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MINIREVIEW

Central Carbon Metabolism and Electron Transport in *Chlamydomonas reinhardtii*: Metabolic Constraints for Carbon Partitioning between Oil and Starch

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The metabolism of microalgae is so flexible that it is not an easy task to give a comprehensive description of the interplay between the various metabolic pathways. There are, however, constraints that govern central carbon metabolism in *Chlamydomonas reinhardtii* that are revealed by the compartmentalization and regulation of the pathways and their relation to key cellular processes such as cell motility, division, carbon uptake and partitioning, external and internal rhythms, and nutrient stress. Both photosynthetic and mitochondrial electron transfer provide energy for metabolic processes and how energy transfer impacts metabolism and vice versa is a means of exploring the regulation and function of these pathways. A key example is the specific chloroplast localization of glycolysis/gluconeogenesis and how it impacts the redox poise and ATP budget of the plastid in the dark. To compare starch and lipids as carbon reserves, their value can be calculated in terms of NAD(P)H and ATP. As microalgae are now considered a potential renewable feedstock, we examine current work on the subject and also explore the possibility of rerouting metabolism toward lipid production.

Photosynthetic algae fix atmospheric carbon (CO_2) by using light energy to drive a series of chemical and redox reactions. This fixed carbon is transformed into reserve molecules that can be broken down at a later time to provide the cell with ATP, reducing power, and carbon skeletons. Starch, a polymer of glucose, is synthesized and degraded as a normal process in a light-dark cell cycle. Starch is also accumulated to prepare for gametogenesis, but it is not a prerequisite. The type and quantity of the reserve depend on environmental and cellular factors: stresses such as nutrient deprivation, salinity, temperature, and high light can be stimuli for starch accumulation and also for triacylglycerol (TAG) synthesis leading to lipid body accumulation in algae. Under nonstress conditions, starch would appear to be a preferential source of reserve in green algae as it is in plant leaves, whereas under stress conditions and in plant oil seeds, storage neutral lipids will accumulate to high levels.

Photosynthesis is a tightly controlled process, from the capture of light energy to the conversion of this energy into ATP and reducing power (NADPH). ATP and NADPH feed the Calvin cycle, which is responsible for CO_2 fixation. In the absence of exogenous carbon supply, photosynthesis is the only source of energy, and CO_2 fixation provides carbon skeletons for all reactions in the cell. Tight control of the photosynthetic reactions is required to fit the downstream metabolic reactions in the chloroplast.

We deliberately oriented our presentation and our choice of references toward *Chlamydomonas reinhardtii*, a unicellular green alga that has garnered much attention from the scientific community, primarily because it is haploid and the genetics have been extensively developed, but it has also proven to be a robust model system for the study of photosynthesis. Now that its genome is fully sequenced (1) and largely annotated, it is accessible for large-scale high-throughput investigative techniques like transcriptomics (2–4), proteomics (5–7), or metabolomics (8, 9). Sometimes, to be as complete as possible, we have drawn reference to literature which is itself a prediction, relies on older techniques, or is available only for other related algae or plants; therefore, we urge the reader to use this minireview as a guide to compartmentalization and energetic constraints, rather than a definitive text.

PHOTOSYNTHESIS, A TIGHTLY CONTROLLED PROCESS

"Linear electron flow" denotes the transfer of electrons from a primary electron donor, H_2O, oxidized at the level of photosystem II (PSII), to a terminal electron acceptor $NADP^+$, reduced at the acceptor side of photosystem I (PSI) (Fig. 1). Electron transfer is mediated in the stroma by ferredoxin (Fd) and ferredoxin-$NADP^+$ reductase (FNR) and at the level of the thylakoid membrane by intersystem electron carriers (inter- PSII and PSI) plastoquinones, cytochrome b_6f complex, and plastocyanin (PC). Many pathways, denoted alternative pathways, connect the stromal electron carriers to the intersystem electron carriers. These alternative pathways operate along the photosynthetic chain to optimize electron transfer in relation to the inputs (light and CO_2) and outputs (metabolites).

Some alternative pathways of electron transfer act as regulatory valves to optimize the stoichiometry of ATP and NADPH re-

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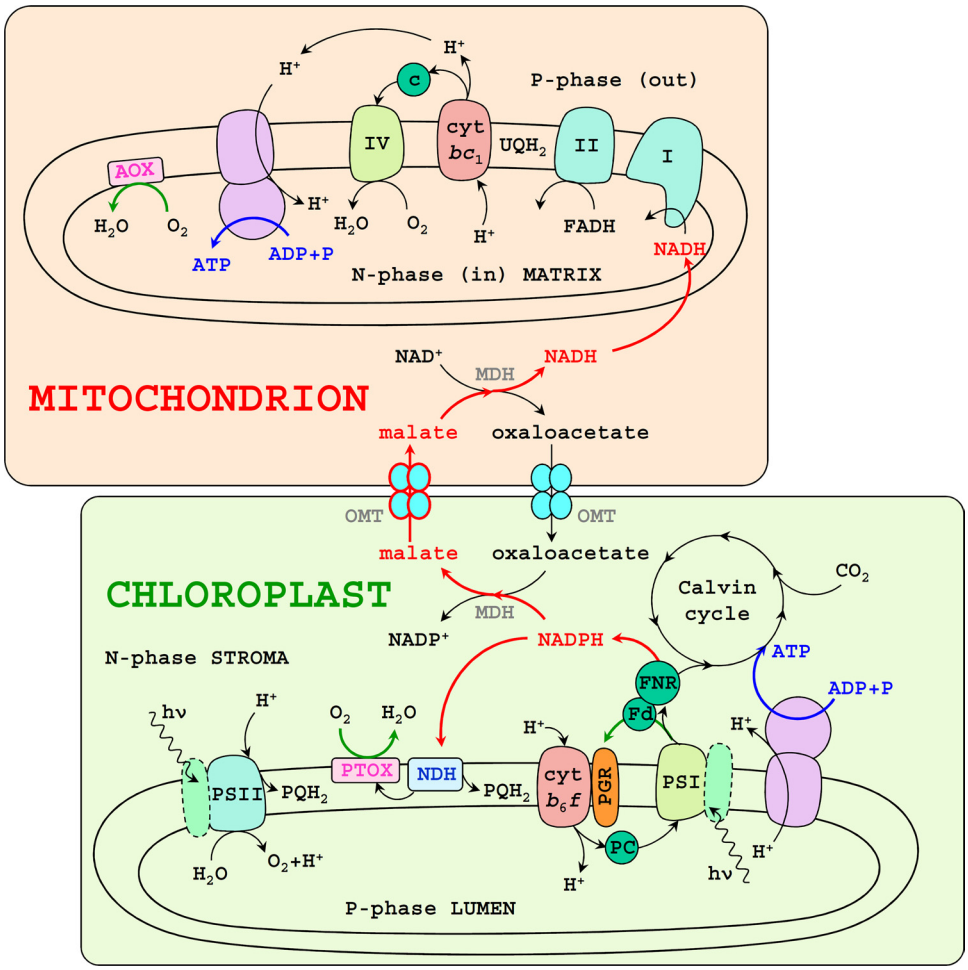

FIG 1 Energetic organelles of *Chlamydomonas*. In the chloroplast, the light reactions and electron transfer chain feed NADPH and ATP toward the carbon fixation pathway (Calvin cycle). In the mitochondria, the major components of the respiratory chain produce ATP at the expense of NADH. *Chlamydomonas reinhardtii* has one chloroplast and multiple mitochondria which appear tightly packed together in electron micrographs. A possible pathway for the transfer of reductants from one organelle to the other, the malate shunt, is shown here. Black arrows denote electron and proton transfer pathways, green and red arrows denote alternative electron transfer pathways, and blue arrows denote ATP synthesis. P-phase, positive phase; N-phase, negative phase; UQH_2, ubiquinol; c, soluble cytochrome; hυ, photon energy; MDH, malate dehydrogenase; OMT, oxoglutarate/malate transporter.

quired for efficient carbon fixation. "Cyclic electron flow" fulfills this role by recycling NADPH. Coupled to proton transfer, it increases the proton gradient across the thylakoid membrane and results in a higher production of ATP (10). There are two pathways for cyclic electron flow in photosynthetic organisms and conserved in *Chlamydomonas*, the PGR5/PGRL1-Fd-mediated pathway (11), recently defined as the ferredoxin-plastoquinone reductase (FQR) in *Arabidopsis* (12) and the NADPH dehydrogenase (NDH, type I or type II) pathway (13). Cyclic electron flow also feeds back on linear electron flow through the cytochrome b_6f complex. Under some conditions, such as high light or when reactions at the acceptor side of PSI become limiting, cyclic electron flow may build a large proton gradient, and that which is not transformed into ATP stimulates the reversible mechanism of nonphotochemical quenching, a process resulting in a de-excitation of chlorophyll and reduced photosynthetic yield (14).

Plastoquinones (PQ) are ubiquitous electron carriers in the thylakoid membrane. The PQ pool can be considered at the intersection of various metabolic pathways: photosynthetic electron flow, alternative pathways and chlororespiration. The redox state of the PQ pool, that is the balance between the reduced form (plastoquinols [PQH_2]) and the oxidized form (plastoquinones [PQ]), is balanced by or involved in the regulation of a number of processes: chlororespiration (15), state transitions (16), PSI-cytochrome (cyt) b_6f supercomplex formation (17), retrograde control of chloroplast protein translation (18), carotenoid biosynthesis (19), and chloroplast biogenesis (20).

Alternative pathways work as valves to dissipate excess NADPH and maintain redox homeostasis. Chlororespiration oxidizes NADPH at the expense of molecular oxygen and plastoquinone via the thylakoid-bound plastid terminal oxidase, plastid terminal oxidase 2 (PTOX2) (21) and the type II NADPH dehydrogenase, NDA2 (13). The malate shunt operates in two directions, allowing for consumption of excess NADPH by the mitochondria or may kick start photosynthetic reactions by supplying reductant from the mitochondria (22). Mitochondrial activity is tightly linked to photosynthetic activity in the green alga *Chlamydomonas*. Alternative oxidase (AOX) (23) is a mitochondrial valve

dissipating NADH without producing ATP; this pathway is proposed to dissipate reducing power under stress conditions (24). These alternative pathways form an important network to regulate and optimize photosynthesis while protecting the chloroplast from the effects of excess energy. However, many of these pathways are interchangeable and flexible.

Because NADPH is a ubiquitous electron carrier for photosynthesis, alternative pathways and chloroplast metabolism, the flux along these pathways and even the directionality of the reactions is almost only dependent upon the relative concentrations of NADPH and $NADP^+$ ([NADPH]/([NADPH] + [$NADP^+$]).

CARBOHYDRATE METABOLISM

Photosynthetic and nonphotosynthetic carbon metabolism. Unlike plants, which possess both photosynthetic and nonphotosynthetic plastids, the *Chlamydomonas reinhardtii* chloroplast retains a fully assembled and functional photosynthetic electron transport chain, even in the dark. *Chlamydomonas* metabolism can rely on different reduced carbon sources, such as endogenous starch accumulated in the chloroplast during the light phase, or exogenous carbon-reduced compounds like acetate (see page 176 of reference 25.) In Fig. 2, acetate is shown incorporated into acetyl coenzyme A (acetyl-CoA) following two possible pathways, both requiring ATP (described in Table 1): a direct conversion with acetyl-CoA synthetase (ACS) or a two-step reaction involving acetate kinase (ACK) and phosphate acetyltransferase (PAT) (25, 26). In the dark, ATP requirements for acetate assimilation are met under aerobic conditions (respiration) but not under anaerobic conditions (fermentation [see reference 27]). In the light, acetate uptake relies mainly on cyclic photophosphorylation (cyclic electron transfer around photosystem I) because the PSII inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), does not inhibit acetate uptake, and the action spectrum of photoassimilation of acetate resembles that of photosystem 1 (28). It accounts for anaerobic acetate photoassimilation in *Chlamydomonas* (27), as well as obligate photoheterotrophic growth on acetate in some other species such as *Chloromonas* sp. (formerly *Chlamydomonas mundana*) (28–30) or *Pyrobotrys* (formerly *Chlamydobotrys*) *stellata* (31, 32).

Acetyl-CoA feeds into the mitochondrial tricarboxylic acid (TCA) cycle that produces 3 NADH molecules, 1 $FADH_2$ molecule, 2 ATP molecules, and 2 CO_2 molecules per acetyl-CoA (C_2). NADH and $FADH_2$ are oxidized in the respiratory electron transport chain and yield ATP (oxidative phosphorylation). Figure 2 shows that when ATP is present, oxaloacetate can be transformed into phosphoenolpyruvate (PEP) by PEP carboxykinase (PCK). PEP is further used for the synthesis of longer-carbon-chain compounds up to glucose, a process known as gluconeogenesis. These biochemical pathways account for the storage of starch by *Chlamydomonas* cells grown in dark aerobic conditions and fed with acetate (33). Photosynthetic carbon fixation also produces starch and shares enzymatic steps with gluconeogenesis. In the dark and in the absence of acetate, starch is broken down to fuel glycolysis (the reverse of gluconeogenesis), yielding ATP and NADH.

Following the oxidative pentose-phosphate pathway (OPPP), glucose-6-phosphate (G6P) can be oxidized to 6-phosphoglucolactone (6-PGL) by G6P-dehydrogenase (GDH). Further yielding to 6-phosphogluconate (6-PG) and then ribulose-5-phosphate (Ru5P), this oxidative phase of the OPPP gives 2 NADPH molecules and 1 CO_2 molecule per C_6. The nonoxidative phase regenerates fructose-6-phosphate (Fru-6-P) and triose-phosphate (C_3) from Ru5P (C_5) in a path reversed to the Calvin cycle.

Metabolic regulation. In aerobic *Chlamydomonas* cells, Fru-6-P is more abundant than fructose 1,6-bisphosphate (Fru-1,6-P2), whereas the situation is reversed in anaerobic cells (34). It reflects the ATP regulation of phosphofructokinase (PFK) activity: phosphofructokinase requires ATP and transforms Fru-6-P into Fru-1,6-P2, but ATP also acts as an allosteric inhibitor of phosphofructokinase. Under ATP-deficient conditions (anoxia or addition of mitochondrial inhibitors), phosphofructokinase is activated, stimulating the glycolytic flux and starch breakdown (27), an effect also referred as "Pasteur effect" (see the "Pasteur effect" section below).

Textbooks report that the pentose-phosphate pathway can operate following different "modes": an ATP-consuming mode (no loss in carbon and net production of ribose-5-phosphate [R5P] as a building block for other molecules), an NADPH-producing mode (all carbon from G6P is released as CO_2), or a mode coupled to glycolysis which produces both ATP and NAD(P)H and CO_2. These schematic modes surely interact and equilibrate with each other in response to the energetic needs of the cell. In any case, the pentose-phosphate pathway can be considered an alternative pathway to the "upper half" of glycolysis: G6P can be converted to Fru-6-P by phosphoglucose isomerase (PGI) (glycolysis) or oxidized to 6-phosphoglucolactone by G6P-dehydrogenase (OPPP). In *Chlamydomonas* chloroplasts, the activity of the former (glycolysis) is found 7 times greater than that of the latter (OPPP) (35). G6P-dehydrogenase activity is inhibited by high levels of NADPH and ribulose-1,5-bisphosphate (RuBP) (36). The negative feedback of NADPH on OPPP is therefore expected to further favor glycolysis over OPPP under reducing ATP-deficient conditions, like anoxia or addition of mitochondrial inhibitors (37).

Compartmentalization of the glycolytic and OPPP pathways. Plants seem to have duplicated the entire glycolytic and oxidative pentose-phosphate pathways in the chloroplast and cytosol (38, 39), and plant chloroplasts also possess an efficient export system for hexoses (glucose and maltose transporters [40]) and triose phosphate (phosphate translocator [41, 42]). Such exported sugars and sugar-phosphates feed into sucrose metabolism and supply the nonphotosynthetic plant tissues with energy (40). In green microalgae such as *Chlamydomonas reinhardtii*, the glycolytic pathway is not duplicated but instead is highly compartmentalized, the "upper half" of glycolysis (from glucose to glyceraldehyde-3-phosphate [G3P]) being exclusively chloroplastic and the "lower half" (from 3-phosphoglycerate [3-PGA] to pyruvate) being localized in the cytosol (see the boxes in Table 1) (for a comprehensive review, see reference 43). Enolase (PGH) (7, 35), phosphoglycerate mutase (PGM) (7, 35), and pyruvate kinase (PYK) (7) are absent from algal chloroplasts, whereas amylase (AMY) (35), starch phosphorylase (SP) (35), and phosphofructokinase (35) are exclusively chloroplastic. Fructose-1,6-bisphosphate aldolase (ALD) is also reported as mainly chloroplastic because it is absent from the cytosol (35, 44), but it has been also found attached to the flagella (6, 45). Outside of the chloroplast, the glycolytic enzymes downstream of glyceraldehyde-3-phosphate, e.g., phosphoglycerate kinase (PGK) and pyruvate kinase, seem attached to the flagella, where ATP production is most needed (6, 45). Chloroplast fractions also contain a pyruvate phosphate dikinase (PPD) (7) that catalyzes the reversible conversion between PEP and pyruvate.

TABLE 1 Enzyme localization in *Chlamydomonas reinhardtii*[a]

Abb.	Enzyme	Reaction	Mit.	Cyt./F.	Chl.
Starch synthesis					
AMY	Amylase	Starch → glucose	—	□a	■ab
SP	Starch phosphorylase	Starch → G1P	—	□a	■ab
GPM	Phosphoglucomutase	G1P ↔ G6P	—	■ac	■ab
Glycolysis / gluconeogenesis					
HK	Hexokinase	Glucose + ATP → G6P	—	—	■b
AGPP	ADP-glucose pyrophosphorylase	ADP-glucose ← G1P + ATP	—	—	■b
PGI	Phosphoglucose isomerase	G6P ↔ Fru-6-P	—	■a	■ab
PFK	Phosphofructokinase	Fru-6-P + ATP → Fru-1,6-P2	—	□a	■ab
FBP	Fructose-1,6-bisphosphatase	Fru-6-P ← Fru-1,6-P2	□e	□e	■be
ALD	Fructose bisphosphate aldolase	Fru-1,6-P2 ↔ G3P	—	□a‡■c	■a‡b
TPI	Triose-phosphate isomerase	G3P ↔ DHAP	—		■ab
GAPDH	Glyceraldehyde phosphate phosphate dehydrogenase	G3P ↔ 1,3BPG + NAD(P)H	—	■ac	■ab†
PGK	Phosphoglycerate kinase	1,3BPG ↔ 3-PGA + ATP	—	■c	■b
PGM	Phosphoglycerate mutase	3-PGA ↔ 2-PGA	—	■c	—
PGH	Enolase	2-PGA ↔ PEP	—	■ac	□a
PYK	Pyruvate kinase	PEP → pyruvate + ATP	—	■ac	□a
PCK	Phosphoenolpyruvate carboxykinase	PEP + HCO_3^- ↔ oxaloacetate + GTP	□e	■ae†	□ae
PEPC	Phosphoenolpyruvate carboxylase	PEP + HCO_3^- → oxaloacetate	—	■#	—
Oxidative pentose-phosphate pathway					
GDH	Glucose-6-phosphate dehydrogenase	G6P → 6-PGL + NADPH	—	■a	■a
6PGDH	6-phosphogluconate dehydrogenase	6-PG → Ru5P + NADPH + CO_2	—	■a	■a
Acetate assimilation					
ACS	Acetyl-CoA synthetase	Acetyl-CoA ← acetate + ATP	■d†	■†	■b†
PAT*	Phosphate acetyltransferase	Acetyl-CoA ← acetyl-P	■de	□e	■b□e
ACK*	Acetate kinase	Acetyl-P ← acetate + ATP	■de	□e	■b□e
Metabolism of acetyl-CoA, pyruvate, phosphoenolpyruvate and C4 compounds					
PDH	Pyruvate dehydrogenase	Pyruvate ↔ acetyl-CoA + NADH + CO_2	■de	□e	■be
PYC	Pyruvate carboxylase	Oxaloacetate ← pyruvate + ATP + CO_2	—	—	■b
PPD	Pyruvate, phosphate dikinase	Pyruvate + ATP ↔ PEP	—	—	■b
MDH	Malate dehydrogenase	Oxaloacetate + NAD(P)H ↔ malate	■d	■c†	■b†
MME	Malic enzyme	Malate ↔ pyruvate + NAD(P)H + CO_2	■d	—	■b
NNT	Transhydrogenase	NADH ↔ NADPH	■d	—	■b
FUM	Fumarase	Fumarate ↔ malate	■d†	□†	□†
Fermentation enzymes					
PDC	Pyruvate dehydrogenase	Pyruvate → acetyl-CoA + CO_2	—	—	■b
PFR	Pyruvate ferredoxin oxidoreductase	Pyruvate → acetyl-CoA + CO_2	—	—	■b
PFL	Pyruvate formate lyase	Pyruvate → formate + acetyl-CoA	■de	□e	■be
PDC	Pyruvate decarboxylase	Pyruvate → acetaldehyde + CO_2	□e	■e	□e
ADH	Alcohol dehydrogenase	Acetaldehyde + NAD(P)H → ethanol	■e	■ce	■be
PAT*	Phosphate acetyltransferase	Acetyl-CoA → acetyl-P	■de	□e	■b□e
ACK*	Acetate kinase	Acetyl-P → acetate + ATP	■de	□e	■b□e
Membrane-bound enzymes					
SDH	Succinate dehydrogenase	Succinate + Q → fumarate + QH_2	■d	—	■†
NDH	NAD(P)H dehydrogenase	NAD(P)H + Q → $NAD(P)^+$ + QH_2	■d	—	■b
PTOX	Plastid terminal oxidase	QH_2 + ½ O_2 → Q + H_2O	—	—	■b
Lipid synthesis					
ACC	Acetyl-CoA carboxylase	Malonyl-CoA ← acetyl-CoA + ATP + HCO_3^-	—	—	■b
KAS	β-ketoacyl synthase, fatty acid synthase	FA + HCO_3^- ← ac-CoA + ma-CoA + NADPH	—	—	■b
GPDH	Glycerol 3-phosphate dehydrogenase	Glycerol-3P ← DHAP + NAD(P)H	□§	□§	■§
Metabolite shuttle across organelles					
MEX	Maltose exporter	(Plastid targeted) 1 gene	—	—	—b
HXT	Hexose (glucose) transporter	(Plastid targeted) 1 gene	—	—	—b
TPT	Triose-P or hexose-P (GPT) translocator	(Plastid targeted) 2 genes	—	—	—b■¶
OMT	Oxoglutarate / malate transporter	(Plastid targeted) 2 genes, see also LCI20	—	—	■b■d
MITC	Mitochondrial carrier	(Mito targeted) 14 genes	■d	—	—
PPT	PEP transporter	(Plastid targeted) 1 gene	—	—	—b
AAA	ATP / ADP transporter (NTT homolog)	(Plastid targeted) 3 genes	—	—	—b■d
ANT	ATP / ADP transporter (AAC homolog)	(Mito targeted) 2 genes	■d	—	—

[a] The enzyme abbreviations (Abb.) are shown in the leftmost column, and the enzymes are shown in the second column. The reactions are shown in the third column; the reactions with an arrow from left to right are generally favorable reactions (glycolysis), and the reactions with an arrow from right to left are generally unfavorable reactions (gluconeogenesis), but this should be checked individually. Q, quinone. Enzyme localization in *Chlamydomonas reinhardtii* in the mitochondrion (Mit.), cytoplasm (Cyt.), flagella (F.), or chloroplast (Chl.) is shown in the three rightmost columns. Symbols: ■, the enzyme is active or a peptide is found in the specified compartment (it may be also found in other compartments); □, enzyme activity is negligible in the specified compartment in comparison with other compartments as a control; —, the peptide has not been found in the specified compartment. The boxes around the localization of the glycolytic and gluconeogenic enzymes highlight the dual compartmentalization of these pathways between chloroplast and cytosol in *Chlamydomonas reinhardtii*. The lowercase letters and symbols next to the squares indicate the reference(s) the data were taken from as follows: a, reference 35; b, reference 7; c, reference 6; d, reference 5; e, reference 64; †, reference 123; ‡, reference 44; §, reference 124; ¶, references 43, 49, and 50; #, reference 62. For the reaction column, when ATP [or NAD(P)H] is shown on one side, ADP + P_i [or $NAD(P)^+$] is also implied on the other side (omitted here for simplicity). The asterisks next to the PAT and ACK enzyme abbreviations indicate that the reactions are essentially reversible and can serve either in acetate assimilation under aerobic conditions for the supply in acetyl-CoA of the glyoxylate and TCA cycles or in fermentative acetate production under anoxic conditions.

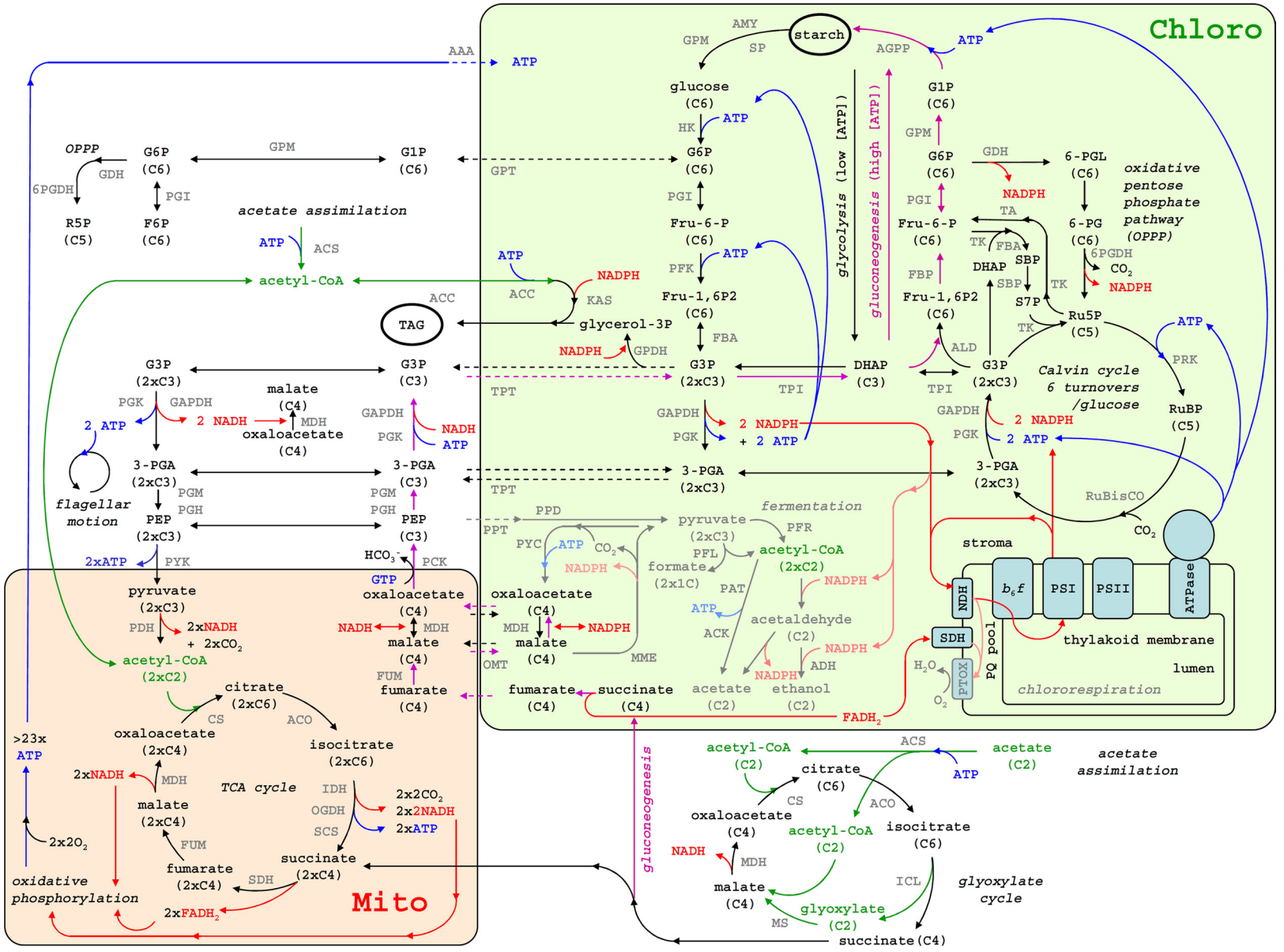

FIG 2 Compartmentalization of central carbon metabolism in *Chlamydomonas reinhardtii*. The abbreviations for enzymes, also specified in Table 1, are written in gray. Glycolysis is localized in the chloroplast (from hexose-phosphates to 3-phosphoglycerate) and the cytosol (from triose-phosphates to pyruvate) (see Table 1 and references in Table 1 for the localization data). Red arrows represent reducing power, NAD(P)H, and blue arrows represent ATP. Acetate uptake (green arrows) can feed into the glyoxylate cycle for carbon assimilation via gluconeogenesis (purple arrows) or into the TCA cycle to sustain respiration and mitochondrial ATP production. Gray arrows represent alternative pathways (chlororespiration and fermentation). Mito, mitochondrion; Chloro, chloroplast.

In *Chlamydomonas reinhardtii*, the localization of the "upper half" of glycolysis in the chloroplast and the lower half in the cytosol is supported by the comparative quantification of metabolites (34), glucose-1-phosphate (G1P), Fru-6-P, and Fru-1,6-P2 being specific to the chloroplast compartment and 2-phosphoglycerate being found only in the cytosol.

Metabolite transport across the chloroplast envelope. Unpublished data from Klein reported in reference 43 show that purified *Chlamydomonas* chloroplasts export 23% 3-PGA, 15% dihydroxyacetone phosphate (DHAP), 20% hexose-phosphates, and 13% glycolate formed in the light from labeled CO_2. The presence of a small accumulation of G6P in the cytosol has also been reported (34). In addition, this compartment contains significant fractions of phosphoglucose mutase (GPM) (35) and phosphoglucose isomerase (35), with lesser amounts of G6P-dehydrogenase (35) and 6-phosphogluconate-dehydrogenase (6PGDH) (35). In the absence of a complete glycolytic pathway (e.g., phosphofructokinase) in the cytosol, the rationale for metabolism of hexose-phosphates in this compartment is unclear; perhaps it is (included in Fig. 2) for the production of NADPH by OPPP for anabolic reactions or the export of carbohydrate precursors, like R5P for nucleotide synthesis or sugars for the synthesis of the cell wall constituted of glycoproteins (page 27 of reference 25).

The induction of glucose metabolism and hydrogen photoproduction by the sole insertion of a hexose uptake protein (hexose uptake protein 1 [HUP1]) in the plasma membrane of *Chlamydomonas* (46) suggests that cytosolic glucose can be imported and broken down in the chloroplast. Two different pathways seem possible, either a direct import via the glucose transporter (hexose transporter [HXT]), or a conversion to G6P and import through a hexose-phosphate (GPT) translocator. The latter pathway would require the presence of hexokinase (HK) in the cytosol. Although hexokinase is found in the chloroplast of *Chlamydomonas* (7), we could not find if it is also present in the cytosol, but a dual localization is possible. Such an induced glucose metabolism appears however limited in *Chlamydomonas* inasmuch as the mutant cells *Stm6Glc4* cannot entirely rely on glucose for heterotrophic growth, although they are capable of one division in the dark (46). Chloroplast proteomics studies could not identify any plastidic hexose or hexose-phosphate transporters (7), suggesting that they are only weakly abundant, which may account for the poor efficiency of glucose feeding (46).

LCI20 (low-CO_2-inducible) protein, orthologous to *Arabidopsis* 2-oxoglutarate/malate translocator of chloroplast envelope membranes (47), is found in the chloroplast proteome (7). Also, one peptide of another 2-oxoglutarate/malate transporter (OMT), predicted to be targeted to the chloroplast, was found in a nonsoluble fraction (5). Although it had to be discarded as contaminant (which is to be expected from the intricate association of mitochondria and chloroplasts), it may suggest the presence of other plastidic malate shuttles.

Triose-phosphate translocator. Triose-phosphate (G3P and DHAP) and the 3-carbon sugar acid, 3-PGA are present in the chloroplast and the cytosol (34), as expected from a shuttle via the TPT (triose-P or hexose-P translocator) (48). Although the genes coding for these metabolite transporters are found in the *Chlamydomonas* genome, the proteins have not been characterized yet. However, active transport of metabolites was evidenced multiple times, independently by various investigators (34, 43, 49).

Glyceraldehyde phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase are localized in the chloroplast and cytosol (35) (Table 1), and the directionality of these reversible reactions is determined by the concentrations in NAD(P)H and ATP in these different compartments. When NAD(P)H and ATP levels are low, glyceraldehyde phosphate dehydrogenase will oxidize G3P into 1,3-bisphosphoglycerate (1,3-BPG), and phosphoglycerate kinase will dephosphorylate 1,3-BPG into 3-PGA (only the sum of these two reactions is shown in Fig. 2); when NAD(P)H and ATP levels are high, the same enzymes will catalyze the reverse reactions, i.e., form G3P at the expense of 3-PGA. These reactions can occur in opposite directions in the chloroplast and cytosol, driving the translocation of G3P and 3-PGA, and therefore of ATP and NAD(P)H, in either one direction or the other.

Those two opposite flows of the TPT are represented in Fig. 3. In the light (Fig. 3A), the large amount of ATP and NADPH formed by photosynthetic reactions promotes the formation of chloroplast G3P. Newly formed G3P can be exported to release NADH and ATP in the cytosol. In heterotrophic conditions (Fig. 3B), oxidative phosphorylation is sustained by acetate assimilation feeding into the TCA cycle, and ATP is exported out of the mitochondrion. If ATP is abundant in the cytosol, it can reverse phosphoglycerate kinase activity in the cytosol and lead to the formation of 1,3-BPG and G3P. Part of the G3P imported into the chloroplast can be used for the synthesis of hexoses (gluconeogenesis); the other part can be oxidized and dephosphorylated in the chloroplast, yielding ATP and 3-PGA, 3-PGA being returned to the cytosol. This would account for starch synthesis in the dark (33).

The operation of the TPT in *Chlamydomonas* has been shown by Klein, as reported in reference 43, and summarized in Fig. 3A, who observed that purified *Chlamydomonas* chloroplasts export 3-carbon sugar acids and triose-phosphates (23% 3-PGA and 15% DHAP) formed in the light from labeled CO_2. Exchange of 3-PGA is reversible in *Chlamydomonas* (50) as in plants (51), inasmuch as 3-PGA added to isolated chloroplasts can be imported and used as an electron acceptor of photosynthetic electron transport (Fig. 3A). G3P can also be imported (Fig. 3B), and it stimulates ATP production in the chloroplast (49) (see below).

CONSEQUENCES ON THE ATP/NADPH BUDGET OF THE CHLOROPLAST IN THE DARK

In *Chlamydomonas*, the "upper half" of glycolysis takes place in the chloroplast, and the "lower half" takes place in the cytosol. Such a disconnection between the "preparatory" (ATP-consuming) and "payoff" (ATP-producing) phases of glycolysis has important consequences on the energy budget of the chloroplast in the dark, with a major control point at the level of the TPT, depending on where (i.e., stroma or cytosol) the oxidation of G3P takes place. (i) If glycolysis proceeds down to 3-PGA in the chloroplast, the balance in ATP is null in this compartment: the two ATP molecules produced by phosphoglycerate kinase (for each two 3-PGA), are required for hexokinase and phosphofructokinase (for each one glucose). It is the same scenario as depicted in Fig. 3B, except that in this instance starch is broken down and not synthesized. (ii) If G3P is exported and oxidized in the cytosol (like in Fig. 3A but in the dark in this instance), the ATP required for the preparatory phase of glycolysis has to be imported into the chloroplast via other pathways.

This balance for ATP is unchanged when taking into account the OPPP: starting with 3 glucose molecules, 3 ATP molecules are

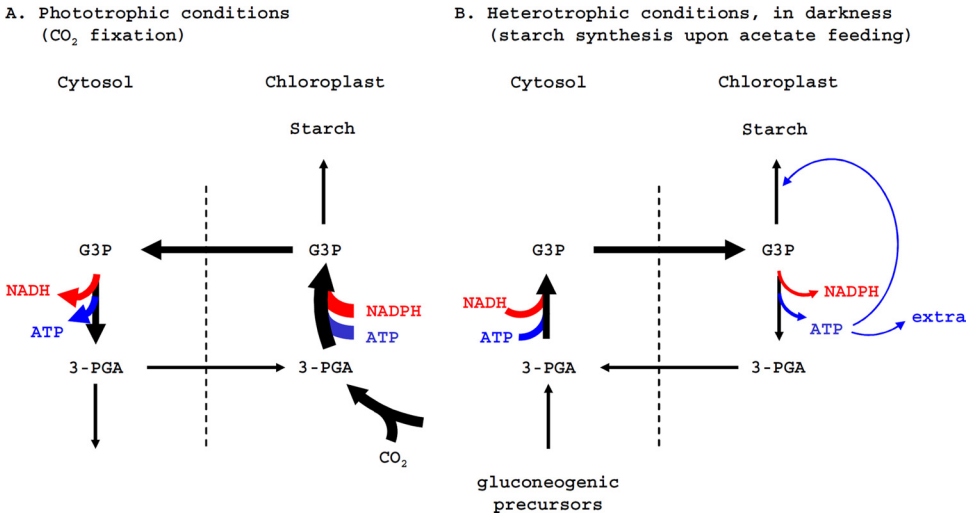

FIG 3 Schematic representation of the reversible action of the TPT. (A) In the light, when photosynthesis produces NADPH and ATP in the chloroplast, G3P is formed from CO_2 and directed toward starch synthesis or exported toward the cytosol. Cytosolic G3P is oxidized into 1,3-BPG by GAPDH, and PGK dephosphorylates 1,3-BPG into 3-PGA. While a fraction of 3-PGA can feed into glycolysis, the rest can be retrieved into the chloroplast where it further accepts ATP and electrons from photosynthesis. The thickness of the arrow represents the amount of flux. (B) In darkness, when ATP levels are higher in the cytosol than they are in the chloroplast, the TPT can operate in the reverse mode. 3-PGA formed from acetate assimilation, glyoxylate cycle, and the first steps of gluconeogenesis, is phosphorylated and reduced into G3P. While part of the G3P translocated into the chloroplast can be further directed toward gluconeogenesis, the rest can be used for ATP production in the chloroplast. NADPH can be reoxidized via chlororespiration to sustain the flux of ATP production.

required for hexokinase but only 2 molecules for phosphofructokinase because one G3P molecule is produced on the side by transketolase (TK), with 3 CO_2 molecules released in the oxidative phase; 5 G3P molecules are therefore produced, regenerating after oxidation and dephosphorylation the 5 ATP molecules required for the upstream steps, again bringing the balance to zero if those reactions occur in the chloroplast.

Glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, and malate dehydrogenase (MDH) are bound to the flagella, the role of phosphoglycerate kinase being to supply the flagella in ATP, while malate dehydrogenase would regenerate NAD^+ for glyceraldehyde phosphate dehydrogenase turnover (6, 45). This mechanism most likely operates in the light when conditions such as those represented in Fig. 3A are reached in the chloroplast: G3P, newly synthesized from CO_2, with ATP and NADPH produced by photosynthetic electron and proton transport, is exported out of the chloroplast. However, in the dark, oxidizing conditions are easily reached in the chloroplast (Fig. 3B). As shown in reference 52, when *Chlamydomonas* cells are treated with DCMU and methyl viologen (MV) and preilluminated (no cyclic or linear electron flow), NADPH is depleted in the chloroplast via the transfer through NDH, cyt b_6f, and PSI to MV. In this case, the dark reduction of the PSI primary electron donor, $P_{700}{}^+$, is limited by the regeneration of NADPH in the stroma and matches the flux of starch breakdown, suggesting that glycolysis can be driven down to 3-PGA in the chloroplast. Following the same reasoning that was used to account for the compartmentalization of the flagellar components (ATP is better off being produced locally) (6, 45), then ATP may have to be produced in the chloroplast by phosphoglycerate kinase to meet the ATP requirements of the preparatory phase of glycolysis. It would minimize the import of ATP via other pathways.

In any case, the rather negative (if nonnull) ATP balance of chloroplast glycolysis leaves little doubt that ATP has to be imported into the chloroplast in the dark.

Chloroplast import of ATP via the triose-phosphate translocator in the dark. Although chloroplast ATPase mutants do not grow under photoautotrophic conditions, they do fine under heterotrophic conditions, assembling a normally developed chloroplast and synthesizing the other major chloroplast proteins (53), a general trait of *Chlamydomonas* (54). Boschetti and Schmid (49) have addressed the question of protein synthesis by chloroplasts devoid of ATPase (isolated from the F54 mutant). They have shown that extra ATP did not induce chloroplast protein synthesis to an appreciable extent, arguing against direct import of ATP via a transporter, whereas addition of triose-phosphate (G3P and DHAP) did promote protein synthesis. The involvement of the TPT in the import of ATP into the chloroplast (Fig. 3B) was further substantiated by the antagonist effects of 3-PGA and by inhibition of metabolite transport (49). Whether protein synthesis was only attributable to ATP produced in the chloroplast by phosphoglycerate kinase or also stimulated by NADPH produced by glyceraldehyde phosphate dehydrogenase was not, however, assessed.

Pasteur effect. The "Pasteur effect" refers to an activation of glycolysis upon inhibition of mitochondrial ATP production (in algae, see references 55 and 37). Because the ATP-regulated steps of glycolysis (hexokinase and phosphofructokinase) are localized in the chloroplast of *Chlamydomonas*, a constant flux of ATP must exist from the mitochondrion to the chloroplast, inhibiting hexokinase and phosphofructokinase under dark aerobic conditions (Fig. 3B). As already mentioned above in the "Metabolic regulation" section, in aerobic *Chlamydomonas*, Fru-6-P accumulates at the expense of Fru-1,6-P2, whereas the equilibrium of the PFK reaction is shifted toward Fru-1,6-P2 under anaerobic conditions (34). It reflects the allosteric inhibition of PFK by ATP.

ATP can be imported directly via a transporter (see "Chloroplast import of ATP via plastidic ATP/ADP transporters" below) or indirectly via the triose-phosphate translocator. The latter pathway has been indirectly evidenced by Klöck and Kreuzberg

(34) who showed that under dark aerobic conditions, G3P is accumulated in the cytosol, while the level of 3-PGA remains low, and the chloroplast contains more 3-PGA than G3P. Those equilibria support an operation of the TPT in the direction depicted in Fig. 3B. Upon inhibition of respiration, the G3P/3-PGA ratio is reversed in the chloroplast and cytosol, showing that the level of cytosolic ATP becomes too low to sustain the phosphorylation of 3-PGA (34). The dark production of triose-phosphate in the chloroplast is then strictly coupled to the glycolytic flux. Under such reducing and ATP-deficient conditions, oxidation of G3P is limited, as is the further breakdown of 3-PGA. Those two intermediates therefore accumulate, as reported in reference 34.

Chloroplast import of ATP via plastidic ATP/ADP transporters. The direct import of ATP into isolated plant chloroplasts has been shown quite a while ago (56). The *Chlamydomonas* genome carries 3 *AAA* genes, homologous to *Arabidopsis NTT* genes, identified as plastidic ATP/ADP transporters (57; for reviews, see references 58 and 59). Although none of these transporters were directly identified in the *Chlamydomonas* chloroplast proteome (7), suggesting their very low abundance, 4 AAA1 (ATP/ADP transporter 1) peptides were found in nonsoluble fractions (5), together with expected larger numbers of ANT1 peptides (ANT is homologous to *Arabidopsis* AAC, the mitochondrial ATP/ADP transporters).

Peculiar and puzzling case of FUD50su. The low abundance of plastidic ATP/ADP transporters found in proteomic studies is supported by studies on mutants devoid of chloroplast ATPase. FUD50 has a deletion in the *atpB* gene of chloroplast ATPase, and although it grows fine under heterotrophic conditions, it does not grow under photoautotrophic conditions (53). For CO_2 fixation, ATP produced in the mitochondrion cannot substitute for ATP produced at the level of the thylakoid membrane. After random mutagenesis of FUD50, a photoautotrophic clone, FUD50su, was recovered, among others, and further characterized (60). Suppression of the acetate-requiring phenotype was attributable to at least two uncharacterized suppressor mutations, which did not restore chloroplast ATPase function but rather made FUD50su phototrophic growth dependent upon mitochondrial ATP production (60).

The fact that phototrophic growth is impeded by mitochondrial inhibitors in FUD50su, but not in the wild type (WT) (60), shows that ATP production via an electron transport pathway in either the chloroplast or the mitochondrion is dispensable, but that at least one of these is required.

Under phototrophic conditions, ATP import into the chloroplast of strain FUD50su is likely to occur via an ATP/ADP transporter. In the light, NADPH is produced by photosynthetic electron transport, forcing glyceraldehyde phosphate dehydrogenase to reduce 1,3-BPG (Fig. 3A) in the chloroplast, the phosphorylation of 3-PGA relying entirely on ATP imported via another pathway.

However, it is possible that there is an alternative mechanism making the phosphate translocator operate in the opposite direction (like in Fig. 3B): the NADPH concentration could remain low in the stroma, even in the light, if the malate shunt was stimulated, allowing for mitochondrial ATP production. Similarly to that proposed for the flagella in (6, 45), NADP-malate dehydrogenase would regenerate $NADP^+$ for a partial G3P oxidation by glyceraldehyde phosphate dehydrogenase and dephosphorylation of 1,3-BPG, allowing for ATP production in the chloroplast. Another fraction of chloroplast G3P would feed into gluconeogenesis, leaving CO_2 fixation active as required for photosynthetic growth.

Although the multiple suppressor mutations in strain FUD50su are unlikely to be easily identified, a deep metabolic and/or proteomic profiling of FUD50su could address the question of the upregulation of biochemical pathways in this strain and reveal this novel mechanism of ATP shunting.

Role of chlororespiration in dark chloroplast metabolism. In *Chlamydomonas*, the likely preponderance of the TPT over the direct import of ATP into the chloroplast seems paralleled by a significant chlororespiratory flux. The difference between these two translocation mechanisms is that the direct transport of ATP/ADP does not involve redox changes, whereas ATP production from dephosphorylation of 1,3-BPG also implies the oxidation of G3P and the production of NAD(P)H. Thus, as is pointed out in references 45 and 6 for the flagella, a sustained ATP production via phosphoglycerate kinase in the chloroplast also relies on the regeneration of $NADP^+$. Similarly to that proposed as an alternative hypothesis in the section above, it is possible to invoke the action of malate dehydrogenase, but it is also possible that chlororespiration participates to a significant extent.

Chlororespiration involves two proteins that have been recently identified in *Chlamydomonas*: a NAD(P)H dehydrogenase, NDA2 (13), and a plastid terminal oxidase, PTOX2 (21). NDA2 reduces plastoquinones from NAD(P)H, and PTOX2 regenerates oxidized plastoquinones at the expense of O_2. The activity of these two chloroplast enzymes, which act in tandem, results in the oxidation of NAD(P)H and the consumption of O_2, and has been denoted chlororespiration (15). Initially, chlororespiration had been thought to provide ATP to the chloroplast in the dark (15). However, and despite contradictory reports (see reference 61 for a discussion of the reports), chlororespiration is probably not electrogenic, i.e., unlike mitochondrial respiration, the electron transfer from NAD(P)H to oxygen is not coupled to the translocation of protons across the thylakoid membrane, which excludes the possibility that ATP could be directly produced by chlororespiration. However, as suggested above, when extended to glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase activity, chlororespiration could participate in sustaining an oxidative flux, associated with ATP production. This may also be the case for a sustained glycolytic flux under starch degradation conditions, and chlororespiration may represent a control point of this flux.

Compartmentalized fermentative pathways. Similarly to mito- and chlororespiration, which oxidize NAD(P)H in the presence of O_2 and sustain the glycolytic flux, in the absence of O_2, *Chlamydomonas reinhardtii* activates fermentative pathways. Recent reviews have described the multiplicity and compartmentalization of these pathways in *Chlamydomonas* (62, 63). In Fig. 2, we have represented a simplified version of the chloroplast fermentative pathways comprising pyruvate phosphate dikinase, pyruvate carboxylase (PYC), malic enzyme (MME), pyruvate ferredoxin oxidoreductase (PFR), pyruvate formate lyase (PFL), phosphate acetyltransferase (PAT), acetate kinase (ACK), and aldehyde/alcohol dehydrogenase (ADH), but other enzymes are found in the cytosol. The enzymes in the cytosol include PEP carboxykinase (PCK), PEP carboxylase (PEPC), pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH), alanine aminotransferase (ALAT), aldehyde/alcohol dehydrogenase (ADH). The mitochondria contain acetate kinase, phosphate acetyltransferase, acetyl-CoA syn-

thase (ACS), pyruvate formate lyase, malic enzyme, fumarate hydratase (FUM), and soluble fumarate reductase (FRD) (62, 64; also see Table 1 for a selection of enzymes). The fact that fermentation enzymes are localized in the chloroplast, mitochondrion, and cytosol shows that reoxidation of NAD(P)H occurs in these different compartments, allowing for a distributed ATP production by glycolysis or TCA. In dark anaerobic conditions, the enzymatic reactions of fermentation would play a similar role to mitochondrial activity or chlororespiration under aerobic conditions, as highlighted in previous sections.

INTERACTION BETWEEN CARBON METABOLISM AND ELECTRON TRANSPORT, A CASE STUDY

Upon dark anaerobic adaptation, the respiratory activity of the cells steadily decreases the oxygen tension in the suspension. Once anoxia is reached, the mitochondrial cytochrome oxidase (65), alternative oxidase (AOX) (65), and the chloroplast alternative oxidases, PTOX1 and PTOX2 (21) are inhibited. As a result, electrons accumulate in the mitochondrial and chloroplast electron transport chains.

Very early experiments have shown the influence of cell metabolism on photosynthesis. As discussed in reference 66, a prolonged anaerobic adaptation induced an inactivation of photosynthesis and it is slowly reactivated upon continuous illumination of the dark anaerobic sample. This slow reactivation shows a pronounced lag, the duration of which increases with the length of the anaerobic incubation. O_2 evolved by PSII possibly feeds back to reoxidize the PQ pool either directly (chlororespiration) or via PSI photochemistry (Mehler reaction) or via the malate shunt, therefore further stimulating the reactivation of PSII photochemistry (67). The synthesis of hydrogenase under anaerobic conditions, likely providing extra electron acceptors at the level of PSI and ferredoxin, also stimulates O_2 evolution and photochemical quenching of chlorophyll fluorescence (68).

In green algae, the reduction of the PQ pool in the dark induces a "state transition," the migration of the type 2 light-harvesting complexes (LHC2) from PSII to PSI (state 2), whereas when the PQ pool is oxidized, LHC2 complexes are attached to PSII (state 1). This mechanism of state transitions is reversible, its role is essentially to poise the light excitation between the two photosystems in order to optimize intersystem electron flow: an overreduction of the PQ pool (too large PSII antenna size) would decrease PSII activity (as witnessed by a reduction of the PSII electron carrier, Q_A), and an overoxidation of the PQ pool and of PSI secondary electron donors (too large PSI antenna size) would decrease PSI photochemical efficiency (accumulation of oxidized P_{700}). State transitions, originally discovered as a chromatic adaptation responding to the difference in absorbance of the photosystems (69, 70), were later shown to depend directly on the redox state of the PQ pool (71, 72). Therefore, they are also expected to occur in the dark in response to metabolic changes.

Antimycin A and salicylhydroxamic acid (SHAM) block mitochondrial electron transport inhibiting cytochrome bc_1 and AOX, respectively. They also indirectly affect photosynthesis, favoring state 2 (37, 55; for a more detailed list of the effects of inhibitors on the redox state of the PQ pool or on state transitions, see reference 73). Inhibition of respiration results in two additive effects: NADH consumption is halted in the mitochondrion, and NADH production increases as a result of the stimulation of glycolysis (Pasteur effect) (for the accumulation of glycolytic intermediates, see reference 34). Similarly, the reduction of the plastoquinone pool is magnified upon inhibition of both mitochondrial and chloroplast oxidases. Addition of either sodium azide (NaN_3, inhibitor of cytochrome oxidase [COX]) or *n*-propylgallate (PG, inhibitor of PTOX) results in an only partial reduction of the PQ pool, while the combined addition of these inhibitors reduces the pool to more than 90% (74). It reflects a general trend, reviewed in reference 73, that either mito- or chlororespiration can reoxidize NAD(P)H produced by glycolysis.

In their kinetics analysis of state transitions, Delepelaire and Wollman (75) investigated the reversibility of state transitions *in vivo*. Their experiments were conducted with a mutant devoid of PSII, but similar results can be easily reproduced in the WT strain or other mutants of interest with the addition of DCMU and hydroxylamine (inhibitor of the donor side of PSII). They show that upon dark anaerobic adaptation, state 2 is formed, due to the reduction of the PQ pool, as discussed above. When the anaerobic sample is illuminated, state 1 is restored, showing that the PQ pool is reoxidized. However, in this instance, this reoxidation of the PQ pool cannot be attributed to the reactivation of chloro- or mitorespiration, or even the Mehler reaction, because O_2 evolution is prevented in the absence of active PSII. Interestingly, and very similarly to that described above (67, 68), Delepelaire and Wollman (75) note the presence of a lag in the kinetics of state 1 formation, the duration of which increases with the time of anaerobic incubation. In the absence of O_2 evolution, the reoxidation of the PQ pool in the light can be slowly initiated although it is probably limited by the pool of electron acceptors at the level of PSI ($NADP^+$). Once the PQ pool starts to become oxidized in the light, electron acceptors are available for cyclic electron flow (NDH pathway and ferredoxin pathway), and ATP is synthesized. This ATP formed in the light is liable to serve as a substrate for many reactions that also consume NAD(P)H, most importantly the Calvin cycle. The newly regenerated $NADP^+$ is an efficient electron acceptor from PSI and amplifies the PQ pool oxidation. This "start-up/amplification" model can explain fairly well the pronounced sigmoid fluorescence rises observed by Delepelaire and Wollman (75). The fact that ATP is required for the formation of state 1 was demonstrated later on by Bulté et al. (76), who used addition of the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and two different chloroplast ATPase mutants to impede the light transition from state 2 to state 1. These authors also showed that this transition was unaffected upon inhibition of mitochondrial respiration (antimycin A and SHAM) or in the respiratory deficient *dum1* mutant, further demonstrating the necessity of ATP production in the chloroplast compartment. Interestingly, chloroplast ATPase mutants treated with antimycin A and SHAM (no mitochondrial ATP production either) could exit from state 2 only when treated with methyl viologen to start up PQ oxidation and ATP production from glycolysis (76).

These observations strongly support a "start-up/amplification" model where in the light, cyclic electron flow stimulates linear electron flow, the newly synthesized ATP initiating the reoxidation of NADPH, further pulling the electrons away from the PQ pool. There is, however, an observation that raises a question: why does the lag phase of the fluorescence rise observed in the transition from state 2 to state 1 increase with the time of anaerobic adaptation? If this lag time really represents the building up of cyclic electron flow, it is indeed expected to increase, as the redox

poise in the stroma decreases (fewer oxidized PSI electron acceptors are available to reoxidize plastoquinones, driving the system to a more inert state [photochemically inactive]). During the time course of dark anaerobic adaptation, reductants are indeed expected to accumulate but only transiently because the activation of fermentative pathways should contribute to the reoxidation of NAD(P)H in the dark. In the light, H_2 production should provide another escape route for electrons out of the photosynthetic chain, so instead of an increase in the lag time upon increasing the anoxic period, a shortening of the lag period is rather expected.

These physiological studies would benefit from an in-depth comparison with time course measurements of transcription and translation upon anaerobic adaptation. This comparison would show if a control is exerted at the genetic level in addition to the metabolic regulation described above.

LIPID METABOLISM

Fatty acid synthesis occurs in the chloroplast. As for starch biosynthesis, fatty acids, the precursors of lipids, are synthesized in the plastid both in flowering plants (77) and algae. The multisubunit chloroplast form of acetyl-CoA carboxylase (ACC) and fatty acid synthase (KAS) complexes are well conserved with their plant and bacterial orthologues and have predicted chloroplast targeting (78). The cytosolic homodimeric form of ACC is also conserved in the *Chlamydomonas* genome. In chloroplasts, the ACC reaction that forms malonyl-CoA from CO_2 and acetyl-CoA to produce long-chain acyl-CoA is the first committed step in fatty acid synthesis, and accumulation of sufficient pools of acetyl-CoA together with the appropriate stromal redox poise to activate ACC have been proposed to orient photosynthate toward fatty acid synthesis (79). While seed oil biosynthesis in chloroplasts has been delineated, the pathway from CO_2 to acetyl-CoA in plant leaves is still not understood; however, in the leaves of tested model species, fixed carbon rather than mitochondrial acetate appears to be the major source for acetyl-CoA (80). In algae, even less is known, although the most direct pathway to acetyl-CoA via 3-PGA, PEP, and pyruvate could potentially be reconstituted between the cytosol, mitochondria, and chloroplast under aerobic phototrophic conditions.

Alternatively, acetate feeding in *Chlamydomonas* will result in direct acetate uptake into the chloroplast from the cytosol, which can be inferred (and will be discussed in greater detail below) from two recent articles: the transcriptomic and ^{13}C labeling data showing downregulation of the glyoxylate cycle in nitrogen-deprived WT cells after 48 h (81) and also the observation of lipid hyperaccumulation induced by acetate boosts in starch-deficient mutants, resulting in both cytosolic and chloroplast lipid bodies (82).

Lipid biosynthesis in *Chlamydomonas reinhardtii*. Only very recently in *Chlamydomonas* have the full range of accumulated polar and nonpolar lipids been identified as well as the genetic resources to produce them. Under nonstress conditions, polar lipids are produced as membrane constituents, with the major species being diacylglyceryltrimethylhomo-Ser (DGTS), which is also found in many other algae, lower plants, and fungi. In all, there are probably over 140 different polyunsaturated polar lipids in *Chlamydomonas* that are accumulated to low levels in nonstress conditions for membranes (83). Triacylglycerols (TAGs) are oils, or neutral lipids, and are made up of three fatty acids esterified to a glycerol backbone. TAG synthesis may occur in *Chlamydomonas* as in plants where it is relatively well understood. Acetyl-CoA is processed in the chloroplast and exported as acyl-CoA or free fatty acids where it goes through synthesis in the endoplasmic reticulum to produce TAG via the action of acyltransferases. Glycerol-3-phosphate and acyl-CoA form diacylglycerol (DAG) via an endoplasmic reticulum multienzyme pathway, then *de novo* TAG synthesis may occur via two different pathways: by transfer of a fatty acid from acyl-CoA to a DAG generated by the action of a phosphatase on phosphatidic acid and diacylglycerol acyltransferase (DAGAT) or by transesterification where a fatty acid from a membrane lipid is transferred by phospholipid diacylglycerol acyltransferase (PDAT) to the free hydroxyl group of DAG (for a review, see reference 84). Recently, it was observed that neutral lipids can be synthesized directly in the chloroplast, but the exact mechanism and enzymes involved are unknown (82, 85).

TAGs can become major storage reserves under certain conditions, including stress or nutrient starvation resulting in the formation of oil bodies. There is strong evidence that these storage units are also required for synthesis, degradation, and homeostasis of lipids, as proteomic studies show that hundreds of proteins localize to these structures (86, 87). The TAG profiles from extracted lipid bodies are enriched in palmitic (16:0), linoleic (18:2), and oleic (18:1) acids.

Due to the complex links between lipid storage and stress response in algae, the lipid accumulation response is expected to be multifactorial. There are multiple diglyceride acyltransferase genes (DAGAT/DGTT) in *Chlamydomonas*, and their expression has been verified, with some of these genes being highly upregulated under N stress and thus exhibiting the lipid accumulation response from type 1 DGAT1 and type 2 DGTT1 (88) and type 2 DGTT4 (89). An experimental approach using the overexpression of three type 2 DAGAT genes, however, had no effect on lipid profiles or lipid productivity in *Chlamydomonas* (90). This mirrors other studies of both diatoms and plant seeds where simply upregulating the TAG biosynthesis genes showed no real increase in lipid productivity. This highlights the complexity of the regulatory pathways and the interactions with other metabolites that are universally found in lipid accumulation.

Ingredients for lipid accumulation in *Chlamydomonas*, an empirical approach. The literature increasingly abounds with examples of the overaccumulation of lipid bodies that can be achieved in starch-devoid mutants of *Chlamydomonas reinhardtii* (82, 91–94). When mutant cells are resuspended in acetate-replete medium, but under nitrogen-starved conditions, the amount of TAGs per cell increases dramatically. This technique is very promising for oil production in microalgae, although there are two factors that beg manipulation. First, upon nitrogen deprivation, cell growth is arrested, so an increase in biomass therefore relies on reserve accumulation within the cell. Second, nitrogen starvation also induces the loss of photosynthetic apparatus, making the cell metabolism dependent upon assimilation of an external carbon source, precluding any cost-effective oil production at this stage.

The mechanisms at work in rerouting carbon metabolism from carbohydrates to fatty acids are still unknown. The present section aims at presenting the possible metabolic pathways involved in reallocating carbon to lipids, and in the following sections, the limitations in the yield of fatty acid production are discussed. A few examples of regulators or stress responses that may also play a role in the process are given.

(i) Nitrogen deprivation. Dissecting the physiological conse-

quences of nitrogen starvation is not an easy task, because depletion of nitrogen in the medium triggers a large number of stress responses, arrests cell division, and engages *Chlamydomonas reinhardtii* in the sexual cycle (see section below). Nitrogen is an essential element that can be assimilated in the form of nitrate (NO_3^{2-}), nitrite (NO_2^{2-}), or ammonium (NH_4^+) (see page 179 of reference 25), where it enters into cell metabolism via amino acid synthesis. From early studies using unicellular algae, we know that there is a link between carbon metabolism and nitrogen metabolism. Autotrophic nitrogen-replete cells will assimilate nitrogen only in the light, while nitrogen-limited cells will assimilate nitrogen in the dark given sufficient stores of starch. The presence of acetate can overcome both the light or starch requirement. This forms the basis of carbon and nitrogen partitioning and shows that access to carbon is essential for nitrogen uptake (95). In nitrogen-replete algae, a significant portion of photosynthate or carbon assimilated from acetate is invested in protein synthesis and cell proliferation. Finally, nitrogen starvation elicits a program in *Chlamydomonas* that stops cell division and engages them in gamete formation, breaking proteins down, oxidizing amino acids, and diverting carbon skeletons to carbohydrate metabolism and starch accumulation (33). The carbon skeletons originating from amino acid oxidation feed into the TCA cycle, leading to an accumulation of intermediates (eventually oxaloacetate) that can be further converted into longer-carbon-chain compounds via gluconeogenesis (glucogenic amino acids). The solely ketogenic amino acids, lysine and leucine, will be converted to acetyl-CoA and will presumably be used as substrate for the TCA cycle or contribute to pools for fatty acid synthesis. Interestingly, recent metabolomic studies that examined the dynamics of metabolite profiles under nitrogen stress identified the lysine degradation intermediate alpha-aminoadipate as a marker for nitrogen stress (8, 9). Under various degrees of nitrogen stress, flux through the lysine pathway is very responsive (9).

(ii) No cell wall, no starch. The starchless mutant from Steven G. Ball (96) (BAFJ5 strain), which is the flagship algal strain for lipid production (82), has major genetic lesions. In addition to the *nit1 nit2* mutations that prevent algal cells from growing when nitrate is the sole nitrogen source (97), this mutant is *cw15 sta6*, which denotes that it lacks both a cell wall and the ADP-glucose pyrophosphorylase; the latter enzyme catalyzes the first committed step in starch biosynthesis (96). The BAFJ5 strain therefore lacks two major carbon sinks, cell wall glycoproteins (with arabinose, mannose, galactose, and glucose being the predominant sugars), and starch granules. Under nitrogen deprivation, cells devoid of cell wall accumulate more triacylglycerols than WT cells do (82, 83). Cell wall hydroxylproline-rich glycoproteins may also be a source of amino acid reserve in nitrogen-stressed strains with a cell wall that is absent in the *cw15* strain. The pronounced increase in neutral lipid deposits in the double mutant devoid of cell wall and starch (82) suggests that carbon skeletons can be diverted through the intermediate acetyl-CoA to fatty acid synthesis. It appears that triacylglycerols and carbohydrates are communicating vessels for reserve storage in *Chlamydomonas*.

(iii) Acetate boost. In plants and most probably in green algae, acetyl-CoA carboxylase is the rate-limiting enzyme of fatty acid synthesis (99). In other eukaryotes, it relies on accumulated pools of acetyl-CoA and citrate (an allosteric activator of ACC). Under normal photoheterotrophic conditions, acetate (C_2) is assimilated to form acetyl-CoA and feeds into the TCA cycle and respiration or into the glyoxylate cycle, which yields succinate (C_4), which is in turn further converted into longer-carbon-chain compounds (gluconeogenesis). Carbon from acetate is eventually stored as starch. In starchless mutants of *Chlamydomonas* (*sta6*), soluble sugars and sugar-phosphates probably accumulate, as they do in the *Arabidopsis pgm* mutant (100). Such an accumulation may halt gluconeogenesis and the glyoxylate cycle and increase the concentration of upstream compounds up to citrate and acetyl-CoA, a situation favorable to fatty acid synthesis. The accumulation of soluble sugar-phosphates, and G6P in particular, is also likely to provide the reductant required for fatty acid synthesis, NADPH, via oxidation of G6P in the pentose phosphate pathway (as in oil seeds [101]). Interestingly, in the wild type, G6P dehydrogenase activity is increased at the onset of nitrate assimilation in nitrogen-limited *Chlamydomonas* cells (95), and its expression is upregulated upon nitrogen deprivation (81).

(iv) Light regulation or residual photosynthesis. Upon nitrogen starvation, in a medium containing acetate, *Chlamydomonas reinhardtii* cells lose chlorophyll and turn yellowish. Photosynthetic electron transport is decreased, cytochrome b_6f being the first of the photosynthetic complexes to be compromised (102, 103). Under such conditions, the contribution of photosynthate to fatty acid synthesis is most likely diminished. Under the experimental conditions used by Fan et al. (85) (late logarithmic cells resuspended in nitrogen-deficient media under 40 $\mu E \cdot m^{-2} \cdot s^{-1}$), TAG levels are shown to be dependent on the presence of both light and acetate, demonstrating a double requirement for fatty acid synthesis under these conditions. Whether light regulation works at the metabolic or genetic level remains to be determined, but it is worth remembering that acetate assimilation is ATP dependent and dependent upon cyclic photophosphorylation (28) and that cyclic electron flow around PSI is stimulated in nitrogen-replete *sta6* cells (52).

(v) Oil accumulation without acetate or mitochondrial activity. Mitochondrial mutants do not store significant reserves of starch under nitrogen stress (103), and strains with mutations in complex I and III genes do not produce lipid reserves like the WT under sulfur-deficient conditions (104). This dependence of starch and lipid synthesis on respiration has not been fully characterized and may be linked to metabolic requirements fulfilled by the mitochondria in a functional Krebs cycle. Intermediates such as malate or acetyl-CoA that serve as major pools of precursors for chloroplast-localized starch and TAG synthesis may be deficient. Alternatively, a loss of respiration has a strong effect on the photosynthetic apparatus by overreducing the PQ pool (104); this may constitutively signal low cellular ATP levels and downregulate reserve accumulation.

Phototrophically grown cells, even those lacking starch, do not show the spectacular yields in oil production achieved when cells are grown to stationary phase, resuspended in nitrogen-deficient media, and given an acetate boost (82). Nevertheless, an accumulation of neutral lipids in minimal medium comparable to that observed for acetate-containing medium has been reported when the cells are exposed to light regimes ranging from 120 to 400 photon flux density (PFD) during nitrogen stress (84, 89, 92). Photoautotrophic growth conditions result in a different physiological response to nitrogen depletion than heterotrophic growth conditions. First, the cytochrome b_6f complex is not lost at the same rate as in an acetate-containing medium (103). This may conserve a flow of photosynthate toward fatty acid synthesis. Sec-

ond, high-light-induced oxidative stress may channel a higher percentage of resources into lipid droplets from oxidized membranes (105). Interestingly, Merchant et al. (84) showed that increasing illumination from 120 to 480 PFD on photoautrophic cultures decreased overall yields of fatty acids; however, the composition of fatty acids at 480 PFD changed over a time course in favor of TAG, lending further support to a "high-light-induced" TAG accumulation pathway via recycling of other lipid components. The experimental quantification of a high-light response for lipid accumulation is challenging, because increased biomass results in shading within the cultures and loss of light intensity. The trend toward the use of controlled biomass bioreactors where light intensity is kept constant to test high-light lipid accumulation should help to further our understanding of this pathway.

Nitrogen starvation engages *Chlamydomonas* in a tremendous change in cell physiology, and the dissection of the mechanism of pumping up the oil content with acetate boosts should be addressed with large-scale techniques, such as transcriptomics, proteomics, and metabolomics. In our last section, we will address the question of the possible relationships between nitrogen starvation, cell cycle, and reserve accumulation, but prior to this, let us investigate the metabolic constraints for the conversion between starch and lipids.

METABOLIC CONSTRAINTS FOR CARBON PARTITIONING BETWEEN OIL AND STARCH

Energy requirements for carbon fixation. Photosynthetic linear electron transfer, from water to CO_2, is tightly coupled to proton transfer and ATP synthesis. Linear photosynthesis is therefore ruled by an inflexible stoichiometry of 9 ATP molecules produced per 7 NADPH molecules (ATP/NADPH ratio = 1.29), which does not allow microalgae to thrive in a changing environment where metabolic needs in ATP and NAD(P)H can vary drastically. The example usually brought to our attention is that linear electron transfer does not even fit the Calvin cycle, which necessitates 3 ATP and 2 NADPH molecules per CO_2 (ATP/NADPH ratio = 1.5). Extra ATP is therefore needed not only for carbon fixation but also for the CO_2-concentrating mechanism (1 or 2 additional ATP molecules per CO_2) and for starch synthesis (1 extra ATP molecule per glucose molecule polymerized). Alternative electron transfer pathways recycle or dissipate reducing power and favor ATP production at the expense of NADPH, therefore providing regulatory mechanisms for carbon fixation.

Energy loss in oil investment. When cells are grown photoautrophically, i.e., with CO_2 as the sole carbon source, starch synthesis is an efficient way to store carbon. In contrast, the synthesis of fatty acids represents a significant loss of carbon. Because acetyl-CoA (C_2) is an obligatory reaction intermediate for lipid synthesis and because photosynthesis produces triose-phosphates (C_3), there is loss of 1 in 3 of the fixed carbons upon the conversion of pyruvate (C_3) to acetyl-CoA (C_2) by the pyruvate dehydrogenase (PDH) complex. The glycolytic yield of 2 ATP molecules and 2 NADH molecules produced in the breakdown of G3P into acetyl-CoA does not compensate for the loss upon decarboxylation (CO_2 fixation requires 3 ATP and 2 NADPH molecules).

Energy payoff in terms of ATP equivalents. From the stoichiometry in ATP, NADH, and $FADH_2$ of biochemical reactions involved in a metabolic pathway, the conversion into "ATP equivalents" or "phosphate/oxygen (P/O) ratios" is a handy method to evaluate the energy cost of an anabolic pathway or the energy yield of a catabolic pathway. NADH is equivalent to 2.5 ATP molecules, and $FADH_2$ is worth 1.5 ATP equivalents. These values are found in textbooks of biochemistry, and they are now commonly accepted. They are not, however, set in stone. They reflect the chemiosmotic coupling of oxidative phosphorylation, i.e., the proton translocation coupled to electron transport in the mitochondrion (or respiration, 10 H^+ pumped per NADH, 6 H^+ per succinate, or $FADH_2$), the number of protons required for the formation of ATP (the *S. cerevisiae* ATPase containing 3 αβ-subunits in F_1 and 10 c-subunits in F_0, 3 ATP molecules are formed each 10 H^+ transferred), the phosphate translocase symporter (1 extra H^+ is used to transport ATP, ADP, and P_i across the mitochondrial membrane), and the shuttle system used to transport cytosolic NADH into the matrix. The values of 2.5 ATP equivalents per NADH and 1.5 ATP equivalents per $FADH_2$ can therefore be considered "optimal" rather than really "operating." Under such optimal conditions, the breakdown of a glucose molecule, giving 4 NADH plus 2 ATP molecules down to 2 pyruvate molecules and 6 NADH plus 2 $FADH_2$ plus 2 ATP molecules through the oxidation of 2 acetyl-CoA molecules in the TCA cycle, would give the most accepted (maximal) value of 32 ATP molecules produced, i.e., $32/C_6 \approx 5.3$ ATP equivalents/C (Table 2).

Upon β-oxidation of an even-numbered fatty acid (C_{2n}), $n - 1$ oxidation steps yield 1 NADH molecule plus 1 $FADH_2$ molecule each, i.e., $(n - 1) \times 4$ ATP equivalents, and n acetyl-CoA molecules. Because acetyl-CoA (C_2) is worth 10 ATP equivalents, it gives a total of $(n - 1) \times 4 + n \times 10 = 14\,n - 4$ ATP equivalents.

As a preliminary conclusion at this stage, although sugars represent a better form of carbon storage than fatty acids, the energy return upon fatty acid oxidation (almost 6.7 ATP equivalents per carbon atom for palmitic acid) is greater than for glucose oxidation (about 5.3 ATP equivalents per C) (see the right column of Table 2). This higher energy/mass ratio for oil may explain why oils are often a preferred storage reserve in seeds: smaller and lighter seeds may confer a selective advantage for dissemination. In microalgae, however, oil accumulation is instead associated with stress conditions like high light or nitrogen deprivation, and the reasons for preferential lipid storage appear less clear.

Unknown ATP equivalence or P/O ratio for NADPH. NADPH is generally used in anabolic pathways, whereas catabolic pathways mostly produce NADH. To compare the energy balance of anabolic versus catabolic pathways, we thus have to address the problematic issue of the calculation of the ATP equivalence of NADPH. Although the standard midpoint potentials of the redox couples NAD^+/NADH and $NADP^+$/NADPH are identical ($E_m = -320$ mV), textbooks of biochemistry generally argue that NADPH is worth one more ATP than NADH. This estimation finds its origin in a cycle involving pyruvate carboxylase (PYC), malate dehydrogenase (MDH), and malic enzyme (MME). Such reactions, reactions in favor of the reduction of oxaloacetate to form malate and the decarboxylation of malate into pyruvate, would produce NADPH at the expense of NADH but would also require an ATP molecule (hence the difference of one ATP equivalent) for the carboxylation of pyruvate to regenerate oxaloacetate and complete the cycle. The involvement of NADP malic enzyme in supplying NADPH for fatty acid synthesis has been evidenced in hepatocytes and adipocytes. It probably stands true in heterotrophic plant tissues (106) but not necessarily in photosynthetic plastids where NADPH can be produced locally in the light by photosynthetic electron transport. Other reactions can also con-

TABLE 2 Rough approximations showing the energy balance between sugars and fatty acids[a]

Process, sugar, or fatty acid	Energy cost of production[a]	Energy return upon breakdown[a]	Yield[b] ATP/C	Yield[b] %
CO_2 fixation	3 ATP + 2 NADPH = *8 ATP eq.*			
C_3 triose-P	9 ATP + 6 NADPH = *24 ATP eq.*	2 ATP + 5 NADH + 1 $FADH_2$ = *16 ATP eq.*	5.3	*66.7*
C_2 acetyl-CoA	7 ATP + 6 NADPH − 2 NADH = *17 ATP eq.*	1 ATP + 3 NADH + 1 $FADH_2$ = *10 ATP eq.*	5	*58.8*
C_2 acetate		3 NADH + 1 FADH = *9 ATP eq.*		
C_6 sugars (glucose)	18 ATP + 12 NADPH = *48 ATP eq.*	4 ATP + 10 NADH + 2 $FADH_2$ = *32 ATP eq.*	5.3	*66.7*
Starch (C_{6n})	*n* (18 ATP + 12 NADPH) + *n* ATP = *49n ATP eq.*	*n* (4 ATP + 10 NADH + 2 $FADH_2$) = *32n ATP eq.*	5.3	*65.3*
C_{2n} fatty acid	*n* acetyl-CoA + (*n* −1) (ATP + 2 NADPH) = 23*n* − 6 *ATP eq.*	*n* acetyl-CoA + (*n* − 1) (NADH + $FADH_2$) = 14*n* − 4 *ATP eq.*		
C_{16} fatty acid (palmitic acid)	8 acetyl-CoA + 7 ATP + 14 NADPH = 63 ATP + 62 NADPH − 16 NADH = *178 ATP eq.*	8 acetyl-CoA + 7 NADH + 7 $FADH_2$ = 8 ATP + 31 NADH + 15 $FADH_2$ = *108 ATP eq.*	6.7	*60.7*
C_{18} fatty acid (oleic acid)	9 acetyl-CoA + 8 ATP + 16 NADPH = 71 ATP + 70 NADPH − 18 NADH = *201 ATP eq.*	9 acetyl-CoA + 8 NADH + 8 $FADH_2$ = 9 ATP + 35 NADH + 17 $FADH_2$ = *122 ATP eq.*	6.8	*60.7*

[a] Regular or roman values show the stoichiometries in ATP and NAD(P)H involved in anabolic or catabolic reactions. These values rather reflect the minimal requirements for synthesis and the maximal return upon breakdown. The overall energies in terms of ATP equivalents (ATP eq.) are shown in italic type. These calculations are based upon chemiosmotic coupling factors of 2.5 ATP produced per NAD(P)H oxidized and 1.5 for succinate ($FADH_2$). Such coupling factors are determined from the stoichiometry of proton to electron transfer in oxidative phosphorylation and from the chemiosmotic coupling of 1 ATP synthesized for 4 H^+ transferred, again a rather optimal value. The difference that may exist between NADH and NADPH is ignored in Table 2. The two ATP molecules required for the formation of palmitoyl-CoA from palmitate are ignored.
[b] The ATP/C yield column shows that, per carbon atom, fatty acids return more ATP than sugars but less energy compared to the energy invested in synthesis, as shown in the percent yield column. The estimates in the right column similarly apply to starch, longer-chain fatty acids (C_{18} or C_{20}) and triacylglycerols (TAGs) because polymerization and synthesis of glycerol-3P and phosphatidic acid involve only a few ATP or NAD(P)H molecules and do not change the ratios much. Simply stated, regular or roman values give reliable data, while italic values show rough estimates.

tribute to the interconversion between NADH and NADPH like the malate shunt and the triose-phosphate translocator. Following these two pathways, which do not produce or consume ATP, NADPH and NADH would have the same ATP equivalence.

Return on investments, the best-case scenario. In the absence of a reliable estimate for the ATP equivalence of NADPH, we cannot determine precise values for the energy costs of synthesis (second column of Table 2), neither can we fully rely on the accuracy of the return on investment ratio (in percentages in the rightmost column of Table 2). However, inasmuch as our purpose here is only to compare sugars to fatty acids, an over- or underestimation of the price of NADPH will offset the ratios in both pathways, still allowing for a side-by-side comparison. We found optimal return on investment values of 66.7% for carbohydrates and 60.7% for fatty acids.

In conclusion, although the energy yield per carbon is greater for fatty acids than for sugars (see above), fatty acids require more energy to produce. The loss of energy upon decarboxylation of pyruvate (C_3) to form acetyl-CoA (C_2) is irremediable. The question of the energy-yielding conversion of carbohydrate to fat has been addressed in a nice study comparing theoretical and measured yields in adipose tissue, where an energy loss of about 10% is similarly estimated (107). These calculations, which are based on common metabolic pathways, may apply as well to algae in darkness or plant heterotrophic tissues. It appears that upon the conversion between sugar and fat, the ATP balance is positive (107). Our estimates show a similar trend: neglecting the needs in ATP for the CO_2-concentrating mechanism, which should contribute equally in both pathways under photoautotrophic conditions, the energy requirement for sugar synthesis is 1.5 ATP molecules per NAD(P)H, while it is around 1.37 ATP molecules per NAD(P)H for fatty acid synthesis. Photosynthetic mechanisms and alternative electron transfer pathways are not designed to accommodate such increased demands in NAD(P)H. The Calvin cycle and cyclic electron flow compete for the utilization of NADPH in *Chlamydomonas* (73, 108) and in plants (109, 110). A decrease of the former translates into an increase of the latter, thereby limiting the production of NADPH per photon captured. This stands true for mutations of genes encoding enzymes involved in sugar metabolism, downstream of the Calvin cycle, like *sta6* (52) or fructose-1,6-bisphosphatase (111, 112), where cyclic electron flow rate is increased. It could explain why an impairment of starch synthesis does not translate into an increase of oil under photoautotrophic conditions. However, the same principles should apply reciprocally to strains with mutations in genes encoding components of cyclic electron flow, *nda2*, *pgr5*, and *pgrl1*, where the ATP/NADPH ratio is expected to decrease, as required for fatty acid synthesis. To our knowledge, however, the question of whether there is carbon partitioning between oil and starch has not yet been addressed in these mutants.

A CELLULAR PROGRAM CONTROLLING RESERVE ACCUMULATION IN MICROALGAE?

Cell cycle and circadian control on starch turnover. Early studies using synchronized, phototrophically grown, cell cultures showed that oscillations in reserve accumulation were regulated by both internal and external cues (113). Synchronization of the cell cultures was achieved by incubating the cultures without acetate in a 12-h light and 12-h dark regime. It was shown that in early G_1 phase, the cell cycle is regulated by light, and this is regulated by photosynthesis because placing the cells in the dark or adding DCMU (inhibitor of PSII) in the light forced the cells to remain in a resting state. Whether this is recognized by the cell as a carbon-depleted state or due to a chloroplast signal remains unknown (113). Interestingly, the cells had starch reserves in early G_1 phase but did not use these reserves, witnessed by the very low rates of

respiration during this period of cell cycle under normal cycling conditions. It was expected that the accumulation of starch and its utilization by respiration were the most pronounced during the latter part of G_1 during the time the cell gains biomass and just before division (113). More recently, it was shown that acetate-grown *Chlamydomonas* cultures synchronized in a 24-h light-and-dark regime exhibit circadian rhythms for starch accumulation and degradation (114). Starch synthesis is tightly correlated with ADP-glucose pyrophosphorylase activity (114). The link between circadian rhythms and breakdown of starch reserves in plants has been interpreted as a way to optimize plant growth and to avoid starvation during the night. Synchronizing starch breakdown with a normal 24-h light cycle may improve growth, whereas abnormal day lengths, outside the normal 24-h cycle, were detrimental to plant yields (115). It may be due to an optimization of reserve mobilization (115) or due to multiple effects affecting growth rates like the circadian control on photosynthetic yield resulting in reduced CO_2 fixation (116). These observations with both *Chlamydomonas* and *Arabidopsis* suggest that a parsimonious utilization of reserves within the context of the environment and the light period is essential to an organism's fitness.

Genetic regulation of reserve accumulation and response to stress. As stated above, high-yield starch and neutral lipid accumulation are stress responses. Nitrogen deprivation has been commonly used as a choice condition that may produce the highest neutral lipid accumulation response in green algae (for a review, see reference 105 and references therein). The response to nitrogen deficiency changes the life cycle from vegetative to reproductive and steers metabolism toward a specific path that stimulates a higher accumulation of storage compounds to prepare for zygote formation. It is worthwhile noting that other nutrient deficiencies or stresses do not stimulate gametogenesis in green algae (25). In the same vein, starch reserves that are rapidly accumulated under nutrient stress, for example sulfur stress, presumably due to cell cycle arrest and reorientation of metabolism, are also rapidly catabolized during the course of the stress (117). Under nitrogen deficiency, starch and lipids are not catabolized or are catabolized at a very low rate, until nitrogen is returned to the medium. This makes nitrogen deprivation a favorable experimental condition to investigate reserve accumulation as well as a way to fix carbon into high-value reserve molecules.

Synchronized cells resuspended in a nitrogen-deficient medium will divide once before the cell cycle is arrested early on, in G_1 phase (118). Under mixotrophic nitrogen limitation, it is the chloroplast that would appear to be specifically targeted for degradation: components of the photosynthetic apparatus, including the b_6f complex and ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), are degraded (89, 103), transcription of almost all photosynthesis-related proteins decreases (81), lipids double and starch is quickly accumulated at the expense of chlorophyll (83). In plants, chloroplasts constitute the major share of total cellular nitrogen (80%) mostly attributable to RuBisCO and other stromal enzymes, while mitochondria represent a much lower expenditure of nitrogen (less than 10% of total cellular nitrogen in plants) (119). Inasmuch as the chloroplast represents a reserve for cellular nitrogen, the fact that plant leaves and *Chlamydomonas* maintain respiration at the expense of photosynthesis may confer a better endurance to nitrogen limitation.

The genes responding to nitrogen deficiency account for around 25% of the *Chlamydomonas* transcriptome, and many of these genes are directly related to nitrogen metabolism and show signs of positive or negative regulation within minutes of transfer (88). One percentage of upregulated genes are related to fatty acid synthesis. One third of the upregulated genes are esterase and lipase genes, reinforcing the observation that a significant proportion of the TAG precursor are recycled from lipids from existing membranes (83). The *NRR1* gene was identified because it showed the same profile of upregulation as the *DGAT1* and *PDAT1* genes did, which are central to *de novo* and transesterification pathways of TAG synthesis. The putative protein, nitrogen response regulator 1 (NRR1), has SQUAMOSA DNA binding motifs and is absent under normal growth conditions; it is upregulated after 12 min of nitrogen deficiency and already 100 times upregulated at the transcriptional level 30 min after the medium is changed. The expression of NRR1 is similar to the level of DGAT1 mRNA, and the *nrr1* mutant also accumulates 50% less TAG than the wild type does. This nitrogen deficiency transcription factor, NRR1, appears to be the first serious candidate to be reported in the literature as a nitrogen regulatory "switch" (88). This type of strategy that uses a combination of metabolic and transcriptome profiling to screen for genes upregulated at the onset of lipid accumulation and then target those genes that have motifs found only in transcription factors is a powerful means to unravel the dense network of control involved in nitrogen stress and lipid accumulation.

Classical genetics remains a good experimental approach to address the question of the interplay between stress responses, cell cycle and reserve accumulation. The disconnection between various elements of the nitrogen deficiency response in knockout mutants is a way to find the links between these various processes. The C4 mutant, deficient in gametogenesis, isolated by Bennoun and reported in references 102 and 103, undergoes a strong accumulation of starch in response to nitrogen limitation but does not lose photosynthetic capacity. The *Dif2* (nonconditional mutant) and *Dif3* (conditional mutant) (120, 121) are class I mutants affected in the gametogenic program. Class I means that they do not respond to nitrogen stress and do not undergo the sexual program. The same group found a number of genetic markers to describe the initial response to nitrogen starvation, and this group of genes is composed of genes involved in both nitrogen-scavenging mechanisms and early gametogenesis response elements (120, 121).

CONCLUSION

That starch and oil could be considered communicating vessels for energy storage is an emerging concept, and although some pieces of evidence seem to support this general trend, others do not or not in the expected direction. For instance, studies on the starchless *Arabidopsis pgm1* mutant (*pgm1* equivalent to *sta6*) (100), embryos were shown to accumulate 40% less oil than the WT (122). In plastids isolated from developing rapeseed embryos, both starch and oil are accumulated, and about 50% of the carbon from G6P is utilized for starch synthesis, with the remainder divided equally between fatty acid synthesis and the OPP pathway. As the embryo switches to oil accumulation, the fluxes of imported G6P to starch and fatty acids then decrease, while oxidation through the OPP pathway increases (101). Depending on culture conditions, unicellular green algae seem to show the metabolic characteristics of plant leaves or oil seed embryos. The regulatory control points that balance sugars and lipids possibly work at the enzymatic and genetic level, and *Chlamydomonas reinhardtii* may

contribute significantly to the dissection of these regulatory mechanisms inasmuch as it is capable of growing under photoautotrophic or heterotrophic conditions and reproducing through the sexual cycle or dividing as a vegetative cell.

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