Why an increase in activity of an enzyme in the Calvin Benson Cycle does not always lead to an increased photosynthetic CO₂ uptake rate? – A theoretical analysis

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Abstract: Overexpressing Calvin Benson cycle (CBC) enzyme shown to limit the flow of CO₂ through the cycle is a major approach to improve photosynthesis. Though control coefficients of CBC enzymes vary under different environmental and developmental conditions, it is usually implicitly assumed that enzymes in the CBC have a monotonic impact on the CBC fluxes. Here, with a dynamic systems model of the photosynthetic carbon metabolism, we show that, for glycerate-3-phosphate kinase (PGAK), fructose-1,6-bisphosphatase (FBPase), fructose-1,6-bisphosphate aldolase (FBA) and transketolase (TKa), individually increasing activity of these CBC enzymes theoretically leads to an initial increase then decrease in the fluxes through the CBC. Also, the inhibition constants of ADP for PGAK and of F6P for FBPase influence the CBC flux in a biphasic manner. These predicted enzymes showing a biphasic manner are always located in different sub-cycles of the CBC, which consume the shared substrates in the early steps in the CBC and produce intermediates used as substrates for enzymes in the later reactions. We show that the excessive increase in activities of enzymes in one sub-cycle consuming the shared metabolite could cause low concentrations of metabolites in the other sub-cycles, which results in low reaction rates of the later reactions and hence lowers overall CBC flux. This study provides a model to explain the underlying reasons that overexpression of enzymes in the CBC sometimes can negatively impact photosynthesis. We find that balanced activities of enzymes in the sub-cycles of the CBC are required to gain a higher efficiency of the CBC.

Key words:

Calvin Benson cycle, Metabolism coordination, Balanced investment, Sub-cycle, Biphasic response
Abbreviations:

Enzyme:

Rubisco  ribulose-1,5-bisphosphate carboxylase oxygenase
PGAK    glycerate-3-phosphate kinase
GAPDH   glyceraldehydes-3- phosphate dehydrogenase
FBA     fructose-1,6-bisphosphate aldolase
FBPase  fructose-1,6-bisphosphatase;
TK      transketolase (including TKa and TKb)
SBPA    Sedoheptulose-1,7-bisphosphate aldolase
SBPase  Sedoheptulose-1,7-bisphosphatase
PRK     ribulose-5-phosphate kinase

Metabolite:

RuBP    ribulose1,5-bisphosphate
PGA     3-phosphoglycerate
DPGA    bisphosphate-glycerate
T3P     triose phosphates (including GAP and DHAP)
DHAP    dihydroxyacetone phosphate
GAP     glyceraldehyde phosphate
FBP     fructose-1,6-bisphosphate
HexP    hexose phosphate (including F6P, G1P, G6P)
F6P     fructose-6-phosphate
G6P     glucose-6-phosphate
G1P     glucose-1-phosphate
E4P     erythrose-4-phosphate
SBP     sedoheptulose-1,7-bisphosphate
| Abbreviation | Description |
|--------------|-------------|
| S7P          | sedoheptulose-7-phosphate |
| Pent         | pentose phosphate (including Ri5P, Xu5P and Ru5P) |
| Ru5P         | ribulose-5-phosphate |
| Xu5P         | xylulose-5-phosphate |
| Ri5P         | ribose-5-phosphate |
| ATP          | adenosine triphosphate |
| ADP          | adenosine diphosphate |
| NADPH        | reduced nicotinamide adenine dinucleotide phosphate |
| NADP         | oxidized nicotinamide adenine dinucleotide phosphate |
| Pi           | orthophosphate |
Introduction

The Calvin-Benson cycle (CBC) fixes CO$_2$ and provides organic compounds for heterotrophic organisms on the earth and is a critical component of the global carbon cycle. The CBC is a classical autocatalytic cycle, where RuBP, which is the compound reacting with CO$_2$, is regenerated by the CBC after CO$_2$ is fixed to generate phosphoglyercate (PGA) (Woodrow and Berry, 1988). The CBC is a complex metabolic network, which includes 13 enzymatic steps and is linked to different branching fluxes exporting intermediates for biosynthesis of compounds, such as thiamine nucleotides, shikimate, sucrose, starch and isoprenoid (Raines, 2011).

In recent years, huge efforts have been investigated in identifying the enzymes that exert a high level of control over fluxes of the CBC and hence can be potentially used as the engineering targets to enhance photosynthesis. The degree of limitation of each step of the CBC efficiency is represented by flux control coefficient, which has been estimated for individual CBC enzymes through transgenic experiments (as summarized by (Raines, 2003; Tamoi et al., 2005; Raines, 2011) or mathematical models (Woodrow and Mott, 1993; Zhu et al., 2007; Zhu et al., 2013). Enzymes with a relative larger control coefficient are considered as primary targets for photosynthesis improvements, such as SBPase and Rubisco (Raines, 2003; Long et al., 2006; Zhu et al., 2010; Raines, 2011). Over-expression of SBPase or FBPase has indeed been shown to be effective in enhancing photosynthesis when they are overexpressed in plants. For example, overexpressing SBPase increases photosynthesis and growth in tobacco at the early stage (Lefebvre et al., 2005; Tamoi et al., 2006; Simkin et al., 2015; López-Calçagno et al., 2020) and under elevated CO$_2$ condition (Rosenthal et al., 2011), enhances photosynthesis and the tolerance of tomato to chilling stress (Ding et al., 2016) and of rice to heat stress (Feng et al., 2007), as well as stimulates photosynthesis and grain yield in wheat under greenhouse condition (Driever et al., 2017). Additionally, in transgenic plants with increased activities of multi-enzymes, such as SBPase/FBPase in tobacco in the greenhouse (Miyagawa et al., 2001) and in soybean in the field with feeding elevated CO$_2$ (Kohler et al., 2017), SBPase/FBPase/Ictb in rice (Gong et al., 2015) and tobacco (Simkin et al., 2015), and SBPase/FBPA/GDCH in Arabidopsis (Simkin et al., 2017), improved photosynthesis and growth are both achieved.
With these studies, it becomes apparent that the flux control coefficient of an enzyme over CBC flux varies depending on the environmental conditions (Stitt and Schulze, 1994; Raines, 2003; Raines, 2011). In the state-of-the-art photosynthesis improvements, one implicit assumption is that overexpression of an enzyme of the CBC will have either a positive or no impact on photosynthetic efficiency. Given this assumption, it comes as a surprise when the transketolase activity was either increased or decreased, the photosynthetic rate showed a certain decrease (Henkes et al., 2001; Khozaei et al., 2015). Though export of intermediates of the CBC may be related to this phenomenon, this observation raises a possibility that the influence of some enzymes on the CBC flux might be non-monotonic.

One option to test whether there are enzymes in the CBC showing non-monotonic responses to changes in enzyme activities is to generate a series of transgenic plants with varying enzyme activities and to test their impacts on photosynthesis, as have been done in the case of transketolase (Henkes et al., 2001; Khozaei et al., 2015). This unfortunately has not been investigated systematically for other enzymes in the CBC. Furthermore, when one enzyme in the CBC is overexpressed in vivo, expression level and activities of many other enzymes are usually changed as well (Price et al., 1995; Haake et al., 1998; Haake et al., 1999; Henkes et al., 2001), which makes it difficult to precisely calculate the control coefficient of a particular enzyme on the CBC in a particular state. Here we use a dynamic systems model of the photosynthetic carbon metabolism (Zhu et al., 2013) to examine which enzyme in the CBC may cause a biphasic change in the overall CBC flux.

The CBC is highly regulated by different manners to gain its operating efficiency (Stitt, 1996). These mechanisms include feedforward regulations of PRK, GAPDH-NADP, SBPase and FBPase performed by the thioredoxin-mediated system (Michelet et al., 2013; López-Calçagno et al., 2014) and Rubisco activation by the Rubisco activase (Slabas and Walker, 1976; Walker, 1976; Stitt, 1996; Raines, 2003; Stitt et al., 2010) in the light. These feedforward regulations are important for the rapid activation of the CBC from low irradiance to high irradiance (Gross et al., 1991; Stitt, 1996; Rascher and Nedbal, 2006; Kaiser et al., 2018). The enzymes of the CBC are also feedback inhibited by intermediates of the CBC (Walker, 1976; Woodrow and Berry, 1988; Stitt, 1996). It is likely that the feedback mechanisms used in the CBC may also help plants in the field to gain higher response speed
of CBC flux and to maintain stable metabolite concentrations in fluctuating environment
(Stitt, 1996). Such a negative feedback regulatory mechanism in fact also widely exists in
genetic regulatory network (Rosenfeld et al., 2002). On one hand, it can accelerate the
response time of the components’ concentrations in the network to external perturbations
(Rosenfeld et al., 2002); on the other hand, it helps to confer the stability of the
concentrations of components in the network under environmental perturbation (Becskei
and Serrano, 2000). So far, the significance of these regulations on the CBC efficiency under
steady state has not been systematically investigated and is the topic of this study. The
major advantage of using a systems modeling approach to determine the impacts on
systems fluxes of the CBC is that each single kinetic parameter of the CBC enzymes can be
changed by a large magnitude without changing the other parameters in the CBC cycle, so
that we can individually examine the impacts of each modified parameter on the CBC fluxes.

The results from this study show that the flux through the CBC indeed interplays a
biphasic response to changes in enzymatic activities for a number of enzymes. And two
regulatory factors of enzymes in the CBC are identified, which also generate biphasic
responses. Both these enzymes and the regulators play important roles in limiting resource
distribution among the sub-cycle in the CBC. This study in theory demonstrated the
importance of balancing the activities of the enzymes to maintain the metabolic
coordination for an efficient CBC.

Method

Model description

The C₃ photosynthetic carbon metabolism module of ePhotosynthesis model constructed
previously (Zhu et al., 2013) was employed in this study. The module includes the metabolic
pathways of the Calvin-Benson cycle, photorespiratory pathway, sucrose synthesis and
starch synthesis. In this module, the ATP and NADPH production through chloroplastic
electron transfer chain is simplified based on equations used in Wang’s model (Wang et al.,
2014). These equations assume that there is obligatory Q cycle, no-cyclic electron transfer:
ADP + Pi -> ATP

\[ v_{16} = \frac{\min\{V_{max16}, D_{LR} \ast J\} \ast ([ADP] \ast [Pi] - [ATP])}{K_{M161} \ast K_{M162} \ast (1 + \frac{[ADP]}{K_{M161}} + \frac{[ATP]}{K_{M162}})} \] ...

NADP -> NADPH

\[ v_{bfn2} = \frac{(\min\{V_{2M}, E_{LR} \ast J\} \ast ([NADP] - [NADPH])}{K_{M2NADP} \ast \left(1 + \frac{[NADP]}{K_{M2NADP}} + \frac{[NADPH]}{K_{M2NADP}}\right)} \] ...

where [ATP], [ADP], [Pi], [NADP], and [NADPH] represent the concentrations of ATP, ADP, Pi, NADP+, and NADPH in chloroplast, respectively; KM represents the Michaelis-Menten constant of an enzyme for its substrate; \( V_{max16} \) and \( V_{2M} \) represent the maximum velocities of ATP synthase and ferredoxin-NADP+ oxidoreductase in the chloroplast, respectively. Since the maximum production rate of ATP and NADPH is also determined by the electron transport rate \( J \), so the maximum production rate of ATP and NADPH are described by \( \min\{V_{max16}, D_{LR} \ast J\} \) and \( \min\{V_{2M}, E_{LR} \ast J\} \), respectively (Wang et al., 2014). The \( D_{LR} \) and \( E_{LR} \) are the stoichiometric ratio of ATP/e- and NADPH/e-, respectively. In our simulations, \( D_{LR} \) is assumed equal to 1 and \( E_{LR} \) is assumed equal to 0.5 (von Caemmerer, 2000). \( J \) is calculated as follows:

\[ j = \frac{I_2 + J_{max} - \sqrt{(I_2 + J_{max})^2 - 4 \ast \theta \ast I_2 \ast J_{max}}}{2 \ast \theta} \] ...

\[ I_2 = I \ast \text{abs} \ast (1 - f) \ast 0.5 \] ...

where \( I \) is the photosynthetic active radiation in the simulation; \( I_2 \) is the useful radiation absorbed by photosystem II; the absorptance (\( \text{abs} \)) of leaf is usually assumed as 0.85; the correction constant for spectral quality (\( f \)) is assumed equal to 0.15; the constant 0.5 means the assumption that irradiance is equally absorbed by photosystem I and photosystem II (values of these parameters are assigned by referencing (von Caemmerer, 2000)); the maximum electron transport \( J_{max} \) in our simulations is assumed equal to 177 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) which is within the range of that in C_3 photosynthesis (Fan et al., 2011); theta (\( \theta \)) is the convexity index, which is assumed as 0.98 according to the value estimation in the single isolated cell of a plant leaf (Terashima and Saeki, 1985).
Simulation

In this study, we investigated the effects of individual kinetic parameters of enzyme in the CBC on RuBP regeneration rate by using a kinetic model of C₃ photosynthesis, which has been constructed previously (Zhu et al., 2013). The response curves of photosynthesis versus fold change of parameter values are shown in this study. Simulations were done using the MATLAB (Mathworks, ver 2012, Natick, MA, USA). All simulations were performed under a PPFD of 1500 μmol m⁻² s⁻¹, a CO₂ concentration of 800 ppm and an ambient O₂ level of 21%. Under these conditions, the changes of maximal photosynthetic CO₂ uptake rate are determined by the kinetic properties of enzymes in the CBC rather than the irradiance or the CO₂ concentration. In each of the simulations, only one parameter under test is perturbed while all other parameters are maintained as their default values. All simulations were conducted by varying the default values of each parameters, $V_{\text{max}}$ and $K_i$ of enzymes in the CBC, by a fold change from $10^{-2}$ to $10^3$. For example, here equations 5 and 6 are used to demonstrate how we modified the $V_{\text{max}}$ and $K_i$, respectively, where $n$ is the fold change in the perturbation.

$$v = (V_{\text{max}} \times n) \times \frac{[S]}{[S] + Km \times \frac{[\text{inhibitor}]}{K_i}} \quad ...(5)$$

$$v = V_{\text{max}} \times \frac{[S]}{[S] + Km \times \frac{[\text{inhibitor}]}{(K_i \times n)}} \quad ...(6)$$

where $v$ is the reaction rate; $V_{\text{max}}$ is the maximum enzymatic capacity; $[S]$ and $[\text{inhibitor}]$ are the concentrations of the substrate and an inhibitor to the enzyme, respectively; $Km$ is the Michaelis-Menten constant of an enzyme for its substrate; $K_i$ is the inhibition constant of enzyme for the inhibitor.

The simulations includes two situations for the perturbations on transketolase, since transketolase catalyzes two reactions in the CBC (TKa: fructose-6-phosphate + triose phosphate $\leftrightarrow$ erythrose-4-phosphate + pentose phosphate; TKb: sedoheptulose-7-phosphate + triose phosphate $\leftrightarrow$ 2 pentose phosphate). The rate equations of Tka (equation 7 and 8) and TKb (equation 7 and 9) catalyzed reactions are described as following (Zhu et al., 2013):

$$v = (V_{\text{max}} \times n) \times \frac{[S]}{[S] + Km \times \frac{[\text{inhibitor}]}{K_i}} \quad ...(5)$$

$$v = V_{\text{max}} \times \frac{[S]}{[S] + Km \times \frac{[\text{inhibitor}]}{(K_i \times n)}} \quad ...(6)$$
\[
\text{TEMP} = \text{Km}8p5p \times \text{Km}5p5p \\
\quad \times \left(1 + \left(1 + \frac{[\text{GAP}]}{\text{Km}5gap} \right) \left(\frac{[\text{F6P}]}{\text{Km}8f6p} + \frac{[\text{S7P}]}{\text{Km}8s7p} \right) \frac{[\text{GAP}]}{\text{Km}8gap} + \frac{1}{\text{Km}8p5p} \right) \\
\quad \times \left(\frac{[\text{Xu}5P]}{\text{Km}5p5p} \left(1 + \frac{[\text{E4P}]}{\text{Km}5p5p} + \frac{[\text{Rl}5P]}{\text{Km}5p5p} \right) + [\text{E4P}] + [\text{e}] \right) \ldots (7)
\]

\[v_7 = PsV7 \times \frac{[\text{F6P}] \times [\text{GAP}] \times PsKE57 - [\text{E4P}] \times [\text{Xu}5P]}{\text{TEMP}} \ldots (8)\]

\[v_{10} = PsV10 \times \frac{[\text{S7P}] \times [\text{GAP}] \times PsKE810 - [\text{Xu}5P] \times [\text{Rl}5P]}{\text{TEMP}} \ldots (9)\]

where \(PsV\) represents the maximum velocity of the reaction and \(Km\) represents the Michealis-Menten constant of enzyme for a substrate. \(PsKE\) represents the equilibrium constant of a biochemical reaction. The symbols with square brackets represent the metabolite concentrations. We perturbed these two reactions by two different strategies. In the first strategy, we individually evaluate the impact of modifying a single enzymatic reaction on the overall CBC flux. Specifically, only \(PsV7\) in equation 8 or \(PsV10\) in equation 9 is perturbated. Under such a scenario, the perturbations on \(Vmax\) of TKa does not influence reaction catalyzed by TKb; similarly, perturbations on \(Vmax\) of TKb does not influence reaction catalyzed by TKa. In the second strategy, the \(Vmax\) of TKa always equals to that of TKb, which means \(PsV7\) and \(PsV10\) are perturbated simultaneously.
Figure 1. The response curves of net photosynthetic CO$_2$ uptake rates versus the maximal activities of enzymes (Vmax) in the CBC. The x-axis represents the fold-change to the default Vmax value, and the y-axis represents the net photosynthetic rate (μmol m$^{-2}$ s$^{-1}$). The red dots represent the maximum net photosynthesis during perturbations. With the increasing activities of enzymes, photosynthetic CO$_2$ uptake rates show either a biphasic (blue) response or a monophasic (black) response.
Results

Increasing the enzyme capacity of the CBC may decrease photosynthesis

The responses of photosynthetic CO₂ uptake rate (A) to an increase in enzymatic activity are grouped into two classes. In the first class, A increases with the increase of enzyme activity until A is close to a plateau; while in the second class, A first increases then decreases with the increase in enzyme activity (Figure 1). In this study, the first class of response is termed as monophasic response and the second class is termed as biphasic response. The enzymes showing a monophasic response in the CBC include Rubisco, GAPDH, SBP aldolase, SBPase, TK_b and PRK; the enzymes showing the biphasic responses in the CBC include PGAK, FBP aldolase and TKA (Figure 1).

Excess capacity of enzymes showing a biphasic response limits RuBP regeneration

Since the PPFD, CO₂ concentration and atmospheric O₂ level are not altered during simulations, changes in A should be attributed to changes in the steady-state RuBP concentration. To better differentiate the influence of enzyme activity or metabolite concentration on the CBC fluxes, we used a ratio of substrate concentration to the Michaelis-Menten constant of the substrate (S/Km_s) in a particular enzyme in the following analysis.

At a steady state of this model, without considering CO₂ released by dark respiration, the net photosynthesis can be calculated by the following equation (von Caemmerer, 2000):

\[
v = V_{cmax} \frac{[RuBP] \cdot \min\{1, \frac{[RuBP]}{E_t}\}}{Km_{RuBP} + [RuBP]} \cdot \alpha \quad \text{(10)}
\]

Where \(V_{cmax}\) represents the maximum carboxylation rate of Rubisco; \(Km_{RuBP}\) represents the apparent Michaelis-Menten constant of Rubisco for RuBP; \(E_t\) represents the Rubisco active sites (2.5mM was assigned in Zhu’s model (Zhu et al., 2013)). In this study, the value of \(E_t\) is equal to the default value (2.5mM). And \(\alpha\) is calculated as follows:
\[
\alpha = \frac{[CO_2]}{[CO_2] + K_c \left(1 + \frac{[O_2]}{K_o}\right)} - 0.5 \times \frac{V_{\text{max}}}{V_{\text{cmax}}} \times \frac{[O_2]}{[O_2] + K_o \left(1 + \frac{[CO_2]}{K_c}\right)} \quad \ldots (11)
\]

where \(K_c\) and \(K_o\) represent the Michaelis-Menten constants of Rubisco for \(CO_2\) and \(O_2\), respectively. \(\frac{V_{\text{max}}}{V_{\text{cmax}}}\) represents the ratio of maximum oxygenation rate to maximum carboxylation rate; it was assumed to be 0.24 in the previous model (Zhu et al., 2013). The constant of 0.5 represents the \(CO_2\) releasing rate through photorespiration pathway is a half of the RuBP oxygenation rate (von Caemmerer, 2000).

In this study, \(\alpha\) is fixed as a constant since the environmental conditions keep constant in all simulations. So that:

\[
\frac{v}{V_{\text{max}}} = \alpha \times \min \left\{ 1, \frac{\text{RuBP}}{E_t} \right\} \times \frac{\text{RuBP}}{K_{m_{\text{RuBP}}}} \times \frac{1}{1 + \frac{\text{RuBP}}{K_{m_{\text{RuBP}}}}} \quad \ldots (9)
\]

The relationship between \(\frac{v}{V_{\text{max}}}\) and \(\frac{s}{K_{m_s}}\), which is also named as enzyme – substrate realationship, was plotted as described in (Fendt et al., 2010). Following Fendt et al. (2010), we examined the reaction in three stages, i.e., in the first stage, the substrate concentration is much lower than its \(K_m\) and the reaction rate is mainly limited by the substrate concentration (the region on the left side of the first dashed vertical line in Figure 2); in the second stage, when the substrate concentration is near its \(K_m\), the reaction rate is limited by both the enzyme capacity and substrate concentration (the middle region bounded by dashed vertical lines in Figure 2); in the third stage, when substrate concentration is times greater than the \(K_m\), the reaction rate is limited by enzyme capacity (the region on the right side of the second dashed vertical line in Figure 2) (Fendt et al., 2010). Following equation 9, we can be informed that the reaction rate of Rubisco is approaching \(V_{\text{max}}\) with the increase of \(\frac{\text{RuBP}}{K_{m_{\text{RuBP}}}}\). In other words, the greater the value of \(\frac{\text{RuBP}}{K_{m_{\text{RuBP}}}}\), the less the limitation by the substrate RuBP, and vice versa.
As to other enzymes of the CBC, increasing their enzymatic capacity influences [RuBP] and further influences the enzyme-substrate relationship of Rubisco (Figure 2). With the increase of capacity of enzymes showing monophasic responses, both $A$ (Figure 1) and $\frac{[\text{RuBP}]}{K_m^{\text{RuBP}}}$ (Figure 2) increase. On the contrary, with the increase of activities for enzymes showing biphasic responses, $\frac{[\text{RuBP}]}{K_m^{\text{RuBP}}}$ initially increases with the increase of $A$ (red dots in the Figure 2 represent the maximum steady state photosynthesis); then, further increase of the enzyme activity leads to decreased $\frac{[\text{RuBP}]}{K_m^{\text{RuBP}}}$ and enhanced the substrate limitation to photosynthesis (Figure 2).

The $\frac{[\text{RuBP}]}{K_m^{\text{RuBP}}}$ and $A$ shows the same responses to changes in catalytic capacity for enzymes in the CBC (Figure 1 and 2), except Rubisco. With a larger catalytic capacity of GAPDH, SBPA, SBPase, TKb and PRK, the $\frac{[\text{RuBP}]}{K_m^{\text{RuBP}}}$ increases and reaches a plateau.
2. The effects of the CBC enzyme capacity on the Rubisco-RuBP relationships. The RuBP/Km<sub>rub</sub> is the ratio of RuBP concentration to the apparent Km of Rubisco for RuBP. The V<sub>c</sub>/V<sub>cmax</sub> is the ratio of carboxylation rate to the maximum carboxylation rate if Rubisco. The x-axis is on a log<sub>10</sub> scale. The color bar means the fold change of the default Vmax of each enzyme. The color of dots in each subgraph represents the fold change (corresponding to the color bar) of enzyme activity. The dark blue means the fold change close to 0.01 and yellow means fold change close to 1000. When the
color of dots changes from blue to yellow, the maximum enzyme activity of the enzyme in each subgraph increases. The gray curve in each subgraph describes the enzyme-substrate relationship of a standard Michaelis-Menten equation for a first-order reaction. Dashed vertical lines in each subgraph separate the subgraph into three regions. The left region, middle region and right region represent substrate limiting stage (stage I), substrate and enzyme co-limiting stage (stage II) and enzymatic capacity limiting stage (stage III), respectively (Fendt et al., 2010). When colors of dots change from blue to yellow, if the corresponding positions of these dots change from left to right, then with the increase of enzyme activity, the limitation over Rubisco carboxylation shifts from RuBP concentration limitation to Rubisco activity limitation. On the other hand, if the corresponding positions of these dots change from right to left, then with the increase of enzyme activity, the limitation shifts from Rubisco capacity limitation to RuBP concentration limitation. The red dot represents a state when photosynthetic CO$_2$ uptake rate is maximized. For GAPDH, SBPA, SBPase, TKb and PRK, the yellow dots overlap with red dot, which means that increasing their enzymatic capacities only slightly increases photosynthesis. For Rubisco, however, with increase in its catalytic capacity, the $\frac{RuBP}{KmRuBP}$ gradually drops. Additionally, the $\frac{RuBP}{KmRuBP}$ has a biphasic response curve with the increase in the activity of PGAK, FBA, FBPase and TKa. Most of the dots are located in the middle region (Figure 2), indicating that CO$_2$ fixation is limited by both the Rubisco catalytic capacity and RuBP concentration in most perturbations. During the perturbations of enzymatic capacity of each enzyme in the CBC, the relationship of enzyme-substrate of every enzymatic reaction is also simulated (Figure S1 to S10).

**The enzymes showing biphasic responses control distribution of substrates in different sub-cycles of the Calvin Benson cycle**

In CBC, there are a few metabolites which are consumed by multiple reactions, e.g. ATP is used as a shared substrate by both PGAK- and PRK-catalyzed reactions, and T3P is used as a shared substrate by 4 steps in the CBC. They are the enzymatic steps catalyzed by FBA, TKa, SBPA and TKb (Figure 3). Existence of substrates used by multiple reactions creates different sub-cycles for the CBC. After careful examination of the location of enzymes generating biphasic responses, we found that these enzymes always catalyze reactions in a sub-cycle consuming one of the shared substrates (Figure 1, Figure 3), meanwhile producing the product which is used as a substrate for a reaction of the next sub-cycle. As a result, with changed capacity of these enzymes, the imbalance of the coordination between
different sub-cycles will lead to the shared substrate being predominantly used in one sub-cycle and thus becoming a limiting substrate for reactions in another sub-cycle. For example, with increase in the capacity of PGAK, the substrate limitation to RuBP regeneration by PRK moves from the short of Ru5P to the insufficient ATP (Figures S2); similarly, with the increase of capacity for FBA or FBPase or Tka, the substrate limitation over TKa moves from limiting availability of S7P to a limitation in the level of T3P (Figure S4, S5 and S6).
Figure 3. The enzymes showing biphasic responses (enzymes in blue) function as the switches coordinating hub reactants distribution among sub-cycles. The blue arrows represent the pathways consuming the shared metabolite. The font size of ATP (a and d) and T3P (b, c, e and f) represent the relative concentration of ATP and T3P, respectively. The thickness of lines represents the relative enzymatic capacity relative to the default value. (a, d), (b, e) and (c, f) represent the shared metabolites distributed by PGAK, FBPA or FBPase, and of TKa, respectively.
Two Negative feedback loops play dominant roles in coordination of the CBC

The above analyses clearly show that, level of activity of enzymes in one sub-cycle consuming shared substrate and producing intermediates required for another sub-cycle play a critical role in determining flux through the CBC (Figure 3). The reaction rate is determined by both enzyme capacity and effectors. Here we used the regulatory constants to describe the regulations in model construction, such as activation constants and inhibition constants for the involved regulators. To test whether there are regulators critical for coordinating the consumption of shared metabolites between sub-cycles, we further tested whether modifying these regulatory constants may also generate biphasic responses in the fluxes through the CBC. In the carbon metabolism module of Zhu’s model, 13 regulators are included (Table 1, Zhu et al, 2013), which are by chance all inhibitors for enzymes. Similar to what we have done for the enzymatic capacity, here we systematically perturbed the inhibition constant by manipulating the default value by $10^{-2} \sim 10^{3}$ folds. We found that out of these 13 regulatory constants, only two inhibitors resulted in biphasic response of photosynthesis. They are the inhibition of ADP to PGAK and inhibition of F6P to FBPase (Figure 4). The enzymes related to these two inhibitions, as expected, locate on the pathways consuming shared metabolites in different sub-cycles.

Table 1 The regulators considered in the current model (Zhu et al., 2013).

| Enzymes | Regulators            | Regulation |
|---------|-----------------------|------------|
| Rubisco | PGA, FBP, SBP, Pi, NADPH | Inhibition |
| PGAK    | ADP                   | Inhibition |
| FBPase  | F6P, Pi               | Inhibition |
| SBPase  | Pi                    | Inhibition |
| PRK     | ADP, PGA, RuBP, Pi    | Inhibition |
Figure 4. The feedback inhibition of ADP to PGAK (a and b) and F6P to FBPase (c and d) are key biochemical regulators for maintaining an efficient CBC. The red dots in a and c represent the maximum net photosynthesis. a and c show the effects of weaker inhibitions of ADP to PGAK and of F6P to FBPase on photosynthesis, respectively. b and d show the working model of the critical inhibitions of ADP and F6P in the CBC; red arrows in b and d represent the pathways consuming the shared metabolites; blue dashed lines and symbols in b and d represent the feedback inhibitions by the products.

A simplified mathematical interpretation of the non-linear responses systems flux to changes in enzyme activities

To gain quantitative insights into what may influence the optimal distribution of shared substrates between sub-cycles, we developed an analytical model representing a simplified metabolic network with sub-cycles competing for shared resources (Figure 5a). The simplified conceptual model only includes two sub-cycles. Substrate element M (we assume M content is saturated in the analysis) is assimilated by the metabolic cycle to produce Product. Here the intermediate C is a shared metabolite; B is a product with the
consumption of C by one sub-cycle (catalyzed by the enzyme E₁); the intermediate B together with C are the substrates for another sub-cycle which is catalyzed by the enzyme E₂. Assuming that that there is a limiting element in C and B with a total of the limiting element being 1, then, if we assume that the steady state concentration of C is x (0<x<1), then the steady state concentration of B is 1-x. Then, on the basis of Michaelis-Menten kinetics equation, the steady-state rate of this cycle, represented here as the flux of production formation, can be described as follows:

\[ v = \frac{V_{\text{max}} \cdot x}{K_1 + x} \cdot \frac{1 - x}{K_2 + (1 - x)} \]  \hspace{1cm} (12)

where \( V_{\text{max}} \) is the maximum velocity of the reaction catalyzed by enzyme E₂, \( K_1 \) and \( K_2 \) represent the Michaelis-Menten constant of E₂ for C and B, respectively. The relationships between reaction rate with the concentration of C (or B) (Figure 5b) show that, there is a biphasic response curve of reaction rates to the increasing concentration of C. The maximum flux rate of this cycle (\( v_{\text{max}} \)) can be obtained.

\[ v_{\text{max}} = \frac{1}{(K_1 + K_2 + 1)^2} \times \left( -2 \times K_1^2 + 2 \times K_2^2 \times P - 4 \times K_1 \times K_2 \times P - 4 \times K_1 \times P - 4 \times K_2 \right) \times P - 2 \times P + 2 \times V_{\text{max}} \times K_1 \times K_2 + V_{\text{max}} \times K_1 + V_{\text{max}} \times K_2 + V_{\text{max}} \right) \]  \hspace{1cm} (13)

where \( P = \sqrt{\frac{V_{\text{max}}^2 \times K_1 \times (K_1 + 1) \times K_2 \times (K_2 + 1)}{(K_1 + K_2 + 1)^4}} \).

The concentration of C corresponding to the maximum flux rate of Product formation at the steady state is:

when \( K_1 = K_2 \),

\[ x_{v_{\text{max}}} = 0.5 \]  \hspace{1cm} (14)

when \( K_1 < K_2 \),

\[ x_{v_{\text{max}}} = \frac{(K_1 - K_2)^2 + (K_1 + 1) \times (K_2^2 + K_2)}{K_1 - K_2} \]  \hspace{1cm} (15)
when $K_1 > K_2$,

$$x_{vmax} = \frac{(K_2-K_1)+\sqrt{(K_1+1)\times K_1\times (K_2^2+K_2)}/(K_1-K_2)^2+K_1+K_2+K_1}{K_1-K_2} \cdots (16)$$

With the increase in the concentration of $C$, $x$ increases, while $(1-x)$ and $\frac{1-x}{K_2+(1-x)}$ decrease. Such that there is a switch of the limitation between $B$ and $C$ over the systems flux. The concentration of $C$ at the maximal systems flux rate, which is represented as $x_{vmax}$ above, depends on Michaelis-Menten constants $K_1$ and $K_2$, but does not depend on catalytic capacity of enzymes(Equation 14, 15 and 16).
Figure 5. The importance of metabolic coordination for an efficient metabolic cycle. (a) The simplified working model of the metabolic cycle with two sub-cycles. This auto-catalytic metabolic cycle assimilates \( M \) to produce \( \text{Product} \). B and C are the intermediates of this cycle, and their total concentration is constrained by the limiting resource \( S_t \). \( E_1 \) is the enzyme converting C to B, and \( E_2 \) catalyzes the reaction consuming B and C for downstream reactions in the cycle. \( K_1 \) and \( K_2 \) are the Michaelis-Menten constants of \( E_2 \) for B and C, respectively. \( x \) and \( (1-x) \) in red color represent the
steady state concentrations of C and B, respectively. (b) The response curves of $v$ versus the limiting resource C. In the panel b, $v$ of the Y-axis has been normalized to the maximum $v$ under the corresponding conditions, and it represents the relative flux of the auto-catalytic cycle. In the simulation: $K_1 = 1$, when $K_1 > K_2$, $K_2 = 0.05$ (red curve); when $K_1 = K_2$, $K_2 = 1$ (green curve); when $K_1 < K_2$, $K_2 = 20$ (purple curve). The dotted line indicates the $x$ corresponding to the maximum $v$. The gradient bars show that, with the increase of $x$ from 0 to 1, the concentration (light green gradient bar) of C increases and concentration (dark green gradient bar) of B decreases.

**Discussion**

It has been proposed that both the enzyme capacity and its regulation are important to determine the efficient operation of the CBC (Stitt, 1996). In this study, *in silico* parameter perturbation experiments were performed and analyzed by employing the systems kinetic model of C₃ photosynthetic carbon metabolism (Zhu et al., 2013). The quantitative simulations theoretically demonstrate that balanced investment in sub-cycles of the CBC, which consume a shared intermediate and produce a substrate used by another sub-cycle, is required to gain a high flux through the CBC. The enzymatic steps (Figure 1) and regulators (Figure 4) contributing to coordinating the metabolic fluxes within the CBC are predicted. This work will promote our better understanding on the CBC operation and its regulations.

In this theoretical study, we didn’t consider the limitation of total enzyme amounts in the CBC but investigated the potential impacts of each enzymatic step or regulator on the CBC efficiency. Our results show that in the CBC, when the enzymatic capacity of the step catalyzed by PGAK, FBA, FBPase or TKa is beyond its optimal level, it decreases rather than increases the flux of the CBC (Figure 1). Since there are a number of limiting resources in the CBC, e.g. the adenylate residue and total phosphate concentration, the level of either total metabolites or single intermediate in the CBC is constrained by the limiting resources. The un-coordinated consumptions of shared metabolites through enzymes of sub-cycles in the CBC may result in the imbalanced distribution of limiting resources (Figure 3 and 4), and ultimately limit the whole CBC efficiency. In theory, PGAK can coordinate ATP consumptions by the sub-cycle with PGAK and by the sub-cycle with PRK, while FBA, FBPase and TK can coordinate the consumption of T3P by different sub-cycles (Figure 3).
So far, conclusive experimental data directly showing such biphasic responses of fluxes in the CBC to the CBC enzyme activities are still absent. On one hand, it’s difficult to estimate the impacts of perturbing a single enzyme on the fluxes within the CBC. This is because, in the transgenic experiments, when the activity of one enzyme is altered, it usually causes the changes in many other enzymes (Haake et al., 1998; Haake et al., 1999; Henkes et al., 2001). However, there are some signs of biphasic responses indeed shown in literatures. For example, in transgenic tobacco overexpressing FBA, increasing FBA activity generally stimulates photosynthesis and growth under either ambient or elevated CO₂ conditions. However, transgenic plants with larger enhancement of FBA relative activity show slightly lower stimulation of photosynthesis and of growth under elevated CO₂ (Uematsu et al., 2012). Consistently, in transgenic Arabidopsis with overexpressing FBA, stimulations of plant growth are also slightly weaker in plants with greater increase in FBA activity (Simkin et al., 2017). Transgenic plants with reduction of FBPase activity show a decrease in A compared with those in WT (Koßmann et al., 1994; Haake et al., 1998; Haake et al., 1999), while over-expressing FBPase increases A and growth in tobacco (Tamoi et al., 2006). In contrast with these observations in plants, under most testing conditions, increasing chloroplastic FBPase activity by overexpressing FBPase in Chlamydomonas reinhardtii dramatically inhibited both the cell growth and biomass accumulations of transgenic strains compared with that of WT (Dejtisakdi and Miller, 2016). For TK, either an increase or a decrease of TK activity in tobacco results in decreased growth, and leaf photosynthesis is decreased in TK antisense lines and in two of three reported TK overexpression lines (Henkes et al., 2001; Khozaei et al., 2015). Here one caveat is that we have simulated the two reactions in the CBC catalyzed by the transketolase independently. Therefore, the conclusions drawn based on TKa and TKb represent conceptual and network topological structural scenarios. When we simulated the TKa and TKb synchronously, the biphasic response of photosynthesis disappears (Figure S12). Interestingly, over-optimal capacity of TK leads to the accumulation of R5P and decreased T3P contents (Figure S13), which promotes the use of R5P to synthesize hydroxymethylpyrimidine pyrophosphate (HMPP), but constrains the synthesis of hydroxyethylthiazole phosphate (HETP), through consuming T3P, ultimately inhibiting thiamine pyrophosphate (TPP) synthesis since both HETP and HMPP are the substrates for TPP production (Henkes et al., 2001; Khozaei et al., 2015).
In this simulation study, PGAK also shows biphasic impacts on $A$. Besides the potential impacts of altering one enzyme on activities of other enzymes, a few other reasons might also contribute to this discrepancy between the experimental observation and the theoretical predictions. First, we discussed the function of enzymes to the CBC metabolism coordination only, while the metabolites are highly connected to other metabolic pathways (Raines, 2011). It may need to be fully considered to accurately predict the impact on systems flux, when activities of these enzymes are modified. Furthermore, the model used for the analysis might still need to be better parameterized, e.g. the activities of enzymes in the CBC model might be far from the real values in one plant species; as a result, it is hard to judge at this point where the enzyme activities from the WT or transgenic plants might resides on the response curve of $A$ to changes in enzyme activities. In addition to these possibilities, experimental results indicate that the impacts of modifying enzyme activity on photosynthesis are also dependent on the environmental conditions (Feng et al., 2007; Uematsu et al., 2012; Ding et al., 2016; López-Calçagno et al., 2020) or developmental stages(Lefebvre et al., 2005; López-Calçagno et al., 2020). Therefore, more studies using plants with greatly enhanced PGAK activity are still needed to test whether their activity might have a biphasic impact on photosynthetic CO$_2$ uptake rate. In previous studies, less than twice activity of that in WT were usually reported in transgenic plants with overexpressing the CBC enzymatic genes (Miyagawa et al., 2001; Lefebvre et al., 2005; Tamoi et al., 2006; Feng et al., 2007; Rosenthal et al., 2011; Uematsu et al., 2012; Gong et al., 2015; Khozaei et al., 2015; Simkin et al., 2015; Driever et al., 2017; Kohler et al., 2017; Simkin et al., 2017; López-Calçagno et al., 2020). Now it is possible to create lines with much higher increase or lower decrease in enzyme activity using synthetic promoters (Cai et al., 2020).

The CBC enzymes are highly regulated by multiple mechanisms in vivo. This study additionally shows that, besides enzymatic capacity, there are two feedback inhibitions playing important roles in gaining a high efficiency of the CBC at steady state, through maintaining the shared metabolite concentration and hence to balance the fluxes through different sub-cycles. They are the auto-negative regulations of PGAK by ADP and of FBPase by F6P (Figure 4a and b). The function of these two inhibitions predicted in the CBC needs to be tested in transgenic experiments. Negative autoregulation is a mechanism to decrease its
own production. Interestingly, at physiological levels, only F6P (near 20 metabolites in chloroplast have been tested) performs an effective and allosteric inhibition for FBPase, which produces a shift from hyperbolic to sigmoidal substrate saturation kinetics for plastic FBPase (Gardemann et al., 1986). This kind of regulation is theoretically demonstrated to be responsible for the homeostasis of metabolites (Hofmeyr and Cornish-Bowden, 2000). This may indirectly reflect the critical role of FBPase activity and its inhibition by F6P in coordinating fluxes among different sub-cycles within the CBC. As to coordinating ATP consumption by PRK and PGAK, the inhibitions of ADP and RuBP to PRK are important, because the affinity of PRK to ATP is greater than that of PGAK (Stitt, 1996). However, considering the greater affinity of PRK for ATP (In current model, the default value of Km of PRK and PGAK for ATP are 0.059 mM and 0.39 mM, respectively (2013)). Simulations show that it is the enzymatic activity of PGAK or ADP inhibition to PGAK more important than that of PRK to coordinate ATP consumption in the CBC (Figure 1 and 5). Interestingly, all the identified reactions or regulators which are responsible for the shared metabolites coordination are located in the sub-cycles of earlier steps in the CBC (Figure 1 and 5). This may be because, the later reaction rates are dependent on not only the shared metabolites concentration, but also the rates of the former steps to supply reactants. In other words, the structural location in the CBC rather than the enzymatic affinity might play a more important role in coordinating the consumption of shared metabolites in this cycle.

In summary, this study shows a new dimension of using a systems model to gain more insights into the operation of the CBC. The stability of the steady state in a metabolic autocatalytic cycle can be influenced by the kinetic properties, i.e., the Michaelis-Menten constant and catalytic number, of involved enzymes (Woodrow et al., 1985; Antonovsky et al., 2016; Barenholz et al., 2017). In this study, analysis for a simplified network shows that the kinetic properties of the enzymes in the sub-cycles, including both their enzyme activities and their regulatory properties, need to be well coordinated to achieve an efficient CBC at steady state.
**Figure legends**

**Figure 1.** The response curves of net photosynthetic CO$_2$ uptake rates versus the maximal activities of enzymes (Vmax) in the CBC. The x-axis represents the fold-change to the default Vmax value, and the y-axis represents the net photosynthetic rate (μmol m$^{-2}$ s$^{-1}$). The red dots represent the maximum net photosynthesis during perturbations. With the increasing activities of enzymes, photosynthetic CO$_2$ uptake rates show either a biphasic (blue) response or a monophasic (black) response.

**Figure 2.** The effects of the CBC enzyme capacity on the Rubisco-RuBP relationships. The RuBP/Km$_{RuBP}$ is the ratio of RuBP concentration to the apparent Km of Rubisco for RuBP. The V$_c$/Vcmax is the ratio of carboxylation rate to the maximum carboxylation rate if Rubisco. The x-axis is on a log$_{10}$ scale. The color bar means the fold change of the default Vmax of each enzyme. The color of dots in each subgraph represents the fold change (corresponding to the color bar) of enzyme activity. The dark blue means the fold change close to 0.01 and yellow means fold change close to 1000. When the color of dots changes from blue to yellow, the maximum enzyme activity of the enzyme in each subgraph increases. The gray curve in each subgraph describes the enzyme-substrate relationship of a standard Michaelis-Menten equation for a first-order reaction. Dashed vertical lines in each subgraph separate the subgraph into three regions. The left region, middle region and right region represent substrate limiting stage (stage I), substrate and enzyme co-limiting stage (stage II) and enzymatic capacity limiting stage (stage III), respectively (Fendt et al., 2010). When colors of dots change from blue to yellow, if the corresponding positions of these dots change from left to right, then with the increase of enzyme activity, the limitation over Rubisco carboxylation shifts from RuBP concentration limitation to Rubisco activity limitation. On the other hand, if the corresponding positions of these dots change from right to left, then with the increase of enzyme activity, the limitation shifts from Rubisco capacity limitation to RuBP concentration limitation. The red dot represents a state when photosynthetic CO$_2$ uptake rate is maximized. For GAPDH, SBPA, SBPase, TKb and PRK, the yellow dots overlap with red dot, which means that increasing their enzymatic capacities only slightly increases photosynthesis.
Figure 3. The enzymes showing biphasic responses (enzymes in blue) function as the switches coordinating hub reactants distribution among sub-cycles. The blue arrows represent the pathways consuming the shared metabolite. The font size of ATP (a and d) and T3P (b, c, e and f) represent the relative concentration of ATP and T3P, respectively. The thickness of lines represents the relative enzymatic capacity relative to the default value. (a, d), (b, e) and (c, f) represent the shared metabolites distributed by PGAK, FBPA or FBPase, and of TKα, respectively.

Figure 4. The feedback inhibition of ADP to PGAK (a and b) and F6P to FBPase (c and d) are key biochemical regulators for maintaining an efficient CBC. The red dots in a and c represent the maximum net photosynthesis. a and c show the effects of weaker inhibitions of ADP to PGAK and of F6P to FBPase on photosynthesis, respectively. b and d show the working model of the critical inhibitions of ADP and F6P in the CBC; red arrows in b and d represent the pathways consuming the shared metabolites; blue dashed lines and symbols in b and d represent the feedback inhibitions by the products.

Figure 5. The importance of metabolic coordination for an efficient metabolic cycle. (a) The simplified working model of the metabolic cycle with two sub-cycles. This auto-catalytic metabolic cycle assimilates \( M \) to produce \( \text{Product} \). B and C are the intermediates of this cycle, and their total concentration is constrained by the limiting resource \( S_t \). \( E_1 \) is the enzyme converting C to B, and \( E_2 \) catalyzes the reaction consuming B and C for downstream reactions in the cycle. \( K_1 \) and \( K_2 \) are the Michaelis-Menten constants of \( E_2 \) for B and C, respectively. \( x \) and \( (1-x) \) in red color represent the steady state concentrations of C and B, respectively. (b) The response curves of \( v \) versus the limiting resource \( C \). In the subplot b, \( v \) of the Y-axis has been normalized to the maximum \( v \) under the corresponding conditions, and it represents the relative flux of the auto-catalytic cycle. In the simulation: \( K_1 = 1 \), when \( K_1 > K_2 \), \( K_2 = 0.05 \) (red curve); when \( K_1 = K_2 \), \( K_2 = 1 \) (green curve); when \( K_1 < K_2 \), \( K_2 = 20 \) (purple curve). The dotted line indicates the \( x \) corresponding to the maximum \( v \). The gradient bars show that, with the increase of \( x \) from 0 to 1, the concentration (light green gradient bar) of C increases and concentration (dark green gradient bar) of B decreases.
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