Design and analysis of synthetic carbon fixation pathways

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Carbon fixation is the process by which CO_2 is incorporated into organic compounds. In modern agriculture in which water, light, and nutrients can be abundant, carbon fixation could become a significant growth-limiting factor. Hence, increasing the fixation rate is of major importance in the road toward sustainability in food and energy production. There have been recent attempts to improve the rate and specificity of Rubisco, the carboxylating enzyme operating in the Calvin–Benson cycle; however, they have achieved only limited success. Nature employs several alternative carbon fixation pathways, which prompted us to ask whether more efficient novel synthetic cycles could be devised. Using the entire repertoire of approximately 5,000 metabolic enzymes known to occur in nature, we computationally identified alternative carbon fixation pathways that combine existing metabolic building blocks from various organisms. We compared the natural and synthetic pathways based on physicochemical criteria that include kinetics, energetics, and topology. Our study suggests that some of the proposed synthetic pathways could have significant quantitative advantages over their natural counterparts, such as the overall kinetic rate. One such cycle, which is predicted to be two to three times faster than the Calvin–Benson cycle, employs the most effective carboxylating enzyme, phosphoenolpyruvate carboxylase, using the core of the naturally evolved C4 cycle. Although implementing such alternative cycles presents daunting challenges related to expression levels, activity, stability, localization, and regulation, we believe our findings suggest exciting avenues of exploration in the grand challenge of enhancing food and renewable fuel production via metabolic engineering and synthetic biology.

metabolic engineering | synthetic biology | photosynthesis | carboxylation | biological optimization

In the process of transforming sunlight into stored chemical energy, plants absorb approximately 10 times more CO_2 from the atmosphere than the total amount emitted by human activities globally (1). Moreover, agriculture, which is effectively a massive carbon fixation industry, makes use of the majority of cultivatable land on earth and accounts for most of the fresh water used by humanity (2). These figures point to the central role that carbon fixation by plants plays in our global ecological footprint. In nature, the factors limiting the growth of photosynthetic organisms vary among habitats and often include the availability of water, light, fixed nitrogen, iron, and phosphorus (e.g., ref. 3). However, in agriculture today, the use of fertilizers and irrigation can make carbon fixation a rate-limiting factor. For example, many C3 plants have shown a significant increase in biomass when exposed to twice the atmospheric CO_2 concentration (4). Impressive growth enhancements have been demonstrated by addressing several biochemical limiting factors, related both to the light-dependent and light-independent reactions (5–9). For instance, transgenic *Arabidopsis* plants that expressed an efficient bacterial photorespiration pathway, instead of the natural photorespiration pathway, grew faster, produced more shoot and root biomass, and contained more soluble sugars (8). In another effort, tobacco plants overexpressing sedoheptulose-1,7-bisphosphatase, an enzyme operating in the reductive pentose phosphate cycle (rPP; also known as the Calvin–Benson Cycle), were characterized by an increased photosynthetic rate and a 30% enhancement in biomass yield (9).

The rPP cycle (Fig. S1), used by the vast majority of autotrophic organisms for CO_2 assimilation, is often limited by the low catalysis rate of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) (6, 7, 10). The negative correlation between the enzyme turnover number and its CO_2 specificity indicates that the enzyme might already be naturally optimized (11–13). Therefore, further optimization of Rubisco may prove difficult and lead to only marginal improvements (7), thereby limiting the potential for increasing the rate of the rPP cycle. As a result, designing and developing alternative (i.e., Rubisco-independent) pathways that can support a higher carbon fixation rate can be highly beneficial.

To date, five natural metabolic pathways that perform carbon fixation in place of the classic rPP cycle have been identified. These are the reductive tricarboxylic acid (rTCA) cycle, postulated in the 1960s (14); the oxygen-sensitive reductive acetyl-CoA (rAcCoA) pathway (15); the extensively researched 3-hydroxypropionate cycle (16); the 3-hydroxypropionate/4-hydroxybutyrate cycle (17); and the recently discovered dicarboxylate/4-hydroxybutyrate cycle (18).

Intrigued by the diversity of solutions found in nature, we were motivated to further explore the space of possible alternative carbon fixation pathways (Fig. 1). We began with analyzing natural metabolic pathways for carbon fixation (Fig. 1*A*) and progressed to the exploration of possible synthetic alternatives (Fig. 1*B*) while restricting ourselves exclusively to naturally occurring enzymes. We considered the entire set of approximately 5,000 known metabolic enzymes (19) as components (Fig. 1*C*) and used a constraint-based modeling approach (20–22) that systematically explored all possibilities that can be devised with these enzymes as building blocks (Fig. 1*D*). Our analysis presents several promising synthetic carbon fixation pathways and implements a methodical set of analysis metrics to compare the natural and synthetic pathways (Fig. 1*E*). Focusing on the cycle kinetics, we developed a proxy measure for the overall specific rates of each pathway, a criterion that can be highly important from an evolutionary point of view. We find that some of the proposed synthetic pathways could have significant kinetic advantages over their natural counterparts, making them promising candidates for synthetic biology implementation (Fig. 1*F*).

Results

Pathway Analysis Metrics Enable a Comprehensive Comparison Between Pathways. Many different aspects of a given metabolic pathway are important for its function. To enable evaluation and comparison of metabolic pathways, we have used several parallel criteria. We be-

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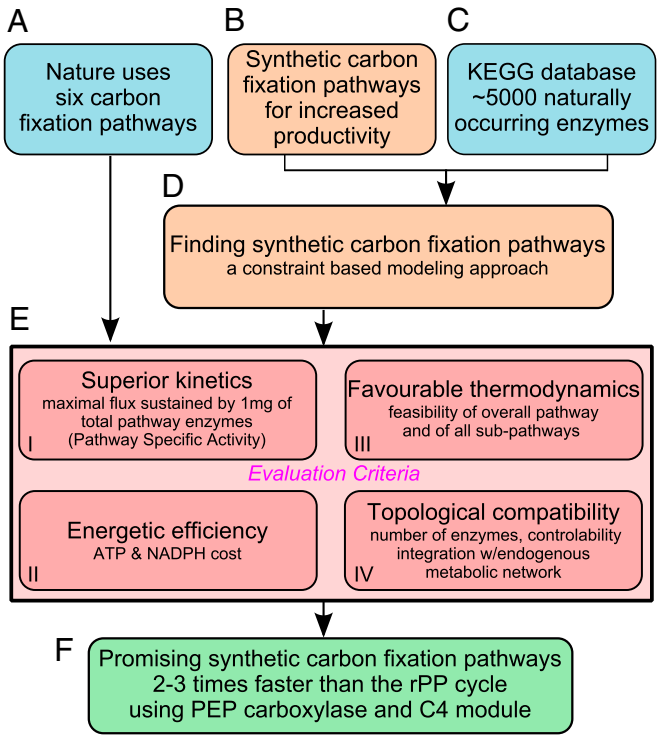

Fig. 1. Our study explores the space of possible carbon fixation pathways. We progress from analyzing natural metabolic pathways for carbon fixation (*A*) through the proposal of possible synthetic pathways (*B*) using the full gamut of naturally occurring metabolic enzymes (*C*). We use a constraint-based modeling approach to detect pathways (*D*) and define evaluation metrics to compare the natural and synthetic pathways (*E*). We find an alternative that makes use of efficient carboxylating enzymes (*F*).

gan by focusing on the pathway kinetics as the central criterion for optimization, as it is often the limiting factor of the carbon fixation process in photosynthetic organisms (5–9). For example, many plants increase the concentration of the relatively slow Rubsico to approximately one third of the soluble protein content in a leaf (23), making it a significant fraction of the cellular protein and nitrogen requirements. Faster pathway kinetics could facilitate a higher rate of carbon fixation given the same amount of resource allocation, or it could free some of the resources in favor of other processes.

The pathway specific activity (criterion I) is analogous to an enzyme's specific activity and is defined to be the maximal rate of product formation by 1 mg of pathway total protein (*Methods*). We have calculated the pathway specific activities for all natural carbon fixation pathways (Table S1). The ubiquitous rPP cycle has a pathway specific activity of 0.25μmol/min/mg (taking into account oxygenase activity), a value that will be used as a benchmark for evaluation of the kinetics of the synthetic pathways (the conditions under which this criterion reflects the pathway flux are discussed in the *SI Appendix*).

Although the kinetics of a pathway are of central importance, they do not give us any information regarding the cellular resources consumed. Different pathways, which ultimately perform the same metabolic conversion, might consume resources to a different degree, thereby unequally affecting the growth of the organism. We analyzed the energetic cost (criterion II) associated with the different pathways, corresponding to the efficiency of using the light-regenerated resources of the cell. The energetic cost can be divided into two subcategories:

NADPH cost. NADPH cost is the number of moles of NADPH equivalents (i.e., redox carriers, such as NAD(P)H, ferredoxins, and $FADH_2$) consumed in the production of one mole of product (GA3P; *Methods*).

ATP cost. ATP cost is the number of moles of ATP equivalents (nonredox energy carriers, e.g., NTPs, phosphate esters, and CoA thioesters) consumed in the production of one mole of product.

The NADPH and ATP costs of all natural carbon fixation pathways are given in Table S1.

The energetic cost of a pathway can be used to determine the energetic feasibility of the pathway as a whole, as well as of any of its parts. A thermodynamically favorable (criterion III) pathway is one for which the free energy change (ΔG^r) associated with the production of one mole of product is negative. We also require that a negative free energy change be obtained for each part of the pathway under the physiological range of metabolite concentrations (*SI Appendix*).

To ensure thermodynamic feasibility, a carbon fixation pathway must involve the hydrolysis of a certain minimal amount of ATP molecules. Yet, hydrolysis of more ATP molecules will decrease the energetic efficiency (i.e., increase the energetic cost). This minimal ATP requirement depends on the identity of the electron donors used by the pathway, as well as the pH and ionic strength (24). In Fig. 2*A*, we analyze the minimal ATP requirement of the prevalent rPP pathway in physiological ionic strengths and pH values, where all of the electron donors are NAD(P)H and under ambient CO_2^{gas} concentration of approximately 390 ppm. As Fig. 2*A* shows, five to six ATPs are the minimal requirement (*SI Appendix*). We note that the rPP uses nine ATPs to support carbon fixation under ambient conditions; the extra ATP molecules consumed are suggested to enhance the kinetics at the expense of ATP efficiency.

Different electron donors, other than NAD(P)H, with lower (i.e., more energetic, e.g., ferredoxin) or higher (e.g., menaquinone) reduction potentials would shift this thermodynamic profile (*SI Appendix*). Fig. 2 *B* and *C* shows the minimal ATP requirement for the rAcCoA pathway and the rTCA cycle, which use these electron

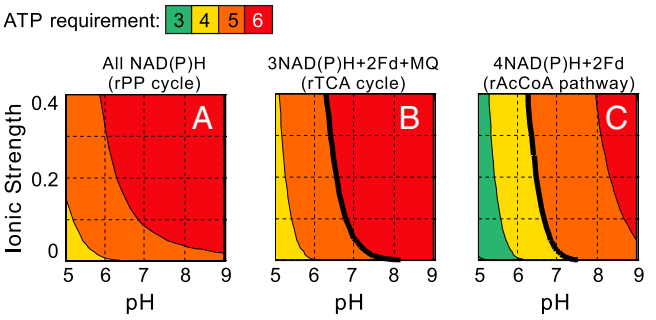

Fig. 2. Thermodynamic constraints on carbon fixation. The minimal number of ATP hydrolysis reactions required to ensure that carbon fixation would be thermodynamically feasible. The cycles' product was assumed to be GA3P. Different electron donor alternatives are analyzed at various pH and ionic strength conditions and under ambient CO_2^{gas} concentration (390 ppm). (*A*) All electron donors are NAD(P)H, as in the case of the reductive pentose phosphate pathway. (*B*) Two of the electron pairs are donated by ferredoxins, one by menaquinone (MQ), and the other three by NAD(P)H, as in the case of the rTCA cycle, where MQ is the electron donor for fumarate reductase (other electron donors are shown in Fig. S2*B*). (*C*) Two of the electron pairs are donated by ferredoxins (Fd) and the other four by NAD(P)H, as in the case of the rAcCoA pathway. Bold lines in *B* and *C* correspond to the feasibility ranges of the pathways, as dictated by their actual ATP requirement: 5 ATP molecules are hydrolyzed by the rTCA cycle and four by the rAcCoA pathway. The pathways are not feasible at pH and ionic strength values corresponding to the areas to the right of those lines (the ATP requirement in those areas is more than hydrolyzed by the pathways). The rPP cycle hydrolyzes nine ATP molecules, well above the minimal thermodynamic requirement (*SI Appendix*).

donors. Interestingly, although these pathways are potentially more efficient (hydrolyzing only four and five ATPs, respectively), we conclude that they are not feasible at pH values higher than 7 under ambient CO_2 concentrations (bold lines in Fig. 2 correspond to the feasibility limits). These results are in agreement with the observation that organisms that operate the rTCA cycle or the rAcCoA pathway usually occupy high CO_2 habitats or operate a carbon-concentrating mechanism (Fig. S2). These organisms are generally anaerobic and energy restricted compared to aerobes (25), which limits their available energy for investment in carbon fixation. This may explain the utilization the rTCA cycle or the rAcCOA pathway, which are more ATP-efficient than the rPP cycle.

Although the kinetics and the energetics of a pathway provide valuable information concerning its function, there are several other factors that can help us further assess and compare the different metabolic alternatives. Of these, we address the topology (criterion IV), corresponding to the internal makeup of a pathway and its integration with the structure of the metabolic network of the cell. The topology criterion incorporates the two following important parameters:

Number of enzymes. The number of enzymes the carbon fixation cycle utilizes as an independent unit, as well as the number of enzymes the complete pathway employs (including the conversion of the cycle's product into triose-phosphate; see *Methods*).

Metabolic compatibility of the synthetic pathways. In designing alternative CO_2 assimilation pathways, it is important to consider how the pathways will integrate into the endogenous metabolic network. We have used a model of central carbon metabolism in the algae *Chlamydomonas* (26) and employed constraint-based analysis [i.e., flux balance analysis and uniform random sampling (27)] to test the compatibility of each cycle with the endogenous metabolic network (28). We have calculated the growth yield supported by each pathway as well as the number of significantly changed fluxes in the modified network compared with the WT model (*SI Text*).

A Systematic Method to Locate Synthetic Carbon Fixation Pathways Reveals the Simplest Carbon Fixation Cycles. We have developed a computational approach to systematically explore all of the possibilities for building carbon fixation cycles of a given number of enzymes from the approximately 5,000 enzymes reported in the Kyoto Encyclopedia of Genes and Genomes database. Each candidate cycle employs one or more carboxylating enzymes, and produces an organic output compound with at least two carbons. This exhaustive search enables us to explore the space of possibilities that were then analyzed for feasibility and functionality using the criteria detailed earlier (*Methods* and *SI Appendix*).

We began our analysis by exploring which of the synthetic carbon fixation pathways employ the simplest (i.e., shortest) cycles. Such pathways can be suggested a priori to be attractive in terms of rate (29, 30). We found several pathways that employ cycles with as few as four to six enzymatic steps (Fig. S3). Fig. 3 presents one of the three cycles that uses only four enzymes. This cycle is a metabolic shortcut of the naturally occurring rTCA cycle; three of its enzymes participate in the rTCA cycle and the enzyme isocitrate lyase metabolically bypasses the rest of the natural cycle. The product of this simple cycle, glyoxylate, is converted to GA3P by the bacterial-like glycerate pathway (8) (Fig. S4).

However, this pathway, as well as others that employ such ultra-short cycles, suffer from a fundamental flaw. Most cycles, as distinct metabolic units that produce glyoxylate, are not thermodynamically feasible when taking into account the physiologically relevant glyoxylate concentrations (*SI Appendix*). In addition, all use an oxygen-sensitive ferredoxin-oxidoreductase enzyme (pyruvate synthase or 2-ketoglutarate synthase) and have a significantly lower pathway specific activity compared with the rTCA cycle (Table S1).

Kinetically Efficient Carbon Fixing Pathways Using the Most Attractive Carboxylating Enzymes. Focusing on the kinetic aspects, we turned our attention to what are often the limiting steps: the carboxylating enzymes. The design of kinetically efficient pathways requires the utilization of high-rate carboxylating enzymes, characterized by high specific activities and affinities toward CO_2 or HCO_3^-. We performed a wide literature survey and compared kinetic properties of known carboxylating enzymes (Table S2). Phosphoenolpyruvate (PEP) carboxylase and pyruvate carboxylase are the most favorable carboxylating enzymes; both have high specific activities and superior affinity toward HCO_3^-. Acetyl-CoA and propionyl-CoA carboxylases follow as the next favorable enzymes. Isocitrate dehydrogenase, which shows a preference for decarboxylation under common physiological conditions (in contrast to the previous options), is also a kinetically acceptable option. All other carboxylating enzymes are rather slow under ambient CO_2/HCO_3^- concentrations (specific activity, <2 μmol/min/mg).

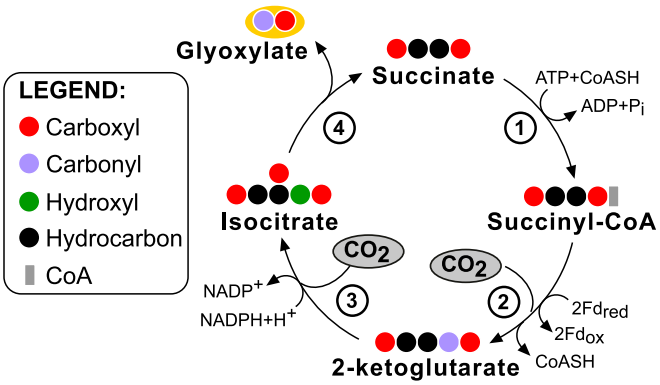

Fig. 3. The shortest possible carbon fixation cycle. Two CO_2 molecules are fixed to give glyoxylate, a two-carbon compound, as the cycle product. Every circle corresponds to a substituted carbon atom. We have used a color notation to display the different functional groups composing the metabolites, which also corresponds to the oxidation states of the carbons: red circles indicate carboxyl, purple corresponds to carbonyl, green to hydroxyl, and black to hydrocarbon. As explained in the text, this cycle is not thermodynamically feasible and does not represent a viable alternative for carbon fixation. Enzymes: 1, succinyl-CoA synthetase; 2, 2-ketoglutarate synthase; 3, isocitrate dehydrogenase; and 4, isocitrate lyase. EC numbers are given in the *SI Appendix*.

We used our systematic search tool to find the shortest pathways that employ different sets of the favorable carboxylating enzymes (*Methods* and *SI Appendix*). We have numerically predicted which of these pathways has the highest pathway specific activity. Notably, we find that all the pathways with the highest specific activities employ similar cycles, with a shared metabolic core structure (Fig. S5). The product of those cycles, glyoxylate, is assimilated by the bacterial-like glycerate pathway. We termed this family of pathways the malonyl-CoA-oxaloacetate-glyoxylate (MOG) pathways. We found that the MOG pathways have a two- to threefold higher pathway specific activity compared with the rPP cycle (Table S1). Fig. 4 *A* and *B* present two MOG pathways that employ only one carboxylating enzyme, the superior PEP carboxylase. We have termed these MOG pathways the C4-glyoxylate cycles, because they overlap with the naturally evolved C4 mechanism. In C4 plants, carbon is temporarily fixed in the mesophyll cells, by the carboxylation of PEP to oxaloacetate. This is followed by the reduction of oxaloacetate to malate. Malate is then transported to the bundle-sheath cells, where it releases the CO_2, which is reassimilated by Rubisco. Pyruvate is recycled to complete the cycle which serves as a "futile cycle" that concentrates CO_2 (31). All these reactions, with the exception of the last step of decarboxylation, appear in the C4-glyoxylate cycle. Therefore, the C4-glyoxylate cycles are an alternative for completing the C4 cycle without "losing" the carbon: replacing the "futile" decarboxylation reaction with an extra carboxylation, accompanied by the export of glyoxylate (Fig. 4*C*).

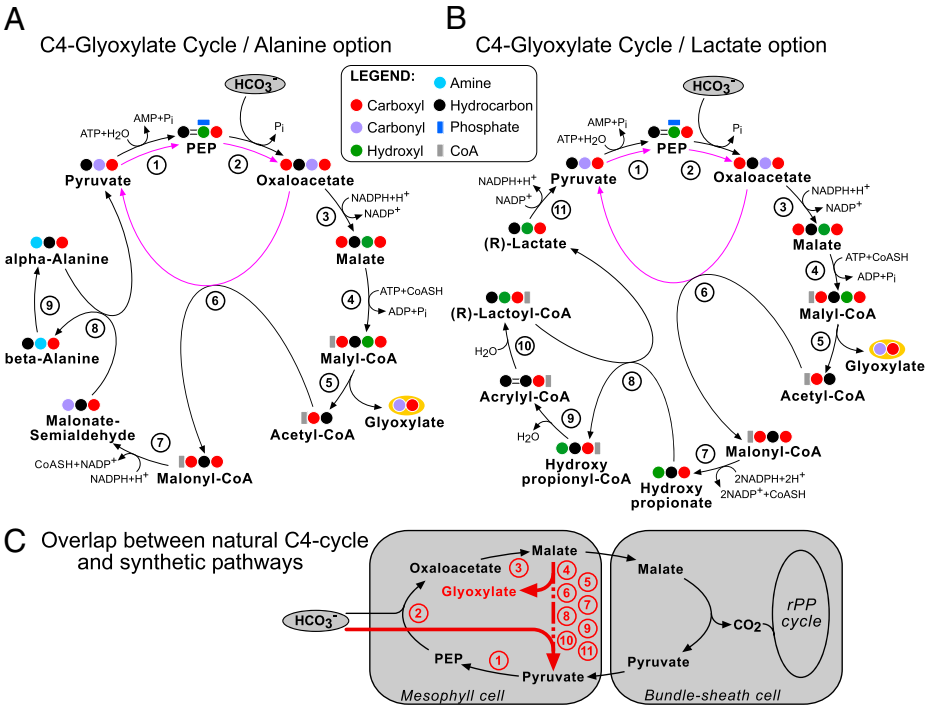

Fig. 4. The C4-glyoxylate cycles. (*A* and *B*) Promising carbon fixation pathways using the most favorable carboxylating enzyme, PEP carboxylase, as the sole carboxylation reaction. The product of the cycles, glyoxylate, can be easily converted into GA3P. Coloring and symbols are as in Fig. 3 with the addition of azure corresponding to an amine and the "=" mark to a double bond. (*C*) The metabolic overlap between the natural C4 cycle and the synthetic C4-glyoxylate cycles (additional reactions marked in red). (*A*) Enzymes: 1, pyruvate water/phosphate dikinase; 2, PEP carboxylase; 3, malate dehydrogenase; 4, malyl-CoA synthetase; 5, malyl-CoA lyase; 6, methylmalonyl-CoA carboxytransferase; 7, malonate-semialdehyde dehydrogenase; 8, β-alanine-pyruvate transaminase; and 9, alanine 2,3-aminomutase. (*B*) Enzymes (1–6) are as in *A*; 7, malonyl-CoA reductase; 8, propionate CoA transferase; 9, enoyl-CoA hydratase; 10, lactoyl-CoA dehydratase; and 11, lactate dehydrogenase. EC numbers are given in *SI Appendix*.

There are several possible variations on the C4-glyoxylate cycles (Fig. S5), each with possible pros and cons. Using pyruvate carboxylase instead of PEP carboxylase results in higher ATP efficiency but somewhat lower pathway specific activity (Table S1 and Fig. S5, module A). In addition, acetyl-CoA carboxylase, another efficient carboxylating enzyme, can be used instead of the carboxytransferase enzyme (Fig. S5, module B). Table S1 presents a comprehensive comparison among all the MOG pathways.

The MOG pathways are equivalent to the rPP cycle in their electron donors types; all donors are NAD(P)H. Hence, Fig. 2*A* presents the ATP requirement for the MOG pathways as well. The MOG pathways hydrolyze eight to 12 ATP molecules (depending on the exact pathway identity; Table S1), and therefore are all thermodynamically favorable, with $\Delta G << 0$ under a wide range of pH and ionic strengths.

By using the central carbon metabolism model of the algae *Chlamydomonas*, we have found that the MOG pathways were able to support maximal growth yield with no further secretion products. Moreover, the flux solution space indicates that the integration of the MOG cycles necessitates the fewest significant changes in the endogenous flux distributions in comparison with other suggested cycles (*SI Appendix*).

Most reactions employed by the MOG pathways are prevalent in many species throughout the tree of life, yet some reactions involved in these pathways are rather unique. For example, the reduction of malonyl-CoA (reaction 7 in Fig. 4) is performed by an enzyme naturally found in thermophilic prokaryotes (32, 33). The hydration of acrylyl-CoA (reaction 10 in Fig. 4*B*) is carried out by the enzyme lactoyl-CoA dehydratase that contains iron-sulfur centers and was found to be oxygen-sensitive (34). Nevertheless, studies indicate that there exist variants of the enzyme that show efficient performance under full aerobic conditions (e.g., ref. 35). Finally, the enzyme alanine aminomutase (reaction 9 in Fig. 4*A*) was evolved from the enzyme lysine 2,3-aminomutase to act on alanine (36). A comprehensive discussion on the unique enzymes of the C4-glyoxylate cycles is given in the *SI Appendix*.

Other promising synthetic carbon fixation pathways that resulted from our analysis appear in Fig. S6. Many of the synthetic pathways are expected to be faster than the rPP cycle (i.e., they have higher calculated pathway specific activities).

Discussion

Analysis and Optimization of Carbon Fixation Pathways. This study has computationally analyzed and compared carbon fixation pathways by focusing on their kinetics. We found synthetic pathways that have the potential to show significantly faster kinetics as evaluated by the pathway specific activity. From a biotechnological point of view, this is probably a major criterion, directly affecting the productivity of a photosynthetic, carbon-fixing organism.

We use the pathway specific activity as a useful, well defined proxy that enables calculation and comparison of alternative pathways, given the limited kinetic data available. The overall flux through a pathway is approximated by the pathway specific activity criterion when (*i*) the enzymes are substrate saturated, (*ii*) the rate of the backward reaction of each enzyme is negligible compared with the rate of its forward reaction, and (*iii*) enzyme expression levels are balanced based on each enzyme's specific activity (i.e., no "surplus" of any enzyme). Obviously, in natural pathways, none of these requirements fully holds; therefore, the pathway specific activity serves as an upper-limit estimation of the pathway overall rate (*SI Appendix*). This metric is not biased, and thus the advantage of the synthetic alternatives over the natural pathways is expected to hold even if the overall rates would be lower than predicted.

Several other optimization methods have been discussed in the literature, based on minimization of overall metabolic intermediate concentration, minimization of transient times, and maximization of enzyme specificity (37). However, most of these seem to us to be less functionally relevant and cannot be systematically employed as a result of the lack of necessary data.

Previous studies have proposed alternatives to central metabolic pathways such as the oxidative and reductive pentose phosphate pathway, the tricarboxylic acid cycle, and glycolysis (e.g., refs. 29, 38, 39). However, those efforts were focused on evolutionary perspectives, with an intention of deciphering the selection imposed on their evolvement. Our work concentrates on biotechnological aims while adhering to a synthetic biology approach. Instead of asking why the existing metabolic pathways evolved the way they did, our goal is to take advantage of the repertoire of known enzymes to design better pathways for human needs.

The life cycle of a photosynthetic organism consists of alternating periods of light saturated and light limited growth. Although some studies have found a correlation between dry matter production and intercepted radiation (40), numerous other studies have shown that, under a wide range of ambient conditions, the plant cells are light-saturated (41–43), and hence ATP and NADPH costs are thought not to be a limiting factor. Indeed, one of the major roles of the mitochondria, under saturating illumination, is to dissipate excess reducing power by alternative oxidase branches, which reduces molecular oxygen without the generation of ATP (44).

In this study, we have referred explicitly only to the efficiency of using ATP-like and NADPH-like resources, both regenerated by light. In reality, however, the water use efficiency, the nitrogen use efficiency, and similar metrics are just as important, even in agricultural settings. We note that the use of a carbon fixation pathway with increased productivity is expected to have a positive effect on these efficiencies as well. For example, higher specific rate of carbon fixation will enable the cell to reallocate its resources, e.g., dedicate less protein for carbon fixation, which in turn will increase the nitrogen use efficiency [photosynthetic rate per unit of N (45)]. In addition, the increased affinity toward inorganic carbon and the absence of the oxygenation reaction will enable the plant to sustain a high carbon fixation rate even when a high fraction of the stomata are closed, which should increase the water use efficiency. Furthermore, there is an energetic cost for de novo synthesis of the catalytic machinery, a cost that is lower for pathways with higher pathway specific activities. Hence, the pathway specific activity is also affecting the energy efficiency. Although the ATP and NADPH costs associated with carbon fixation probably reflect the major energy fluxes within the cell, this type of energy utilization can have a further effect on the cell in vivo. Finally we note that our analysis was performed for static environmental conditions. One of the future challenges is to incorporate the effect of fluctuations in, for example, temperatures, water availability, and CO_2 concentration into the modeling framework.

Implementing the Synthetic Carbon Fixation Pathways. The MOG pathways are the most promising synthetic carbon fixation pathways revealed in our analysis. This is because of the unique use of the best carboxylating enzyme, PEP carboxylase (Fig. 4). Considering the apparent effectiveness of the proposed pathways, why are they not found in nature? We can only speculate about this intriguing question. In many organisms, the rPP cycle is responsible for diverse metabolic needs apart from carbon fixation (46, 47). Several side pathways that branch out of the main cycle produce important metabolites, such as sugars, amino acids, and other central cellular metabolites. The centrality of carbon fixation within the overall metabolic network of the photosynthetic cell, as well as the branching routes, diminishes the "evolvability" of the reductive pentose phosphate cycle as a carbon fixation metabolic unit, while leaving room for only minor adaptations to specific enzymes within the pathway.

The in vivo implementation and assimilation of the proposed synthetic carbon fixation pathways in photoautotrophs will certainly involve numerous difficulties. The assimilation of the foreign pathway can be regarded as a "metabolic heart transplant," which the host cell may well reject. The most immanent problems may arise from negative interactions of the pathway with the endogenous metabolic network of the host, nonoptimal expression, activity and stability of the recombinant proteins, and erroneous regulation and localization. However, by forcing the use of these alternative pathways as the sole option for growth, natural selection might be harnessed to overcome these obstacles. A genetically modified photosynthetic organism might not have a selective advantage in nature, but may hold great promise from a biotechnological point of view, increasing crop yields under controlled and optimized agricultural conditions.

Materials and Methods

Algorithm for Finding Carbon Fixation Cycles. Using all reactions in the Kyoto Encyclopedia of Genes and Genomes database (19), we have created a universal stoichiometric matrix (48). We developed an algorithm that is a variant of constraint based modeling (20–22), which finds for a given substrate (CO_2 or HCO_3^-) and product (GA3P), the shortest carbon fixation cycle that will achieve this transformation using a prescribed carboxylating enzyme or enzymes. Many of the reactions have more than one substrate and/or product as well as cofactors and thus required a custom approach implemented using Phyton and GLPK (http://www.gnu.org/software/glpk/), a linear programming solver, to find the solutions (*SI Appendix*).

Calculating Pathway Specific Activity. To maintain a product flux of 1 μmol/min through a given enzyme i, one needs $1 / V_i$ mg of that enzyme, where V_i is the enzyme's specific activity, in units of μmol/min/mg. $1 / V_i$ is therefore defined as the enzyme cost of that reaction (milligrams of enzyme needed to achieve 1 μmol/min flux). In the case of a linear pathway, the fluxes through all enzymes are equal. Therefore, to maintain an overall flux of 1 μmol/min, the enzyme cost of the whole pathway will be the sum of the individual enzyme costs, i.e., $\Sigma(1 / V_i)$. The pathway specific activity is defined to be the maximal flux (μmol/min) sustained by 1 mg of pathway total protein; therefore, it equals 1 divided by the enzyme's cost of the whole pathway, which is $1 / (\Sigma(1 / V_i))$. The general case of nonlinear pathways is analyzed in the *SI Appendix*, alongside a comprehensive discussion on the characteristics of this criterion.

Pathway Evaluation and Comparison. To enable pathway evaluation and comparison in terms of kinetic, energetic, and topological criteria, we defined a common pathway product. We chose GA3P as the end product metabolite because it is regarded as the product of the reductive pentose phosphate cycle. In addition, it is the simplest sugar leading to the biosynthesis of larger transport metabolites, such as glucose or sucrose. Each pathway is therefore composed of a cycle and an assimilation subpathway that converts the cycle's product into GA3P (Fig. S4). We note that the selection of such a common product is useful to enable comparison, but choosing a different compound as the common product would not have a qualitative difference on the results of the analysis.

Table S1 presents a comparison between the pathway specific activities of the natural carbon fixation pathways. Some of the natural pathways use ferredoxin-oxidoreductase enzymes to perform a carboxylation reaction. These enzymes are sensitive to oxygen and their specific activities were scarcely measured in the carboxylation direction (49, 50). Therefore, the pathways that contain those enzymes are shown in a separate section in Table S1 and their pathway specific activities cannot be compared with those of the non–ferredoxin-oxidoreductase–containing pathways.

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