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Anaerobic Metabolism in the N-Limited Green Alga *Selenastrum minutum*[1]

I. Regulation of Carbon Metabolism and Succinate as a Fermentation Product

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ABSTRACT

The onset of anaerobiosis in darkened, N-limited cells of the green alga *Selenastrum minutum* (Naeg.) Collins elicited the following metabolic responses. There was a rapid decrease in energy charge from 0.85 to a stable lower value of 0.6 accompanied by rapid increases in pyruvate/phospho*enol*pyruvate and fructose-1,6-bisphosphate/fructose-6-phosphate ratios indicating activation of pyruvate kinase and 6-phosphofructokinase, respectively. There was also a large increase in fructose-2,6-bisphosphate, which, since this alga lacks pyrophosphate dependent 6-phosphofructokinase, can be inferred to inhibit gluconeogenic fructose-1,6-bisphosphatase activity. These changes resulted in an approximately twofold increase in the rate of starch breakdown indicating a Pasteur effect. The Pasteur effect was accompanied by accumulation of D-lactate, ethanol and succinate as fermentation end-products, but not malate. Accumulation of succinate was facilitated by reductive carbon metabolism by a partial TCA cycle (GC Vanlerberghe, AK Horsey, HG Weger, DH Turpin [1989] Plant Physiol 91: 1551–1557). An initial stoichiometric decline in aspartate and increases in succinate and alanine suggests that aspartate catabolism provides an initial source of carbon for reduction to succinate under anoxic conditions. These observations allow us to develop a model for the regulation of anaerobic carbon metabolism and a model for short-term and long-term strategies for succinate accumulation in a green alga.

Changes in levels of glycolytic intermediates in response to anoxia have been reported in higher plants (4, 11, 14, 19). Such studies have clearly pointed to PFK[2] and PK as key regulatory enzymes in glycolysis because these reactions are far removed from equilibrium *in vivo* and increased glycolytic flux during anoxia (the Pasteur effect) is accompanied by large increases in FBP/F6P and pyr/PEP ratios (37). A Pasteur effect also has been shown to occur in green algae (13, 28) but these observations have not been accompanied by a detailed analysis of changes in glycolytic intermediates. There has been speculation that PFK is also important in regulating anaerobic carbon metabolism in green algae (13).

Fru-2,6-P_2 is an important effector of plant PFP and FBPase and therefore a key regulator of plant respiration (2). Studies have reported increases in Fru-2,6-P_2 in response to anoxia in higher plants (22, 27) yet it is unclear how Fru-2,6-P_2 contributes to increased glycolytic flux under these conditions. One possibility is that by inhibiting the cytosolic FBPase it stops gluconeogenic carbon flow. A second possibility is that by stimulating PFP, the forward (glycolytic) reaction may provide FBP for glycolysis or conversely, the reverse (gluconeogenic) reaction may provide PPi for use in sucrose mobilization (1, 2, 33, 34). In the green alga *Selenastrum minutum*, there is no PFP (5, 38), but a cytosolic FBPase does exist and is strongly inhibited by Fru-2,6-P_2 (6, 38). The absence of PFP in this alga means that a role of Fru-2,6-P_2 in regulating cytosolic FBPase during changes in glycolytic flux can be tested unambiguously.

In higher plants, anaerobiosis has been shown to lead to an accumulation of succinate (9, 25, 26), and Ala (9, 20, 25, 26, 30, 36) and a decline in Asp (9, 36). Recently, we have shown that succinate is synthesized in *S. minutum* in response to anoxia via a partial reductive TCA cycle (39). This is analogous to the pathway of succinate accumulation in facultatively anaerobic invertebrates (8). In these organisms, Asp catabolism provides a mechanism whereby succinate and Ala accumulate as fermentation products (8). The possibility that Asp, Ala, and succinate metabolism may be linked has been suggested for plants under anoxia (36) but no studies have examined the response of all three metabolites to anoxia.

In the present study we examined changes in the levels of key metabolic intermediates and Fru-2,6-P_2 in cells of the green alga *S. minutum* during a transition to anoxia. This enabled us to identify the regulatory enzymes responsible for the Pasteur effect in this alga and to examine the role of Fru-2,6-P_2 in regulating anaerobic carbon metabolism in an organism lacking PFP. We also show that succinate is a product of fermentation in this alga and that Asp catabolism accounts for the initial accumulation of succinate and Ala under anoxic conditions. We show that D-lactate and ethanol are important fermentative end-products but that malate is not.

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[2] Abbreviations: PFK, 6-phosphofructokinase; AEC, adenylate energy charge; DHAP, dihydroxyacetone phosphate; FBP, fructose-1,6-bisphosphate; FBPase, fructose-1,6-bisphosphatase; F6P, fructose-6-phosphate; Fru-2,6-P_2, fructose-2,6-bisphosphate; OAA, oxaloacetate; PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase; PFP, pyrophosphate dependent 6-phosphofructokinase; 3-PGA, 3-phosphoglycerate; PK, pyruvate kinase; pyr, pyruvate; TP, triose phosphate.

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 (39).

Experimental Conditions

Experiments were performed with cells in the dark, after they had been taken from the chemostat, concentrated by centrifugation, and resuspended to approximately 40 μg Chl·mL^{-1} in culture supernatant. These cells were not resupplied with any N source after being taken from the chemostat. The cells were put in a darkened, water-jacketed (20°C) cuvette equipped with a magnetic stirrer and a sparger through which the cells were bubbled with humidified, temperature equilibrated (20°C) air. Cells were preincubated in this way for 40 min before sampling for metabolites began. Strict anaerobiosis was achieved by use of a O_2 scavenging system (10) consisting of 5 mM glucose, 40 μg·mL^{-1} catalase, and 400 μg·mL^{-1} glucose oxidase and by blowing the headspace of the cuvette with N_2. The glucose was added during the 40 min preincubation. It has been shown previously that these cells are unable to utilize exogenous glucose (39). All data reported are the means of triplicate experiments unless otherwise indicated.

Metabolite Extraction

Cell samples (1 mL) were killed and extracted by one of two methods. For ATP, ADP, AMP, pyr, PEP, ethanol, and D-lactate determinations the samples were injected into $HClO_4$ (final concentration 10%), frozen in liquid N_2 and neutralized with 5 N KOH/1 M triethanolamine. For the other metabolites the samples were immediately frozen in liquid N_2 and lyophilized. The lyophilized samples were then resuspended in a mixture containing 1.5 mL $CHCl_3$, 3.5 mL methanol, and 0.6 mL 20 mM Hepes (pH 8.5) with 5 mM EGTA and 50 mM NaF. They were then incubated at 4°C for 40 min. After adding 3 mL H_2O the samples were centrifuged and the upper aqueous phase dried (10 min, 38°C) by rotary evaporation and resuspended in H_2O.

Metabolite Analysis

ATP, ADP, AMP, pyr, PEP, F6P, FBP, 3-PGA, TP, and malate were all measured using coupled enzymatic assays and a dual-wavelength spectrophotometer (ZFP 22, Sigma Instruments, FRG) as previously described (38). D-Lactate, ethanol, and succinate were measured enzymatically as outlined in the Boehringer Mannheim test kit Nos. 139084, 176290, and 176281, respectively. Amino acids were analyzed employing HPLC of *o*-phthaldehyde derivatives on a C-18 reverse-phase column (Waters Scientific, Missisauga, Ontario).

Fru-2,6-P_2 was measured enzymatically as outlined by Van Shaftingen *et al.* (41) using a Milton Roy Spectronic 3000 diode array spectrophotometer equipped with a multiple cuvette holder. The assay buffer contained 90 mM Tris-HCl (pH 8.1), 4.5 mM $MgCl_2$, 2 mM F6P, 260 μM NADH, 520 μM PP_i, glycerol-3-phosphate dehydrogenase (1 unit), triose-P isomerase (1 unit), aldolase (0.15 unit) and PFP (0.17 unit). The PFP was purified from potato as previously described (38). For each sample determination, a linear standard curve for Fru-2,6-P_2 was produced simultaneously. This was done using acid treated sample to make up three internal standards and a blank. The assay was started by addition of sample or standard and the reaction was followed for 20 min during which time the reaction rate remained linear. At two different times during the course of the experiments duplicate samples were taken and one was immediately spiked with a known concentration of Fru-2,6-P_2. From these samples Fru-2,6-P_2 recovery was determined to be 72.4 ± 4.3% (for samples spiked at 10 min, SD, n = 3) and 66.2 ± 9.1% (for samples spiked at 25 min, SD, n = 3).

Starch Breakdown

Cells were taken from the chemostat, concentrated by centrifugation, and resuspended to approximately 10 μg Chl·mL^{-1} in culture supernatant. They were then preincubated in the dark for 1 h (bubbling with air). The aerobic treatment was then carried out in the darkened cuvette (described above) while the anaerobic treatment was carried out in capped 1.5 mL mini-centrifuge tubes with the glucose oxidase/catalase system present. The tubes were incubated at 20°C. Triplicate samples for starch analysis were taken over time and killed in $HClO_4$ (final concentration 10%). After 15 min on ice, the samples were centrifuged and the supernatant discarded. The pellet was washed once with H_2O and then resuspended in H_2O and lyophilized. It was then resuspended into 0.02 N NaOH, autoclaved for 100 min (121°C, 14 psi), sonicated for 2 × 10 s and then autoclaved another 100 min. Autoclaving in 0.02 N NaOH solubilizes the starch and destroys contaminating glucose present in the cells and in the medium (glucose is used in the glucose oxidase/catalase system). The samples were then treated with α-amylase (20 units) and amyloglucosidase (2 units) for 15 h at 55°C in 200 mM Na-acetate (pH 5.0). The glucose produced was analyzed as previously described (32).

Other Methods

Chl was measured after extraction in 100% methanol (16). AEC was calculated as follows: AEC = (ATP + 0.5 ADP)/(ATP + ADP + AMP) (29). *In vivo* dark carbon fixation via

Table I. *Carbon Budget in S. minutum Cells under Anoxia*

Metabolite	Triose Equivalents[a]	
	Consumed	Produced
	μmol·mg^{-1} Chl·h^{-1}	
Starch	50.6	
D-Lactate		29.9
Ethanol		8.5
Succinate		4.5
	50.6	42.9

Carbon recovery = (42.9/50.6)*100 = 84.8%

[a] One glucose consumed is equivalent to 2 trioses consumed. Each molecule of D-lactate, ethanol, or succinate produced consumes 1 triose.

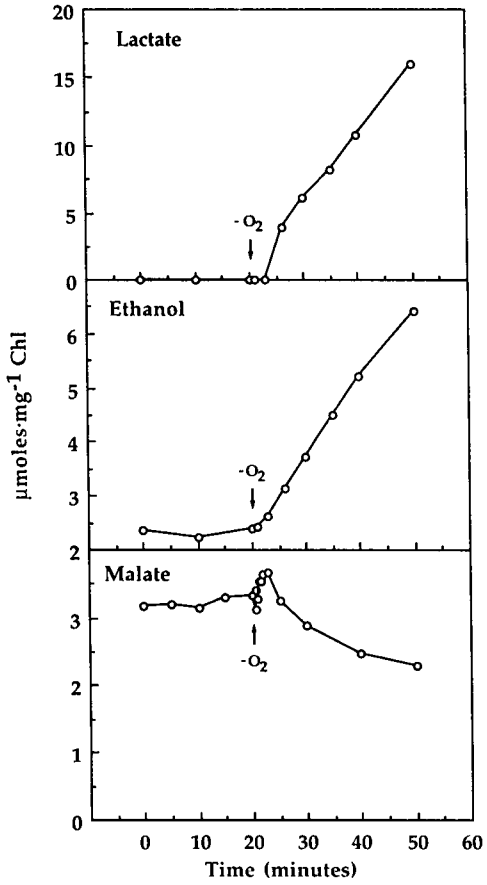

Figure 1. Changes in D-lactate, ethanol, and malate in *S. minutum* during anaerobiosis. The anaerobic treatment began at 20.5 min where indicated by the arrow. The data is the average of three experiments.

PEPC was measured by following the incorporation of $H^{14}CO_3^-$ into acid stable products as previously described (39, 40).

RESULTS

Starch Breakdown

Dark aerobic starch breakdown occurred at a rate of 13.0 μmol glucose·mg^{-1} Chl·h^{-1}. The rate of dark anaerobic starch breakdown was 25.3 μmol glucose·mg^{-1} Chl·h^{-1}. Accumulation of D-lactate, ethanol, and succinate (see below) accounts for 84.8% of the starch degraded over the 30 min anaerobic treatment.(Table I).

D-Lactate, Ethanol, Malate

D-Lactate and ethanol are both important fermentative products (Fig. 1). Production of these metabolites was linear over the first 30 min of anaerobiosis. D-Lactate accumulated at a rate of 29.9 μmol·mg^{-1} Chl·h^{-1} while ethanol accumulated at a rate of 8.5 μmol·mg^{-1} Chl·h^{-1}. Enzymatic determination of lactate requires either D-lactate dehydrogenase or L-lactate dehydrogenase. No lactate was found with L-lactate dehydrogenase and we therefore conclude that only the D isomer of lactate is produced in this alga.

There is a transient increase in malate over the first 2 to 3 min of anoxia. This is followed, however, by a gradual, long-term decline in malate level (Fig. 1).

Succinate, Aspartate, Alanine

Anaerobiosis resulted in a rapid drop in Asp and increases in succinate and Ala (Fig. 2). Close examination of the data showed that the first metabolite to change was Asp. The stoichiometric relationship between changes in these metabolite levels over the first 10 min of anaerobiosis was determined by plotting levels of the following pairs of metabolites: Asp *versus* Ala; Asp *versus* succinate; and succinate *versus* Ala. Linear regression analysis indicated that there is an inverse relationship between Asp and Ala levels and between Asp and succinate levels. A stoichiometry of ~1.19 mol of Asp depleted per mol of Ala accumulated ($r^2 = 0.91$; $n = 10$) was observed. Similarly, ~0.81 mol of Asp were depleted per mol of succinate accumulated ($r^2 = 0.71$; $n = 10$). Succinate and Ala levels increase in the same manner with a stoichi-

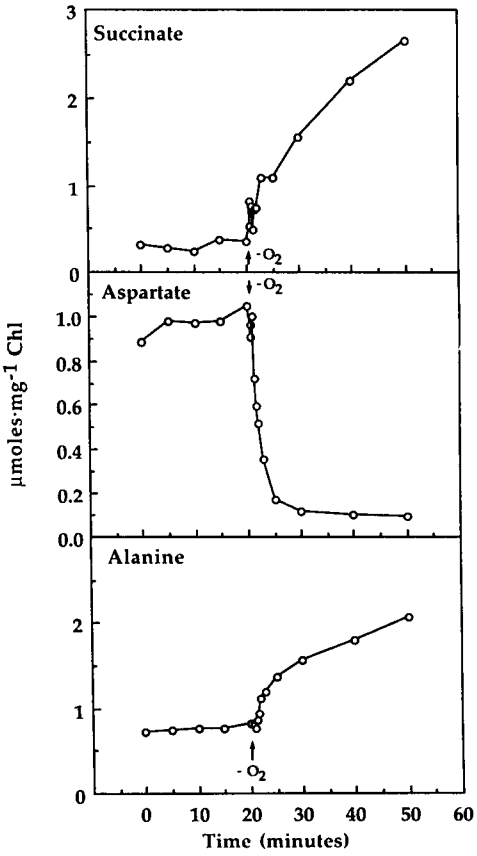

Figure 2. Changes in succinate, Asp, and Ala in *S. minutum* during anaerobiosis. The anaerobic treatment began at 20.5 min where indicated by the arrow. The sixth point on the graph is 10 s after the anoxic treatment has begun. The data is the average of three experiments.

Table II. *Carbon Fixation in Darkened Cells of S. minutum under Aerobic and Anaerobic Conditions*

Treatment	Dark Carbon Fixation
	$\mu mol\ CO_2 \cdot mg^{-1}\ Chl \cdot h^{-1}$
Aerobic	3.9
After 5 min anaerobic	0.4
After 20 min anaerobic	1.6

ometry of ~1.12 mol succinate per mol Ala ($r^2 = 0.76$; $n = 10$). After 10 min of anaerobiosis, Asp was depleted and Ala accumulation dropped off to a low rate (~1.5 $\mu mol \cdot mg^{-1}$ $Chl \cdot h^{-1}$) while succinate continued to accumulate (Fig. 2). Over the first 30 min of anaerobiosis succinate accumulated at an average rate of 4.5 $\mu mol \cdot mg^{-1}$ $Chl \cdot h^{-1}$.

Dark Carbon Fixation

The rate of aerobic dark carbon fixation was ~3.9 μmol $CO_2 \cdot mg^{-1}$ $Chl \cdot h^{-1}$ (Table II). Within 5 min after going anaerobic this declined dramatically to ~0.4 μmol $CO_2 \cdot mg^{-1}$ $Chl \cdot h^{-1}$. After 20 min of anaerobiosis, however, the rate had increased to ~1.6 μmol $CO_2 \cdot mg^{-1}$ $Chl \cdot h^{-1}$.

Adenylates

After 30 s of anoxia, ATP levels had dropped by 38% and ADP and AMP rose 1.5-fold and 6-fold, respectively (Fig. 3). Over this same time, AEC decreased from 0.85 to 0.6 and then remained stable at 0.6 over a 30 min period of anaerobiosis (Fig. 3).

PEP, pyr, F6P, FBP

After 30 s of anoxia the level of PEP dropped by 20% and pyr increased 68% resulting in a twofold increase in the pyr/PEP ratio (Fig. 4). This ratio continued to rise rapidly and had increased sixfold after 9.5 min of anoxia.

After 30 s of anoxia, F6P levels dropped 14% and FBP levels increased 13% resulting in a 1.3-fold increase in the FBP/F6P ratio (Fig. 5). This ratio continued to rise rapidly and after 9.5 min of anoxia had increased threefold.

Fru-2,6-P_2

The level of Fru-2,6-P_2 almost doubled after 20s of anaerobiosis. The level then rose for another 10 min to stabilize at approximately 3.4-fold its aerobic level (Fig. 6).

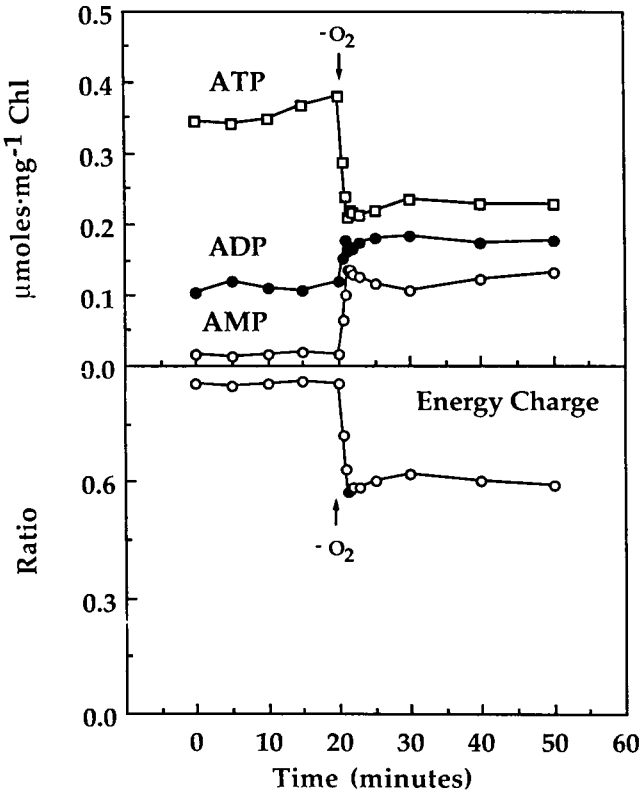

Figure 3. Changes in ATP, ADP, AMP, and the AEC in *S. minutum* during anaerobiosis. The anaerobic treatment began at 20.5 min where indicated by the arrow. The sixth point on the graph is 10 s after the anoxic treatment has begun. The data is the average of three experiments.

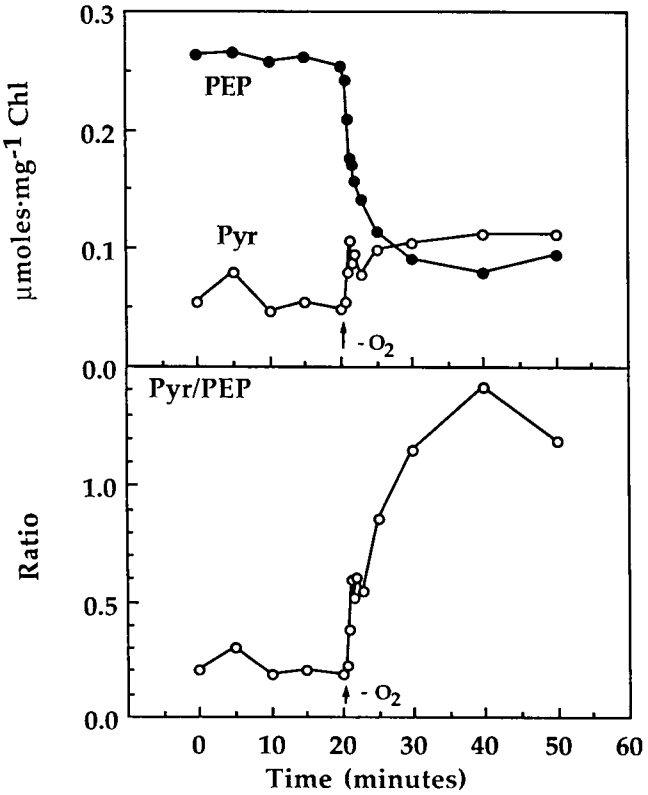

Figure 4. Changes in PEP, pyr, and the pyr/PEP ratio in *S. minutum* during anaerobiosis. The anaerobic treatment began at 20.5 min where indicated by the arrow. The sixth point on the graph is 10 s after the anoxic treatment has begun. The data is the average of three experiments.

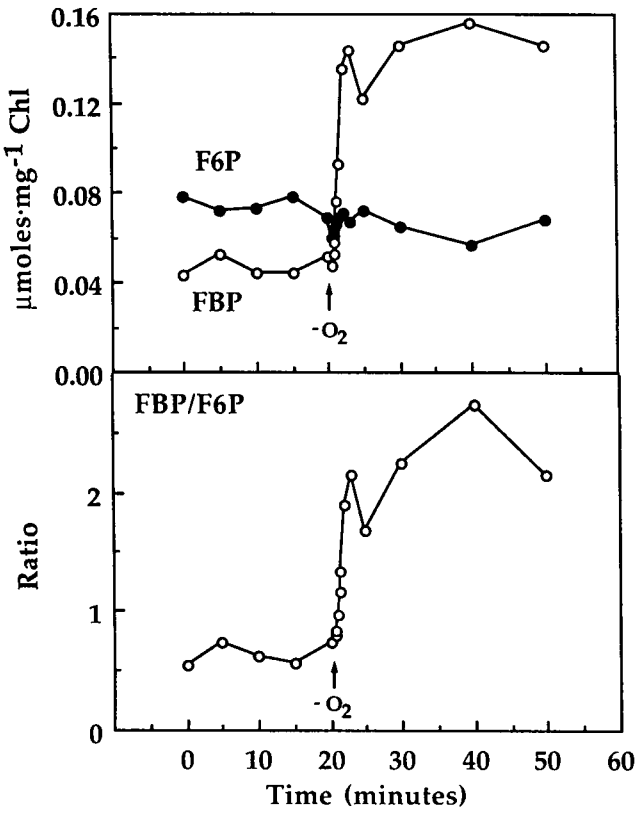

Figure 5. Changes in F6P, FBP, and the FBP/F6P ratio in *S. minutum* during anaerobiosis. The anaerobic treatment began at 20.5 min where indicated by the arrow. The sixth point on the graph is 10 s after the anoxic treatment has begun. The data is the average of three experiments.

3-PGA, TP

Anoxia resulted in a large drop in 3-PGA which paralleled the drop in PEP (Fig. 7). Both metabolites had declined by more than 50% after 2.5 min of anaerobiosis. TP (predominantly DHAP) levels doubled after 1.5 min of anoxia (Fig. 7).

DISCUSSION

Pasteur Effect

The Pasteur effect is the acceleration of glycolysis under anoxia, believed to occur so that the cell can meet its ATP utilization requirements despite the much lower efficiency of ATP production by fermentation compared to aerobic respiration. Only recently has a Pasteur effect been demonstrated in green algae (13, 28). In the present work with the N-limited green alga *S. minutum,* the onset of anaerobiosis resulted in an approximately two-fold increase in the rate of starch breakdown (see "Results").

Fermentation Products

D-Lactate and Ethanol

D-Lactate and ethanol are important fermentation products in this alga (Fig. 1). Both begin to accumulate almost imme-

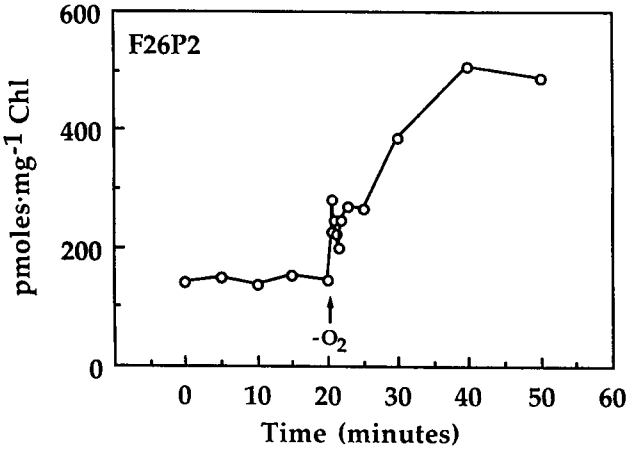

Figure 6. Changes in Fru-2,6-P_2 in *S. minutum* during anaerobiosis. The anaerobic treatment began at 20.5 min where indicated by the arrow. The sixth point on the graph is 10 s after the anoxic treatment has begun. The data is the average of three experiments.

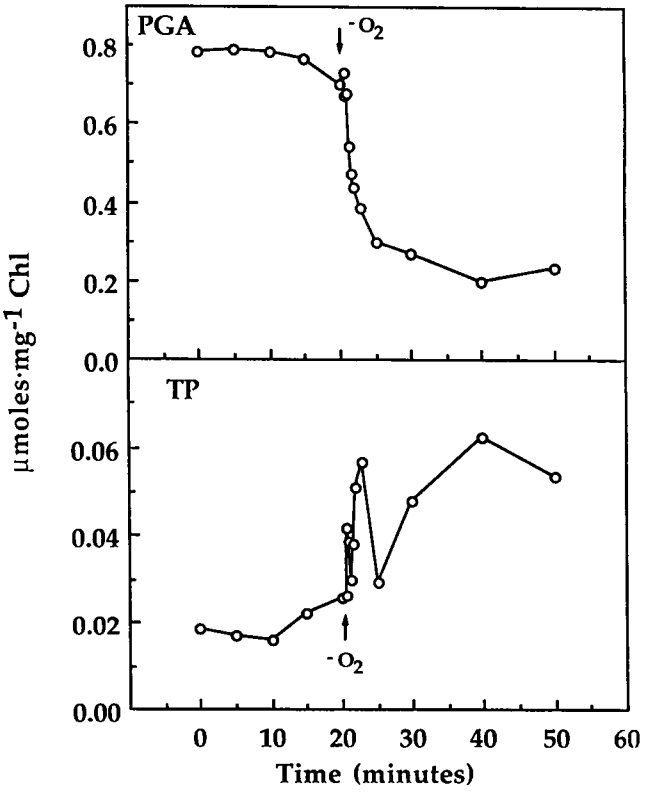

Figure 7. Changes in 3-PGA and TP (predominantly DHAP) in *S. minutum* during anaerobiosis. The anaerobic treatment began at 20.5 min where indicated by the arrow. The sixth point on the graph is 10 s after the anoxic treatment has begun. The data is the average of three experiments.

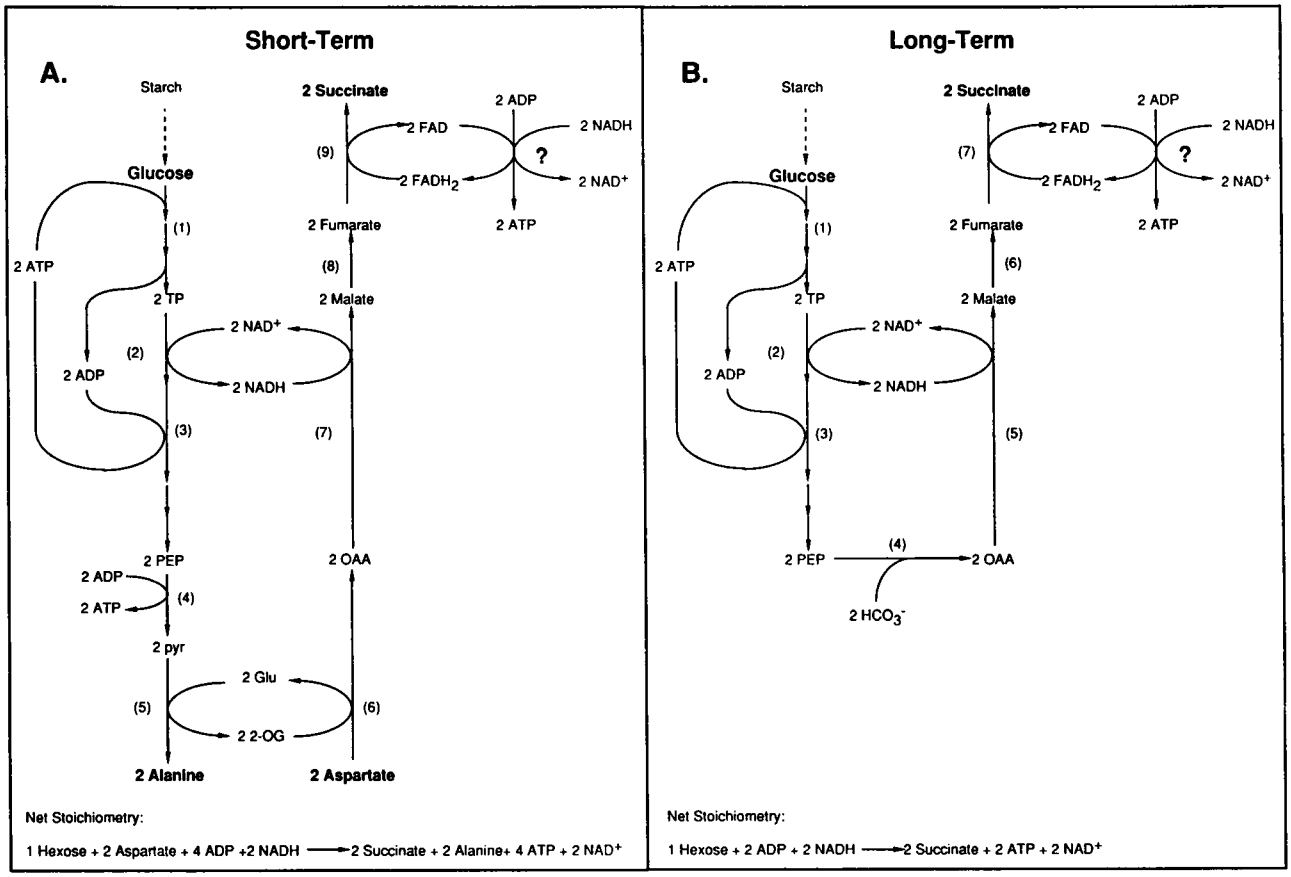

Figure 8. Proposed pathways to provide OAA for reduction to succinate. In the short-term (A) Asp provides the carbon skeleton for OAA. This pathway results in a 1:1 relationship between the production of succinate and Ala and the depletion of Asp and occurs until available Asp is depleted. Enzymes designated in (A) are: (1) hexokinase, phosphoglucose isomerase, PFK; (2) glyceraldehyde 3-phosphate dehydrogenase; (3) phosphoglycerate kinase; (4) PK; (5) glutamate-pyruvate aminotransferase; (6) glutamate-oxaloacetate aminotransferase; (7) malate dehydrogenase; (8) fumarase; (9) succinate dehydrogenase/fumarate reductase (see 39 and "Discussion"). The long-term pathway (B) occurs once Asp is depleted. In this pathway OAA is synthesized via the carboxylation of PEP by PEPC (39). Enzymes designated in (B) are (1) hexokinase, phosphoglucose isomerase, PFK; (2) glyceraldehyde 3-phosphate dehydrogenase; (3) phosphoglycerate kinase; (4) PEPC; (5) malate dehydrogenase; (6) fumarase; (7) succinate dehydrogenase/fumarate reductase (see 39 and discussion). Both of the above pathways assume starch degradation via an amylase. However, if starch is degraded via a starch phosphorylase, ATP yield will increase by 0.5 ATP per succinate produced due to bypassing of hexokinase.

diately upon anaerobiosis. Production of only the D isomer of lactate is similar to what has been found in other green algae (15, 18, 21).

Succinate

This study demonstrates an increase in succinate pool size under anaerobic conditions (Fig. 2) showing its role as a fermentation product in this alga. Others have recently reported succinate as a fermentative end-product in higher plants (25, 26). The present study is, to our knowledge, the first report of such a finding in a green alga.

We previously have shown that under anaerobic conditions, succinate is synthesized via a partial reductive TCA cycle in which OAA is reduced to malate, fumarate, and succinate (39). In facultatively anaerobic invertebrates, the production of succinate under anoxia is linked to the catabolism of Asp (8). In this scheme, Asp provides the OAA for reduction to succinate. The amino group of Asp is conserved by transaminating pyr to Ala. Glycogen provides the pyr required for Ala synthesis. According to this pathway there should be a 1:1 relationship between the production of succinate and Ala and the depletion of Asp. Analysis of metabolite data suggests that a similar pathway of Asp catabolism occurs in *S. minutum* over the first 10 min of anaerobiosis, until available Asp is depleted. The stoichiometries of Asp decline and Ala and succinate increases are reasonably close to the expected 1:1 relationship (see "Results"). Figure 8A depicts how Asp catabolism is linked to Ala and succinate synthesis. Since succinate accumulation continues after Asp is exhausted there must be another source of OAA for this reductive pathway. As shown previously in radiotracer studies (39), an important source of OAA is the carboxylation of PEP by PEPC (Fig. 8B). Table II shows that carbon fixation by PEPC is very low (~0.4 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$) after 5 min of anaerobiosis, when Asp catabolism is providing the OAA for reduction to succinate.

However, once Asp is depleted, carbon fixation increases ($\sim$1.6 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$) indicating increased synthesis of OAA by PEPC. This rate of synthesis of OAA by PEPC along with the long-term rate of decline of malate (Fig. 1) would provide the carbon required to support the observed rates of succinate synthesis.

Figure 8, A and B, compares the short-term and long-term strategies to produce OAA for reduction to succinate. Succinate accumulation is an excellent strategy for anaerobic fermentation because fumarate reduction may be linked to NADH oxidation and possibly to ATP synthesis (12). It is envisioned in this scheme (Fig. 8) that NADH oxidation is linked to reduction of FAD through Complex I and Complex II of the mitochondrial electron transport chain. The proton gradient produced can be used to synthesize ATP. $FADH_2$ oxidation then is linked to fumarate reduction producing succinate. If OAA is generated via Asp catabolism, there is a production of 2 NAD^+ and 4 ATP per hexose (Fig. 8A). This pathway would provide a two-fold increase in the ATP yield per hexose as compared to production of the more classical fermentative end-products (*i.e.* lactate and ethanol) while at the same time providing 2 NAD^+. If OAA is synthesized via the carboxylation of PEP, there is a production of 2 NAD^+ and 2 ATP per hexose (Fig. 8B). Therefore, unlike lactate, ethanol, or malate accumulation, accumulation of succinate, with either source of OAA, provides a net sink for reductant (39) (Fig. 8, A and B).

Malate, often considered a normal end product of glycolysis in plants, has been reported to accumulate in anoxic plant tissue (7, 24). Other studies, however, have not found any evidence that malate is a significant fermentative end-product (3, 31). In *S. minutum* there is no evidence of malate accumulation following a transition to anaerobiosis. There is a transient increase in malate over the first 2 to 3 min of anaerobiosis but this is followed by a long-term decline (Fig. 1). The transient increase may be related to the fact that malate is an intermediate in the pathway leading to succinate accumulation (39) (Fig. 8, A and B). As mentioned above, the long-term decline would appear to indicate its consumption for succinate synthesis.

Carbon Budget during Fermentation

An analysis of carbon recovery (Table I) indicates that $\sim$84.8% of starch degraded under anoxia can be accounted for by the accumulation of D-lactate, ethanol, and succinate. This indicates that no major fermentative end-products have been unaccounted.

Regulation of Fermentative Carbon Metabolism

Anaerobiosis results in an immediate drop in AEC from 0.85 to 0.60 (Fig. 3). Accompanying this is a rapid activation of both PK and PFK as evidenced by rapid increases in the pyr/PEP and FBP/F6P ratios (Figs. 4 and 5). The kinetic and regulatory properties of the *S. minutum* cytosolic and plastidic PKs and the single PFK (plastidic) have been examined in detail (5, 23). The physiological concentrations of metabolite effectors of PK and PFK have been shown to be in the range to effect enzyme activity (38). Also, recent work has suggested that the *in vivo* activity of *S. minutum* PK may be limited by the availability of ADP (38). Based on this information and on examination of short-term metabolite changes following a transition to anaerobiosis, we are able to hypothesize the following mechanism for the activation of PK and PFK. The rapid increase in ADP following transition to anaerobiosis (1.5-fold increase in 30 s, Fig. 3), releases PK from adenylate control. The observation that the PEP decline and pyr increase are the first changes in glycolytic metabolism is consistent with initial activation of PK (Fig. 4). Over the same time period there is little change in F6P or FBP (Fig. 5). Since PEP is in equilibrium with 3-PGA the level of 3-PGA also drops but with a lag compared to PEP (Fig. 7). The drop in PEP and 3-PGA, both strong inhibitors of *S. minutum* PFK (5, 38) could then facilitate activation of PFK (Fig. 5). This would result in an increase in DHAP (Fig. 7), a potent activator of *S. minutum* PK, thus reinforcing its activation. This hypothesized mechanism for activation of PK and PFK following a transition to anaerobiosis is similar to that described for activation of glycolysis to provide carbon skeletons for NH_4^+ assimilation (38).

Accompanying the activation of PFK by anaerobiosis is a large increase in the level of Fru-2,6-P_2 (Fig. 6). Since this alga has no PFP (5, 38) and since PFK is plastidic and its activity is unaffected by Fru-2,6-P_2 (5, 38), a role for Fru-2,6-P_2 in activating glycolysis is unlikely. However, Fru-2,6-P_2 may serve an important role in preventing gluconeogenesis in the cytosol under anoxia (22). *S. minutum* has been shown to contain both a plastidic and cytosolic FBPase (6, 38) and the increased FBP/F6P ratio would favor gluconeogenesis if FBPase activity remained unaffected. However, the large increase in Fru-2,6-P_2 concentration, presumably in the cytosol (Fig. 6), along with the increased AMP (Fig. 3) would serve to inhibit the cytosolic FBPase, preventing the consumption of TP by gluconeogenesis (6, 35, 38). This would ensure all TP exported from the plastid was available for fermentative metabolism. As these experiments were carried out in the dark, the plastid FBPase isozyme most likely remained inhibited due to the lack of thioredoxin activation (17).

CONCLUSION

Anaerobic cells of the green alga *Selenastrum minutum* produce D-lactate, ethanol, and succinate as fermentation end-products. Malate does not accumulate following a transition to anaerobiosis but it is an intermediate in the pathway leading to succinate accumulation. Asp catabolism provides an initial source of OAA for reduction to succinate. The Pasteur effect is supported by an apparent initial activation of PK. The resulting decline in PEP and 3-PGA corresponds with the apparent activation of PFK. This is accompanied by an increase in Fru-2,6-P_2 which could serve to inhibit gluconeogenesis.

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