Anaerobic Carbon Metabolism by the Tricarboxylic Acid Cycle

Evidence for Partial Oxidative and Reductive Pathways during Dark Ammonium Assimilation

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ABSTRACT

Nitrogen-limited cells of Selenastrum minutum (Naeg.) Collins are able to assimilate NH₄⁺ in the dark under anaerobic conditions. Addition of NH₄⁺ to anaerobic cells results in a threefold increase in tricarboxylic acid cycle (TCAC) CO₂ efflux and an eightfold increase in the rate of anaplerotic carbon fixation via phosphoenolpyruvate carboxylase. Both of these observations are consistent with increased TCAC carbon flow to supply intermediates for amino acid biosynthesis. Addition of H¹³CO₃⁻ to anaerobic cells assimilating NH₄⁺ results in the incorporation of radiolabel into the α-carboxyl carbon of glutamic acid. Incorporation of radiolabel into glutamic acid is not simply a short-term phenomenon following NH₄⁺ addition as the specific activity of glutamic acid increases over time. This indicates that this alga is able to maintain partial oxidative TCAC carbon flow while under anaoxia to supply α-ketoglutarate for glutamate production. During dark aerobic NH₄⁺ assimilation, no radiolabel appears in fumarate or succinate and only a small amount occurs in malate. During anaerobic NH₄⁺ assimilation, these metabolites contain a large proportion of the total radiolabel and radiolabel accumulates in succinate over time. Also, the ratio of dark carbon fixation to NH₄⁺ assimilation is much higher under anaerobic than aerobic conditions. These observations suggest the operation of a partial reductive TCAC from oxaloacetic acid to malate, fumarate, and succinate. Such a pathway might contribute to redox balance in an anaerobic cell maintaining partial oxidative TCAC activity.

The assimilation of NH₄⁺ into amino acids requires carbon skeletons provided by the TCAC (25). In N-limited cells of Selenastrum minutum (Naeg.) Collins, the aerobic assimilation of added NH₄⁺ results in a dramatic increase in oxidative TCAC carbon flow and mitochondrial ETC activity (28–30). Weger and Turpin (30) have shown that under anaoxia, these N-limited cells are able to assimilate NH₄⁺, although at a much reduced rate as compared with aerobic conditions. Under anoxia, the transfer of electrons from Cyt oxidase to O₂ cannot take place and reductant (NADH and FADH₂) produced by the TCAC is no longer oxidized by the mitochondrial ETC. It was therefore of interest to determine whether carbon skeletons for NH₄⁺ assimilation could be supplied by partial oxidative TCAC activity under anoxia. Clearly, if this were the case, the metabolism would have to be altered to accommodate the regeneration of NAD⁺. Typical fermentative end-products such as lactate, ethanol, or malate can only provide an electron sink for the NADH produced by glycolysis and are not able to support active oxidative TCAC carbon flow.

In the present study, we provide evidence that cells of S. minutum are able to maintain partial oxidative TCAC activity under anaerobic conditions for the supply of αKG for net glutamate production. We provide data which suggest that maintenance of partial oxidative TCAC activity under anaerobic conditions may be due, in part, to a reductive pathway from PEP through to OAA, malate, and succinate.

MATERIALS AND METHODS

Organism and Culture Conditions

The green alga Selenastrum minutum (Naeg.) Collins (UTEX 2459) was grown autotrophically in N-limited chemostats as previously described (4). The medium was buffered to pH 8.0 with 25 mM Heps, the growth rate was 0.3 d⁻¹, and steady state Chl concentrations in the chemostats were 1.2 to 1.4 µg mL⁻¹. Growth under N-limitation maximizes the potential biosynthetic demand for TCAC intermediates once the alga is resupplied with a N-source (28).

Experimental Conditions

All experiments were performed with cells in the dark, after they had been taken from the chemostat, concentrated by centrifugation, and resuspended to the desired density in culture supernatant (the only exception to this is in the CO₂ efflux experiments in which a different resuspension buffer is used). The cells were put into a sealed, N₂-purged, water-jacketed (20°C), dark cuvette equipped with a magnetic stirrer and a serum-stoppered sampling port. The cuvette was
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Table in expressed 1.

conditions were maintained by use of a catalase/H₂O₂ system. Strict anaerobiosis was achieved by use of an O₂ scavenging system consisting of 5 mM glucose, 20 μg·mL⁻¹ catalase and 200 μg·mL⁻¹ glucose oxidase (6). After 10 min preincubation under these conditions (dark-aerobic or dark-anaerobic), the experiment was started, usually by injection of NH₄Cl or H¹⁴CO₃⁻ through the sampling port. Samples were then taken from the cuvette for different analyses. Where necessary, samples were taken with a blackened syringe to ensure dark conditions were maintained. The rate of uptake of glucose equiped so that, upon removal of a sample, the sample volume was displaced by an equal volume of N₂. Aerobic conditions were maintained by use of a catalase/H₂O₂ system. Strict anaerobiosis was achieved by use of an O₂ scavenging system consisting of 5 mM glucose, 20 μg·mL⁻¹ catalase and 200 μg·mL⁻¹ glucose oxidase (6). After 10 min preincubation under these conditions (dark-aerobic or dark-anaerobic), the experiment was started, usually by injection of NH₄Cl or H¹⁴CO₃⁻ through the sampling port. Samples were then taken from the cuvette for different analyses. Where necessary, samples were taken with a blackened syringe to ensure dark conditions were maintained. The rate of uptake of glucose

Figure 2. Ethanol production following the transition from aerobic to anaerobic conditions and following NH₄⁺ addition. The steady state concentration of ethanol seen during the aerobic phase (before addition of glucose oxidase) was probably produced when the cells were being concentrated by centrifugation and therefore going anaerobic in the pellet.

Table II. Proportion of Radiolabel in Different Metabolites in Cells Pulsed with H¹⁴CO₃⁻ during NH₄⁺ Assimilation in the Dark

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Total Aqueous Extract Radiolabel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td>Malate</td>
<td>1.9</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.0⁴</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.0⁴</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.4</td>
</tr>
<tr>
<td>Glutamate</td>
<td>5.2 (81.5)⁵</td>
</tr>
<tr>
<td>Glutamine</td>
<td>20.6</td>
</tr>
</tbody>
</table>

⁴ No spot detected on autoradiogram. ⁵ Percent label in the α-carboxyl carbon.

(determined using d-[U-¹⁴C]glucose) was determined to be less than 0.1 μmol·mg⁻¹ Chl·h⁻¹. This rate is insignificant when compared to the three order of magnitude higher rates of starch breakdown in these cells (our unpublished data).

### Dark Carbon Uptake

Cells (5 μg Chl·mL⁻¹) were supplied with Na₂ [⁴CO₃] (specific activity of approximately 0.45 μCi·μmol DIC), and samples were withdrawn over time for determination of total counts fixed. NH₄Cl (1 mM) was then added to the cells and further samples were taken. Samples were immediately injected into 1 mL of kill solution (80% [v/v] aqueous ethanol, 5% [v/v] HCOOH) and the unfixed counts were removed by sparging with a gas stream for 1 h. The remaining acid stable radioactivity was then counted in Aquasol II (DuPont) liquid scintillation cocktail. Samples were also injected into alkaline H₂O for determination of total radioactivity. Total DIC (approximately 3.5–4.0 mM) was measured by IR gas analysis.
Table III. Proportion of Radiolabel in Different Metabolites in Dark Anaerobic Cells Pulsed with H\textsuperscript{14}CO\textsubscript{3} \textsuperscript{-} at Different Times during NH\textsubscript{4}\textsuperscript{+} Assimilation in the Dark

In each case the cells were exposed to H\textsuperscript{14}CO\textsubscript{3} \textsuperscript{-} for 5 min before being killed. Enough NH\textsubscript{4}\textsuperscript{+} was added so that it would not all be assimilated within 45 min.

<table>
<thead>
<tr>
<th>Time after NH\textsubscript{4}\textsuperscript{+} Pulse before H\textsuperscript{14}CO\textsubscript{3} \textsuperscript{-} Added</th>
<th>Total Aqueous Extract Radiolabel</th>
<th>% Label in the (\alpha)-Carboxyl Carbon of Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>Malate</td>
<td>Fumarate</td>
</tr>
<tr>
<td>0</td>
<td>51.0</td>
<td>0.7</td>
</tr>
<tr>
<td>5</td>
<td>48.0</td>
<td>1.0</td>
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<tr>
<td>10</td>
<td>41.0</td>
<td>1.0</td>
</tr>
<tr>
<td>20</td>
<td>43.0</td>
<td>1.5</td>
</tr>
<tr>
<td>40</td>
<td>39.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Respiratory CO\textsubscript{2} Efflux

Respiratory CO\textsubscript{2} efflux was monitored by stable carbon isotope mass spectrometry as described previously (29, 30). This method measures gross CO\textsubscript{2} efflux rather than simply net CO\textsubscript{2} exchange. Cells were resuspended to 3.5 \(\mu\)g Chl mL\textsuperscript{-1} and 22 \(\mu\)g Chl mL\textsuperscript{-1} in aerobic and anaerobic experiments, respectively. CN\textsuperscript{-} was added at a concentration of 40 \(\mu\)M.

Metabolism of H\textsuperscript{14}CO\textsubscript{3} \textsuperscript{-}

Na\textsubscript{14}CO\textsubscript{3} was added to cells immediately following or at different times after the addition of NH\textsubscript{4}Cl. Aliquots of the cells were then withdrawn and killed by injection into hot (90°C) 80% (v/v) ethanol. After two extractions in hot ethanol, the aqueous extract was acidified with formic acid and dried down to remove unfix H\textsuperscript{14}CO\textsubscript{3} \textsuperscript{-}. The extract was then resuspended in H\textsubscript{2}O, partitioned five times against chloroform, dried down, and then resuspended in H\textsubscript{2}O.

Analysis of Radiolabeled Products

An aliquot of the radiolabeled extract (200,000 dpm) was spotted onto paper (Whatman No. 3, 25 \(\times\) 25 cm) for two-dimensional paper chromatography. Chromatograms were developed twice in 95% (v/v) aqueous ethanol:CH\textsubscript{3}OH:H\textsubscript{2}O (80:5:15, v/v/v) in the first dimension and then twice in n-butanol:formic acid:H\textsubscript{2}O (10:2:15, v/v/v, upper phase) in the second dimension. This system allowed good separation of both the amino and organic acids. The position of labeled metabolites on the paper was determined by autoradiography.

Labeled compounds were identified both by cochromatography with authentic radiolabeled compounds and by the use of detection reagents to visualize authentic compounds. Amino acids were detected with 0.5% (w/v) ninhydrin in 95% (v/v) ethanol. The papers were heated to 80°C for 10 min after spraying. Organic acids were detected using an ammoniacal silver nitrate reagent (1).

After they were identified, the radioactive spots were cut out and added to 5 mL of H\textsubscript{2}O. After 1 h, 10 mL of scintillation cocktail was added and radioactivity determined by liquid scintillation counting.

The proportion of radiolabel in the \(\alpha\)-carboxyl carbon of glutamic acid was determined by ninhydrin decarboxylation (5) after elution of the glutamic acid from the paper.

Metabolite Determinations

To determine the specific activity of glutamic acid, a portion of the radiolabeled extract was used to measure glutamic acid concentration. This was done by an enzymatic method (31) using glutamate dehydrogenase (Boehringer Mannheim).

Ethanol was measured in neutralized perchloric acid extracts of cells using an enzymatic kit from Boehringer Mannheim (No. 176290)

Other Methods

Ammonium assimilation was measured by following the disappearance of NH\textsubscript{4}\textsuperscript{+} from the medium over time (30). Chl was measured after extraction in 100% methanol (15).

RESULTS AND DISCUSSION

Oxidative TCAC Carbon Metabolism during Anaerobiosis

Previous work on NH\textsubscript{4}\textsuperscript{+} assimilation by Selenastrum minutum has highlighted the role of the TCAC and the mitochondrial ETC in the provision of carbon skeletons for amino acid biosynthesis (28–30). The addition of NH\textsubscript{4}\textsuperscript{+} to aerobic N-limited S. minutum results in a dramatic increase in the rate of TCAC CO\textsubscript{2} release (Fig. 1A; ref. 29). This is consistent with increased TCAC carbon flow to provide \(\alpha\)KG for the GS-GOGAT reaction which is the principal pathway by which plants and green algae assimilate NH\textsubscript{4}\textsuperscript{+} into amino acids (25). Associated with the increased biosynthetic demand for TCAC carbon is an increase in dark CO\textsubscript{2} fixation (4). This is due to increased PEPC activity (11), an anaplerotic reaction which serves to replenish TCAC intermediates (20, 21). This increase in biosynthetic TCAC activity is accompanied by an increase in mitochondrial ETC activity (29).

The ability of S. minutum to assimilate NH\textsubscript{4}\textsuperscript{+} anaerobically implies maintenance of partial TCAC carbon flow under anaerobic conditions. If this is the case, assimilation of NH\textsubscript{4}\textsuperscript{+} by anoxic cells should result in an increase in the rate of dark
carbon fixation. Table I shows that there is a 20-fold and an 8-fold increase in the rate of dark carbon fixation during aerobic and anaerobic NH₄⁺ assimilation, respectively.

If the TCAC is providing carbon skeletons for amino acid synthesis in anaerobic cells, then NH₄⁺ addition should also stimulate TCAC CO₂ efflux. Figure 1 shows that there is a dramatic increase in the rate of CO₂ efflux when either aerobic or anaerobic cells assimilate NH₄⁺. Aerobic CO₂ efflux is inhibited by cyanide (29), but under anaerobic conditions CO₂ efflux is unaffected (Fig. 1B). This is expected since aerobic regeneration of TCAC generated reductant is via the Cyt ETC (29) whereas the ETC cannot serve this function under anoxia.

As this alga has a high rate of ethanol production under anoxia (Fig. 2), it was necessary to confirm that the increased CO₂ efflux observed upon NH₄⁺ addition to anaerobic cells was due to an increase in the rate of ethanol production via pyruvate decarboxylase. Previous work with other green algae has suggested that ethanol may be produced via pyruvate formate lyase and acetaldehyde dehydrogenase and therefore
not result in CO₂ production (9, 18). Regardless of the pathway of ethanol synthesis in *S. minutum*, Figure 2 shows that NH₄⁺ had no appreciable effect on the rate of ethanol production, implying that the NH₄⁺ induced increase in CO₂ release could not be due to increased ethanol production.

The final line of evidence for anaerobic oxidative TCAC carbon metabolism during NH₄⁺ assimilation was obtained by examining the synthesis of glutamic acid. Synthesis of glutamic acid via either glutamate dehydrogenase or GS-GOGAT requires αKG (25). If αKG is being supplied by the TCAC under anaerobic conditions, then fixation of H^14CO₃⁻ by PEPC should result in radiolabel incorporation into the α-carboxyl carbon of glutamic acid. When dark, anaerobic cells were pulsed with NH₄⁺ and H^14CO₃⁻, radiolabel appeared in glutamic acid (Tables II, III, IV). Ninhydrin decarboxylation of this glutamic acid showed that the majority of incorporated radiolabel was indeed in the α-carboxyl carbon regardless of whether the cells were aerobic or anaerobic (Tables II, III).

Evidence that TCAC carbon flow to glutamate synthesis was sustained during long periods of anaerobiosis was provided by experiments in which H^14CO₃⁻ was added at different times following the addition of NH₄⁺ (Table III). Again, radiolabel appears in glutamic acid, even when H^14CO₃⁻ is added 40 min after the NH₄⁺ addition. The majority of radiolabel is again localized in the α-carboxyl carbon of glutamic acid (Table III). The proportion of the total aqueous extract radiolabel found in glutamic acid decreases if the addition of H^14CO₃⁻ is delayed (Table III), but this is due to the dramatic decrease in the pool size of glutamic acid during anaerobic NH₄⁺ assimilation (Fig. 3). The important observation is that the specific activity of glutamic acid continues to increase over the course of the experiment, suggesting continued flux of radiolabel into this compound (Fig. 3). We therefore conclude that this alga is capable of maintaining partial oxidative TCAC activity under anoxic conditions to supply αKG for net glutamate synthesis.

Studies of anaerobic metabolism in *Echinochloa crus-galli* has provided some evidence for anaerobic TCAC activity. Feeding experiments with [14C]sucrose and [14C]glucose resulted in radiolabel incorporation into TCAC intermediates (26) and there was considerable in vitro activity of mitochondrial enzymes in *Echinochloa* seedlings grown under anaerobic conditions (8, 17). To our knowledge, however, the present study is the first study demonstrating oxidative TCAC carbon flow to αKG occurring in a photosynthetic tissue under anaerobic conditions.

### Table IV. Proportion of Radiolabel in Different Metabolites in Dark Anaerobic Cells Assimilating NH₄⁺

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Total Aqueous Extract Radiolabel*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Malate</td>
<td>44.4</td>
</tr>
<tr>
<td>Succinate</td>
<td>4.6</td>
</tr>
<tr>
<td>Fumarate</td>
<td>1.6</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.6</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* The total aqueous extract radiolabel represented 86% (5 min), 90% (10 min), 89% (15 min), and 92% (20 min) of the total acid stable radioactivity incorporated at that time (first experiment) and 78% (5 min), 84% (10 min), 93% (15 min), and 88% (20 min) of the total acid stable radioactivity incorporated at that time (second experiment).

### Reductive TCAC Carbon Metabolism during Anaerobiosis

Maintenance of partial oxidative TCAC activity to αKG requires oxidation of NADH produced in the reactions catalyzed by glyceraldehyde 3-phosphate dehydrogenase, pyruvate dehydrogenase, and isocitrate dehydrogenase (Fig. 4). Glycolytic fermentative end products, such as lactate or ethanol, produced under anaerobiosis are unable to sustain oxidative TCAC activity as they only provide an electron sink for the NADH produced in glycolysis. Malate has often been considered as a normal end product of glycolysis in plant tissue (19). Moreover, it has frequently been reported to accumulate in anoxic plant tissue (2, 22). However, like lactate or ethanol fermentation, malate can only provide an electron sink for the NADH produced in glycolysis (20). For partial oxidative TCAC carbon flow to be maintained under anoxia, there must be other electron sink(s) capable of regenerating NAD⁺.

Radiolabeling experiments have shown that the flow of carbon from dark H^14CO₃⁻ fixation differs dramatically in aerobic versus anaerobic cells (Table II). Under aerobic conditions, glutamate and glutamine contained a high proportion of the total aqueous extract radiolabel. No label was detected in succinate or fumarate because decarboxylation of αKG to produce succinyl-CoA removes the labeled carbon (Fig. 4). Malate contains some label, probably because the equilibrium between malate and OAA lies heavily in favor of malate. Under anaerobic conditions, glutamate and glutamine still become labeled, but the greatest proportion of label is found in malate, fumarate, and succinate (Tables II, III, IV). As with glutamic acid, the incorporation of radiolabel into these metabolites is sustained during prolonged periods of anoxia (Table III). Also, time course experiments indicate that there is radiolabel accumulation in succinate over time (Table IV). This suggests that during anaerobic NH₄⁺ assimilation, PEPC
serves not only an anaplerotic function supplying carbon for amino acid synthesis, but it also provides OAA which serves as substrate for a reductive path to malate, fumarate, and succinate (Fig. 4). In this situation, the rate of dark anaerobic carbon fixation during NH₄⁺ assimilation should be higher than would be expected if PEPC was functioning simply to provide anaplerotic substrate to the TCAC. Under aerobic conditions, when PEPC serves only the anaplerotic function, the ratio of dark carbon fixation per NH₄⁺ assimilated is approximately 0.3 (Table V). During anaerobic NH₄⁺ assimilation, the ratio increases to approximately 1.0.

There have been only a handful of reports on the anaerobic accumulation of succinate in plants, but there is little evidence for any particular metabolic pathway leading to its accumulation (2, 23, 24). Reductive TCAC carbon flow has been hypothesized as a mechanism by which the host cells of root nodules might produce C₆-dicarboxylic acids such as succinate to supply bacteria (32). Operation of a reductive pathway from OAA to succinate has been extensively studied in facultatively anaerobic invertebrates (3, 13, 14). Succinate is also a major by-product in the fermentation of yeast where its synthesis can occur via an oxidative or reductive TCAC (12). Although we have provided evidence of a reductive pathway from OAA to succinate in this alga during anaerobiosis, we do not know the relative flux of carbon between the oxidative and reductive paths of the TCAC. We therefore do not know the significance of the reductive pathway in the maintenance of oxidative TCAC carbon flow relative to other potential processes such as H₂ evolution (9, 10). We also do not know the mechanism by which fumarate is reduced to succinate. A fumarate reductase unique from succinate dehydrogenase has not yet been shown to occur in plants. It is possible that the enzyme responsible for fumarate reduction is succinate dehydrogenase since it catalyzes a freely reversible reaction (ΔG°' ~ 0). Succinate dehydrogenase has been implicated in the reduction of fumarate in facultatively anaerobic animals (16) and in Euglena (27). It has been shown that fumarate reduction may be linked to NADH oxidation and ATP synthesis (7 and references therein) thereby providing an excellent strategy for anaerobic fermentation.

CONCLUSIONS

We have provided evidence that the N-limited green alga Selenastrum minutum is capable of maintaining partial oxidative TCAC activity during anaerobic NH₄⁺ assimilation. We have also provided evidence that a reductive pathway from OAA to malate, fumarate, and succinate may contribute to overall redox balance.

ACKNOWLEDGMENTS

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LITERATURE CITED