System Response of Metabolic Networks in *Chlamydomonas reinhardtii* to Total Available Ammonium

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Drastic alterations in macronutrients are known to cause large changes in biochemistry and gene expression in the photosynthetic alga *Chlamydomonas reinhardtii*. However, metabolomic and proteomic responses to subtle reductions in macronutrients have not yet been studied. When ammonium levels were reduced by 25–100% compared with control cultures, ammonium uptake and growth rates were not affected at 25% or 50% nitrogen-reduction for 28 h. However, primary metabolism and enzyme expression showed remarkable changes at acute conditions (4 h and 10 h after ammonium reduction) compared with chronic conditions (18 h and 28 h time points). Responses of 145 identified metabolites were quantified using gas chromatography-time of flight mass spectrometry; 495 proteins (including 187 enzymes) were monitored using liquid chromatography-ion trap mass spectrometry with label-free spectral counting. Stress response and carbon assimilation processes ( Calvin cycle, acetate uptake and chlorophyll biosynthesis) were altered first, in addition to increase in enzyme contents for lipid biosynthesis and accumulation of short chain free fatty acids. Nitrogen/carbon balance metabolism was found changed only under chronic conditions, for example in the citric acid cycle and amino acid metabolism. Metabolism in *Chlamydomonas* readily responds to total available media nitrogen with temporal increases in short-chain free fatty acids and turnover of internal proteins, long before nitrogen resources are depleted. *Molecular & Cellular Proteomics* 11: 10.1074/mcp.M111.016733, 973–988, 2012.

Algae are being investigated as a potential source for biofuel production (1–3). Temperature, light intensity, salinity, and nutrient availability are the major external determinants of algal growth (4–6), reproduction (4, 5), and morphology, including lipid contents. Nitrogen availability is one of the most important factors affecting cellular physiology and lipid metabolism in algae (7–9). An imbalanced carbon-to-nitrogen ratio alters activities of a wide range of metabolic enzymes not only through the control of nitrogen assimilation but also by modulating photosynthetic capacity and starch and sugar accumulation (10). At the same time, algae reduce their growth and divert their metabolic fluxes toward the protection of cellular structures by synthesizing more lipids (11). In algal biofuel research, identifying metabolic modification and associated biological consequences under nutrient stress conditions may substantially help achieve an optimized balance between cellular growth and lipid production.

Algal growth and metabolism are tightly coordinated by regulatory biochemical networks to increase the survival probability of algae in nutrient deprivation conditions (12–16). Advances in molecular profiling technologies such as microarray-based transcriptomics and mass spectrometry-based proteomics enable studies of the cellular responses to stress conditions on different molecular levels (17–22). However, alterations in gene expression may not translate to alterations in proteins abundances. There are many ways how proteins, and especially enzymes, can be regulated to affect metabolic fluxes and ultimately, phenotypes, comprising protein translation and degradation, post-translational modifications and allosteric actors (metabolites). Hence, the outcome of cellular regulation should encompass metabolite measurements to truly depict a systemic view of stress responses, called metabolomics. Metabolomics uses a range of platforms for surveying combinations of chemical compounds in biological systems (23–26). Although measurements of proteins, especially enzymes, concomitant with their substrates, products and allosteric modifiers appear to be a logical choice of tools to study metabolic response to nutritional stress, there have been only a few investigations on metabolomics and proteomics data integration to enable a system level understanding of cellular metabolism. We present here such integrative analysis, investigating the response of *Chlamydomonas reinhardtii* to limitations of total nitrogen availability. *C. reinhardtii* is a microalga that has importance as model for algal biofuels (11). Availability of a sequenced genome (27), molecular biology protocols (28, 29), a proteomic database (25), and metabolomics protocols (30, 31) benefit the exploitation of *Chlamydomonas* to investigate global metabolic changes in response to environmental or genetic alterations (32, 33).

When sources of nitrogen are fully depleted in culture media of microalgae, growth of biomass is strictly limited
CC125 was used for all studies. The strain was cultivated in TAP medium (4) at 23 °C under constant illumination with cool-white fluorescent bulbs at a fluence rate of 70 μmol m⁻² s⁻¹ and with continuous shaking (120 rpm). Cryopreserved stocks (41) were used to inoculate a starter culture, which was harvested at late log-phase and used to inoculate a new culture at a starting density of 5.0 × 10⁵ cells/ml. All cell numbers were counted with a Z2 Coulter Counter cell and particle counter (Beckman-Coulter, Brea, CA). Coulter cell counts were verified for representative samples using microscopy. After 48 h, cells were harvested by centrifugation, washed twice with sterile 20 mM TRIS-buffered media (42) under five different growth conditions (standard condition for 400 mg/L ammonium chloride, and decreased contents of 300 mg/L, 200 mg/L, 100 mg/L, and 0 mg/L of starting ammonium availability) using 20 ml total volume in 125 ml flasks. Eight independent cultures per condition were used for metabolomic studies in response to total ammonium availability from lag phase to the entry of stationary phases ensuring that ammonium uptake was not the limiting factor in cellular responses and that cell division remained unaltered. Using mass spectrometry for quantifying cellular responses on the level of protein and metabolite abundances, we show that C. reinhardtii readily responds to the total available ammonium content in its local environment, and that there are a range of biochemical modules that differ in a dynamic manner to cultures grown with “standard” starting ammonium conditions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Harvest**—The Chlamydomonas reinhardtii strain CC125 was used for all studies. The strain was cultivated in TAP medium (4) at 23 °C under constant illumination with cool-white fluorescent bulbs at a fluence rate of 70 μmol m⁻² s⁻¹ and with continuous shaking (120 rpm). Cryopreserved stocks (41) were used to inoculate a starter culture, which was harvested at late log-phase and used to inoculate a new culture at a starting density of 5.0 × 10⁵ cells/ml. All cell numbers were counted with a Z2 Coulter Counter cell and particle counter (Beckman-Coulter, Brea, CA). Coulter cell counts were verified for representative samples using microscopy. After 48 h, cells were harvested by centrifugation, washed twice with sterile 20 mM TRIS-buffered media (42) under five different growth conditions (standard condition for 400 mg/L ammonium chloride, and decreased contents of 300 mg/L, 200 mg/L, 100 mg/L, and 0 mg/L of starting ammonium availability) using 20 ml total volume in 125 ml flasks. Eight independent cultures per condition were used for metabolomic studies plus four cultures per condition for protein studies. 1 ml of cell culture was harvested at four different points of growth phase (4 h, 10 h, 18 h, and 28 h). At the incubation site 1 ml cell suspensions were injected into 1 ml of −70 °C cold quenching solution composed of 70% methanol in water and pellets were lyophilized and stored at −80 °C further analysis. Ammonia contents in media were measured by spectrophotometry using the salicylate method (43) with the TNTplus™ 832 ammonia detection kit (Method 10205, Hach Company, Loveland, CO) in reference to a 5-point calibration curve. Media samples were diluted with blank media to the recommended range for quantification (2 to 47 mg/L). Protein content was determined using the Bradford assay. Total chlorophyll content was measured by spectrophotometry using the following equation: Chlorophyll (a+b) = 20.04 x (A649) + 6.1 x (A665).

**Extraction Method and Sample Preparation of Metabolites and Proteins**—For metabolites, procedures followed the protocol we had previously validated (30). Briefly, lyophilized cells were disrupted using a single 5 mm i.d. steel ball, followed by the addition of 0.75 ml extraction solvent of methanol:chloroform:water (5:2:2) and vortexing. After 2 min centrifugation at 16,100 rcf, 0.70 ml extracts were collected and concentrated to dryness for further analysis. A mixture of internal retention index markers was prepared using fatty acid methyl esters of C8, C9, C10, C12, C14, C16, C18, C20, C22, C24, C26, C28, and C30 linear chain length, dissolved in chloroform at a concentration of 0.8 mg/ml (C8–C16) and 0.4 mg/ml (C18–C30). 2 μl of this RI mixture were added to the dried extracts. 5 μl of a solution of 20 mg/ml of 98% pure methoxyamine hydrochloride (CAS No. 593–56–6, Sigma, St. Louis, MO) in pyridine (silylation grade, Pierce, Rockford, IL) was added and shaken at 30 °C for 90 min to protect aldehyde and ketone groups. 45 μl of MSTFA with 1% TMCS (1 ml bottles, Pierce) was added for trimethylsilylation of acidic protons and shaken at 37 °C for 30 min.

Proteins were extracted from lyophilized cell pellets with 0.75 ml of 45 mM Tris buffer with protease inhibitor (Halt Protease Inhibitor Mixture, Pierce, Rockford IL). Supernatants were collected after centrifugation at 16,100 rcf (2 min, 4 °C), proteins concentration were measured using the Bradford assay and the supernatants were further concentrated for trypsin digestion. 50 mM ammonium bicarbonate with 10% acetonitrile (final concentration) was added to concentrates and the disulfide bonds contained in the proteins were reduced in 10 mM final concentration of TCEP (Bond breaker, Pierce) for 10 min and alkylated with 55 mM iodoacetamide (Sigma) for 1 h in a dark place. The alkylation was quenched by addition of DTT (5 mM final concentration, Sigma) followed by an overnight trypsin digestion (Trypsin Gold, Madison, WI) at 37 °C using a ratio of 1:30. The digestion was stopped by addition of formic acid and sonication for 10 min, and the digests were desalted and concentrated prior to reconstitution with 5% acetonitrile in water with 0.1% formic acid, which equaled to initial condition for liquid chromatography injection.

**GC-TOF MS Analysis for Metabolites and LC-tandem MS/MS Analysis for Proteins**—A Lecos Pegasus III time of flight mass spectrometer (St. Joseph, MI) with a Gerstel automatic liner exchange and cold injection system (Baltimore, MD, CO) was coupled to an Agilent 6890 gas chromatograph (Santa Clara, CA) (44). Data acquisition was controlled by the Leco ChromaTOF software versus 2.32. Result files were preprocessed directly after data acquisition and stored as ChromaTOF-specific `.peg files, as generic `.txt result files and additionally as generic ANDI MS `.cdf files. For more details see Lee and Fiehn (30) and Kind et al. (45).

Liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) was performed on linear ion trap (LTQ) mass spectrometer (Thermo Electron, San Jose, CA) coupled with an Agilent 1200 Series binary HPLC pump (Agilent Technologies). Shotgun proteomics was performed for four replicates of the 100, 75 and 50% N conditions after trypsin digestion. 60 μl of the trypsin digests were separated on a 2.1 mm i.d. x 150 mm RP-C18 resin column with 1.8 μm particle size and 102Â pore size (Waters, Milford, MA). Separation was performed on a gradient from 5 to 95% acetonitrile in 0.1% formic acid at a flow rate of 150 μl/min for 140 min by alternating sample running and washing. MS and MS/MS data were acquired in a data-dependent mode: 10 LTQ MS/MS spectra were acquired on the most abundant ions detected master spectrum. DTA were created using DTSupercharge (version 2.16) and extract_msn (Thermo).

**MS/MS Data Analysis and Estimation of Protein Abundance**—Tandem mass spectra were extracted using Bioworks 3.0 and analyzed using the XITandem GPM Tornado version 2.0.0.4 (2008.02.01.2 spectrums modeler) (http://www.theqpm.org). XITandem was configured to search against NCBI nonredundant protein sequence (nr) database for Chlamydomonas reinhardtii (31,207 entries on August 1, 2008). One missed cleavage was allowed, and searches were performed with trypsin as the digestion enzyme, fixed carbamidomethylation of cysteines and potential modifications of oxidation of methionine and tryptophan, deamidation of asparagine and glutamine in round 1, and dioxidation of methionine and tryptophan in round 2. XITandem was configured to allow parent ion mass error of 1.8 Da and fragment mass error of 0.6 Da. Scaffold (version Scaffold-3.3.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide, protein identifications, and spectral counting-based semi-quantitation. Peptide identifications with greater than 95.0% were accepted as specified by the PeptideProphet
algorithm (46) and protein identifications with higher than 99.0% probability containing at least two identified peptides. Proteins that contained similar peptides and could not be differentiated on the basis of MS/MS analysis alone were grouped to satisfy the principles of parsimony. False discovery rates (FDR) were calculated by using the probabilistic method in the Scaffold application. For quantification by spectral counting, low-scoring versions of identified peptides were used at >80% matching probability to the corresponding spectra of confidently identified peptide sequences (supplement Proteins) (47–49). The Scaffold protein report containing the protein identification probabilities, number of unique peptides, unique spectra, assigned spectra and total spectra is provided in Supplement Proteins. The resultant 498 proteins were finally assigned to 495 UniprotKB identifiers (unique proteins) for authentic protein sequence, characterization, and further statistical analysis (supplement Proteins). All of the UniProtKB identifiers corresponding to each NCBI gene identifier are listed in Supplement Proteins.

Statistical Data Analysis—Statistical analyses were performed on all continuous variables using the Statistica software versus 8.0 (StatSoft, Tulsa OK). Univariate statistics for multiple study design classes was performed by t-tests and one-way analysis of variance. Data distributions were displayed by box-whisker plots, giving the arithmetic mean value for each category, the standard error as box and whiskers for 1.96 times the category standard error.

For the identification of differentially expressed proteins, protein abundances were assigned using normalized spectral abundance factor values (50). Normalized spectral abundance factor data sets were introduced into the R environment for statistical calculation (51) and parsed into each “exprSet” object for the recognition by specific Bioconductor packages (52). The Bioconductor package power law model was used to detect differentially expressed proteins with delta 0.05 by performing model-based estimates of standard deviation (53). The range of protein expression levels was partitioned in 10 intervals, and the model was fit at the 0.5 quantile of standard deviations in each partition. All proteins were considered if they were detected at least three times in one nitrogen condition and at least once in another condition. If proteins were detected at least three times in one nitrogen condition and not in any other condition, they were counted as complete induction or suppression.

Multivariate statistics and clustering analysis were performed by supervised partial least square (PLS) statistics within the Statistica data miner 8.0 version software. The metabolite dataset was pre-treated by Pareto scaling for multivariate analysis (54) while the protein data set was imported as expression abundance set with normalized spectral abundance factors (55).

MetaMAP—Molefile (56) encoded chemical structures of all the identified metabolites were retrieved from PubChem Compound database (57) using compound identifiers and the NCBI Batch Entrez utility (58, 59). The retrieved structures were clustered using an online structural clustering tool at PubChem website. The tool uses Tanimoto chemical similarity co-efficient (60) (range 0.0 to 1.0, where high score means high similarity between two metabolites) for calculation of similarity among the encoded structures which are decomposed into a substructure matrix defined by a 881 bit substructure key fingerprint (9, 61). Next, a pair-wise chemical similarity matrix was downloaded from the structural clustering result web-page. The similarity matrix and the list of associated metabolites that were found in the KEGG biochemical database were used as an input into the MetaMapp (62) software for generation of Cytoscape (63) network files in simple interaction format (.SIF). A threshold of 0.7 Tanimoto score was used to define the similarity cut-off among metabolites. A KEGG Rpair (64) reaction network graph was created using a single-metabolic step neighbor finding algorithm in MetaMapp (http://metamapp.rieknlab.ucdavis.edu). An additional similarity threshold of 0.85 Tanimoto score was used for lipids species. The final network graphs were imported into Cytoscape and merged into a single network graph. Results of differential statistics were converted into Cytoscape node attribute files, and were imported into Cytoscape. The graph was visualized using an organic layout algorithm in Cytoscape. Fold change was mapped to node size, and direction (up/down) was mapped to node color. Metabolites with p > 0.05 remained unlabeled. Metabolic mapping and its visualization are provided in supplement section as Cytoscape session file.

Integrated Biochemical Map of Detected Proteins and Metabolites in C. reinhardtii—An integrated biochemical map consisting of primary enzymes and metabolites was generated using Visualization and Analysis of Networks containing Experimental Data (VANTED) (65). The backbone of the metabolic pathway was constructed based on glycolysis, citric acid cycle (TCA) and amino acid metabolism available in the software. According to the list of enzymes and proteins of interest, we reconstructed the map to include only those metabolites and enzymes that showed significant differences in at least one experimental condition compared with the standard ammonium control. The resulting graph was visually enhanced using squares for enzymes and circles for metabolites, and colors were used to indicate the direction of differential expression. Dotted edges indicate several reaction steps between network nodes while solid edges indicate direct biochemical interactions.

RESULTS

Physiological Responses of Chlamydomonas Cells from Ammonium Limitation to Depletion—Metabolism responds in a highly dynamic way to environmental fluctuations that could affect the cellular growth. Use of algae in biotechnology industry relies on growth conditions that yield optimal results between input parameters (such as nitrogen) and output parameters (such as growth and product yields). We have hence selected four time points (lag, early and late exponential, and the entry of stationary phases) to resolve the time dependence of metabolic responses to different nitrogen regimes (standard replete-N condition (400 mg/l) and 25%, 50%, 75%, and 100% reduction of available nitrogen source). Fig. 1A shows that the ammonium uptake rates from media were nearly identical, independent of the starting concentrations. 57 mg/L ammonium chloride was still available for the 25%-reduced culture after 28 h. Importantly, cellular growth curves were identical among normal nitrogen conditions and the 25 and 50% deficient ammonium contents (Fig. 1B) for 28 h of nitrogen deficiency. Media ammonium was fully depleted after 10 h for the 75%-deficient culture, and consequently, growth of the 75% and the 100% (fully depleted) cultures stopped after 18 h, when intracellular protein recovery could not sustain further cell divisions. Despite a very similar uptake of ammonium from the medium, overall protein contents in the 25%- and 50% N-deficient Chlamydomonas cultures were significantly decreased after 10–28 h time points (p < 0.05) compared with the intracellular protein content at fully N-replete cultures (Fig. 1C), despite the readily available ammonium content in these cultures. Drastic reductions in total

1 The abbreviations used are: VANTED, Visualization and Analysis of Networks containing Experimental Data; EC, Enzyme commission.
available ammonium (75% to 100% N-deficient cultures) led to immediate and sustained reductions in cellular protein contents. Similarly, chlorophyll contents were rapidly decreased in 100% or 75% N-depleted cultures at 4–28h (Fig. 1). In contrary, the 50% N-depleted cultures showed chlorophyll reduction only at more than 18h of growth, and no significant reductions in chlorophyll contents were observed when comparing the mild 25% reduction in total available nitrogen with standard ammonium contents. However, it should be noted that in batch-fed cultures, even the standard conditions of 400 mg/L available ammonium chloride led to a decrease in cellular protein contents (after 28 h of growth) compared with initial contents at 4 h of growth, pointing to a high adaptability of metabolism in algae.

With less amounts of chlorophyll, less light energy is absorbed to be used for carbon fixation and cell division. With lower cellular amounts of proteins, available nitrogen contents can be used to sustain growth under unfavorable conditions by turning over less important proteins to ones that are critical for response to current environmental conditions. Both findings support the notion that intracellular mechanisms are activated to sense total nitrogen levels in the medium to balance use of nitrogen for protein or nucleotide biosynthesis in support of growth toward maximal cell density, and to decrease the absorption of light energy in anticipation of capacity limits of cell division.

Quantification of 187 Enzymes and 145 Identified Metabolites by Untargeted Mass Spectrometry Profiling—The genome-scale reconstruction of the *Chlamydomonas reinhardtii* metabolic network consists of 263 pathways and 1419 reactions (25), catalyzed by 713 enzymes that have been annotated experimentally or computationally to a DNA sequence of *C. reinhardtii*. In order to perform comprehensive analysis of primary metabolite responses to nitrogen depletion, we have used mass spectrometry-based profiling of proteins and metabolites in these cultures in a semiquantitative way.

To this end, we have identified 187 *C. reinhardtii* enzymes (out of a total of 495 identified proteins), or 26% coverage of the currently annotated biochemical network, using LC-ion trap tandem mass spectrometry and bioinformatics algorithms. All proteins were quantified by spectral counting, relative to the counts observed in samples from standard replete N-conditions culture. While proteomics by spectral counting only yields rough estimates of protein quantities (in comparison to quantification by target peptides, e.g. by isotope labeling), it has been shown to be equal or superior to SDS-PAGE gel separation with Coomassie blue staining (66). A total of 718,948 spectra were detected by using 140 min long liquid chromatography separation and data-dependent tandem mass spectrometry for 48 samples of N-replete and 25 and 50% N-deprived cultures. Using X!tandem, 43,879 unique peptides were assigned to detected spectra by MS/MS spectral matching, and were annotated as 2,802 unique proteins. To retain only high-confidence protein identifications, data were further filtered by a peptide probability threshold of >95% and a protein probability threshold of >99.0% with a
minimum of 2 peptides/protein. The filtration resulted in 498 proteins with NCBI sequence identifiers (GI), and the IDs were finally assigned to 495 UniprotKB identifiers (unique proteins) for authentic protein sequence and characterization (supplement Proteins). Among these proteins, 148 proteins were annotated by enzyme commission numbers (EC numbers) via KEGG database queries and 39 enzymes were manually added using Blast2go. The resulting 187 enzymes constitute 129 reactions and 64 pathways that were used for metabolic mapping. All proteins were further analyzed using Blast2go and Gene Ontology tools. 383 proteins were assigned to at least one category of biological process, molecular function and cellular component based on its sequence similarity to other proteins (67); (http://www.blast2go.de/).

We have further used cold injection/liner exchange gas chromatography-time of flight mass spectrometry in conjunction with automatic mass spectral deconvolution and BinBase database annotations for profiling of primary metabolites. From a total of 1,787 unique metabolic peaks that were positively detected at s/n >5 in at least one Chlamydomonas extract, 242 non-redundant metabolites were structurally identified, not counting internal standards and known chemical artifacts. In order to obtain high-reliability identifications by filtering out noisy and inconsistent signals, only metabolites were considered that were positively detected in at least 20 samples by our open-source BinBase algorithm (68). Quantifications for true negative or false negative peak detections were obtained from the raw GC-MS data on each specific quantification m/z-value as published before (68). Overall 564 compounds remained of which 145 unique metabolites were structurally identified using the Fiehnlib mass spectral/retention index libraries (45). We have mapped all identified metabolites to the KEGG database for pathway annotations and found that all major primary metabolic pathways were covered such as glycolysis, TCA cycle, pentose phosphate pathway, amino acids, urea cycle, carbon fixation, nucleotide metabolism, and fatty acid biosynthesis. However, 22 metabolites were not mapped to any pathway map in KEGG database, including important lipids such as 1- and 2-monopalmitin. Therefore, we used a recently proposed visualization method (62, 69) that combined biochemical similarity (via the KEGG Rpair database) with chemical similarity (via decomposing chemical structures into sets of substructures and subsequently calculating the Tanimoto similarity (60)). Using Cytoscape software (63), all identified metabolites were then mapped as nodes in network graphs to highlight the temporal dynamics of metabolic responses toward nitrogen limitation. These networks efficiently cluster novel metabolites (that are not yet annotated to Chlamydomonas enzymes) to known metabolic modules constructed from the KEGG Rpair database.

Chlamydomonas Metabolism Responds to Total Exogenous Ammonium Contents—Because C. reinhardtii’s ammonium uptake rate does not depend on the total available ammonium in the medium, it had always been implicitly assumed that metabolism remains unaltered as long as sufficient ammonium is present in the medium. On contrary, we have found that a range of metabolites were detected at significantly different levels (p < 0.05) even under subtle reduction of initial ammonium chloride concentrations to 300 mg/L, compared with replete nitrogen conditions. Counting all unique metabolites, i.e. unidentified compounds as well as structurally known metabolites, the number of significantly altered metabolites (p < 0.05) increased for each level of ammonium reduction from 19–109 compounds at 4 h to 172–272 compounds at 28 h (Table I). The same trend was found for structurally known compounds and for unknowns, and detailed results are given as supplement metabolites. Compared with cultures grown under standard ammonium contents, over half of all identified metabolites were significantly different after 28 h of complete nitrogen depletion, suggesting dramatic reshuffling of metabolic pathways. As expected, many compounds were found significantly down-regulated under nitrogen depletion in comparison to all other ammonium-level cultures, such as key intermediates of the citric acid cycle (Fig. 2 top left panel, malic acid), or the chlorophyllide building block phytol and the glycolytic intermediate 3-phosphoglycerate (supplement metabolites). In addition to differences in regulation of metabolite abundances we found differences in co-regulation of metabolites. For example, all metabolites of the citric acid cycle (supplement metabolites) showed the same temporal regulation as malic acid (Fig. 2 top left panel), with maximum metabolite levels found at the late exponential growth phase (18 h growth) for all nitrogen-containing conditions. This co-regulation of metabolites of the citric acid cycle was completely absent under fully depleted nitrogen conditions (Fig. 2, top left panel). Using Pearson’s correlation analysis, a range of further differences in temporal co-regulation of metabolism were observed (data not shown), especially when comparing replete nitrogen conditions to drastically reduced or depleted nitrogen levels. This finding suggests that complete nitrogen depletion affects metabolism and overall algal growth so drastically that conclusions may not be useful for applications in biotechnology.

Interestingly, a range of metabolites showed gradual differences in both temporal regulation and overall metabolite pools, such as galactonic acid and thronic acid (both involved in ascorbate metabolism) (Fig. 2 mid panel), levels of

| Table I
| Number of differentially expressed metabolites in temporal response to reduction in ammonium availability, compared to standard nitrogen conditions (400 mg/L NH4Cl) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | 4 h             | 10 h            | 18 h            | 28 h            |
| 300 mg/L NH4Cl | 19              | 83              | 85              | 172             |
| 200 mg/L NH4Cl | 29              | 75              | 73              | 145             |
| 100 mg/L NH4Cl | 83              | 204             | 175             | 212             |
| 0 mg/L NH4Cl   | 109             | 175             | 242             | 272             |
free fatty acids, amino acids or the lysine-pathway intermediate 2-ketoadipic acid and several other metabolites (supplement metabolites). In a different type of regulation, large increases in metabolic pools were observed under low nitrogen levels. For example, biosynthesis of some compounds such as the plant hormone salicylate was only triggered under chronic exposure to full nitrogen depletion (Fig. 2, lower panel). Other metabolites showed an earlier response to low availability of nitrogen, such as sophorose and guanosine. As such compounds are not used in central carbon metabolism and are not known to be directly used as sources for production of RNA, proteins or cell walls during cell division, it is an intriguing possibility that these metabolites might work as intracellular signaling molecules. For instance, the drastic alteration of guanosine levels might be involved in differential RNA splicing in which the 5’ cap of mRNA, 7 methyl-guanosine triphosphate (m7G) is required to initiate protein synthesis (70).

Based on the measurements of ammonium contents in the media, the disaccharide sophorose (PubChem CID 92797; 2-O-beta-D-Glucopyranosyl-D-glucose) was induced whenever ammonium amounts were decreased to below 100 mg/L for several hours. Sophorose, a novel algal metabolite with yet unknown biochemical origin, was identified with high reliability. GC-TOF MS technology is particularly well suited to distinguish many different disaccharides simultaneously. For example, sophorose is well separated from the structurally related glucosyl-glycosides trehalose, kojibiose, laminaribiose, maltose, isomaltose, trehalose, cellobiose, lactose and others, using retention-index based mass spectral libraries (45).

Synthesis of guanosine was triggered drastically in cultures with less than ca. 150 mg/L ammonium (Fig. 2, lower panel). This increase in guanosine in response to low ammonium levels was far more pronounced and found at much earlier time points than increases in adenosine levels (supplement...
metabolites), which was found to be elevated only after media were devoid of ammonium for more than 4h of growth (supplement metabolites). Conversely, adenine, the precursor and catabolite of adenosine, showed exactly the opposite temporal response to nitrogen deprivation (supplement metabolites). Adenine shows a steep increase from 4h to 28h under standard N-conditions and (less pronounced) temporal increases under reduced ammonium concentrations, but adenine remains at very low levels under fully N-depleted culture conditions, without any temporal response. The profiling platform used here could not detect the—much lower abundant—known signaling molecules cAMP and cGMP; hence, it is not clear if the observed increases in guanosine and adenine are functionally related to known intracellular signaling pathways. At the very least, these findings may constitute novel hypotheses for nitrogen sensing in algae: metabolism in Chlamydomonas is regulated by the total available amount of nitrogen available, possibly orchestrated by novel signaling molecules like sophorose and guanosine, and not by the classic plant stress hormone salicylate which is only induced after prolonged lack of ammonium.

Metabolic Maps Show Increase in Short-chain Fatty Acids as Short Term Response to Lower N-availability—Although the detailed investigation of responses of individual metabolites is important, metabolic overview is lost. Classic multivariate statistics such as heatmaps, PCA or OPLS statistics helps ordering data with regards to covariance of variables (metabolites) and parameters (time and scope of ammonium availability) but hardly provides biochemical overview. Using Pearson’s correlation thresholds of \( r \geq 0.8 \) (71) for the metabolic dataset presented here correctly clusters the TCA metabolites malate, fumarate and citrate. Pearson’s correlation \( (r > 0.8) \) also clusters free fatty acids and corresponding lipid backbones (inositol-monophosphate, phosphoethanolamine, linoleic acid, linolenic acid, and heptadecanoic acid. However, metabolite covariance may be driven by a range of factors (72, 73), e.g. enzymes working at equilibrium (e.g. phosphogluco-isomerase), pathway regulation via committing enzymes, functional roles (lipid backbone and fatty acid biosynthesis), substrate availability, or transcriptional regulation by master transcription factors. Hence, data-driven clustering may miss genuine biochemical relationships but may instead co-cluster biochemically diverse molecules such as sophorose and guanosine \( (r > 0.8) \) based on their physiological function under a given biological situation.

We have thus employed a novel strategy for obtaining biochemical overview of metabolic regulations while maintaining visual clarity: MetaMAPP, a biochemical graph approach that uses biochemical reaction pair information (Fig. 3, red lines) and chemical similarity (Fig. 3, blue lines), see method section for details. We contrasted these metabolic regulation networks under reduced ammonium contents for all time points in comparison to standard starting levels of 400 mg/L ammonium chloride (Fig. 3 for 200 mg/L and 300 mg/L conditions, supplement MetaMapp for 0 and 100 mg/L ammonium chloride). Between 10h and 18h of nitrogen deprivation, the number of up-regulated metabolites (red nodes) was increased and the number of down-regulated metabolites was simultaneously decreased (blue nodes) for both the 50% and the 75% N-cultures (Fig. 3). Notably, it is apparent that many nucleosides were up-regulated as a cluster of compounds at 18 h of treatment, while this up-regulation of nucleoside biosynthesis was starting at even earlier time points for the 25% nitrogen cultures (10h) and prevalent for the fully depleted 0% N-cultures already at 4h (supplement MetaMapp). Importantly, it becomes clearly visible that proteinogenic amino acids were not differentially regulated at 4h under any degree of nitrogen deprivation. Lysine pathway intermediates (homoserine, lysine, 2-aminoadipate and 2-ketoadipate) responded most sensitively to nitrogen deprivation by increasing metabolite pools relative to cultures grown under replete ammonium media, e.g. at 18 h treatment for all nitrogen deprivation cultures but even earlier at 4 h and 10 h treatment under fully N-depleted conditions. Only under more severe and sustained ammonium deprivation, other proteinogenic amino acids underwent down-regulation of metabolic pools, starting with the key amino donor glutamate, then asparagine and glutamine, before further amino acid pools were affected such as alanine, threonine, serine or isoleucine. Down-regulation of metabolism was most strongly observed under completely absent ammonium cultures that also showed lack of growth starting 4h after treatment. Both the magnitude and the coverage of metabolome down-regulation were most evidently different after 18 h and 28 h in fully N-depleted cultures, while cultures that initially started with 75–25% ammonium-containing media showed increased metabolite contents compared with replete N-conditions for a range of biochemical modules, in particular for sugar alcohols and, surprisingly, free fatty acids. Indeed, the short chain, saturated free fatty acids C08:0–C12:0 were the first biochemicals to be up-regulated under even modest nitrogen deprivation at early time points (4 h) compared with fully replete conditions (supplement fatty acids), whereas at late-exponential growth, the 75–25% ammonium cultures provided increased levels of longer chain free fatty acids C16:0–C20:0, including several monounsaturated fatty acids. We found short-chain free fatty acid levels (C08-C10) to be inversely regulated to long chain free fatty acids (C18-C20) when comparing early time points (4 h, 10 h) with later time points (18 h, 28 h), see supplement fatty acids. Under severe nitrogen deprivations, especially for the 25% N-deprived cultures, up-regulation of fatty acids already started with longer chain free fatty acids during the lag and early exponential phases, while additional free fatty acids, including monooleylglycerol, were found increased at late exponential and early stationary phases. Intermediates of the glycolysis/glucoseogenesis pathway were found slightly up-regulated under 25% nitrogen conditions and on early time
Fig. 3. Metabolic networks of biochemical reaction pairs (red edges) and chemical similarity (blue edges) showing the regulation of all identified metabolites in C. reinhardtii under nitrogen limitation. Blue = down regulated metabolites, red = up-regulated metabolites at $p < 0.05$. Ball sizes reflect magnitude of differential metabolite expression. Metabolites that were not significantly different were left unnamed in order to keep visual clarity.
points under complete nitrogen depletion but down-regulated or indifferent under slight nitrogen reduction.

In summary, many different metabolic pathways sensitively responded to decreases in total ammonium contents, far before the available nitrogen was depleted from the media. Fold-changes in metabolite pools induced by nitrogen depletion (0%N), however, were far lower to what was observed in nitrogen starvation experiments in non-photosynthetic microbes (31, 74).

**Enzymes in Carbon Uptake and Carbon Fixation are Downregulated at Early Time Points of Low Nitrogen Availability**—Differential changes in metabolic pool levels can be explained by a variety of different factors, e.g. increases in biosynthetic capacity, decreases in metabolite catabolism, acceleration of breakdown of storage biopolymers (complex lipids, proteins or even polynucleotides) or decrease in building biopolymers. We have therefore used shotgun proteomics with spectral count semiquantification to investigate, which of the detected metabolic changes might be caused by changes in enzyme concentrations. 495 proteins were identified in the proteomic screens of which 187 proteins were annotated with enzymatic functions using gene ontology analysis. Statistically significant changes in protein abundance suggested a large range of biochemical adaptations to nitrogen deficiency. Using the top 215 proteins that were positively detected in at least 80% of the samples, cluster analysis by Partial Least Square discrimination showed that overall biochemical phenotypes were separated by the combination of three linear discriminant vectors (Fig. 4). Vectors in supervised linear dimension reductions were ordered by the degree of variance in protein abundance that can be explained by the study parameters. Vector 1 (14.7% total explained variance) discriminated protein profiles at late exponential growth from early phases (Fig. 4), while vector 2 (5.4% total explained variance) mostly separated clusters based on different total ammonium availability and vector 3 (3.3% total explained variance) displayed the differential regulation of protein abundances at early growth stages (4h and 10h). For visual clarity of the discriminatory impact of individual vectors, two-dimensional plots are given as supplement proteomic clusters. The clusters in Fig. 4 show that most proteomic changes were due to overall temporal changes along growth phases, with only subtle additional regulation in biochemical phenotypes due to reduced nitrogen availability. Interestingly, however, these subtle differences were more prominent under 50% nitrogen reduction compared with the 25% reduction in nitrogen availability, similar to our findings in metabolite abundances. Hence, both proteomic and metabolomic phenotypes showed that biochemical responses were directly associated to the total nitrogen availability in the cultures, way before nitrogen was depleted.

Subsequently, all proteins were analyzed for differential expression by univariate statistics and time course analysis. Using the power law global error model (53), we found a higher number of differentially expressed proteins under 50% nitrogen limitation than under 25% reduced nitrogen availability (Table II) which concurred with the overall quantitative biochemical phenotypes observed in Partial Least Square discriminant models. We then investigated which proteins had specific differences in response to reduced nitrogen availability either at early or late time points. Selected examples are given in Fig. 5, while all data are presented as numerical values in supplemental information (supplement proteins). At 25% or 50% reduction in ammonium levels both acetate uptake and CO$_2$ fixation were down regulated by a two-fourfold magnitude in comparison to standard ammonium concentrations. This effect was only observed at early time points, but not at late exponential growth phases (Fig. 5 top panel). For CO$_2$ fixation, abundances for several proteins were markedly down regulated in early response to lowered ammonium levels, namely rubisco and rubisco activase (RCA1)
but also enzymes needed to complete the Calvin Cycle, namely Calvin cycle protein (CP12), transketolase (TRK1), ribulose bisphosphate carboxylase (rbcL) and chloroplastic phosphoribulokinase (PRKA) (supplement proteins). Down-regulation of acetate uptake was evidenced by lower acetyl-CoA synthetase, (Fig. 5 top panel). In addition, acetyl CoA acyltransferase and carbonic anhydrase were down regulated more than 6-fold (supplement proteins). Overall, the down-regulation of carbon uptake at early time points can be interpreted as preparation of cellular regulation to slower overall growth rates due to limited total ammonium availability. In fact, we found acetylCoA synthetase and phosphoenolpyruvate carboxykinase to be perfectly correlated ($r_{xy} = 0.93$), suggesting that acetate import and gluconeogenesis are tightly controlled in Chlamydomonas.

Corresponding to the down-regulation of carbon fixation and carbon uptake, we found constitutive reduction at all time points in expression of glutamate-1-semialdehyde aminotransferase (GSA), the committing step in Chlamydomonas for production of porphyrin and chlorophyll biosynthesis. Similarly, other enzymes in chlorophyll biosynthesis were also found to be significantly down-regulated under the power law global error model, such as uroporphyrinogen-III decarboxylase (UROD1), uroporphyrinogen-III synthase (UROS), uroporphyrinogen decarboxylase (UROD2), coproporphyrinogen III oxidase (Cpx1), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (supplement proteins). This repression of chlorophyll biosynthesis ultimately resulted in the decreased amount of chlorophyll contents starting at 18 h after inoculation (Fig.1D).

Enzymes Involved in Nitrogen/Carbon Balance Metabolism are Up-regulated Under Late Exponential Growth Stages of Total Ammonium Limitations—In contrary to carbon uptake metabolism, and maybe also in contrast to naïve hypotheses on the ‘first responders’ to low ammonium availability, enzymes of carbon and nitrogen use were mostly affected at late growth stages and not during lag phase and early exponential phases of cellular growth. Key control proteins of carbon-nitrogen integration were selectively up-regulated starting at 18 h after inoculation, and more strongly overexpressed in the 50%-reduced ammonium content than at the 25% reduction.

**Fig. 5. Regulation of protein levels for selected enzymes in response to available ammonium in culture media and time after inoculation.** Protein intensities are given as MS/MS spectral counts, summed for all peptides of each protein and normalized to the total sum of all identified proteins per sample ($n = 4$).
level. Turnover and re-use of nonessential proteins, as established by lowered protein contents especially after 18–28h at lowered nitrogen levels (Fig. 1C), was shown to be caused by overexpression of periplasmic L-amino acid oxidase (LAO1) (supplement proteins) (75, 76). This enzyme releases ammonium in addition to yield supply of carbon backbones that feed back into the TCA cycle and acetyl-CoA. Uptake of ammonium for use in amino acid metabolism was activated by overexpressing glutamine synthetase (GLN1) and carbamoyl-phosphate synthase (CMPL1) (Fig. 5 lower panel) as well as arginosuccinate synthase (AGS1), aspartate transaminase and 4-aminobutyrate aminotransferase (supplement proteins).

Under prolonged reduction of ammonium availability, higher levels of enzymes of the citric acid cycle were found such as mitochondrial malate dehydrogenase (Fig. 5, mid panel) and isocitrate dehydrogenase (IDH1,2), 2-oxoglutarate dehydrogenase, citrate synthase, succinate dehydrogenase and dihydrolipoyl succinyltransferase (supplement proteins). For all these citric acid cycle enzymes, up-regulation was strongest under 50% ammonium reduction and at late time points, despite the overall trend toward protein turnover and lower absolute protein contents. Hence, the up-regulation of citric acid cycle enzymes supports the notion of increasing the capacity to produce carbon backbone substrates for amino acid biosynthesis, in response to decreased levels of these substrates. Many glycolytic enzymes were found to be differentially regulated under nitrogen limitation, but results were not consistent with a unified response pathway. Fructose-1,6-bisphosphate aldolase as well as phosphoglycerate kinase and enolase were found at lower expression levels at early time points (supplement proteins), whereas enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Fig. 5, mid panel) were both up-regulated at late exponential growth phases, especially under 50% ammonium availability conditions.

GAPDH is an enzyme known to be responsive to stress conditions (77), and indeed, a range of further stress response proteins were found at increased levels under nitrogen limiting conditions such as the plastid lipid-associated protein fibrillin (PLAP10), plastid vesicle inducing protein (VIPP1), mitochondrial inner membrane translocase (TIM 9), cytochrome c and mitochondrial cytochrome c oxidase (COX12), cytosolic cell wall protein pherorbin-C2 (PHC2), FKBP-type peptidyl-prolyl cis-trans isomerase, NaCl-inducible protein (NIP1), protein disulfide isomerase 1 (PDH1), rieske ferrodoxin, thioredoxin-related protein (CITRX), chaperonin 10 (CPN 10), flagellar flavodoxin (AGG4), and high mobility group protein (HMG1) (supplement proteins).

Integration of Metabolite and Enzyme Response Demonstrates a Large Biochemical Shift Between Early and Late Exponential Growth Under Low Nitrogen Availability Conditions—A functional integration of metabolome and proteome data can best be performed on the level of metabolic pathways. Here, we have used the VANTED software for displaying selected metabolite and protein data in combination for 40 enzymes and 54 small molecule intermediates. Calvin cycle enzymes as well as glycolytic and chlorophyll-pathway enzymes were largely down-regulated at lag phase and early exponential growth conditions at 50% lower ammonium availability in comparison to standard nitrogen contents (Fig. 6). Similar effects were found in 25% reduced ammonium concentrations (supplement proteins). At these time points, short chain free fatty acids were found up-regulated concomitant with up-regulation of the central hub protein for lipid biosynthesis, the acyl-carrier protein ACP2. For cultures grown in media with either 25 or 50% reduced ammonium contents, a dramatic shift in biochemical regulation was detected in many metabolic modules (supplement VANTED). Calvin cycle as well as glycolytic enzymes and metabolites were no longer differentially regulated from 18h after inoculation onwards. Lipid biosynthesis was not up-regulated any longer but in fact, both acyl-carrier protein and a subunit of the committing enzyme acetyl-CoA carboxylase were down-regulated in comparison to control nitrogen conditions. Instead, biosynthesis of purines and pyrimidines was found to be activated, and several enzymes involved in biosynthesis and transformation of amino acids were found up-regulated, including enzymes of the TCA cycle that provide carbon skeletons for amino acid metabolism. It is important to note that despite the up-regulation of enzymes, amino acids were still found at lower concentrations such as aspartate, asparagine, glutamate, glutamine, and alanine. This finding suggests that up-regulation of some parts of the molecular network do not necessarily imply that downstream effects follow. In fact, one could rather argue that the up-regulation of enzymes is a cellular response to the lowered amino acid levels but that these enzymes are not active or overexpressed enough to fully replenish the amino acid pools due to the continuing lowered availability of ammonium; despite the higher protein turnover.

**DISCUSSION**

The combined analysis of proteins and metabolites in our study presents the most comprehensive functional and quantitative determination of the metabolic regulation of *Chlamydomonas* in response to total available nitrogen. It is remarkable that *Chlamydomonas* cells accurately sense and respond to moderate reduction of total available ammonium in its environment (25 and 50% N-reduction), despite lack of any difference in ammonium uptake rates compared with 100% ammonium levels (400 mg/L NH₄Cl). This nitrogen-sensing is transduced as an integral signal to carbon- and nitrogen-responsive pathways (59) which leads to a metabolic adjustments that first seem to mimic the typical cellular responses to N-deprivation; down-regulation of carbon assimilation and chlorophyll biosynthesis, increase of lipid biosynthesis and activation of oxidative stress response enzymes. A range of
Calvin cycle enzymes were suppressed in lag and early exponential phase under reduced nitrogen conditions, such as PRKA, rbcl, PGK1, TRK1, FBA3 and MDH5, in addition to lower abundance of Rubisco activase (78). This reduction in carbon assimilation is an effort to balance anticipated slower cell growth with lower amounts of nitrogen available in order to prepare for optimal carbon/nitrogen ratios. Specifically, carbon anhydrase (CAH1) and acetyl-CoA synthetase (ACS3) showed a most dramatic down-regulation during lag and early exponential phases. Both enzymes are involved in carbon concentration mechanisms (33, 79). A 20-fold decrease in CAH1 levels implies that active Ci diffusion to the cell surface is turned off (80) which was found in accordance to lowered levels of chlorophyll-producing enzymes. Down-regulation of acetyl-CoA synthetase reduces uptake of acetate as carbon source from heterotrophic and mixotrophic environments (4, 81, 82). This early response of lowered carbon uptake was accompanied by an increase in production of short-chain fatty acids, but not long chain or unsaturated fatty acids (83). Our proteomic data suggest that this early-phase increase in free fatty acids is not due to degradation of complex lipids by lipases, which in accordance to gene expression data found under N-deprived condition (39). Instead, we identified an up-regulation of fatty acid biosynthesis of the acyl-carrier protein under early time points under reduced ammonium availability, which confirms the up-regulation of ACP gene expression that was found under drastic nitrogen deprivation (39). There may be two lines of reasoning for the early increase in short-chain fatty acids: First, the pathway of omega-hydroxylation of caprate and laurate within the MetaCyc database suggests that the increase could be related to Cyp-450 hydroxylase activity for plant defense (84). This could be an evolutionary conserved defense mechanism against abiotic stress. Secondly, at least in some vascular plants, thus, maybe also in *Chlamydomonas*, thioesterases with specificity for shorter chain length acyl-ACPs are capable of prematurely...
terminating fatty acid synthesis, thereby regulating the chain length of the products incorporated into lipids. For example, thioesterases specific for medium-chain fatty acids are responsible for high levels of C10 and C12 fatty acids in triacylglycerols of California Bay, Cuphea, and other species (85). For late growth stages, we cannot exclude the possibility that the increase in long-chain fatty acids may be derived from partial decomposition of membranes since a range of stress responses were observed in proteins levels which can partially drive the degradation/reconstitution of membrane lipids.

In contrast to full nitrogen deprivation, however, the cellular system in Chlamydomonas manages to balance cell growth rates with lipid production, and no further accumulation of fatty acids or lipid biosynthesis is observed under late stages of reduced ammonium availability. Indeed, under exponential and stationary growth stages, this initial reduction in carbon assimilation is reversed and replaced by up-regulation of enzymes involved in amino acid metabolism, including carbon backbones, leading to no overall difference in cell division and biomass growth. This rapid adaptation and metabolic flexibility shows the robustness of the alga to unfavorable environments (27). The induced levels of glutamate synthase and glutamine synthetase directly lead to a higher capacity for ammonium assimilation. Aspartate aminotransferase and ATP citrate synthase together control the use of oxaloacetate and acetate for producing 2-oxoglutarate in the citric acid cycle, the precursor for glutamate, or use of oxaloacetate directly for aspartate metabolism. Carbamoyl phosphate synthetase further recycles the ammonium released by protein breakdown and periplasmic L-amino acid oxidase. Carbamoyl phosphate is a precursor in both pyrimidine and arginine biosynthesis (86) and it directly links to other up-regulated enzymes in arginine and polyamine biosynthesis, namely N-acetyl-gamma-glutamyl-phosphate reductase, argininosuccinate synthase and argininosuccinate lyase. The latter enzymes connect citric acid cycle metabolites to amino acid metabolism via fumarate and aspartate (87).

In addition, no further phenotypic differences were observed such as degradation of chlorophyll and pigments, unlike observed under 75–100% ammonium reduction (88). Considering the moderate level of reduction in total available nitrogen, the degree of metabolic adaptations seem to be even higher than what the cells actually need for control of cell growth and division, and the initial reduction of carbon assimilation might be actually an overshoot response which is only later balanced by increases in amino acid metabolic enzymes. Potentially, the presence of acetate as additional carbon source from the media buffer changes the cellular sensitivity toward maintaining optimal C/N ratios (89), which accelerated the down-regulation of carbon assimilation and photosynthesis, and in turn induced oxidative stress responses (90).

A range of further questions arise from the presented data. For example, the regulation of metabolism could be further detailed by measuring carbon/nitrogen fluxes in central metabolism (89), photosynthetic activity, and total contents of membrane lipid and carbohydrates. We have here focused on regulation of metabolism by enzyme abundance, but other regulatory elements from gene expression (39), post-translational modification (91) or modeling the effect of allosteric interactions could deepen our understanding of dynamic adaptations of cellular metabolism to changing microenvironments on a systems scale. More important for applications in biotechnology, however, are assessments of total biomass production and distribution of carbon and nitrogen into different metabolic compartments. Although our study did not aim at optimizing parameters for biofuel production, our results suggest that reduced nitrogen contents in media may increase fatty acid biosynthesis while maintaining cellular growth (92), potentially even in high density fermentations (93) and continuous cultures with constant lower input levels of nitrogen source (94). For economic feasibility of algae growth in biofuel productions, ideally, higher levels of lipid production in early exponential growth stages under nitrogen limitation should be combined with high protein turnover and an overall minimal protein content, potentially with an increase in total polysaccharide pools that could serve as additional input for fermentable sugars (95). Analysis of metabolic regulation on the molecular level can then be used to tune engineering solutions to harvest optimal yields under minimal costs from photoautotrophic or mixotrophic microalgae.

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