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Reliable estimation of biochemical parameters from C_3 leaf photosynthesis–intercellular carbon dioxide response curves

LIANHONG GU[1], STEPHEN G. PALLARDY[2], KEVIN TU[3], BEVERLY E. LAW[4] & STAN D. WULLSCHLEGER[1]

[1]*Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA,* [2]*Department of Forestry, University of Missouri, Columbia, MO 65211, USA,* [3]*Department of Integrative Biology, University of California – Berkeley, Berkeley, CA 94720, USA,* [4]*College of Forestry, Oregon State University, Corvallis, OR 97331, USA*

ABSTRACT

The Farquhar–von Caemmerer–Berry (FvCB) model of photosynthesis is a change-point model and structurally overparameterized for interpreting the response of leaf net assimilation (A) to intercellular CO_2 concentration (Ci). The use of conventional fitting methods may lead not only to incorrect parameters but also several previously unrecognized consequences. For example, the relationships between key parameters may be fixed computationally and certain fits may be produced in which the estimated parameters result in contradictory identification of the limitation states of the data. Here we describe a new approach that is better suited to the FvCB model characteristics. It consists of four main steps: (1) enumeration of all possible distributions of limitation states; (2) fitting the FvCB model to each limitation state distribution by minimizing a distribution-wise cost function that has desirable properties for parameter estimation; (3) identification and correction of inadmissible fits; and (4) selection of the best fit from all possible limitation state distributions. The new approach implemented theoretical parameter resolvability with numerical procedures that maximally use the information content of the data. It was tested with model simulations, sampled A/Ci curves, and chlorophyll fluorescence measurements of different tree species. The new approach is accessible through the automated website leafweb.ornl.gov.

Key-words: A/Ci curve fitting; change-point model; leaf gas exchange measurements; leafweb; photosynthesis.

INTRODUCTION

The Farquhar–von Caemmerer–Berry (FvCB) biochemical model of photosynthesis (Farquhar, von Caemmerer & Berry 1980; Farquhar & von Caemmerer 1982; von Caemmerer 2000) is commonly used to model CO_2 assimilation at spatial scales from leaf to canopy to terrestrial biosphere and at atmospheric CO_2 concentration levels of the past, present, and future. Yet, the estimation of parameters in the FvCB model can be rather difficult and uncertain. Typically, its key parameters are inferred from analysis of leaf gas exchange measurements (A/Ci curves) and numerous A/Ci fitting methods have been developed. Depending on the methods used and their implementation, estimated parameter values can differ markedly for the same data set (e.g. Manter & Kerrigan 2004; Miao *et al*. 2009). It is often difficult to tell which fitting method, if any, is superior based on the measures of goodness of fit calculated from regression residuals. This difficulty is attributable to the fact that there are almost as many free parameters as points in a typical A/Ci curve, and hence a high fitting precision can be obtained even when the estimated parameter values have uncertain biochemical/physiological meaning (Sharkey *et al*. 2007).

This study was undertaken to identify theoretical and procedural difficulties in fitting A/Ci curves and to develop strategies to overcome them. The objective was to further improve the accuracy and utility of A/Ci data analysis in leaf photosynthesis research. The apparently straightforward FvCB model has a number of previously unrecognized complexities. For example, it belongs to a type of so-called change-point models in the statistics literature, which possess peculiar behaviours for parameter estimation (Hudson 1966; Khodadadi & Asgharian 2008) and is structurally overparameterized with respect to A/Ci data in the submodels of individual limitation states. Analysis of these complexities led to the development of a new estimation method that is tailored to the structural characteristics of the FvCB model and can, in theory, estimate up to eight parameters in the ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco)-, RuBP regeneration- and triose-phosphate utilization (TPU)-limited states from an adequately measured A/Ci curve. Simulations, measured A/Ci curves, and chlorophyll fluorescence measurements of multiple tree species were utilized to test the reliability and utility of the new method. Based on insights gained from these analyses, guidelines for informative A/Ci curve measurements were proposed.

To enable researchers to apply the new method, an interactive website has been set up. Users can upload their data

Correspondence: L. Gu. Fax: +1 804 982 2137; e-mail: lianhong-gu@ornl.gov

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and obtain an automated analysis of A/Ci curves through http://www.leafweb.ornl.gov.

The FvCB model with CO_2 internal transfer conductance

According to the conventional treatment of the FvCB model, the following equations describe photosynthesis and its limitations (Main symbols are defined in Appendix 1):

FOR $C_c > (1+3\alpha)\Gamma^*$,

$$A = \min\{W_c, W_j, W_p\}(1-\Gamma^*/C_c) - R_d \tag{1a}$$

For $C_c \le (1+3\alpha)\Gamma^*$,

$$A = \min\{W_c, W_j\}(1-\Gamma^*/C_c) - R_d \tag{1b}$$

$$W_c = \frac{V_{cmax}C_c}{C_c + K_{co}}, \tag{2}$$

$$W_j = \frac{JC_c}{4C_c + 8\Gamma^*}, \tag{3}$$

$$W_p = \frac{3T_P C_c}{C_c - (1+3\alpha)\Gamma^*}, \tag{4}$$

$$C_c = C_i - A/g_i. \tag{5}$$

K_{co}in Eqn 2 is a composite parameter:

$$K_{co} = K_c\left(1 + \frac{O}{K_o}\right). \tag{6}$$

In Eqn 3, the potential electron transport rate (J) is related to J_{max} through empirical relationships such as the following (Farquhar & Wong 1984):

$$J = \frac{\sigma I + J_{max} - \sqrt{(\sigma I + J_{max})^2 - 4\theta\sigma I J_{max}}}{2\theta}, \tag{7}$$

where I is the incident light level, σ is a composite parameter accounting for leaf absorptance, spectral quality of the available light as well as the splitting of the available light between photosystem I and II, and θ is a curvature parameter.

In the literature, the frequently used form alternative to Eqn 1 is $A = \min\{A_c, A_j, A_p\} - R_d$. That form deviates from the one in the original FvCB paper (Farquhar *et al.* 1980) and strictly speaking, is incorrect (G. D. Farquhar, personal communication). It chooses the wrong limitation state for $C_c < \Gamma^*$ and erroneously creates two transitions between the Rubisco- and RuBP regeneration-limited states in the A–C_c relationship. To be valid for all conditions, the choice of the limitation state should be based on the carboxylation rate (V_c), not the net assimilation rate.

For $C_c < (1+3\alpha)\Gamma^*$, the TPU-limited V_c (that is, W_p) is negative and therefore, if the TPU-limited state is still included in the comparison, it will always be selected. Additionally, there is a singularity for W_p at $C_c = (1+3\alpha)\Gamma^*$. Thus, the TPU-limited state is not considered for $C_c \le (1+3\alpha)\Gamma^*$.

The expression for W_p is taken from von Caemmerer (2000) after correcting a typographical error (the factor $3\alpha/2$ in the original expression should be 3α, von Caemmerer, personal communication). Frequently though, α is set to zero, leaving $W_p = 3T_p\, C_c/(C_c - \Gamma^*)$ and

$$A_p = 3T_p - R_d. \tag{8}$$

A_c and A_j can be expressed as a function of C_i (Ethier & Livingston 2004):

$$A = \frac{b - \sqrt{b^2 - 4c}}{2} \tag{9}$$

For A_c:

$$\begin{aligned} b &= V_{cmax} - R_d + (C_i + K_{co})g_i \\ c &= [(C_i - \Gamma^*)V_{cmax} - (C_i + K_{co})R_d]g_i \end{aligned} \tag{10}$$

For A_j:

$$\begin{aligned} b &= J/4 - R_d + (C_i + 2\Gamma^*)g_i \\ c &= [(C_i - \Gamma^*)J/4 - (C_i + 2\Gamma^*)R_d]g_i \end{aligned} \tag{11}$$

The solution for A_p as a function of C_i is more complicated. It can be shown that, in order to obtain the true solution, one should switch between the two roots of the quadratic equation resulting from substituting Eqn 5 into Eqn 4:

$$\begin{aligned} &A_p = \frac{b - \sqrt{b^2 - 4c}}{2}, \\ &\quad \text{if } C_i \ge (1+3\alpha)\Gamma^* + (3T_p - R_d)/g_i + 6\sqrt{T_p\alpha\Gamma^*/g_i} \\ &A_p = \frac{b + \sqrt{b^2 - 4c}}{2}, \\ &\quad \text{if } C_i \le (1+3\alpha)\Gamma^* + (3T_p - R_d)/g_v - 6\sqrt{T_p\alpha\Gamma^*/g_i}, \end{aligned} \tag{12}$$

subject to $C_c > (1+3\alpha)\Gamma^*$, where

$$\begin{aligned} b &= 3T_p - R_d + [C_i - (1+3\alpha)\Gamma^*]g_i \\ c &= \{[C_i - \Gamma^*]3T_p - [C_i - (1+3\alpha)\Gamma^*]R_d\}g_i \end{aligned} \tag{13}$$

Among the possible combinations of the quadratic roots, the solution Eqn 12 is the only formulation that can be correctly reduced to Eqn 4 in the case of an infinite g_i ($C_i = C_c$) and to $A_p = 3T_p - R_d$ in the case of $\alpha = 0$. The two inequalities in Eqn 12 differ in the sign of the last term and are subject to the condition $C_c > (1+3\alpha)\Gamma^*$. Mathematically, there is no real root for A_p (that is, the export limitation cannot occur) if C_i falls between the two thresholds. In the case of $\alpha = 0$, the second equation as well as the equivalence in the inequality of the first equation in Eqn 12 are disregarded as they violate the condition $C_c > (1+3\alpha)\Gamma^*$ for the TPU-limited state to be considered.

With the basic equations described, we can now move to discuss the structural features of the FvCB model from a parameter estimation standpoint.

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Characteristics of the FvCB model crucial for parameter estimation

The FvCB model is structurally overparameterized with respect to A/Ci curves in individual limitation states

If a model has more parameters than can be resolved from a given set of measurements, then the model is said to be overparameterized with respect to the given data set. Overparameterization can be caused by parameter redundancy in the model structure, an insufficient quantity of data, large error in the measurement, or a combination of these factors. For typical A/Ci curves, which are measured by controlling leaf temperatures and light levels, the FvCB model is structurally overparameterized in both straightforward and subtle ways.

The overparameterization in the expressions for K_{co} (Eqn 6) and J (Eqn 7) is straightforward. At best only the composite parameter K_{co} could be estimated because leaf temperatures are kept constant and the chloroplastic oxygen partial pressure O is assumed to equal the atmospheric oxygen partial pressure. The individual values of K_c and K_o could not be resolved as there is an infinite number of pairs of K_c and K_o to satisfy the same K_{co}. Similarly, σ and θ in Eqn 7 could not be estimated by fitting A/Ci curves because the light level I is constant. J_{max} could be estimated only if the values of parameters σ and θ are given *a priori*. However, J can be treated as a constant parameter to be estimated directly from A/Ci curves without *a priori* information of σ and θ (Sharkey *et al*. 2007).

Other occurrences of overparameterization in the FvCB model are subtle, but can be inferred through a mathematical analysis of the model structure. The analysis is presented in Appendix 2 and its results are summarized in Table 1. The Rubisco-limited state equation contains two state-specific parameters V_{cmax} and K_{co} and three common parameters g_i, R_d, and Γ^* that are shared across states. As explained in Appendix 2, this state is overparameterized. Thus suppose one has an A/Ci curve whose points are all limited by Rubisco and fit the Rubisco-limited submodel to this data set, one will find that no matter how many points this data set contains and how accurate the measurement is, only K_{co} and g_i can be uniquely resolved. Other parameters can only be resolved to the expressions of $(V_{cmax} - R_d)$ and $(\Gamma^* V_{cmax} + K_{co} R_d)$. At least one of the unknown parameters V_{cmax}, R_d, and Γ^* must be assigned an *a priori* value in order to resolve all five parameters. Furthermore as shown in Appendix 2, the best choice for assigning a value is Γ^*, because an error in the assigned value of Γ^* should only minimally affect the accuracy of the estimated V_{cmax}.

The TPU-limited state is also overparameterized. This state has two state-specific parameters T_p and α and three common parameters g_i, R_d and Γ^* when $\alpha \neq 0$, and one state-specific parameter T_p and one common parameter R_d when $\alpha = 0$. If the TPU-limited submodel is fit to a data set that contains only TPU-limited points, only g_i and the expressions of $(3T_p - R_d)$, $(1 + 3\alpha)\Gamma^*$, and $[3T_p - (1 + 3\alpha)R_d]\Gamma^*$ can be uniquely resolved when $\alpha \neq 0$. When $\alpha = 0$, only the expression of $(3T_p - R_d)$ can be determined.

When J is treated as a parameter to be estimated directly, the RuBP regeneration-limited state is not overparameterized. Consequently, if the RuBP regeneration-limited submodel is fit to a data set that contains RuBP regeneration-limited points only, the state-specific parameter J and the common parameters g_i, R_d and Γ^* can all be resolved uniquely.

The overparameterization of the FvCB model in the Rubisco- and TPU-limited states with respect to A/Ci curves has important implications for how the parameters should be estimated. Even if the limitation states of points in an A/Ci curve are known exactly, any method that fits the three states separately cannot succeed unless some parameters are given precise *a priori* values. A proper approach will pool information contained in the data of different limitation states so that the common parameters g_i, R_d and Γ^* can be constrained. Better constrained g_i, R_d and Γ^* will allow the state-specific parameters V_{cmax}, K_{co}, J, T_p and α to be uniquely resolved. In particular, it is advantageous to treat J, instead of J_{max}, as a parameter to be estimated because doing so, the RuBP regeneration-limited state is not overparameterized and can be used to constrain not only the common parameters but also state-specific parameters in the Rubisco and TPU limitations.

The FvCB model is a change-point model

A change-point model consists of a set of different submodels each of which applies to a non-overlapping subregion of the independent variable with unknown transitions (change points) (Hudson 1966; Khodadadi & Asgharian 2008). This type of model is sometimes presented under other names such as segmented, piecewise and multiphase (e.g. Hinkley 1971; Hawkins 1976; Lerman 1980). Parameters of a change-point model cannot be estimated in the same manner as those of a regular non-linear model. With the change points unknown, the model-wise cost function (also called objective function or penalty function, usually the sum of squared difference between the measured and calculated response variables) constructed for parameter estimation is not smooth and may possess many local minima some of which may not be stationary (Lerman 1980; Khodadadi & Asgharian 2008). Even when all submodels are linear in parameters and independent variables, the model-wise cost function can still have multiple minima. With non-linear submodels, the shape of the model-wise cost function can be extremely complex (e.g. Figs 1–3 in Lerman 1980 and Figs 2 & 4 in Hudson 1966). As a result, it is very difficult to obtain a global minimization of the model-wise cost function for a change-point model.

The type of change-point model that has been studied extensively by the statistics community has a unique submodel for each segment and a unique set of parameters for each submodel (that is, no common parameters across segments) and is continuous at the change points. Within this type of change-point model, the submodels are often

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Table 1. Theoretical resolvability of parameters and parameter expressions under different groups of limitation state distributions for an A/Ci curve

Limitation group	Resolvable parameters	Resolvable parameter expressions	Non-resolvable parameters	Non-involved parameters	Best parameter to be assigned a value
Rubisco, RuBP, TPU	V_{cmax}, K_{co}, J, T_p, α, g_i, Γ^*, R_d	NA	NA	NA	NA
Rubisco, RuBP	V_{cmax}, K_{co}, J, g_i, Γ^*, R_d	NA	NA	T_p, α	NA
Rubisco, TPU					
$\alpha = 0$	g_i, K_{co}	$V_{cmax} - R_d$, $3T_p - R_d$, $\Gamma^* V_{cmax} + K_{co} R_d$	V_{cmax}, T_p, Γ^*, R_d	J	Γ^*
$\alpha \neq 0$	g_i, K_{co}, (V_{cmax}, T_p, α, Γ^*, R_d)	NA	NA	J	(Γ^*)
RuBP, TPU	J, T_p, α, g_i, Γ^*, R_d	NA	NA	V_{cmax}, K_{co}	NA
Rubisco only	K_{co}, g_i	$V_{cmax} - R_d$, $\Gamma^* V_{cmax} + K_{co} R_d$	V_{cmax}, Γ^*, R_d	J, T_p, α	Γ^*
RuBP only	J, g_i, Γ^*, R_d	NA	NA	V_{cmax}, K_{co}, T_p, α	NA
TPU only					
$\alpha = 0$	NA	$3T_p - R_d$	T_p, R_d	V_{cmax}, K_{co}, J, g_i, Γ^*	R_d
$\alpha \neq 0$	g_i	$3T_p - R_d$, $(1 + 3\alpha)\ \Gamma^*$, $[3T_p - (1 + 3\alpha)R_d]\Gamma^*$	T_p, α, Γ^*, R_d	V_{cmax}, K_{co}, J	R_d

Resolvable parameters or parameter expressions are those that can be uniquely determined if an A/Ci curve contains an adequate number of data. Non-resolvable parameters are those that cannot be uniquely determined with the given data set but can be uniquely determined if another non-resolvable parameter is assigned an *a priori* value. A parameter expression, which is an algebraic function of resolvable or non-resolvable parameters, can be uniquely resolvable even if it contains non-resolvable parameters. Note that non-resolvable parameters are different from non-involved parameters, which are limitation state-specific parameters whose state does not occur in the given data set. For any given A/Ci data set, there are seven groups of possible limitation state distributions because any limitation could be missing from the data set: (1) (Rubisco, RuBP, TPU); (2) (Rubisco, RuBP); (3) (Rubisco, TPU); (4) (RuBP, TPU); (5) (Rubisco); (6) (RuBP); and (7) (TPU). Under the group (Rubisco, TPU), the parameters of V_{cmax}, T_p, α, Γ^*, and R_d are resolvable, but only weakly so because the re-parameterized system of independent equations is ill-conditioned; they are put in parenthesis to indicate this fact and we recommend Γ^* be assigned an *a priori* value under this situation. Note that what is considered here is only the overparameterization caused by the FvCB structural features and the resolvability really represents the best case. Overparameterization may also occur if the data set is inadequate. To deal with overparameterization caused by an inadequate data set, additional parameters may need to be fixed. In particular, K_{co} may need to be assigned a value in order to resolve V_{cmax} if the A/Ci curve is inadequately sampled.
NA, not applicable.

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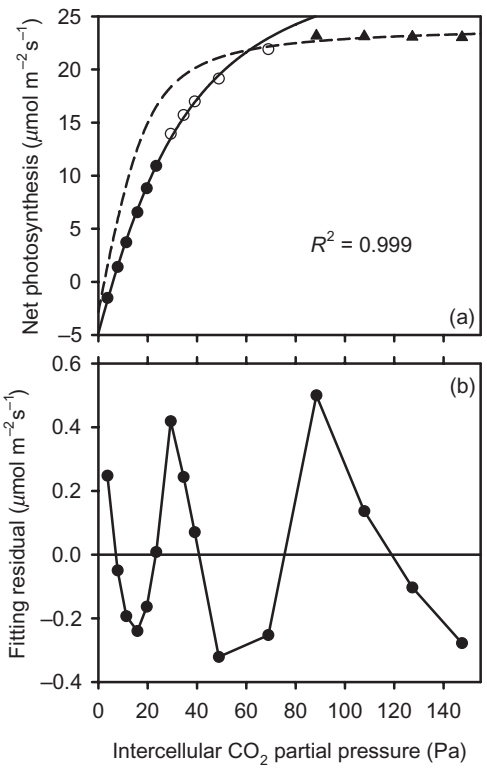

Figure 1. Apparent good agreement (a) hides a deficiency (b) in fitting a synthetic A/Ci curve. A Type II method is used in the fitting. The synthetic A/Ci curve is generated with the FvCB model and the parameters in Table 2. In (a), filled circles denote ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco)-limited points, unfilled circles RuBP regeneration-limited, filled triangles TPU-limited and the solid and dash lines represent, respectively, Rubisco- and RuBP regeneration-limited photosynthesis calculated with the optimized parameters. The fitting fails to detect the presence of TPU-limited points. In (b), a plot of residual photosynthesis against intercellular CO_2 partial pressure (C_i) shows fitting anomalies more clearly.

polynomials and their first derivatives are forced to be smooth at the change points as these restrictions lead to desirable properties for regression (Gallant & Fuller 1973; Zhan, Dean & Routledge 1996).

The FvCB model is a change-point model. However, it does not conform to the type of change-point model commonly studied by the statistics community. Firstly, it has both submodel-specific and common parameters. Secondly, in a typical change-point model, the behaviour of a submodel in a region it does not describe has no relevance for parameter estimation. In the FvCB model, the behaviour of a submodel is relevant even in a region it does not describe because its carboxylation rate must be larger than that of the submodel that describes the region (Eqn 1). Thirdly, forcing the FvCB model to be smooth at the change points so that fitting can be done relatively easily may lead to the loss of biological relevance of the estimated parameters. Fourthly, in a typical change-point model, the number of segments or sub-regions equals the number of submodels. However, in the FvCB model, the number of segments could be less than the number of submodels because any limitation state could be missing from the data set and, further, one does not know when this happens. Thus, parameter estimation for the FvCB model is even more complex than that for typical change-point models.

The FvCB model dictates the order of the three limitation states along the C_i *(*C_c*) axis*

It can be proven that the three biochemical limitation states in the FvCB model do not occur randomly along the C_i (C_c) axis (Appendix 3). They either do not exist in the same curve or exist together in the following order: the Rubisco-limited state occupies the lowest C_i (C_c) values, the RuBP regeneration-limited state the intermediate C_i (C_c) values, and the TPU-limited state the highest C_i (C_c) values. Furthermore, when the three limitation states exist together, the following conditions hold:

$$4V_{\mathrm{cmax}} > J > 12T_{\mathrm{p}} \tag{14}$$

The three characteristics of the FvCB model outlined above provide the basis for discussing the problems associated with extant methods of A/Ci curve analysis.

Problems with extant methods of A/Ci curve analysis

Detailed descriptions of extant methods of A/Ci curve analysis can be found in Ethier & Livingston (2004), Dubois *et al.* (2007), Miao *et al.* (2009), and Yin *et al.* (2009). These methods differ in some details but can be broadly divided into two types. Type I methods assign the domains of limitation states in advance so that the limitation state of each point is fixed throughout the parameter estimation process. Type II methods determine the domains of limitation states with parameter values at each iterative step of minimizing the *model-wise* cost function so that the limitation state of each point changes freely within the parameter estimation process. Most analyses of A/C_i curves have been conducted by applying Type I methods although Type II methods have been presented more recently. Both Type I and II methods have potential problems in fitting A/Ci curves. Some problems are specific to Type I, some to II and others to both.

Specific problems with Type I methods

Type I methods assume that the transition between the Rubisco- and RuBP regeneration-limited states ($C_{\mathrm{i_CJ}}$) occurs, for example, around 25 Pa intercellular CO_2 and the TPU-limited state is not present. The cost function is typically constructed in a manner analogous to the following:

$$f_{\mathrm{c}} = \frac{1}{2}\sum_{i=1}^{n_{\mathrm{c}}}(A_{\mathrm{ci}} - A_{\mathrm{cmi}})^2, \text{ for } C_{\mathrm{i}} < C_{\mathrm{i_CJ}}, \tag{15a}$$

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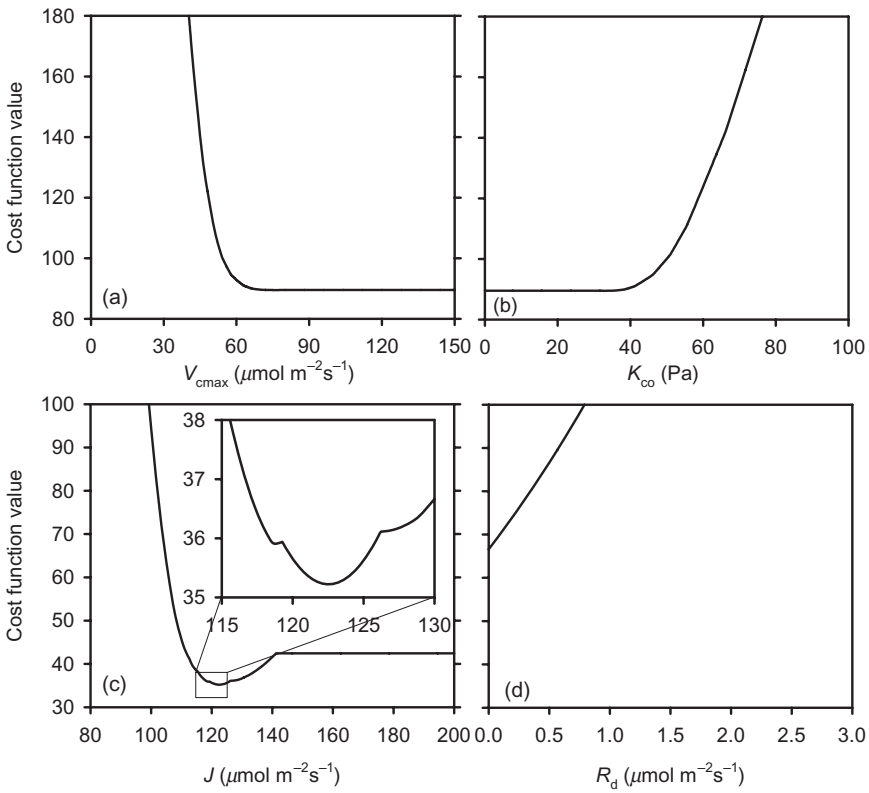

Figure 2. Undesirable shapes of the model-wise cost function of a Type II method for fitting the synthetic A/Ci curve in Fig. 1. The cost function exhibits: flat bottomed-behaviour without an identifiable minimum (a and b), erratic behaviour with multiple minima (see the inset in c), and monotonic descending behaviour without a defined minimum within the realistic parameter space (d). These plots were produced by slicing through the high dimensional space of the cost function (that is, changing only one parameter at a time).

$$f_j = \frac{1}{2}\sum_{i=1}^{n_j}(A_{ji} - A_{jmi})^2, \text{ for } C_i \geq C_{i_CJ}, \tag{15b}$$

where A_{ci} is the predicted Rubisco-limited net assimilation rate for the point i assigned to be Rubisco-limited, A_{cmi} is the measured value for this point, and n_c is the number of Rubisco-limited points; A_{ji}, A_{jmi} and n_j are the counterparts for the RuBP regeneration-limited state. Parameter estimation involves minimizing f_c and f_j separately although sometimes common parameters estimated by minimizing f_c may be used to reduce the number of parameters in the process of minimizing f_j. A potential exception is the approach of Sharkey *et al.* (2007) in which the method apparently minimizes a joint cost function.

One of the problems with Type I methods is that there is evidence indicating that both C_{i_CJ} and the transition between the RuBP regeneration- and TPU-limited states (C_{i_JP}) is not constant. Wullschleger (1993) suggested C_{i_CJ} could take a range of values between 20 and 25 Pa. Xu & Baldocchi (2003) used measurements with C_i less than 15 Pa for the Rubisco-limited state and those with C_i greater than 25 Pa for the RuBP regeneration-limited state. Sharkey *et al.* (2007) suggested that preliminary fitting is conducted first with the data between 20 and 30 Pa excluded in order to gain some understanding on the distribution of limitation states and further analysis could then proceed with the aid of this understanding. Ethier *et al.* (2006) used a value of 40 Pa for C_{i_CJ}. Manter & Kerrigan (2004) found that C_{i_CJ} values ranged from 25 to 152 Pa for 19 woody species and showed that the estimation of key photosynthetic parameters can be significantly affected if incorrect C_{i_CJ} values are used.

The practice of setting the limitation state transition in advance is equivalent to subjectively forcing J to be proportional to V_{cmax} and their ratio to be a constant. This consequence is not widely known (but see Dubois *et al.* 2007) and may have led to some circular arguments in A/Ci curve analyses. For example, a conserved J_{max}/V_{cmax} ratio, which is determined by the J/V_{cmax} ratio, has been interpreted as of biological origin and has been used to support a conserved C_{i_CJ} assumption. How the J/V_{cmax} ratio is related to C_{i_CJ} can be seen in a special case for which g_i is infinite and C_i equals C_c:

$$\frac{J}{V_{cmax}} = \frac{4C_{i_CJ} + 8\Gamma^*}{C_{i_CJ} + K_c(1 + O/K_o)} \tag{16}$$

Most analyses assume the kinetic properties of Rubisco are conserved across species and thus assign *a priori* values to Γ^*, K_c and K_o. Consequently, C_{i_CJ} is the only variable that determines J/V_{cmax} and therefore J_{max}/V_{cmax}. Once C_{i_CJ} is set in advance, J/V_{cmax} and thus J_{max}/V_{cmax} is fixed in advance, without input from data. Of course a particular A/Ci curve may not have a point right at C_{i_CJ} and thus the obtained ratio of J/V_{cmax} or J_{max}/V_{cmax} may vary somewhat, depending on the actual distribution of sampled C_i values in the data set. It is possible that there may be a truly conserved biological relationship between J (J_{max}) and V_{cmax} in nature. If so, the true relationship may be distorted or concealed by

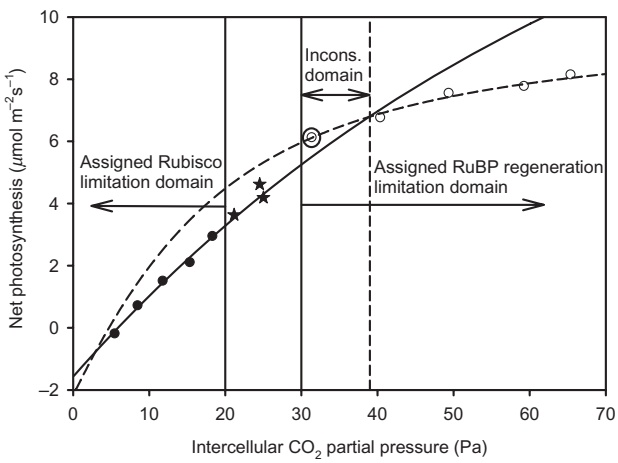

Figure 3. An example of inadmissible fit demonstrating a previously unreported anomaly in fitting A/Ci measurements for parameters in the Farquhar–von Caemmerer–Berry (FvCB) model. In this example, the curve was from a sugar maple (*Acer saccharum* Marsh.) leaf and the fitting approach of Sharkey *et al.* (2007) was used. Filled circles denote points assigned in advance to be ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco)-limited. Unfilled circles denote points assigned in advance to be RuBP regeneration-limited. Points represented by stars have intercellular partial pressures between 20 to 30 Pa (range marked by the two solid vertical lines) and thus are not used in the fitting, following the recommendation of Sharkey *et al.* (2007). The fitted lines for the Rubisco-limited and RuBP regeneration-limited photosynthesis are also shown. The dashed vertical line denotes the transition between the Rubisco- and RuBP regeneration-limited state calculated with the optimized parameters. Note that in the region labeled with 'Incons. domain', the limitation state calculated with the FvCB model and the optimized parameters does not agree with the limitation state assigned in advance for the fitting. The point in this inconsistent domain is circled.

fixing the limitation state transition for parameter estimation at some value of C_i.

Several authors have attempted to objectively determine C_{i_CJ}. Manter & Kerrigan (2004) tried four C_{i_CJ} values (30, 40, 50 and 60 Pa) sequentially and set the transition at the value that had the smallest regression mean square statistic. The approach of Manter & Kerrigan (2004) is an improvement over the conventional, fixed C_{i_CJ} approach in that it tried to bring some objectivity into the determination of C_{i_CJ}. However, if as shown in the literature, the true C_{i_CJ} value can be as low as less than 20 Pa and as high as over 150 Pa, testing a limited number of C_{i_CJ} values is not a complete solution to proper assignment of the limitation state transition point. Ethier *et al.* (2006) set C_{i_CJ} to the value at which convergence was achieved between the two g_i values estimated independently from each of the two sides of an A/Ci curve. A potential drawback of the approach is because of the fact that the Rubisco-limited photosynthesis region is more sensitive to C_i and C_c and therefore g_i than the RuBP regeneration-limited region. Consequently, the values of g_i estimated from the two sides might not converge at all or converge for the wrong reason. Further, there is no obvious reason why convergence on the values of g_i is a better criterion for setting the transition than convergence on the values of other parameters such as day respiration R_d, but the results based on R_d and g_i may not agree with each other.

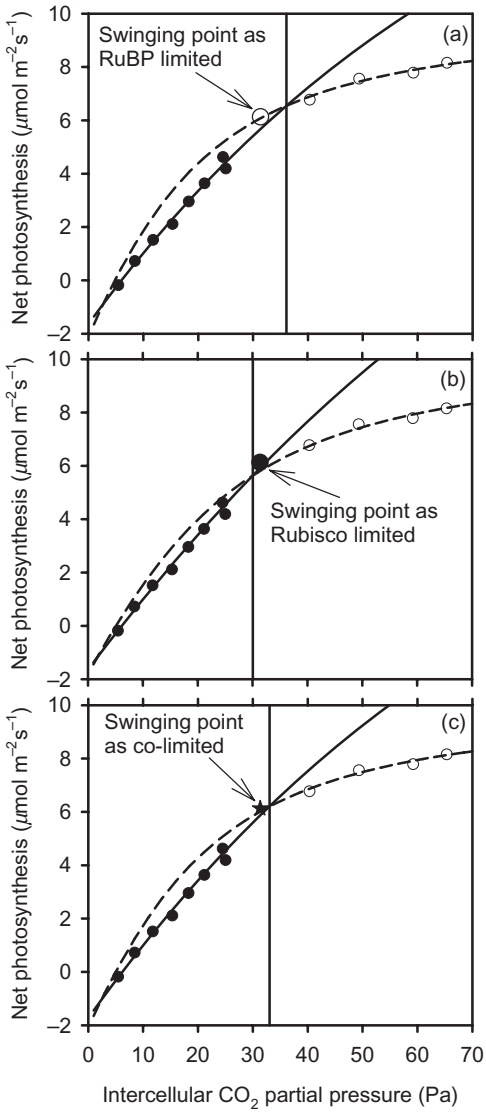

Figure 4. An example demonstrating the presence of a 'swinging' point and the effect of treating it as a co-limited point. This is the same leaf as in Fig. 3 but the new approach is used for the fitting. Filled circles, unfilled circles, and star denote ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco)-limited, RuBP regeneration-limited and co-limited points, respectively. Fitted Rubisco- (solid) and RuBP regeneration-(dashed) limited lines are also shown. The vertical lines denote the transition between the Rubisco-limited and RuBP regeneration-limited states calculated with the optimized parameters. The 'swinging' point is indicated by an arrow and is the same point in each plot. In (a), the 'swinging' point is assigned in advance to be limited by RuBP regeneration for the fitting, but calculation with the optimized parameters indicates it should be limited by Rubisco. In (b), the situation is reversed. In (c), the 'swinging' point is designated as co-limited, moving the calculated state transition to be between the ones calculated in (a) and (b).

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These is another issue with Type I methods that fit each limitation state separately. The structural overparameterization in the FvCB model affects these methods directly. Unless precise *a priori* values of some parameters are given (Table 1), it is difficult for them to succeed in the estimation of key parameters. In addition, fitting each limitation state separately amounts to division of information. Division of information weakens the constraining power of the A/Ci data set for both common and state-specific parameters, making parameter estimation prone to the influence of measurement noise.

Specific problems with Type II methods

Type II methods use the model-wise cost function, usually constructed in the following manner (Dubois *et al*. 2007; Miao *et al*. 2009, and Yin *et al*. 2009):

$$f = \frac{1}{2}\sum_{i=1}^{n}(A_i - A_{mi})^2, \tag{17}$$

where A_i is the net assimilation rate at the C_i value of the *i*th point calculated directly with the full FvCB model, that is, Eqn 1; A_{mi} is the measured net assimilation rate of this point; *n* is the total number of points in the data set. Type II methods take advantage of the fact that parameter estimation of non-linear models starts from an initial set of guessed parameter values and iteratively improves this set until the cost function is minimized. Therefore at each iterative step, parameter values are always given and can be used to calculate the limitation state and *A* at each sampled C_i value. The limitation state at a C_i can be determined in two ways (Dubois *et al*. 2007). One way is to calculate the carboxylation rate under each of the three limitation states and then select the one with the smallest carboxylation rate. The second is to calculate the transition points C_{i_CJ} and C_{i_JP} first and then determine the limitation state domains based on the transition points. A benefit of these limitation state 'auto-identifying' strategies is that there is no need to specify and fix the limitation domains of the A/Ci curve in advance.

Problems with Type II methods are best demonstrated with an example. We generated a synthetic A/Ci curve (Fig. 1a) with the full FvCB model and the parameters in Table 2. No noise was introduced into the synthetic data. All three limitation states were represented in the synthetic curve. We then fit the full FvCB model to it with the model-wise cost function in an attempt to retrieve the parameters used. To improve the odds of correctly retrieving the parameters used, the initial guess was selected by conducting a dense grid search with nearly half a million points, following the recommendation of Dubois *et al*. (2007) and Miao *et al*. (2009). During the iteration, carboxylation rates are calculated and used to determine limitation states of individual points. At first glance (Fig. 1a), the fitting appeared to be good with $R^2 = 0.999$ (actually a pseudo R^2 since strictly speaking, it only applies to linear models). But the residual plot (Fig. 1b) showed fluctuating departures of the fitting from 'measurements'. Also, only the presence of Rubisco- and RuBP regeneration-limited states in the data set was identified; the presence of TPU-limited points was not detected at all. Comparing the original and retrieved parameters, no parameter was retrieved correctly except for g_i (Table 2); V_{cmax} was underestimated by more than 20%, K_{co} underestimated by nearly 60%, *J* overestimated by more than 10%, R_d overestimated by over 800% and Γ^* underestimated by nearly 80%. These large errors in estimated parameters indicate how problematic an apparently good fit (i.e. a high pseudo R^2) can be for A/Ci analysis.

Table 2. Values of parameters used to generate the synthetic A/Ci curve of Fig. 1a and values retrieved with different fitting methods

Parameters/RMS	True value	Type I method	Type II method	New approach
V_{cmax} (μmol m^{-2} s^{-1})	55	84.10	42.89	55.00
K_{co} (Pa)	35	106.22	14.75	35.00
J (μmol m^{-2} s^{-1})	120	95.84	136.64	120.00
T_p (μmol m^{-2} s^{-1})	8	N/A	N/A	8.00
α	0.05	N/A	N/A	0.05
g_i (μmol m^{-2} s^{-1} Pa^{-1})	1.5	998612.14(C) 0.54(J)	1.50	1.50
R_d (μmol m^{-2} s^{-1})	1.1	3.75 (C) 0.00 (J)	10.00	1.10
Γ^* (Pa)	5	0.8791	1.10	5.00
RMS (μmol m^{-2} s^{-1})		0.01 (C) 0.28 (J)	0.25	0.00

The Type I method fits the Rubisco- and RuBP regeneration- limited regions separately with the transition at 25 Pa intercellular CO_2. This method results in two values for g_i and R_d (C–Rubisco-limited, J–RuBP regeneration-limited). Similarly, the root mean square (RMS) error has two values. Also for the Type I method, the value of Γ^* is derived from the fitting in the RuBP regeneration-limited region because for fitting in the Rubisco region, Γ^* must be fixed because of the presence of overparameterization (see the text). The Type II method uses the smallest carboxylation rate to determine the limitation state of each point during the optimization.

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The reason for this behaviour is that Type II methods do not account for an important fact about the FvCB model: it is a change-point model, not a regular smooth non-linear model. With the use of the model-wise cost function, Type II methods make no distinction between these two fundamentally different models for parameter estimation. Successful minimization requires the cost function to be bowl-shaped and smooth with only one minimum within the realistic ranges of the parameters. As a change-point model, the model-wise cost function Eqn 17 does not have these necessary properties (Fig. 2). Slicing through the space of the cost function along different planes, which is done by changing only one parameter at a time and fixing all others, we see that the cost function simply has no minimum for V_{cmax} (Fig. 2a), K_{co} (Fig. 2b) and R_d (Fig. 2d) and has a substantial flat region for J (Fig. 2c). Figure 2c also shows that the cost function is not smooth and contains multiple minima, which invalidates the use of any gradient-based optimization approach. Direct grid search does not work either: no matter how densely one searches the space of the cost function, no single minimum could be found because a single minimum simply does not exist.

Problematic shapes of the model-wise cost function are not unique to the particular example used here. They are inherent in Type II methods. This can be understood from the following basic argument: Any time an If-Then condition is used to calculate a mathematical function, a discontinuity is introduced to the function. Irrespective of how the limitation states of different points are determined by Type II methods, If–then conditions always have to be used. As a parameter changes, some points may jump from one limitation state to another, causing abrupt changes in the value of the cost function. Also, a parameter affects the calculated photosynthesis and therefore the cost function only when its state is limiting. If its state is not limiting, a change in this particular parameter has no influence on the cost function and the cost function flattens out with respect to it (Fig. 2a–c). These problems affect all limitation state-specific parameters (V_{cmax}, K_{co}, J, T_p and α). They also cascade to the common parameters (R_d, g_i and Γ^*), causing them to be monotonic (e.g. Fig. 2d).

For the synthetic A/Ci example discussed above, the fitting process underestimated the number of limitation states in the data set. The opposite can happen too. If a limitation state is actually missing from the A/Ci curve, Type II methods may still give an optimally estimated set of parameters in that limitation state. Additionally, we have found that Type II methods may produce optimal fits in which the number of estimated specific parameters of a limitation state is equal to or more than the number of data of that state in the curve. Such fits lack statistical validity.

Problems common to both Type I and II methods: inadmissible fits

We define 'inadmissible fits' as cases where the 'optimized' parameters lead to an inconsistent or incorrect identification of the limitation states of the points in the A/Ci curve data set. The phrase is borrowed from the literature of change-point models (Lerman 1980). Inadmissible fits can be produced by both Type I and II methods.

Figure 3 demonstrates one example of an inadmissible fit that may occur with Type I methods. The example is an actual A/Ci curve of a leaf of sugar maple (*Acer saccharum* Marsh.). The A/Ci data sets and study site are described in the Section 'Testing the new approach with actual measurements'). In this example, the fitting follows the method of Sharkey *et al*. (2007) and uses the web-based tool http://www.blackwellpublishing.com/plantsci/pcecalculation. Points with C_i less than 20 Pa are designated as limited by Rubisco and those with C_i above 30 Pa by RuBP regeneration. Points between 20 to 30 Pa are not used in the fitting. Figure 3 displays the fitting results. Note that in Fig. 3, the circled point has a C_i value above 30 Pa and is thus designated to be in the domain of the RuBP regeneration-limited state. However, the carboxylation rate at this point calculated with the optimized parameters under the RuBP regeneration limitation is larger than under the Rubisco limitation and according to the formulation of the FvCB model this point should have been in the Rubisco limitation state. Thus, there is a contradiction between the limitation states designated in advance and the limitation states calculated with the optimized parameters based on the designated limitation states. In fact, there is a range of C_i (or C_c) values within which the fitting outcome is inconsistent with the formulation of the FvCB model (the range marked with 'Incons. Domain' in Fig. 3). This type of inconsistency may happen because the FvCB model produces three segments of an A/Ci curve and the change from one segment to the next is not smooth in terms of the derivatives of A with respect to C_i. It is not unique to the method of Sharkey *et al*. (2007). All Type I methods can exhibit it as they assign the domains of limitation states in advance and have no mechanism to ensure that the obtained parameters can actually reproduce the assigned domains of limitation states with the full FvCB model.

Two scenarios of inadmissible fits can be produced with Type II methods, depending on how the limitation states of different points are determined during the iterative process. If the limitation states are determined with transition points C_{i_CJ} and C_{i_JP}, then the carboxylation rate of a limitation state calculated with the optimized parameters outside the domain of this limitation state may be smaller than that of the limitation state that is supposed to be limiting. For example, in a region that has $C_i > C_{i_CJ}$ and is supposed to be limited by RuBP regeneration, the carboxylation rate of Rubisco limitation may be the smaller. This scenario is similar to the one discussed above for Type I methods. If the limitation states are determined with carboxylation rates, then Type II methods can produce inadmissible fits in which the positions of limitation states along the C_i axis are out of order. This can occur because the fitting process allows the three limitation states to appear in any order along the C_i axis. For example, we have found that optimal fits can sometimes be produced in which the RuBP regeneration-limited

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state occurs at C_i values smaller than those in the Rubisco-limited state.

A new fitting approach

Based on the examination of the FvCB model structure and problems associated with extant methods, we propose further development of A/Ci curve analysis utilizing a new approach. The new approach is better suited to the characteristics of the FvCB model and overcomes some major problems of extant methods. It consists of the four main steps:

1. Enumeration of all possible distributions of limitation states allowed by a given A/Ci data set.
2. Fitting the FvCB model to each limitation state distribution.
3. Identification and correction of inadmissible fits.
4. Selection of the best fit from all possible limitation state distributions.

These steps are described as follows.

Enumeration of all possible distributions of limitation states

The possible distributions of limitation states of a given A/Ci data set are enumerated according to the following rules:

1. Any of the three limitation states may or may not be present in the given A/Ci data set.
2. If a distribution contains multiple limitation states, their positions along the C_i axis follow the order dictated by the FvCB model.
3. The minimum number of points in a particular limitation state is one more than the number of specific parameters of that limitation state to be estimated.

As a demonstration, Appendix 4 lists all possible limitation state distributions for an A/Ci data set that contains 10 points.

The limitation state distributions are divided into seven distribution groups based on the presence or absence of the three limitation states: (Rubisco, RuBP, TPU), (Rubisco, RuBP), (Rubisco, TPU), (RuBP, TPU), (Rubisco), (RuBP) and (TPU). Each of the last three groups contains only a single distribution. Each of the first four groups contains many distributions that represent all possible combinations of points in the limitation states of the group. The set of parameters to be estimated differs among the groups. However, different distributions within the same group share the same set of parameters to be estimated. Parameters estimable in theory for each group are listed in Table 1.The position of a limitation state inside the parentheses (Rubisco, RuBP, TPU) (Rubisco, RuBP) (Rubisco, TPU) and (RuBP, TPU) reflects the order along the C_i axis dictated by the FvCB model. The number of parameters estimable must be less than the number of data. Thus the Rubisco-limited state either does not occur at all or occurs with at least three points if both V_{cmax} and K_{co} are to be estimated and with at least two points if only V_{cmax} is to be estimated. The RuBP regeneration-limited state either does not occur at all or occurs with at least two points. The TPU-limited state either does not occur at all or occurs with at least two points. When there are only two TPU export-limited points, α is always fixed at zero.

Fitting the FvCB model to each limitation state distribution

The new approach fits the FvCB model to each limitation state distribution individually. For a limitation state distribution in the group (Rubisco, RuBP, TPU), the corresponding set of parameters is estimated by minimizing the following cost function:

$$f = \frac{1}{2}\left[\sum_{i=1}^{n_c}(A_{ci} - A_{cmi})^2 + \sum_{i=1}^{n_j}(A_{ji} - A_{jmi})^2 + \sum_{i=1}^{n_p}(A_{pi} - A_{pmi})^2\right], \tag{18}$$

where A_{ci} is the calculated Rubisco-limited net assimilation rate for the Rubisco-limited point i, A_{cmi} is the measured value for this point, and n_c is the number of Rubisco-limited points; A_{ji}, A_{jmi} and n_j are the counterparts for the RuBP regeneration-limited state; A_{pi}, A_{pmi} and n_p are for the export-limited state. For other distribution groups, the cost function is formulated accordingly.

When a limitation state distribution contains multiple limitation states, the cost function Eqn 18 represents the joint sum of the sums of squares for individual limitation states. There is a fundamental difference between Eqn 18 and the model-wise cost function Eqn 17. In the evaluation of Eqn 18, the transition points C_{i_CJ} and C_{i_JP} are never calculated and the carboxylation rates under different limitation states are never compared, that is, the min{} in the FvCB model is never used. The net assimilation rate at a point is calculated with the submodel of the limitation state to which the point is assigned. In contrast, the evaluation of the model-wise cost function Eqn 17 involves the use of either C_{i_CJ} and C_{i_JP} or the carboxylation rates under different limitation states to determine the limitation states of data. To differentiate the cost function Eqn 18 from the model-wise cost function Eqn 17, we call Eqn 18 the distribution-wise cost function.

The cost function is minimized with an algorithm that is a hybrid of gradient and non-gradient approaches. The gradient approach is essentially that provided in the ODRPACK FORTRAN subroutine package from http://www.netlib.org. The non-gradient approach is a combination of the Powell and Simplex approaches with subroutines either developed by the authors or adapted from Press *et al.* (1992). The gradient and non-gradient approaches are combined in series with the output from the gradient approach being fed into the non-gradient approach. Because the FvCB model is not continuous in the derivatives of A with respect to C_i at limitation state transitions, this hybrid strategy proved more effective than either the gradient or

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non-gradient approach alone. Sometimes it helps if the optimized parameters are used again as an initial guess until a final convergence between the initial guess and the optimized parameters is achieved. This treatment is particularly useful when the cost function changes very gradually around the minimum or when the landscape of the cost function is complicated, which could occur when no or too few RuBP regeneration-limited points are in the data set.

Identification and correction of inadmissible fits

After the distribution-wise cost function for a limitation state distribution is minimized, an admissibility check is conducted if the distribution belongs to one of the four distribution groups that contain multiple limitation states. This is a step to ensure the consistency of the obtained parameters with the formulation of the FvCB model. To conduct the admissibility check, the obtained parameters and the full FvCB model are used to calculate the limitation state of each point in the A/Ci data set, which involves the comparison of carboxylation rates of different limitation states. If the calculated limitation state distribution agrees with the assigned limitation state distribution, then the fit is admissible; if not, it is inadmissible.

If the fit is inadmissible, there are several additional procedures to go through in order to obtain a new fit that is admissible. The first procedure is to fit the calculated limitation state distribution to estimate a new set of parameter values. The new set of estimated parameter values is then used to recalculate the limitation state distribution. If the recalculated limitation state distribution agrees with the assigned limitation state distribution, that means there are some points that alternate between two limitation states. These data are termed 'swinging' points. Figure 4a and b shows an example. When the swinging point is assigned to be in the RuBP regeneration-limited state, subsequent calculation with the optimized parameters puts it in the Rubisco-limited state. But when it is assigned to be in the Rubisco-limited state, calculation puts it back in the RuBP regeneration-limited state. Swinging points are located around the C_i transitions between limitation states. If there is only one swinging point around a transition, we treat the swinging point as co-limited and the assigned limitation state distribution is adjusted to allow this point to be indexed as co-limited. The distribution-wise cost function is reformulated such that the co-limited point is counted in two states to calculate the square sums. The adjusted assigned limitation state distribution is then refit. See Fig. 4c for an example with a co-limited point.

If the recalculated limitation state distribution does not agree with the assigned limitation state distribution or if there is more than one swinging point around a limitation state transition, we force the fit of the assigned limitation state distribution to be admissible. This is achieved by adding an additional penalty term to the distribution-wise cost function. The additional penalty term penalizes the parameter values that render the calculated limitation state distribution to be different from the assigned limitation state distribution. Its only role is to make sure an inadmissible set of parameter values is not selected by the iterative optimization process. It could be either a fixed large value (say 10^5) or made proportional to the number of points that have different assigned and calculated limitation states. We found that the latter treatment is more effective.

Selection of the best fit from all possible limitation state distributions

To select the best fit from all possible limitation state distributions, we take into consideration the fact that different groups of limitation state distributions have different numbers of parameters and thus different degrees of freedom (Table 1). Within the same group, the same set of parameters is estimated and therefore the best fit is the one with the smallest value of the minimized distribution-wise cost function. However, when we compare across different groups of limitation state distributions, two fits are considered equally effective if the difference between their values of the minimized distribution-wise cost function is less than the machine precision of the computer used. Machine precision is the smallest number ε such that the computer can distinguish between 1 and $1 + \varepsilon$. For a 32 bit computer, ε is about 10^{-16} for double precision numbers. If two fits are equally effective, the one with fewer parameters should be selected based on the principle of parsimony. Thus, among the seven distribution groups, the order in decreasing preference for selection in case of equal fitting performance is (RuBP), (Rubisco), (TPU), (Rubisco, RuBP), (RuBP, TPU), (Rubisco, TPU), (Rubisco, RuBP, TPU).

Strengths and weaknesses of the new approach

The new approach eliminates problems that we have identified with extant A/Ci curve fitting methods. A tenet of the new approach is that FvCB parameters can be estimated reliably only if the limitation state distribution of points in the A/Ci data set is estimated reliably. This tenet is implemented by using two distinct, nested optimizations: the limitation state distribution optimization (LSDO) and the parameter optimization (PO). Because LSDO is achieved via exhaustively enumerating all possible distributions of limitation states for a given A/Ci data set, the real limitation state distribution cannot be missed, at least in theory. Thus LSDO is able to optimally estimate whether a given A/Ci data set contains a single limitation state or a combination of different limitation states and, if more than one limitation state exists, how these states are distributed among the points. The application of LSDO eliminates the need to assume that a given A/Ci data set contains only Rubisco- and RuBP regeneration- limited points and that the transition between states occurs at some subjectively assigned static point. Thus it does not artificially determine which parameters are estimable and does not cause relationships between key parameters to be fixed, a problem suffered by Type I methods.

Note that LSDO in our new approach does not require extra information because a limitation state distribution is

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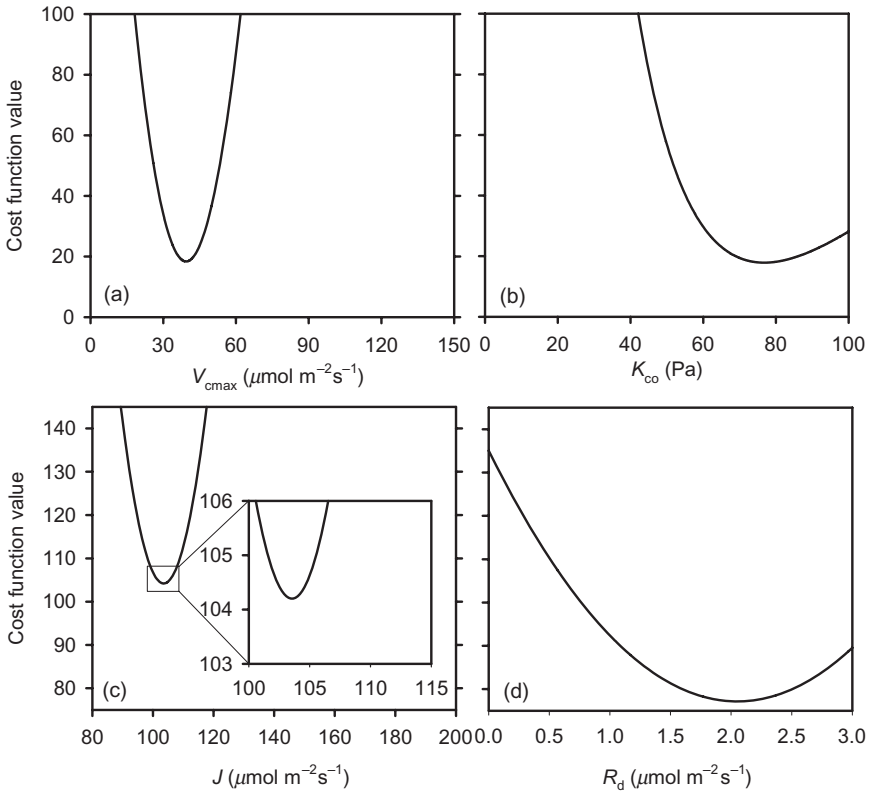

Figure 5. Desirable properties of the distribution-wise cost function of the new approach for parameter estimation. It is bowl-shaped and smooth, in contrast to the model-wise cost function of Type II methods (cf. Fig. 2). These plots are produced with the synthetic A/Ci curve of Fig. 1a in the same way as in Fig. 2. The new approach's bowl-shaped cost function with a clearly defined minimum explains why it is able to accurately retrieve the parameters of the synthetic curve.

completely determined by a set of FvCB parameters. Hence the information needed for LSDO is already contained in the data set. Extant methods conduct PO but not LSDO and thus do not use the information in the data set to the fullest extent.

The new approach takes into consideration the structural overparameterization in the FvCB model. The formulation of the distribution-wise cost function Eqn18 pools information contained in points of different limitation states to better constrain unknown parameters. This is an advantage over the separate formulation of the cost function Eqn 15 under Type I methods and represents an effective solution to the structural overparameterization in the FvCB model. Also, because for each enumerated limitation state distribution, the limitation type of each point is known, the new approach can always match the right model with the limitation state distribution while a mismatch between model and data can happen for Type II methods. Furthermore with the new approach, the theoretical guidance on parameter resolvability summarized in Table 1, the principle that the number of estimable parameters must be less than the number of data, and the principle of parsimony can all be applied dynamically within the optimization process to determine which parameters should be estimated and which parameters should be fixed, another advantage over both Type I and II methods.

The distribution-wise cost function of the new approach has desirable shapes and properties for parameter estimation. This is demonstrated with the A/Ci curve of Fig. 1a. The corresponding distribution-wise cost function is shown in Fig. 5. Compared with the model-wise cost function of Type II methods (Fig. 2), the distribution-wise cost function is bowl-shaped and smooth, an important property for reliable parameter estimation. The good behaviour of the distribution-wise cost function is attributable to the fact that the limitation states of all data are not changeable during its calculation, which ensures the value of the cost function varies smoothly and non-monotonically with the parameters.

To our knowledge, the new approach is the first to recognize and develop a solution to solve the problem of inadmissible fits in A/Ci curve analysis. The elimination of inadmissible fits ensures that values of estimated parameters are consistent with the formulation of the FvCB model and are biologically meaningful.

The ability to identify and incorporate co-limited points is also a strength of the new approach. Co-limited points are located in the important curvature region of an A/Ci curve and are close to the transition between limitation states (Fig. 4). A point right at the transition is by definition a co-limited point and belongs to two limitation states simultaneously. Information about state transitions is a powerful constraint for fitting an A/Ci curve. From a practical standpoint, a leaf surface may be heterogeneous in underlying photosynthetic machinery or in stomatal conductance. Thus different parts of the leaf may not be in the same limitation state (Cheeseman 1991; Chen, Zhu & Long 2008). Data near the transition in A/Ci measurements are more likely than other data to reflect such heterogeneity (Sharkey *et al.* 2007). Being able to identify such data and treating them

properly as having double limitations should make the parameter estimation more robust.

As compared with previous methods the new approach is computationally much more expensive. It requires many regression operations for a single curve fitting (Appendix 4) and the number of required regressions grows rapidly as the number of points in A/Ci data set increases. However, with modern computer speeds, the new method is entirely feasible.

Some implementation issues of the new approach

In theory our new approach can estimate up to eight parameters, including five state-specific parameters (V_{cmax}, K_{co}, J, T_p and α) and three common parameters (g_i, R_d and Γ^*). But as in any parameter estimation, the number and type of parameters that can be estimated depend on not only the fitting method but also the quality and quantity of the data. A parameter estimation method can only be as good as the data set provided. For an actual A/Ci curve, which unavoidably contains measurement errors, there is no guarantee that all eight parameters can be estimated even when all three limitation states are present in the data set. Thus, it is always a good idea to check if the data are adequate for the set of parameters estimated. There are at least two ways to accomplish this check. In one approach, the first and second partial derivatives of the cost function with respect to estimated parameters may be computed. If the data set is adequate for the set of parameters estimated, then the first partial derivatives should be approximately zero while the second partial derivatives should be non-zero. A non-zero first partial derivative indicates the parameter is not really optimized whereas a near-zero second derivative indicates that around the final value of the parameter the cost function is flat (Fig. 2a–c). A second approach is to use those optimization schemes that allow specification of parameter bounds. If the set of parameters is not adequately constrained by the data, some parameters may end up at their bounds. We refer to Tarantola (2005) for the logic behind these ideas.

If it is found that the data set is not adequate for the set of parameters estimated, a reduced set of parameters must be used. Note that even if a parameter has a non-zero first partial derivative or a near-zero second partial derivative, it doesn't necessarily mean that that parameter should be dropped from optimization and be assigned a value; lack of constraint on one parameter could be caused by the lack of constraint on another parameter. In the practical implementation of the new approach in leafweb.ornl.gov, A/Ci curve fitting is done with K_{co}, α and Γ^* either fixed or estimated. A good fitting agreement coupled with biological reasonableness of estimated parameter values is a basic criterion to determine whether estimating K_{co}, α and Γ^* is warranted.

Testing the new approach with simulations

The new approach accurately retrieved the parameters used in the synthetic A/Ci curve of Fig. 1a (Table 2). We generated 100 additional synthetic A/Ci curves so that the new approach can be tested for a wide range of parameter values and be compared with extant methods. It may be desirable to introduce artificial errors into the synthetic curves because real measurements always contain noise. However, it would be difficult to attribute without ambiguity the cause of failure in parameter estimation if error-containing synthetic A/Ci curves are used. The failure could be attributable to improperly introduced errors or to an inadequate fitting method. Further, how measurement noise affects parameter estimation critically depends on the number of points the data set contains and the distribution of these points along the C_i axis. A treatment of this dependency, which would be required if error-containing synthetic data are used, is beyond the scope of this present study. Consequently we decided to use error-free synthetic A/Ci curves with the understanding that success in fitting these curves only establishes a theoretical feasibility in the estimation of the eight parameters in the FvCB model.

The values of the parameters for the 100 synthetic A/Ci curves were generated from a random number generator. Each curve contained 15 points. V_{cmax} was from 5 to 150 μmol m^{-2} s^{-1}, J_{max} from 5 to 250 μmol m^{-2} s^{-1}, T_p from 0.5 to 15.5 μmol m^{-2} s^{-1}, g_i from 0.1 to 30.1 μmol m^{-2} s^{-1}Pa^{-1}, Γ^* from 0 to 5 Pa, K_{co} from 1 to 151 Pa, α from 0 to 1, and R_d from 0.01 to 5.01 μmol m^{-2} s^{-1}. No correlations were introduced to these parameters. The 15 C_i values in Pa were randomly drawn from the following ranges: 0–4; 4–9; 9–14; 14–19; 19–23; 23–27; 27–32.5; 32.5–39.5; 39.5–48.5; 48.5–59.5; 59.5–72.5; 72.5–87.5; 87.5–104.5; 104.5–123.5; and 123.5–144.5. It was required that the limitation states must occur with at least 3 Rubisco-, 2 RuBP regeneration- and three TPU-limited points in the sequence of (Rubisco, RuBP regeneration, TPU) along the C_i axis.

To test the new approach against the 100 additional synthetic curves, the initial guess was set arbitrarily. For comparison, we also conducted parameter estimation by minimizing the model-wise cost function as in a previous Type II method. For the parameter estimation by the Type II method, a grid search of nearly half a million points was conducted first to select the best initial guess and carboxylation rates were used to determine the limitation states of individual points. The distribution-wise cost function of the new approach and the model-wise cost function of the Type II method were both minimized with the same gradient–non-gradient hybrid optimization technique described earlier.

Figures 6 and 7 compare the estimated parameters against the true parameters used in generating the 100 curves. The new approach correctly identified the limitation state distributions of all 100 curves and retrieved all parameters perfectly (the right column of panels in Figs 6 and 7). In contrast to the success of the new approach, the Type II method was only able to identify correctly the limitation state distributions of 13 out of 100 curves. Thus for a majority of the 100 synthetic A/Ci curves, the Type II method could not retrieve their parameters correctly (the left column of panels in Figs 6 and 7).

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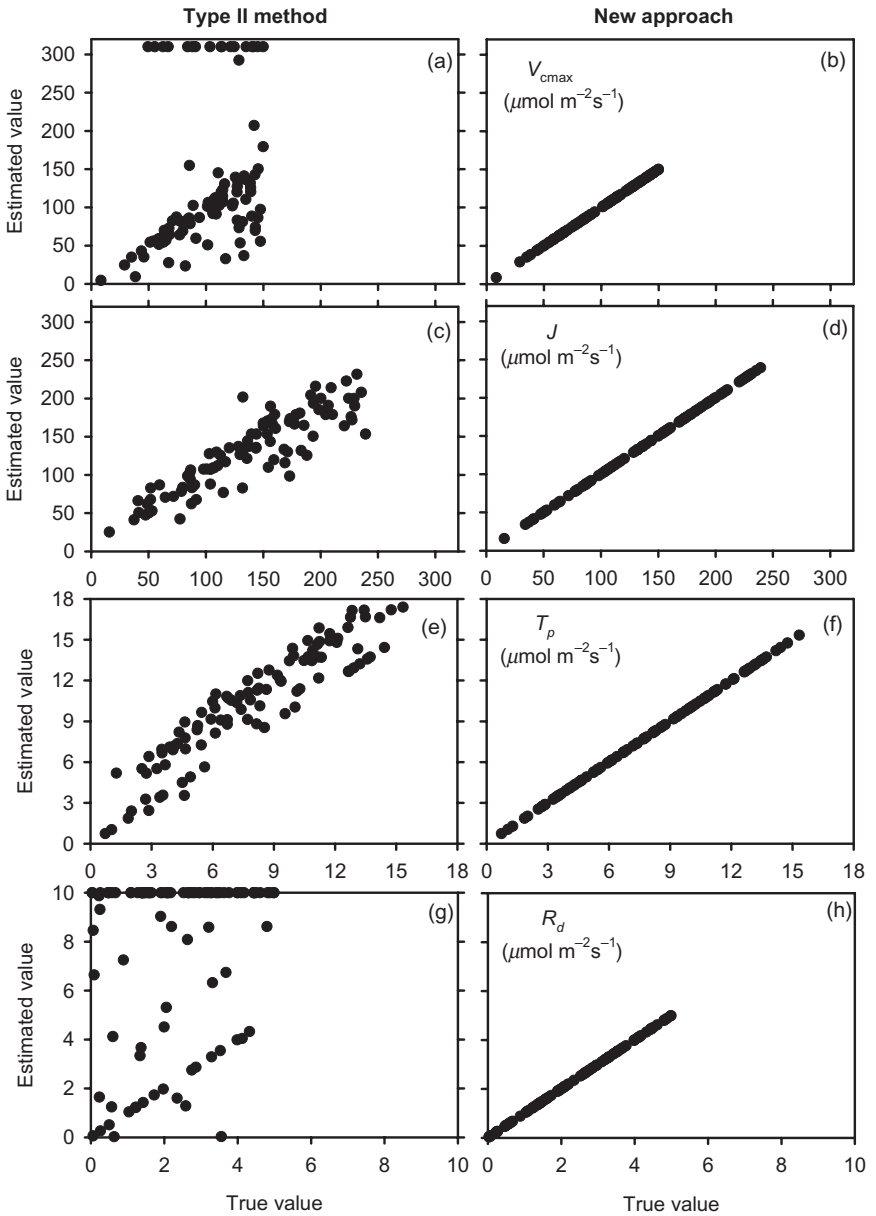

Figure 6. Comparison of the parameters estimated with a Type II method and the new approach against the true parameters for 100 synthetic A/Ci curves. Parameters shown are V_{cmax} (a and b), J (c and d), T_p (e and f), and R_d (g and h). The left column is for the Type II method and the right for the new approach. Some parameters estimated with the Type II method are at their bounds. Also some plots in the left column may have less than 100 points because the Type II method does not always detect the presence of all three limitation states.

Testing the new approach with actual measurements

Although the true values of the FvCB parameters for a real leaf are not known, it is possible to independently test the new approach with actual measurements. The foundation of the new approach is its optimization for the limitation state distribution among all possible limitation state distributions allowed by the data of an A/Ci curve. The parameter optimization is nested within, and a reproducible product of, the optimization for the limitation state distribution. Thus, a direct verification of the optimized limitation state distribution represents an independent test of the new approach. The limitation state of a point can be identified with the chlorophyll fluorescence technique (Harley *et al*. 1992; Baker, Harbinson & Kramer 2007), and we utilized this technique to test the new approach.

A/Ci and chlorophyll fluorescence measurements were made on a number of tree species at the Missouri Ozark AmeriFlux (MOFLUX) site in 2008. MOFLUX is located in the Baskett Research and Education Area (formerly the Ashland Wildlife Area) in central Missouri (38.76°N, 92.20°W) owned and managed by the University of Missouri. For details about this site, see Bahari, Pallardy & Parker (1985), Gu *et al*. (2006) and Gu *et al*. (2007). A Li-Cor LI-6400 Portable Photosynthesis System (Li-Cor, Inc., Lincoln, NE, USA) fitted with a CO_2 mixing apparatus and a 6400–40 Leaf Chamber Fluorometer were used in the measurement of the photochemical efficiency of photosynthesis (Φ_{PSII}) and the generation of A/Ci curves. Sample cuvette (ambient) CO_2 values for A/Ci curve generation

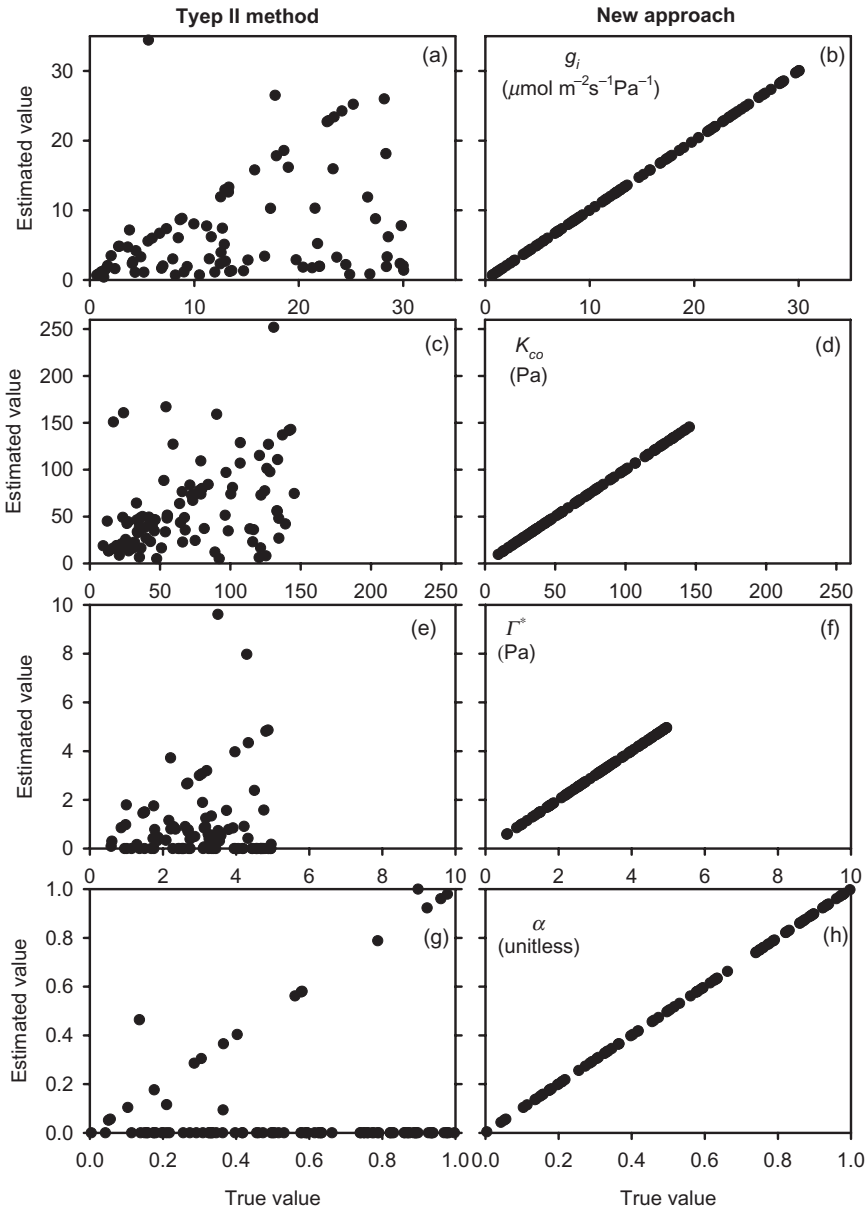

Figure 7. Same as in Fig. 6 except for parameters g_i (a and b), K_{co} (c and d), Γ^* (e and f), and α (g and h).

were about 120, 100, 80, 60, 47.5, 37, 30, 25, 20, 15, 10 and 5 Pa. Saturating photosynthetically active photon flux density (1200 μmol m^{-2} s^{-1}) was provided by the fluorescence attachment.

The photochemical efficiency Φ_{PSII} was calculated as $(F'_m - F_s)/F'_m = \Delta F/F'_m$, where F'_m is the maximal fluorescence during a saturating pulse of light and F_s is the steady state fluorescence. As CO_2 increases from sub-ambient values, Φ_{PSII} initially increases, reflecting increased actual rates of electron transport as high energy intermediates involved in CO_2 reduction after fixation are regenerated. At higher CO_2 partial pressures, Φ_{PSII} saturates as the electron transport system reaches the limit of capacity, J, and remains constant. Beyond this point, any increase in A reflects a greater rate of carboxylation attributable to higher rate of delivery of CO_2 to Rubisco relative to that of O_2. Thus in a plot of Φ_{PSII} against C_i, the Rubisco-limited points are located in the region where Φ_{PSII} increases with C_i whereas the RuBP regeneration-limited points are located where Φ_{PSII} does not change with C_i.

The FvCB model was fit to the measured A/Ci curves with the new approach. An examination of the fitting results revealed that some sampled curves were not adequate for estimating all eight parameters. To be consistent across the samples, we did not optimize for Γ^* and K_{co}. Instead, their values were calculated based on temperature functions in von Caemmerer (2000) and used for optimizing other parameters.

Figures 8–10 display six leaf samples from three tree species (white oak, *Quercus alba* L., Fig. 8; shagbark hickory, *Carya ovata* (Mill.) K. Koch, Fig. 9; black oak, *Quercus velutina* Lam., Fig. 10). The parameters estimated

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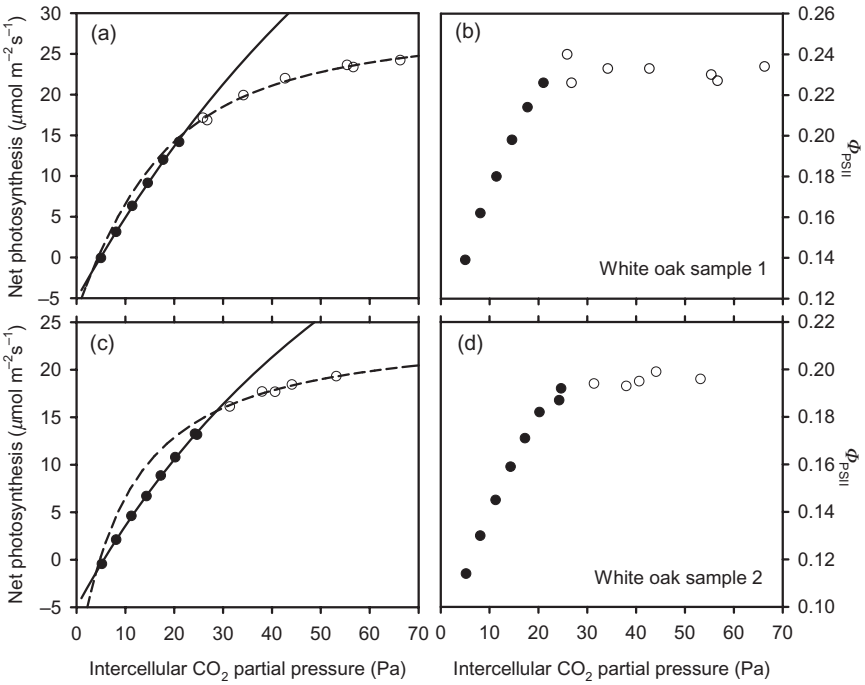

Figure 8. Two white oak (*Quercus alba* L.) leaf A/Ci samples demonstrating the agreement in limitation states predicted through the new approach (a and c) and identified with chlorophyll fluorescence (b and d). Plots (a) and (b) are for the same leaf and (c) and (d) for the other. In (a) and (c), the Rubisco-limited points predicted through optimization are denoted with filled circles and the RuBP regeneration-limited points with unfilled circles. The fitted Rubisco-limited photosynthesis (solid lines) and RuBP regeneration-limited photosynthesis (dashed lines) are also shown. In (b) and (d), the photochemical efficiency (Φ_{PSII}) of each point corresponding to the same point in (a) or (c) is shown as a function of intercellular CO_2 partial pressure (C_i). The photochemical efficiency of the RuBP regeneration-limited points predicted through optimization shows little change with C_i whereas that of the Rubisco-limited points increases with C_i, validating the optimization of the limitation state distribution in the new approach.

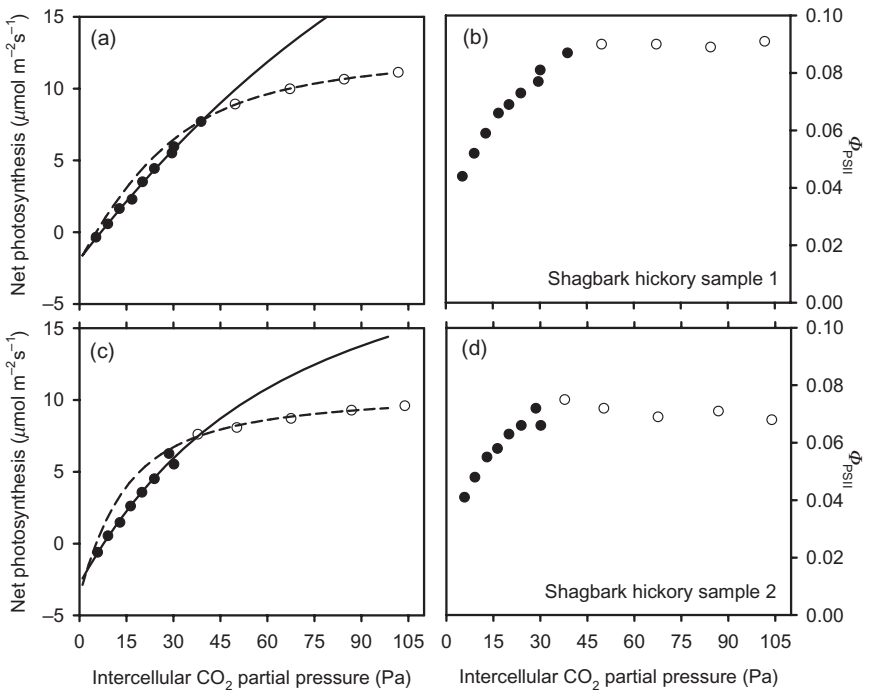

Figure 9. Same as in Fig. 8 except for shagbark hickory [*Carya ovata* (Mill.) K. Koch.].

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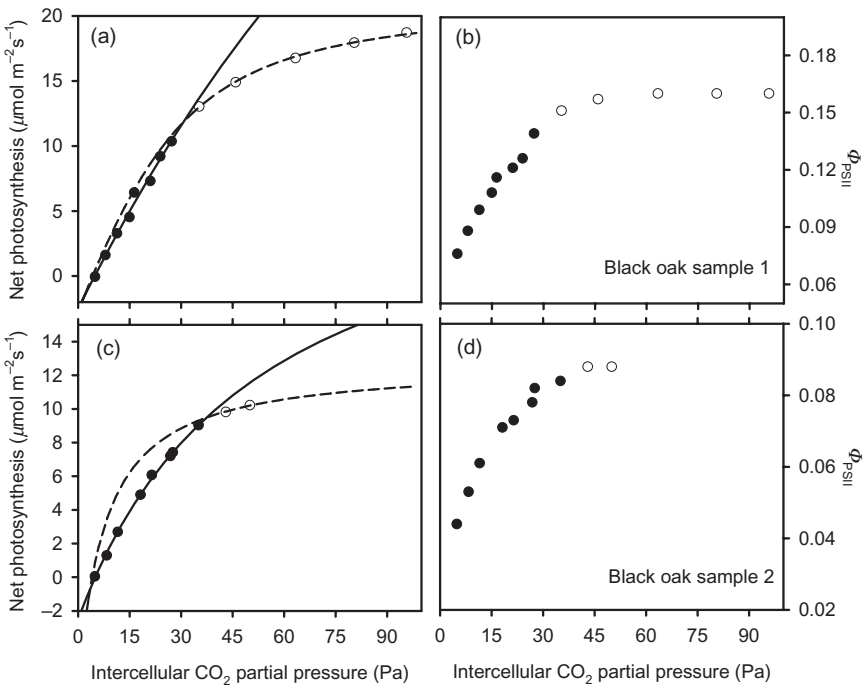

Figure 10. Same as in Fig. 8 except for black oak (*Quercus velutina* Lam.).

are given in Table 3. For these samples, points in the region for which photochemical efficiency does not change with C_i, that is, RuBP regeneration-limited points, can be visually differentiated from points in the region for which photochemical efficiency varies, that is, Rubisco-limited points (Figs 8b, d, 9b, d, 10b, d). The limitation state distribution for each sample identified from the fluorescence data can be compared with the corresponding limitation state distribution predicted by the new approach (Figs 8a, c, 9a, c, 10a, c). Overall, points predicted to be limited by Rubisco are located within the region with steadily increasing photochemical efficiency whereas those predicted to be limited by RuBP regeneration are located within the region with relatively stable photochemical efficiency. The predicted limitation transition points are also located where they should be according to visual examination of the plots of photochemical efficiency against C_i. Thus, the predicted limitation states of the new approach agree well with chlorophyll fluorescence pattern.

DISCUSSION: RECOMMENDATIONS FOR INFORMATIVE A/C$_i$ CURVE MEASUREMENTS

The test earlier with actual A/Ci curves indicates that whether the theoretical capacity offered by the new approach can be achieved in application ultimately depends on the information content of the data set. An informative data set is measured according to procedures that take the FvCB structural characteristics into consideration. As only a limited number of data can be measured at a time without risk of physiological changes in the leaf, sample points should be strategically distributed within the reasonable range of C_i.

It is essential to have a sufficient number of points in the RuBP regeneration-limited state. The RuBP regeneration-limited state is the only state that does not suffer from the problem of structural overparameterization when J is treated as the only state-specific parameter for the RuBP regeneration limitation (Table 1). Points in the RuBP

Table 3. Parameters estimated with the new approach for the six leaf samples in Figs 8–10

Leaf sample	V_{cmax} μmol m^{-2} s^{-1}	J μmol m^{-2} s^{-1}	g_i μmol m^{-2} s^{-1} Pa^{-1}	R_d μmol m^{-2} s^{-1}	K_{co} Pa	Γ^* Pa
White oak 1 (Fig. 8a)	101.6	126.9	2.97	1.81	61.2[a]	3.74[a]
White oak 2 (Fig. 8c)	78.3	103.9	13.54	1.33	80.2[a]	4.12[a]
Shagbark hickory 1 (Fig. 9a)	42.5	58.5	0.56	1.59	71.1[a]	3.95[a]
Shagbark hickory 2 (Fig. 9c)	26.7	50.8	1.63	1.92	51.1[a]	3.50[a]
Black oak 1 (Fig. 10a)	63.4	91.9	0.93	1.46	50.8[a]	3.50[a]
Black oak 2 (Fig. 10c)	26.7	53.5	27.8	0.71	50.9[a]	3.50[a]

Values with the superscript 'a' are fixed (calculated based on leaf temperature).

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regeneration limitation region can constrain the common parameters g_i, R_d and Γ^* better than those in either the Rubisco or TPU limitation regions. Strong constraint on g_i, R_d, and Γ^* will help constrain parameters specific to the Rubisco and TPU limitation states. Ideally, an informative A/Ci curve data set should contain at least five points in the RuBP regeneration-limited state since it is described by four parameters.

Rubisco-limited points at high C_i levels are more informative than those points of this state at low C_i levels. Points at high C_i levels will have their corresponding C_c levels comparable with K_{co} (~60 Pa) so that $(C_c - \Gamma^*)V_{cmax}/(C_c + K_{co})$ cannot be closely approximated by $(C_c - \Gamma^*)(V_{cmax}/K_{co})$. These high-level C_i points of the Rubisco-limited state will allow both V_{cmax} and K_{co} to be resolved. In contrast, if in the data set, Rubisco-limited points all have C_c values that are very small compared with K_{co}, then $(C_c - \Gamma^*)V_{cmax}/(C_c + K_{co})$ can be closely approximated by $(C_c - \Gamma^*)(V_{cmax}/K_{co})$, leaving only the ratio V_{cmax}/K_{co} resolvable. Under this situation, K_{co} will have to be given an *a priori* value in order to resolve V_{cmax}. von Caemmerer *et al*. (1994) came to the same conclusion in earlier work.

Admittedly, the limitation state domains are not known beforehand. Hence, how can optimal placement of A/Ci curve measurements be accomplished? It is obvious that all limitation states must be sampled for biologically meaningful results. However, our analysis suggests that a sampling strategy weighting to some extent in the curvature region provides more informative data for parameter estimation. When an A/Ci curve is developed under normal measurement conditions, the RuBP regeneration-limited state occupies the intermediate range of C_i and links with the Rubisco-limited state in the low range and with the TPU-limited state in the high range of C_i. Thus, as a linkage between the Rubisco- and TPU-limited states, the RuBP regeneration-limited state has at least part of its domain in the curvature region of the A/Ci curve. If this region is sampled densely, the likelihood of having a sufficient number of RuBP regeneration-limited points for robust parameter estimation is increased. Also if Rubisco-limited points can occur at all with C_i (C_c) close to K_{co}, these points would be in the curvature region. Thus by sampling the curvature region somewhat more densely, the likelihood of having a number of points in the Rubisco limitation state with C_c values comparable with K_{co} is increased. Finally, the transition between the Rubisco and RuBP regeneration-limited states is located in the curvature region. When the region is sampled more densely, the likelihood of having a point close to the transition is increased. Since the new approach is able to identify and treat co-limited points, this will allow better constraint of the limitation state distribution and therefore more accurate estimation of the parameters.

Thus, we recommend the following strategy for leaf gas exchange measurements. Firstly, prior knowledge (e.g. A/Ci data from literature) about the photosynthetic CO_2 response of the interested species should be used to establish the likely overall shape of the A/Ci curve. If prior knowledge is not available, preliminary measurements may be needed. The key is to identify the C_i range within which the curvature of the curve is located. Then sample somewhat more preferentially within the curvature region. Obviously, care is needed to make sure there are adequate numbers of points in the two relatively straight regions of low and high C_i ends to establish the overall pattern. Such a sampling objective would also require some prior knowledge of appropriate levels of cuvette CO_2 to specify to obtain informative values of C_i given the stomatal conductance and mesophyll photosynthetic capacity of the target species.

Finally, A/Ci curves have been generally measured at saturating light levels, which tend to produce more Rubisco-limited points. One way to obtain a relatively large number of RuBP regeneration-limited points is to measure at a constant but non-saturating light level for the A/Ci curve. Of course, if the light level is too low, both the Rubisco- and TPU-limited states might not occur at all. Even when the Rubisco-limited state does occur under this situation, it might occur in the initial straight region of the A-Ci curve with small C_i (C_c) values, leading to irresolvable V_{cmax} and K_{co} (the threshold C_{i_CJ} decreases as J decreases, see Eqn A6). Thus, as a balance, some intermediate light level may be more appropriate in some circumstances.

CONCLUSION

The FvCB model has received wide application in modeling photosynthesis from leaf to canopy to global scales. However, it has remained a challenge to reliably estimate the needed parameters from leaf-level gas exchange measurements. Many attempts have been made to address this challenge, but because these attempts have not accounted for the structural uniqueness of the FvCB model, they have not been completely successful. In this paper, we analysed the structural characteristics of the FvCB model from a parameter estimation standpoint. This analysis led to the identification of previously unrecognized complexities in fitting for the model parameters from A/Ci curves. Strategies to overcome these complexities were developed and implemented in a new approach for A/Ci curve analysis. The new approach was tested against model simulations, sampled A/Ci curves, and chlorophyll fluorescence measurements of different tree species. An interactive website, http://www.leafweb.ornl.gov, has been constructed for researchers to apply the new approach.

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APPENDIX 1. THE DEFINITIONS OF KEY SYMBOLS

Symbol	Definition	Unit
A	Net assimilation rate	μmol m^{-2} s^{-1}
A_c	Net assimilation rate assuming Rubisco limitation	μmol m^{-2} s^{-1}
A_j	Net assimilation rate assuming RuBP regeneration limitation	μmol m^{-2} s^{-1}
A_p	Net assimilation rate assuming TPU limitation	μmol m^{-2} s^{-1}
C_c	Chloroplastic CO_2 partial pressure	Pa
C_i	Intercellular CO_2 partial pressure	Pa
C_{i_CJ}	C_i transition between the Rubisco and RuBP Regeneration limitations	Pa
C_{i_JP}	C_i transition between the RuBP Regeneration and TPU limitations	Pa
g_i	Internal (mesophyll) conductance to CO_2 transport	μmol m^{-2} s^{-1} Pa^{-1}
J	Potential electron transport rate at the measurement light level	μmol m^{-2} s^{-1}
J_{max}	Maximal electron transport rate	μmol m^{-2} s^{-1}
K_c	Michaelis-Menten constant for RuBP carboxylation	Pa
K_o	Michaelis-Menten constant for RuBP oxygenation	Pa
K_{co}	$= K_c\ (1 + O/K_o)$	Pa
R_d	Day respiration	μmol m^{-2} s^{-1}
O	Oxygen partial pressure	Pa
T_p	Rate of triose phosphate export from the chloroplast	μmol m^{-2} s^{-1}
V_{cmax}	Maximal Rubisco carboxylation rate	μmol m^{-2} s^{-1}
W_c	Rubisco-limited carboxylation rate	μmol m^{-2} s^{-1}
W_j	RuBP regeneration-limited carboxylation rate	μmol m^{-2} s^{-1}
W_p	TPU-limited carboxylation rate	μmol m^{-2} s^{-1}
Γ^*	Chloroplastic CO_2 photocompensation point	Pa
α	Non-returned fraction of the glycolate carbon recycled in the photorespiratory cycle	N/A

APPENDIX 2. THE STRUCTURAL OVERPARAMETERIZATION OF THE FVCB MODEL WITH RESPECT TO A/CI MEASUREMENTS

The structure of the FvCB model does not lend itself easily to parameter estimation even when an adequate A/Ci data set is provided because the Rubisco- and TPU-limited states are structurally overparameterized. First, we examine the formulation for the Rubisco-limited state. We reorganize b and c in Eqn 10 into the following:

$$\begin{aligned} b &= C_i g_i + (V_{cmax} - R_d + K_{co} g_i) = C_i g_i + p \\ c &= C_i (V_{cmax} - R_d) g_i - (\Gamma^* V_{cmax} + K_{co} R_d) g_i = C_i q + u \end{aligned}.$$

As C_i is the only independent variable that changes and is measured, the Rubisco-limited part of the FvCB model can be re-parameterized with only four truly free independent parameters g_i, p, q and u:

$$\begin{cases} g_i = g_i \\ p = V_{cmax} - R_d + K_{co} g_i \\ q = (V_{cmax} - R_d) g_i \\ u = -(\Gamma^* V_{cmax} + K_{co} R_d) g_i \end{cases}. \quad \text{(A1)}$$

But there are five unknowns in the above re-parameterized algebraic system: g_i, V_{cmax}, R_d, K_{co} and Γ^*. So the number of free independent parameters (equations) is less than the number of unknowns. Therefore the Rubisco-limited state is overparameterized. The significance of this overparameterization is the following: Suppose we have an A/Ci curve whose points are all limited by Rubisco and we fit the model of the Rubisco-limited state to this data set, we will find that not all parameters can be estimated uniquely no matter how many points this data set contains and how accurate the measurement is. At least one parameter must be given in advance in order to obtain a unique solution for the remaining four parameters. But which parameter is the best candidate if one parameter has to be assigned a value? The answer lies in the re-parameterized system A1.

The common practice in A/Ci curve fitting is to assign a value to K_{co} by setting K_c and K_o in advance. It turns out this common practice cannot provide sufficient constraint for other parameters. This is because a pre-determined K_{co} only results in two redundant equations (that is, the expression for p and q in the system A1), and thus cannot help resolve V_{cmax}, R_d, Γ^* and g_i.

A careful examination of the re-parameterized system A1 indicates a subset of parameters can always be resolved uniquely even with overparameterization (in theory, assuming there are a sufficient number of points in the data set). Because g_i appears alone in the system, it can always be resolved uniquely. Consequently, the difference ($V_{cmax} - R_d$) can be resolved uniquely even though the estimated values of individual V_{cmax} and R_d may not be reliable ($V_{cmax} - R_d = q/g_i$), which means that K_{co} can also be resolved uniquely $\{K_{co} = [p - (V_{cmax} - R_d)]/g_i\}$. Finally, the parameter expression $\Gamma^* V_{cmax} + K_{co} R_d$ equals $-u/g_i$ and is thus resolvable. Therefore, if one fits the Rubisco-limited state to a data set that contains a sufficient number of only Rubisco-limited points, g_i and K_{co} can be uniquely estimated.

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This leaves the potential candidate for value assignment to V_{cmax}, R_d and Γ^*. Clearly, one wants to estimate V_{cmax} from the data, so one has to decide between R_d and Γ^*. Since $(V_{cmax} - R_d)$ is uniquely resolvable, an error in the assigned value of R_d is linearly translated to an error in the estimated V_{cmax}: $\Delta V_{cmax} = \Delta R_d$. Since $(\Gamma^* V_{cmax} + K_{co} R_d)$ is uniquely resolvable, combining the last two equations in the system A1, we have:

$$V_{cmax} = \frac{K_{co} q - u}{g_i(\Gamma^* + K_{co})} \approx \frac{K_{co} q - u}{g_i K_{co}}. \tag{A2}$$

The approximation holds because a typical K_{co} (~60 Pa) is at least an order of magnitude larger than a typical Γ^* (~5 Pa). (For typical values of $\Gamma^*$and K_{co}, see von Caemmerer 2000.) Thus, an error in the assigned value of Γ^* should only minimally affect the accuracy of the estimated V_{cmax}. Thus Γ^* is the best candidate to be assigned a value if one parameter has to be fixed in advance.

It is worth emphasizing that overparameterization in the Rubisco-limited state is not caused by an additional need to fit for g_i. Even if g_i is neglected (i.e. g_i is infinite and $C_i = C_c$), overparameterization still exists as can be seen from the following reorganization:

$$A = \frac{V_{cmax}(C_i - \Gamma^*)}{C_i + K_{co}} - R_d = \frac{C_i(V_{cmax} - R_d) - \Gamma^* V_{cmax} - K_{co} R_d}{C_i + K_{co}}. \tag{A3}$$

In Eqn A3, there are really only three independent composite parameters $(V_{cmax} - R_d)$ $(\Gamma^* V_{cmax} + K_{co} R_d)$, and K_{co} but with four unknowns. As in the case for which g_i is considered, assigning a value to K_{co} in advance is not sufficient to constrain V_{cmax}, R_d and Γ^* because these three unknowns appear in only two composite groups in A3. In fact, K_{co} is the only parameter that can be uniquely resolved, whereas other parameters can only be resolved in parameter expressions $(V_{cmax} - R_d)$ and $(\Gamma^* V_{cmax} + K_{co} R_d)$. The best parameter to be assigned a value in advance is still Γ^*.

Like the Rubisco-limited state, the TPU-limited state is also overparameterized, which can be seen from the re-parameterized system corresponding to this state:

$$\begin{cases} g_i = g_i \\ t = 3T_p - R_d - (1+3\alpha)\Gamma^* g_i \\ v = (3T_p - R_d) g_i \\ k = -[\Gamma^* 3T_p - (1+3\alpha)\Gamma^* R_d] g_i \end{cases}. \tag{A4}$$

Similar to the system A1, there are only four independent equations but with five unknowns. If the model of the TPU-limited state is fit to a data set that contains only TPU-limited points, the parameter and parameter expressions that can be uniquely resolved are g_i, $(3T_p - R_d)$, $(1+3\alpha)\Gamma^*$, and $[\Gamma^* 3T_p - (1+3\alpha)\Gamma^* R_d]$. Assigning a value to either Γ^* or R_d can lead to the resolution of the rest. However, the system A4 is ill-conditioned as α approaches zero (the equations for v and k become redundant). In fact when α equals zero, that is, when the simpler form for the TPU-limited photosynthesis (Eqn 8) is used, only $(3T_p - R_d)$ can be resolved and since Γ^* and g_i are not involved at all, R_d must be assigned in advance in order to resolve T_p. Even when α does not equal zero, the system A4 is difficult to solve because it is extremely non-linear as indicated by the four-parameter product $\alpha\Gamma^* R_d g_i$ in the equation for k. Thus, if the data set contains only the TPU-limited state, assigning a value to R_d in advance is always preferred regardless of whether Eqn 4 or 8 is used.

Among the three limitation states in the FvCB model, only the RuBP regeneration-limited state is not overparameterized. This assertion assumes J is the only state specific parameter to be estimated for the RuBP regeneration limitation. The re-parameterized system corresponding to the RuBP regeneration-limited state is as follows:

$$\begin{cases} g_i = g_i \\ x = J/4 - R_d + 2\Gamma^* g_i \\ y = (J/4 - R_d) g_i \\ z = -(\Gamma^* J/4 + 2\Gamma^* R_d) g_i \end{cases}. \tag{A5}$$

This system has four independent equations with four unknowns and is uniquely resolvable. Therefore there is no redundant parameter in the RuBP regeneration-limited state and all four parameters can be uniquely determined if this state is optimized independently.

Although the FvCB model is structurally overparameterized and key parameters are not estimable when the model is fit to the Rubisco- and TPU-limited data separately, it is possible to overcome the structural overparameterization by joining the limitation states together in the parameter estimation process. When the three limitation states are joined together (A1 + A4 + A5), there are 10 independent equations with eight unknowns and thus the joint system is overdetermined, which means all eight unknowns can be uniquely resolved. Similarly when the RuBP regeneration-limited state is joined with the Rubisco-limited state (A1 + A5) or the TPU-limited state (A4 + A5), the joint systems are also overdetermined, and therefore all unknowns in these joint systems can be uniquely resolved. The only problematic joint system is the Rubisco-TPU system (A1 + A4). When $\alpha = 0$, the joint system has five independent equations with six unknowns and thus the unknowns cannot be resolved uniquely without fixing at least one parameter in advance. When $\alpha \neq 0$, the joint system has seven equations with seven unknowns and thus in theory the seven unknowns could be resolved uniquely. But because the TPU-limit state is highly non-linear when $\alpha \neq 0$, solving for all the unknowns in the Rubisco-TPU system could still prove to be extremely difficult. Thus for the Rubisco-TPU joint system, at least one parameter should be assigned an *a priori* value. For a general understanding of parameter estimation theory, we refer to Tarantola (2005). Table 1 summarizes the resolvability of parameters and parameter expressions under different limitation groups.

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APPENDIX 3. THE ORDER OF THE THREE LIMITATION STATES ALONG THE C_c AND C_i AXES DICTATED BY THE FVCB MODEL

According to the FvCB model, the A–C_c relationship within each limitation state is monotonic and there is one and only one intersection between any of the two limitation states within the whole range of C_c. This means that in the A–C_c relationship, no points of any limitation state can be separated by points of other limitation states. Furthermore, the three limitation states follow a fixed order along the C_c axis. To illustrate, let $W_c < W_j$ and solve for the corresponding range of C_c: $\frac{V_{cmax}C_c}{C_c + K_{co}} < \frac{JC_c}{4C_c + 8\Gamma^*}$, $(4V_{cmax} - J)C_c < JK_{co} - 8V_{cmax}\Gamma^*$. Thus if $4V_{cmax} > J$, then

$$C_c < \frac{JK_{co} - 8V_{cmax}\Gamma^*}{4V_{cmax} - J}, \tag{A6}$$

that is, the Rubisco-limited state occurs at lower C_c values than the RuBP regeneration-limited state does. Under this condition, however, if $J < 8V_{cmax}\Gamma^*/K_{co}$, the C_c at the intersection is negative, which means the Rubisco-limited state will not occur at all. If $4V_{cmax} < J$, then $C_c > \frac{JK_{co} - 8V_{cmax}\Gamma^*}{4V_{cmax} - J}$, and it therefore appears that the Rubisco-limited state may occur at higher C_c values than the RuBP regeneration-limited state does. However, if $J > 8V_{cmax}\Gamma^*/K_{co}$, the C_c value at the intersection is negative, which means, the RuBP regeneration-limited state does not exist at all. If $J < 8V_{cmax}\Gamma^*/K_{co}$, the C_c value at the intersection is positive, but this also means that $4V_{cmax} < J < 8V_{cmax}\Gamma^*/K_{co}$ and $K_{co} < 2\Gamma^*$. Typically $K_{co} \sim 60$ Pa whereas $\Gamma^* \sim 5$ Pa (see data in for example von Caemmerer 2000). Thus it seems unlikely that the condition $K_{co} < 2\Gamma^*$ can be met. Therefore, in the A–C_c relationship, the Rubisco- and RuBP regeneration-limited states either do not exist together or exist with the Rubisco-limited state first and the RuBP regeneration-limited state next along the C_c axis. Furthermore, when both states exist, the following condition holds:

$$4V_{cmax} > J.$$

The relative order between the RuBP regeneration- and TPU-limited states can be determined similarly. Let $W_j < W_p$, we have $(J - 12T_p)C_c < 24\Gamma^*T_p + (1 + 3\alpha)\Gamma^*J$ (note that for the TPU-limited state to be considered, $C_c > (1 + 3\alpha)\Gamma^*$). If $J > 12T_p$, then

$$C_c < \frac{24\Gamma^*T_p + (1 + 3\alpha)\Gamma^*J}{J - 12T_p}. \tag{A7}$$

That is, the RuBP regeneration-limited state occurs before the TPU-limited state along the C_c axis. If $J < 12T_p$, the C_c value at the intersection is negative, which means, the TPU-limited state does not exist at all. Therefore, in the A–C_c relationship, the RuBP regeneration- and TPU-limited states either do not exist together or exist with the RuBP regeneration-limited state at lower C_c values than the TPU-limited state. Furthermore, when both limitation states exist together, the following condition must hold:

$$J > 12T_p.$$

Combining the deductions presented earlier together, the following regarding the relative orders of the three limitation states along the C_c axis can be ascertained. The three limitation states either do not exist together or exist in the following order: the Rubisco state occupies the lowest C_c values, the RuBP regeneration-limited state the intermediate C_c values, and the TPU-limited state the highest C_c values. Furthermore, when the three limitation states exist together, the following conditions hold:

$$4V_{cmax} > J > 12T_p. \tag{A8}$$

The order of the three limitation states along the C_i axis should be the same as along the C_c axis as the $C_i - C_c$ relationship should be orderly, that is, a higher C_c should correspond to a higher C_i and vice versa. This assertion assumes a stable g_i. Consequently for typical A/Ci curves it is reasonable to assume that along the C_i axis the Rubisco-, RuBP regeneration- and TPU-limited states appear in sequence and there can be no more than one continuous section within which a limitation state operates. For example, a RuBP regeneration-limited section cannot be sandwiched between two Rubisco-limited sections. This order has been implicitly or explicitly assumed in previous studies (e.g. von Caemmerer 2000; Sharkey *et al*. 2007), but has never been mathematically evaluated. This prior knowledge can greatly reduce the number of possible limitation state distributions allowed by a given A/Ci data set.

However, for measurements in which C_i, light level and temperature all vary, any limitation state can occur at any value of C_i and a limitation state can be flanked by another limitation state at both sides, that is, a RuBP regeneration-limited section could be sandwiched between two Rubisco-limited sections. Since we focus on conventional analysis of A/Ci curves only, we do not consider this possibility in this paper.

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APPENDIX 4. ALL POSSIBLE LIMITATION STATE DISTRIBUTIONS FOR AN A/CI CURVE WITH 10 POINTS

In the following table, the 10 points are labeled A_i, $i = 1$ to 10 and 'C', 'J' and 'P' denote the Rubisco-, RuBP regeneration- and TPU export-limited states, respectively. The data are assumed to have been ordered according to C_i from low to high. Additionally, it is assumed that all parameters (V_{cmax}, K_{co}, J, T_p, g_i, α, Γ^* and R_d) are to be estimated, and therefore the minimum number of points for a limitation state to be enumerated in a distribution is three, two, and two for Rubisco-, RuBP regeneration- and TPU-export limited states, respectively. When there are only two TPU-export limited points, α is fixed at zero.

A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}
C	C	C	C	C	C	C	C	C	C
C	C	C	C	C	C	C	C	J	J
C	C	C	C	C	C	C	J	J	J
C	C	C	C	C	C	J	J	J	J
C	C	C	C	C	C	J	J	P	P
C	C	C	C	C	J	J	J	J	J
C	C	C	C	C	J	J	J	P	P
C	C	C	C	C	J	J	P	P	P
C	C	C	C	J	J	J	J	J	J
C	C	C	C	J	J	J	J	P	P
C	C	C	C	J	J	J	P	P	P
C	C	C	C	J	J	P	P	P	P
C	C	C	J	J	J	J	J	J	J
C	C	C	J	J	J	J	J	P	P
C	C	C	J	J	J	J	P	P	P
C	C	C	J	J	J	P	P	P	P
C	C	C	J	J	P	P	P	P	P
J	J	J	J	J	J	J	J	J	J
J	J	J	J	J	J	J	J	P	P
J	J	J	J	J	J	J	P	P	P
J	J	J	J	J	J	P	P	P	P
J	J	J	J	J	P	P	P	P	P
J	J	J	J	P	P	P	P	P	P
J	J	J	P	P	P	P	P	P	P
J	J	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P
C	C	C	P	P	P	P	P	P	P
C	C	C	C	P	P	P	P	P	P
C	C	C	C	C	P	P	P	P	P
C	C	C	C	C	C	P	P	P	P
C	C	C	C	C	C	C	P	P	P
C	C	C	C	C	C	C	C	P	P

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