"Enhancing Carbon Fixation by Metabolic Engineering: A Model System of Complex Network Modulation"

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FINAL REPORT

Summary

In the first two years of this research we focused on the development of a DNA microarrayS for transcriptional studies in the photosynthetic organism *Synechocystis* and the elucidation of the metabolic pathway for biopolymer synthesis in this organism. In addition we also advanced the molecular biological tools for metabolic engineering of biopolymer synthesis in *Synechocystis* and initiated a series of physiological studies for the elucidation of the carbon fixing pathways and basic central carbon metabolism of these organisms. During the last two-year period we focused our attention on the continuation and completion of the last task, namely, the development of tools for basic investigations of the physiology of these cells through, primarily, the determination of their metabolic fluxes. The reason for this decision lies in the importance of fluxes as key indicators of physiology and the high level of information content they carry in terms of identifying rate limiting steps in a metabolic pathway.

While flux determination is a well-advanced subject for heterotrophic organisms, for the case of autotrophic bacteria, like *Synechocystis,* some special challenges had to be overcome. These challenges stem mostly from the fact that if one uses ^{13}C labeled CO_2 for flux determination, the ^{13}C label will mark, at steady state, *all carbon atoms of all cellular metabolites,* thus eliminating the necessary differentiation required for flux determination. This peculiarity of autotrophic organisms makes it imperative to carry out flux determination under *transient conditions,* something that had not been accomplished before. We are pleased to report that we have solved this problem and we are now able to determine fluxes in photosynthetic organisms from stable isotope labeling experiments followed by measurements of label enrichment in cellular metabolites using Gas Chromatography-Mass Spectrometry. We have conducted extensive simulations to test the method and also are presently validating it experimentally using data generated in collaboration with a research group at Purdue University. As result of these studies we can now determine, for the first time, fluxes in photosynthetic organisms and, eventually, in plants.

Metabolic Flux Analysis

Metabolic flux analysis (MFA) using stable isotope tracers is a powerful platform for metabolic engineering and systems biology. The goal of MFA is the quantitative determination of intracellular fluxes. Fluxes describe the rates at which compounds are inter-converted through specific biochemical routes and thus provide key indicators of pathway kinetic bottlenecks, network regulation and overall cellular phenotype. Metabolic fluxes are key indicators of cell physiology and have been used recently extensively to elucidate cellular metabolism and identify rate-limiting steps for product overproduction.

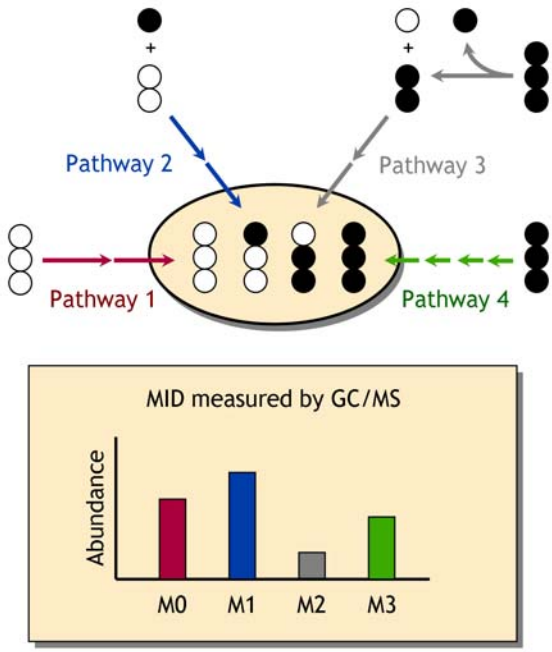

Figure 1: Simple MFA example. Each pathway produces a unique labeling pattern in the final product, and the resulting mass isotopomer distribution (MID) provides a direct measure of relative flux in the network. Mass isotopomers are molecules with the same chemical formula but different molecular weights due to varying incorporation of heavy isotopes. They are denoted M0, M1, M2, etc. in order of increasing weight.

Metabolic fluxes are determined by culture labeling using stable isotopic tracers. The labeling patterns of metabolic intermediates and byproducts that emerge upon feeding an isotopically labeled substrate are directly determined by the relative pathway fluxes (see Figure 1). These labeling patterns can be measured by mass spectrometric methods such as GC/MS or LC/MS, and the resulting data can be computationally analyzed to reconstruct comprehensive metabolic flux maps. In other words, metabolic fluxes and the pathway structure uniquely determine the labeling pattern of metabolic intermediates. By solving the inverse problem, fluxes can be estimated from the measurement of the labeling patterns of metabolites following the introduction of isotopic labeling compounds.

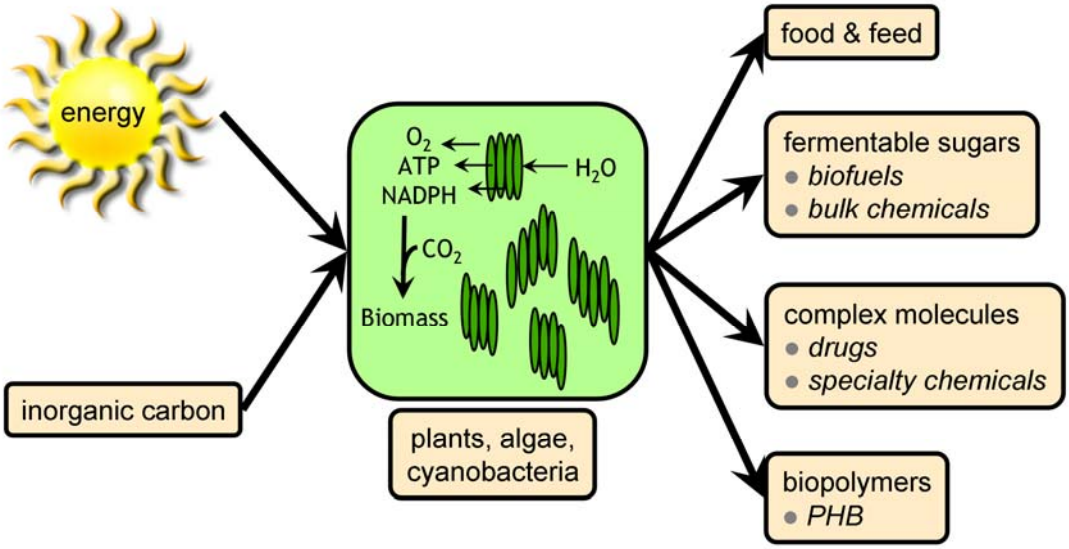

Figure 2: Photoautotrophic metabolism involves harnessing sunlight to generate chemical energy in the form of ATP and reducing power in the form of NADPH. These cofactors then drive the conversion of inorganic CO_2 into biomass, which provides a renewable source of many agricultural and biotechnological products.

Photoautotrophic Metabolism

Photoautotrophic metabolism involves the process by which plants and other organisms use energy from sunlight to convert inorganic carbon sources, like carbon dioxide, into organic biomolecules. This represents the primary source of all food on earth as well as raw materials for bio-based production of fuels and chemicals (see Figure 2). The efficiency of these carbon-fixing and product-forming metabolic pathways can be improved by applying methods of metabolic engineering. Metabolic engineering of microbial systems is accomplished using either rational, directed methods identifying genetic targets or combinatorial methods to construct and screen large libraries of mutant strains. However, the latter approaches cannot be easily extended to plants where growth rates are much slower and genetic tools are relatively limited. Therefore, the ability to perform quantitative studies of metabolism using isotope tracers and MFA is critical to the development of directed approaches for plant metabolic engineering that depend upon a detailed understanding of flux regulation. Fluxes

are therefore imperative for rational metabolic engineering of autotrophic organisms. However, fluxes have never been determined for autotrophic systems due to some special problems encountered with autotrophic systems as described below.

^{13}C-Labeling in an Autotrophic System

^{13}C is the preferred isotope tracer for studying central carbon metabolism. For heterotrophic organisms, the labeling studies are carried out using a ^{13}C-labeled substrate (such as glucose) and measurements of label enrichment patterns in metabolites using GC-MS. Unlike heterotrophic systems, however, the steady-state ^{13}C-labeling in an autotrophic system does not convey flux information (see Figure 3). The reason is that at steady state, all carbon atoms of all metabolites will be labeled by the ^{13}C marker of the CO_2 label used in photosynthetic organisms. We note that the ability to estimate fluxes relies on the differential intracellular metabolite labeling resulting from carbon atom distribution along carbon metabolic pathways of carbon sources like glucose. Molecules such as glucose (containing 6 carbon atoms) are broken into smaller units that are distributed among the various metabolic pathways, thus labeling different metabolites differently according to the pathway fluxes. This will not be the case when CO_2 is the sole carbon source, as it will label equally all metabolites at steady state. However, before a steady state is reached (transient conditions)

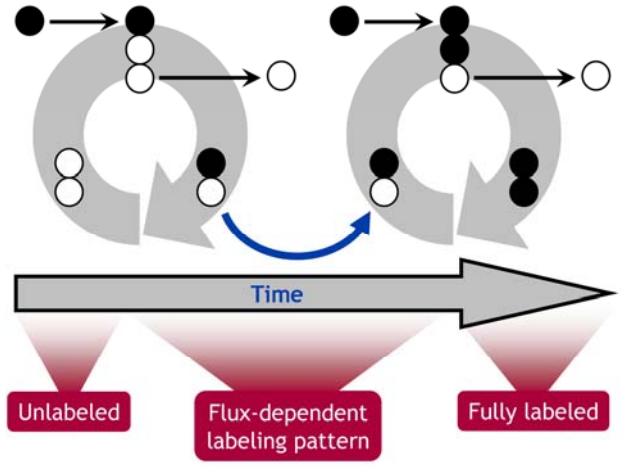

Figure 3: Carbon labeling in an autotrophic system. Because CO_2 is the sole carbon source, the **steady-state** labeling of all carbon atoms is identical to that of CO_2 and is not sensitive to fluxes. However, the **transient** labeling trajectory is sensitive to fluxes and to metabolite pool sizes.

metabolites will be labeled differently and according to the fluxes of the pathways synthesizing and destroying such metabolites. For this reason it has been suggested that fluxes in photosynthetic organisms can be determined from transient measurements of isotope incorporation following a step change from unlabeled to labeled CO_2. To accomplish this task, new techniques for isotopically nonstationary MFA (INST-MFA) had to be developed. We undertook this task and build a new software for INST-MFA that turned out to be significantly more sophisticated than that which is needed for conventional MFA, due to the fact that the isotopic labeling must be described by differential rather than algebraic equations. We have developed a package of Matlab® routines based on an Elementary Metabolite Unit decomposition of the underlying isotopomer network that achieves more than 5000-fold speedup relative to previous INST-MFA approaches (Antoniewicz et al., 2007; Young et al., 2008). These computational tools now permit INST-MFA of biologically relevant networks such as those of photosynthetic bacteria, algae and plants. Until now, MFA studies of these organisms have been done only under heterotrophic or mixotrophic conditions, since the more conventional MFA approaches that rely on steady-state labeling cannot be applied to the autotrophic case.

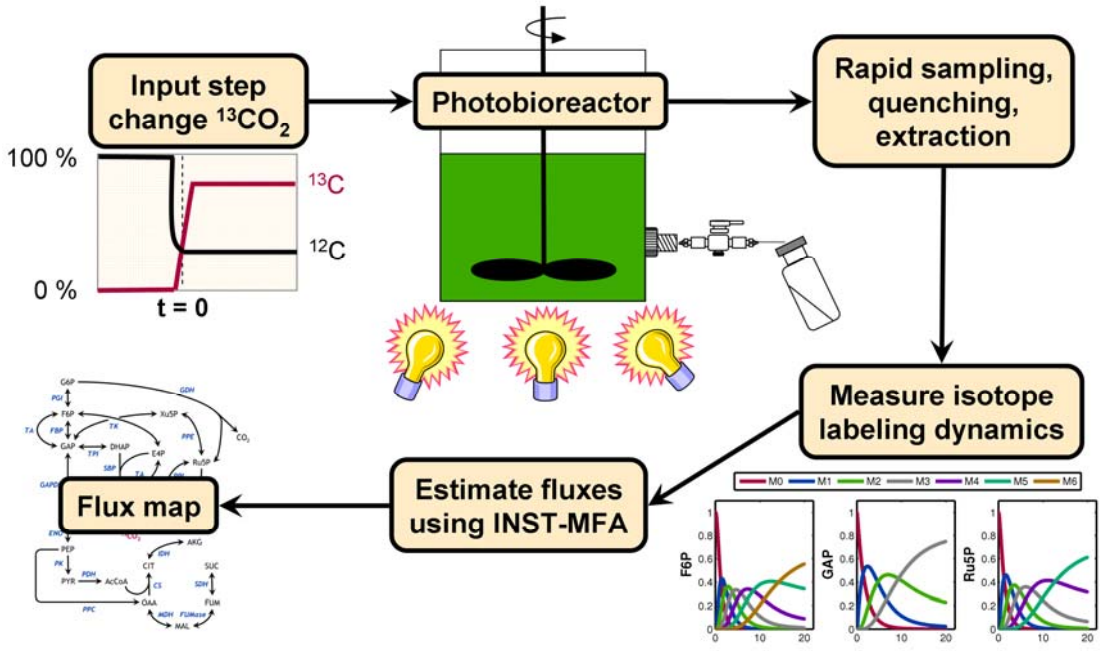

Figure 4: ^{13}C INST-MFA experimental procedure. A step change in CO_2 labeling is introduced to a *Synechocystis* culture at metabolic steady state. Biomass samples are withdrawn at several time points following tracer introduction, and the cells are rapidly quenched in a -40ºC methanol/water solution. Intracellular metabolites are subsequently extracted and their isotopic labeling is measured using a combination of GC/MS and LC/MS/MS. This data is then processed using our custom INST-MFA analysis package to reconstruct a comprehensive flux map that quantitatively describes the photoautotrophic metabolism of *Synechocystis*.

Synechocystis Photoautotrophic Metabolism

We are currently applying the INST-MFA approach to study the metabolism of *Synechocystis* sp. PCC 6803 under photoautotrophic conditions. This cyanobacterium is a model photosynthetic organism that has been investigated as a host for several biotechnology applications including polyhydroxybutyrate (PHB) synthesis. It was the first photosynthetic organism to have a fully sequenced genome. Experiments are being performed by collaborators at Purdue University using ^{13}C-labeled bicarbonate as an isotopic tracer under batch growth conditions in a photobioreactor (see Figure 4). Labeling data are obtained by withdrawing biomass samples at a series of time points following

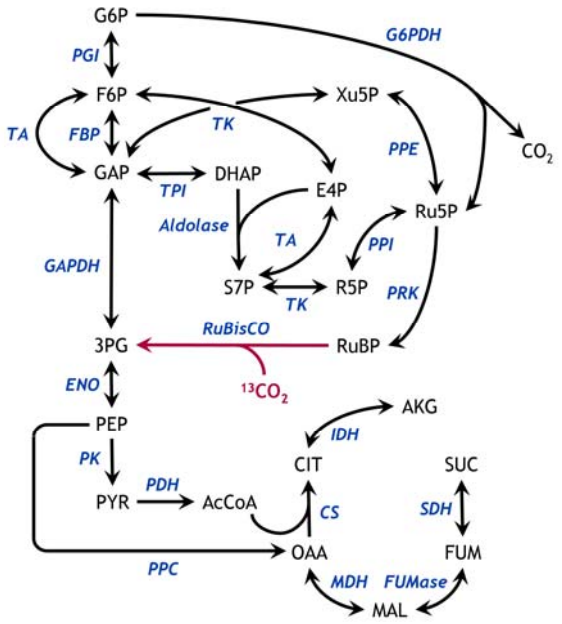

Figure 5: *Synechocystis* metabolic network. The RuBisCO reaction is highlighted, showing the entry of CO_2 into the Calvin cycle. Also note that the alpha-ketoglutarate dehydrogenase enzyme is absent, leading to an incomplete TCA cycle.

tracer introduction. The biomass is cold-quenched and intracellular metabolites are extracted and subsequently analyzed using both GC/MS and LC/MS/MS. An identifiability study has been completed using simulated data, which confirmed that accurate and precise flux estimates can be extracted from the transient isotope labeling dynamics in this system.

Synechocystis MFA Results

An initial MFA study has been completed that will be the focus of an upcoming publication (Shastri et al., in prep.). This work will provide the first comprehensive flux map of autotrophic metabolism in *Synechocystis* or any other photosynthetic organism. The metabolic network is shown schematically in Figure 5. A mathematical model of the isotopomer balance equations derived from this network was used to estimate flux values using the INST-MFA approach. The experimental labeling data is shown in Figure 6 along with the best-fit model simulation, which provided a statistically acceptable description of the observed measurements.

Table 1 summarizes a few notable results from the ^{13}C flux analysis with comparisons to values predicted by a linear programming (LP) method (Shastri and Morgan, 2005). The LP solution does not require experimental

measurements but instead assumes that carbon and light resources are consumed in a way that optimizes growth. Although the LP predicts that there should be no flux through the oxidative pentose phosphate pathway (G6PDH reaction), the experimental results indicate that around 10% of the fixed carbon is lost via G6PDH. Due in part to these losses, 142 ± 12 moles of CO_2 must be fixed to yield a net gain of 100 C-moles of biomass. This is significantly more than the 111 moles of CO_2 predicted by the LP model, indicating that growth is suboptimal with respect to carbon utilization.

Another notable result of the flux analysis is that *Synechocystis* appears to use transaldolase rather than aldolase as the primary route to regenerate S7P in the Calvin cycle. This is interesting since plants are known to use aldolase for this purpose. Further work to measure the activities or expression levels of these enzymes is planned to corroborate these findings.

Table 1: Comparison of flux values predicted by linear programming with experimentally determined values obtained using ^{13}C INST-MFA. Fluxes are relative to a net CO_2 uptake rate of 100. Standard errors are indicated for the ^{13}C INST-MFA results.

Reaction	*Linear Program*	*^{13}C INST-MFA*
G6PDH	**0**	**13 ± 3**
Transaldolase	**0**	**43 ± 4**
Aldolase	**37.2**	**0 ± 0.2**
RuBP oxygenation	**0**	**0.3 ± 3**
RuBisCO	**111**	**142 ± 12**

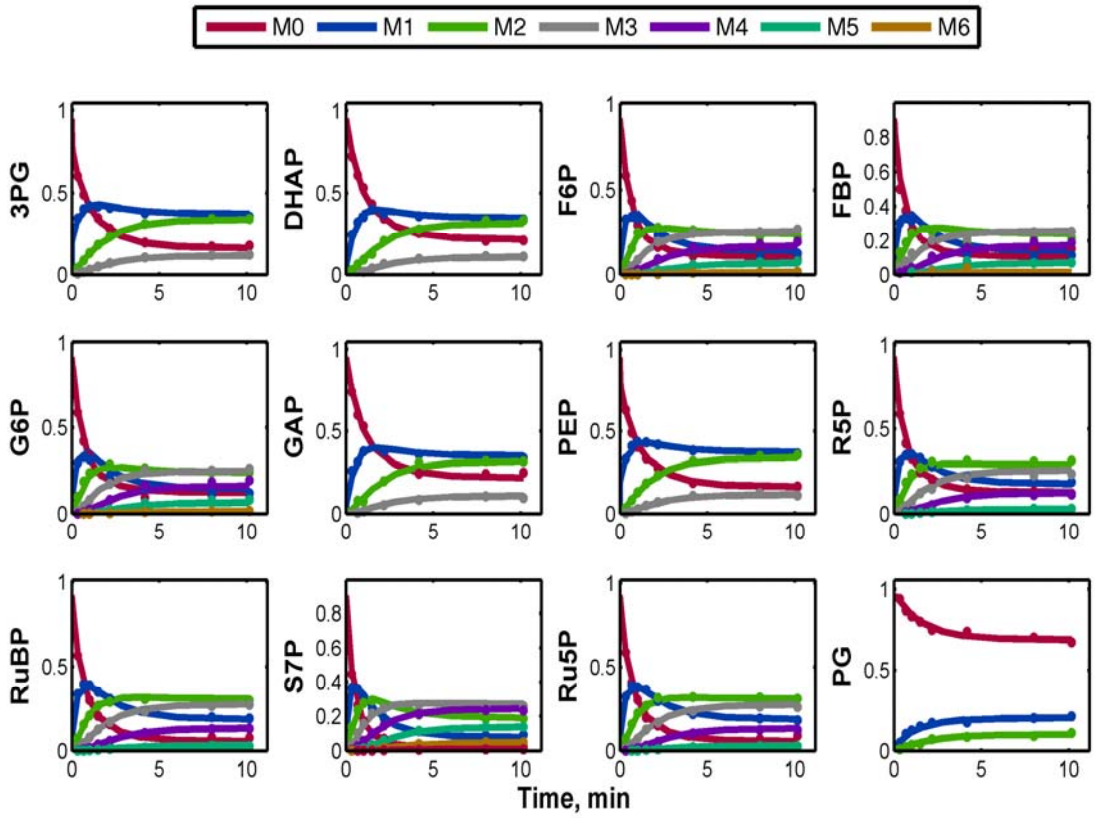

Figure 6: Time course of transient isotope labeling in Calvin cycle intermediates compared to best-fit model simulation. As the metabolite pools accumulate ^{13}C label from the $^{13}CO_2$ tracer, higher weight mass isotopomers are formed in a sequential manner. The optimized solution achieved a sum-of-squared residuals (SSRES) value of 348.7, which is within the acceptable range of 311.2–416.6 based on the 95% confidence range of a χ^2 random variable with 362 degrees of freedom.

Lastly, the experimental results confirm the LP prediction that RuBP oxygenation is insignificant. This reaction results from lack of RuBisCO specificity for CO_2 over O_2, which can result in the formation of the wasteful by-product phosphoglycolate. In plants, this is known to be a major drain on biomass productivity as the phosphoglycolate must be recycled via a series of energy-consuming reactions. However, *Synechocystis* possesses a potent mechanism for concentrating CO_2 in the vicinity of RuBisCO which is thought to greatly increase its specificity. The results confirm this hypothesis as indicated by the negligibly low RuBP oxygenation flux shown in Table 1.

Summary, future directions

The development of advanced tools for INST-MFA has enabled the translation of transient isotope labeling data into quantitative estimates of flux. These methods are required to determine autotrophic fluxes from ^{13}C tracer experiments since steady-state approaches cannot be used. We have successfully applied INST-MFA to reconstruct a comprehensive flux map of a photoautotrophic system for the first time. Comparisons to an LP solution reveal that *Synechocystis* metabolism does not make optimal use of fixed carbon due in part to activity of the oxidative pentose phosphate pathway. Interestingly, the results show that transaldolase is an active Calvin cycle enzyme instead of aldolase which is preferred in plants. Our results also confirm that the specificity of RuBisCO for CO_2 is extremely high in *Synechocystis*, and reactivity with oxygen occurs at a negligible rate.

Future extensions of this work will be to apply the methods of INST-MFA to quantify fluxes in higher autotrophs such as crop plants or algae. These methods will be of particular value to plants where, as it was mentioned, most combinatorial approaches applicable to microorganisms cannot be used and it is imperative to identify rational targets of pathway modulation. We envision application of these methods in fully enclosed chambers where plants are grown in the presence of ^{13}C-labeled CO_2 and where plant parts are sampled and analyzed for ^{13}C enrichments of their metabolites at various points in time. These measurements can then be used for metabolic reconstruction and flux determination.

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