for the maintenance of respiratory carbon flow may occur, in part, by either the Cyt or alternative electron transport chains. Cyt pathway activity is coupled to the synthesis of 3 ATP per electron pair passed to O2, while operation of the alternative pathway yields a maximum of 1 ATP per electron pair (generated via site I [11]). There has been considerable discussion as to the role of the alternative pathway in plant metabolism, but a satisfactory picture has yet to emerge (15). One hypothesis is that the alternative pathway serves to maintain TCA cycle carbon flow in support of biosynthesis when mitochondrial electron transport chain activity is restricted by ADP supply (2, 12, 14).

Maintenance of TCA Cycle Carbon Flow during Dark Ammonium Assimilation

The onset of dark ammonium assimilation resulted in a large increase in respiratory CO2 release in wild-type cells, consistent with an increase in glycolytic and TCA cycle carbon flow necessary for the provision of carbon skeletons for amino acid biosynthesis (Figs. 1 and 2; cf. ref. 16). Concomitant with the increase in respiratory CO2 release was an increase in mitochondrial O2 consumption. During dark ammonium assimilation by wild-type cells, the addition of SHAM had a little effect on respiratory CO2 release or O2 consumption (Fig. 1A), and only a 30% inhibition of NH4^+ assimilation (Table IV).

In the Cyt ox^- strain, the ammonium assimilation-induced stimulation of respiratory CO2 release and O2 consumption (Fig. 1B) was much less than observed in the wild type. This was consistent with the lower rates of ammonium assimilation exhibited by Cyt ox^- cells in the dark (Table IV). Both O2 consumption and TCA cycle CO2 release were SHAM-sensitive in Cyt ox^- cells. Furthermore, the addition of SHAM completely inhibited dark ammonium assimilation (Table IV).
The addition of ammonium to KCN-treated wild-type cells also resulted in a stimulation of O2 consumption and CO2 release, which was shown to be SHAM-sensitive (Fig. 2).

These results imply that respiratory carbon flow to amino acid synthesis was supported by the Cyt pathway in wild-type cells. Only in the Cyt ox− mutant, or in wild-type cells in the presence of KCN, did the alternative pathway play a role in maintaining carbon flow for biosynthesis. These results do not support the hypothesis that the alternative pathway serves to maintain biosynthetic TCA cycle carbon flow.

The use of inhibitors in evaluating the engagement of the alternative pathway may cause artefacts (13). Recently it has been shown that the alternative and Cyt pathways discriminate differentially against 18O2 (7, 19). By examining the discrimination against 18O2 by whole cell respiration it is possible to determine the partitioning of respiratory electron flow between the Cyt and alternative pathways in the absence of inhibitors. Use of this technique confirms the conclusions reached from the inhibitor-based work: the stimulation of mitochondrial O2 consumption during ammonium assimilation by wild-type cells was mediated entirely via the Cyt pathway, and that the alternative pathway was not engaged (Table I). Not surprisingly, the ammonium-induced stimulation of mitochondrial O2 consumption by Cyt ox− cells was mediated via the alternative pathway.

### TCA Cycle Carbon Flow during Photosynthetic Ammonium Assimilation

Although the above results have shown that the alternative pathway did not support respiratory carbon flow to amino acid synthesis in the dark unless the Cyt pathway was absent or inhibited, it was conceivable that the alternative pathway could play a role in supporting carbon flow for biosynthesis during photosynthesis. This apparently is not the case in wild-type cells as there was no SHAM-sensitive O2 consumption in the absence of ammonium (Table II) or during the ammonium assimilation-enhanced respiration (Fig. 3). These results imply that even during photosynthesis the Cyt pathway supports biosynthetic TCA cycle carbon flow.

In the Cyt ox− strain, NH4+ assimilation was nearly fourfold higher in the light than in the dark (Table IV), possibly due to inadequate ATP supply due to the absence of a functional Cyt pathway. Ammonium addition in the light resulted in both increased mitochondrial O2 consumption and TCA cycle CO2 release (Fig. 3B). As expected, this increase was SHAM-sensitive, indicating that it was mediated by the alternative pathway. The addition of SHAM also resulted in approximately a 50% decline in the rate of ammonium assimilation (Table IV).

### Role of Alternative Pathway Respiration

The results presented in this paper indicate that the alternative pathway can indeed function to maintain TCA cycle carbon flow for the provision of intermediates during biosynthesis when the Cyt pathway is nonfunctional (+KCN, Cyt ox− cells). Other work with Euglena has shown that alternative pathway capacity and activity can be induced by growing cells in the presence of antimycin A, which inhibits Cyt pathway activity (3). However, in the presence of a functional Cyt pathway, we were unable to demonstrate engagement of the alternative pathway under any conditions tested. In wild-type cells, the continued reductant oxidation during ammonium assimilation-enhanced TCA cycle activity was mediated exclusively via Cyt pathway activity. This suggests that, under the conditions tested, the alternative pathway is not involved in the maintenance of TCA cycle carbon flow for the provision of biosynthetic intermediates in wild-type cells.

### ACKNOWLEDGMENTS

We wish to thank Dr. R. G. Smith for useful discussions.

### LITERATURE CITED


### Table IV. Ammonium Assimilation Rates by Wild-Type and Cyt ox− Cells under Various Conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NH4+ Assimilation Rate (μmol NH4+·mg−1 Chl·h−1 ± se (n))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>247 ± 19 (3)</td>
</tr>
<tr>
<td>Light + SHAM</td>
<td>208 ± 10 (3)</td>
</tr>
<tr>
<td>Dark</td>
<td>205 ± 10 (4)</td>
</tr>
<tr>
<td>Dark + SHAM</td>
<td>146 ± 7 (5)</td>
</tr>
<tr>
<td>Dark + CCP</td>
<td>0 (2)</td>
</tr>
</tbody>
</table>

IV). The addition of ammonium to KCN-treated wild-type cells also resulted in a stimulation of O2 consumption and CO2 release, which was shown to be SHAM-sensitive (Fig. 2).