RESEARCH PAPER

Temperature response of Rubisco kinetics in Arabidopsis thaliana: thermal breakpoints and implications for reaction mechanisms

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Abstract

Enhancement of Rubisco kinetics could improve photosynthetic efficiency, ultimately resulting in increased crop yield. However, imprecise knowledge of the reaction mechanism and the individual rate constants limits our ability to optimize the enzyme. Membrane inlet mass spectrometry (MIMS) may offer benefits over traditional methods for determining individual rate constants of the Rubisco reaction mechanism, as it can directly monitor concentration changes in CO₂, O₂, and their isotopologs during assays. However, a direct comparison of MIMS with the traditional radiolabel method of determining Rubisco kinetic parameters has not been made. Here, the temperature responses of Rubisco kinetic parameters from Arabidopsis thaliana were measured using radiolabel and MIMS methods. The two methods provided comparable parameters above 25 °C, but temperature responses deviated at low temperature as MIMS-derived catalytic rates of carboxylation, oxygenation, and CO₂/O₂ specificity showed thermal breakpoints. Here, we discuss the variability and uncertainty surrounding breakpoints in the Rubisco temperature response and the relevance of individual rate constants of the reaction mechanisms to potential breakpoints.

Keywords: Arabidopsis, kinetic breakpoints, membrane inlet mass spectrometry, reaction mechanisms, Rubisco, temperature.

Introduction

The enzyme Rubisco catalyzes the reaction of CO₂ or O₂ with ribulose-1,5-bisphosphate (RuBP) initiating the photosynthetic carbon reduction cycle or photorespiratory cycle, respectively (Bowes et al., 1971; Andrews et al., 1973). Kinetic studies on Rubisco typically report the Michaelis–Menten constants for carboxylation (Kₐ) and oxygenation (Kₒ), the catalytic rate of carboxylation (k_catCO₂) and oxygenation (k_catO₂), and the specificity of the enzyme for CO₂ over O₂ (S_C/O₂) as these parameters are used for modeling leaf gas exchange (von Caemmerer, 2000). Each of the above Michaelis–Menten parameters is a combination of elementary rate constants that describe the reaction mechanism; however, the rate constants are less well studied as the nature of the chemical mechanism and their intermediates are uncertain (Tcherkez, 2013, 2016).

Optimization of Rubisco kinetics for enhanced CO₂ reduction has been proposed (Spreitzer and Salvucci, 2002), but this effort is limited by our current understanding of the reaction mechanism (Tcherkez et al., 2006; Tcherkez, 2013).

The carboxylation and oxygenation reaction mechanisms can be separated into elementary rate constant as originally proposed by Farquhar (1979), reviewed by Tcherkez (2013), and reproduced in Fig. 1. Since the initial description of the reaction mechanism (Hurwitz et al., 1956), there has been slow progress in defining rate constants due to experimental...
Fig. 1. Elementary reactions of Rubisco-catalyzed carboxylation and oxygenation (Farquhar, 1979). Each reaction, forward and reverse, is numbered in a filled circle following the numbering from Farquhar (1979). Steps 2 and 5 are written as irreversible reactions. Step 8 includes cleavage, hydration, and retronolation as a single step. Step 5 includes cleavage and hydration as a single step. Each step is associated with a rate constant (k) and energy of activation (ΔAG°) following the same numbering as shown in the filled circles. Abbreviations are as follows: E, free activated enzyme; RuBP, d-ribulose-1,5- bisphosphate; E-RuBP, enzyme-bound RuBP; E-Enediol, enzyme-bound 2,3-enediolate form of RuBP; CO2, carbon dioxide; E-CKABP, enzyme-bound carboxylketone intermediate; PGA, 3-phospho-D-glycerate; O2, oxygen; E-PKABP, peroxo intermediate; PGO, 2-phosphoglycolate.

difficulties in isolating their individual effects. However, the use of membrane inlet mass spectrometry (MIMS) to study Rubisco kinetics may hold promise. The traditional radiolabel method used in most Rubisco publications relies on 14C assays to determine kcatCO2, KC, and K0, a separate 3H assay to determine Sc/O2, leaving kcatO2 to be calculated. Alternatively, the MIMS assay simultaneously measures changing concentrations of CO2 and O2, and can therefore determine all kinetic parameters with a single assay (Cousins et al., 2010; Boyd et al., 2015). An advantage of the MIMS method is that in addition to the abundant isotopologs of CO2 (12CO2) and O2 (16O2), the system can monitor less abundant stable isotopologs such as 13CO2 and 16O18O. Measurements of primary kinetic isotope effects have been useful in defining enzyme reaction mechanisms (O’Leary et al., 1992); therefore, the MIMS system may provide new information regarding the individual rate constants. At 25 °C the MIMS method has been used for determining both Rubisco carbon fractionation (McNevin et al., 2006, 2007; Tcherkez et al., 2013), and Michaelis–Menten constants of the carboxylation (νc) and oxygenation (νo) reactions (Cousins et al., 2010). Additionally, it was used to determine the temperature dependencies of the Rubisco kinetic parameters in the C4 species Setaria viridis, where the Arrhenius energy of activation (Ea) is used to describe the temperature dependence of chemical reaction rates (Boyd et al., 2015). However, previous work using the radiolabel method suggests lower Ea values for Vmax in C4 species than that measured by Boyd et al. (2015) (Sage, 2002; Kubien et al., 2003; Perdomo et al., 2015; Sharwood et al., 2016). This suggests that comparisons between the MIMS Ea values and the traditional radiolabel method are needed.

Here we measured the temperature response of Rubisco kinetic parameters from Arabidopsis thaliana using two methods. First, we used the traditional method involving the use of radiolabeled substrate and analysis of labeled products following the reaction in known concentrations of CO2 and O2 (Jordan and Ogren, 1981). Secondly, we used the MIMS method following the simultaneous consumption of CO2 and O2 over time, giving a direct measure of νc, νo, CO2, and O2, leading to simultaneous determination of kcatCO2, kcatO2, KC, K0, and Sc/O2 (Cousins et al., 2010; Boyd et al., 2015). Additionally, for the radiolabel method, we compared curve fitting CO2 responses to determine KC and kcatCO2 simultaneously in an O2-free buffer, and kcatO2 determined at a single bicarbonate concentration in open air. The latter is a common practice for determining kcatCO2 temperature responses (Tieszen and Sigurdson, 1973; Sage et al., 1995; Crafts-Brandt and Salvucci, 2000; Pittermann and Sage, 2000; Sage, 2002; Kubien et al., 2003; Perdomo et al., 2015).

Recently, the existence of thermal breakpoints in the kcatCO2 temperature response was highlighted as a source of variability in the Rubisco temperature response literature (Sharwood et al., 2016). Thermal breakpoints occur when E values differ between temperature ranges. Initial observations of breakpoints in Vmax temperature responses were determined to be a methodological artifact due to the use of a single bicarbonate concentration at all temperatures and were corrected by varying the bicarbonate concentration with temperature (Björkman and Peary, 1970). However, breakpoints were later observed for kcatCO2, kcatO2, and KC at 15 °C using a curve fitting method (Badger and Collatz, 1977). It was suggested that these breakpoints could be due to changes in rate-limiting steps of the reaction mechanism caused by changes in enzyme conformation (Badger and Collatz, 1977). An additional breakpoint was reported in the kcatCO2 of Oryza sativa at 22 °C (Sage, 2002), and Kubien et al. (2003) observed different temperature responses when kcatCO2 was measured from 0 °C to 12 °C compared with 18 °C to 42 °C in Flaveria bidentis. Most recently, Sharwood et al. (2016) observed breakpoints in kcatCO2 at 25 °C for Panicoid grasses when using a curve fitting method. Inconsistencies are evident between studies, and it is unclear if breakpoints are universal to all temperature response studies of plant Rubisco. Here, we discuss the possible causes of breakpoints, focusing on the three previously proposed causes of breakpoints: erroneous bicarbonate concentrations, changes in the rate-limiting step of the reaction mechanism, and deactivation of Rubisco at low temperature, using the radiolabel and MIMS data sets reported here.

Materials and methods

Plant growth

Plants for the radiolabel method were grown and assayed at the University of New Brunswick, Fredericton, Canada. Arabidopsis thaliana (Col-0) seeds were stratified for 3 d at 4 °C on Promix (Plant Products, University of New Brunswick, Fredericton, Canada. Arabidopsis thaliana using two methods. First, we used the traditional method involving the use of radiolabeled substrate and analysis of labeled products following the reaction in known concentrations of CO2 and O2 (Jordan and Ogren, 1981). Secondly, we used the MIMS method following the simultaneous consumption of CO2 and O2 over time, giving a direct measure of νc, νo, CO2, and O2, leading to simultaneous determination of kcatCO2, kcatO2, KC, K0, and Sc/O2 (Cousins et al., 2010; Boyd et al., 2015). Additionally, for the radiolabel method, we compared curve fitting CO2 responses to determine KC and kcatCO2 simultaneously in an O2-free buffer, and kcatO2 determined at a single bicarbonate concentration in open air. The latter is a common practice for determining kcatCO2 temperature responses (Tieszen and Sigurdson, 1973; Sage et al., 1995; Crafts-Brandt and Salvucci, 2000; Pittermann and Sage, 2000; Sage, 2002; Kubien et al., 2003; Perdomo et al., 2015).

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Brampton, Canada), transferred to a growth chamber (E-15, Conviron, Winnipeg, Manitoba, Canada), and grown under a photoperiod of 10 h light and 14 h dark, day/night temperatures of 20/18 °C, and a photo-synthetic photon flux density (PPFD) of 300 μmol m⁻² s⁻¹. Plants were watered with modified Hoagland’s solution as needed.

Plants for MIMS were grown and assayed at Washington State University, Pullman, Washington, USA. Seeds of *A. thaliana* ecotype Col-0, were placed in 2 liter pots containing commercial soil (LC1 Sunshine Mix, Sun Gro Horticulture, Vancouver, Canada) and watered as needed.

**Sampling for radiolabel analysis**

Leaf punches were obtained at mid-day, flash-frozen in liquid nitrogen, and stored at −80 °C until extraction. Leaf tissue was ground (1.1 cm² disks, ~20 mg) in a Tenbroek glass tissue homogenizer containing 3 ml of ice-cold extraction buffer [100 mM HEPES pH 7.6, 2 mM Na-EDTA, 5 mM MgCl₂, 5 mM DTT, 10 mg ml⁻¹ polyvinylpyrrolidone (PVPP), 2% (v/v) Tween-80, 2 mM NaH₂PO₄, 12 mM amino-n-capronic acid, and 2 mM benzamidine] and 50 μl of Protease inhibitor cocktail (Sigma, St. Louis, MO, USA). This leaf homogenate was centrifuged at 16 000 g at 4 °C for 60 s. The resulting supernatant was then desalted using an Econo Pac 1-DEG column (Bio-Rad), and aliquots were incubated with 20 mM MgCl₂ and 10 mM NaHCO₃ at 30 °C for 20 min to carbamylate Rubisco fully; Rubisco content (number of active sites) was quantified using the [¹⁴C]carboxy-arabinitol bisphosphate ([¹⁴C]ABP)-binding assay (Rusnak et al., 1998; Kubien et al., 2011).

**Sampling for MIMS analysis**

The youngest fully expanded leaves of plants 30–40 d after planting were sampled for Rubisco extraction. The mid vein was removed and ~2 g of leaf tissue was ground in 2 ml of ice-cold extraction buffer [100 mM HEPES pH 7.8, 10 mM EDTA, 25 mM MgCl₂, 1 mM DTT, 10 mM NaHCO₃, 1% (g ml⁻¹) PVPP, 0.5% (v/v) Triton X-100] with a mortar and pestle on ice. Protease inhibitor cocktail (P9599, Sigma-Aldrich), 67 μl per g of fresh leaf tissue, was added to the extraction buffer before grinding. The homogenized extract was centrifuged at 4 °C, for 10 min, at 17 000 g. The supernatant was collected and desalted using an Econo Pac 10DG column (Bio-Rad), filtered through a Millex GP 33 mm syringe-driven filter unit (Millipore), and then centrifuged using Amicon Ultra Ultracel 30K centrifugal filters (Millipore) at 4 °C for 1 h at 3000 g. The layer maintained above the filter unit was collected, brought to 20% (v/v) glycerol (v/v), flash-frozen in liquid nitrogen, and stored at −80 °C until measured. Rubisco content was determined as described above.

**Radiolabel measurement of Rubisco kinetic parameters**

The maximum carboxylation rate of fully activated Rubisco (*Vₘₐₓ*) was measured following the methods of Kubien et al. (2011) from 0 °C to 35 °C, by the incorporation of ¹³C into acid-stable products. This method is later referred to as the ‘single point’ method. Assays were initiated by the addition of 50 μl of activated extract (as described above) to 250 μl of assay buffer [100 mM Bicine-NaOH (pH 8.2), 1 mM Na-EDTA, 20 mM MgCl₂, 5 mM DTT, 400 μM RuBP, and 11 mM NaH¹⁴CO₃ (~700 Bq nmol⁻¹)] and stopped after 30–60 s by adding 250 μl of 1 M formic acid. Samples were dried at 90 °C, and ¹³C acid-stable products were counted using a scintillation counter (LS-6500, Beckman-Coulter). The catalytic rate of carboxylation (*k_{catCO₂}*), was calculated using the equation

\[ k_{catCO₂} = \frac{V_{ₘₐₓ}}{\text{active sites}} \]  

where active sites are measured by the [¹⁴C]ABP method described above. It was assumed that there is a one to one relationship between the moles of [¹⁴C]ABP and active sites, resulting in units for *k_{catCO₂}* of mol CO₂ mol⁻¹ active site s⁻¹ that simplifies to s⁻¹.

Michaelis–Menten parameters for CO₂ (*K_C*), and apparent *K_C* at 21% O₂ ([K_{C[21%O₂]}) were determined by assaying the initial rate of Rubisco carboxylation (*v*) in 7 ml septum-sealed, N₂-sparged vials over a range of seven NaH¹⁴CO₃ concentrations (Paul et al., 1994; Kubien et al., 2008). Concentrations of NaHCO₃ varied depending on temperature (e.g. 0.01–3.0 mM at 10 °C, versus 0.3–13.0 mM at 35 °C). The temperature effect on pH using p*K* values (Edsall and Wyman, 1958) to calculate the CO₂ concentration was incorporated and the Henry coefficients (Sander, 2015) were used to account for the temperature effect on CO₂ solubility (see Supplemental Table S1 at JXB online). Assays were initiated by injecting 50 μl of the activated extract into vials containing CO₂-free assay buffer [100 mM Bicine-NaOH (pH 8.2 at 25 °C), 20 mM MgCl₂, 1 mM Na₂EDTA, 400 μM RuBP, and 10 μg ml⁻¹ carbonic anhydrase], stopped after 30–60 s by adding 250 μl of 1 M formic acid, and counted as described above. The response of *v* to partial pressures of CO₂ were fit to the Michaelis–Menten equation

\[ v = \frac{V_{ₘₐₓ} \cdot CO₂}{CO₂ + K_C} \]  

in SigmaPlot (Systat Software, San Jose, CA, USA) solving for *V_{ₘₐₓ}* and *K_C*. This analysis, referred to as the ‘curve fitting’ method, gave a separate temperature response of *k_{catCO₂}* from the single point method described above. From the same extract, the apparent *K_C* at 21% O₂ ([K_{C[21%O₂]}) was determined, and the Michaelis constant for oxygenation (*K_O*) was calculated from the relationship

\[ K_{C[21%O₂]} = K_C \left(1 + \frac{O_2}{K_O}\right) \]  

Rubisco specificity for CO₂ over O₂ (*S_{CO₂/O₂}) was determined following the method described by Kane et al. (1994). Septa-sealed vials containing Rubisco, buffer [30 mM triethanolamine-acetate (pH 8.3), 20 mM Mg-acetate], and 0.2 mg ml⁻¹ carbonic anhydrase were incubated in humidified gas (0.1% CO₂ in O₂, with a total flow rate of 2000 ml min⁻¹; G400 gas mixing system, Qubit Systems, Kingston Canada) at each measurement temperature, with oscillatory shaking. Reactions were initiated by injecting 2 nmol of [³H]RuBP (3 kcal mol⁻¹) into the vial and terminated after 60 min by the addition of alkaline phosphatase. To prepare the sample for separation, the reaction products were applied to a 0.5 ml column of BioRad AG1-X8 anion exchange resin (200–400 mesh, formate form), washed with 10 column volumes of ddH₂O, and radiactivly labeled glycerate and glycolate eluted with 10% H₂SO₄. The [³H] glycerate and [³H]glycolate were separated via HPLC (system described in Shay and Kubien, 2013) on an Aminex HPX-87H column (BioRad, Canada) maintained at 60 °C. The mobile phase was 7.5 mM H₂SO₄, and the flow rate was 0.4 ml min⁻¹. Glycerate and glycolate fractions were collected in drop-synchronization mode (Fraction Collector III, Waters), and the amount of [³H] in each fraction was determined via scintillation counting.

\[ S_{CO₂/O₂} = \frac{k_{catCO₂} \cdot K_O}{k_{catO₂}} \]  

MIMS measurement of Rubisco kinetic parameters

Rubisco assays were conducted in a 600 μl temperature-controlled cuvette linked to an isotope ratio mass spectrometer (Thermo-Fischer DeltaV) and calibrated as previously described (Cousins et al., 2010; Boyd et al., 2015). Samples were measured similarly to Boyd et al. (2015); four
oxygen concentrations ranging from 40 μM to 1600 μM, and five CO2 concentrations ranging from 0 μM to 200 μM at each oxygen level were made. Measurements were made in 5 °C intervals from 10 °C to 40 °C, and the same three replicates were measured at each temperature. The assay buffer consisted of 200 mM HEPES (pH 7.7 at each measurement temperature), 20 mM MgCl2, 0.1 mM α-hydroxyisopropylmethanesulfon ic acid (α-HPMS), 8 mg ml−1 carbonic anhydrase (Sigma), and 0.6 mM RuBP A 10 μl aliquot of extract was added per measurement. Rubisco was activated by leaving the extract at room temperature for 10 min prior to returning to ice before measurement.

The measured \( v_c, v_o \), and the corresponding CO2 and O2 concentrations were fit simultaneously to the following equations

\[
\begin{align*}
\frac{v_c}{v_{max}} &= \frac{V_{\text{comb CO}_2}}{CO_2 + K_C(1 + O_2 / K_O)} \\
\frac{v_o}{v_{\text{max}}} &= \frac{V_{\text{comb O}_2}}{O_2 + K_O(1 + CO_2 / K_C)}
\end{align*}
\]

(5) (6)

solving for the parameters \( V_{\text{comb CO}_2}, V_{\text{comb O}_2}, K_C, \) and \( K_O \). All model fits were performed in the software package Origin 8 (OriginLab) using the non-linear curve-fit function NLMft. \( S_{\text{CO}_2} \) was calculated using Equation 4. The \( k_{\text{catCO}_2} \) was calculated according to Equation 1 and the \( k_{\text{catO}_2} \) was calculated using the analogous equation

\[
k_{\text{catO}_2} = \frac{V_{\text{max}}}{\text{activates}}
\]

(7)

It should be noted that plant growth temperature, photoperiod, extraction protocol, and assay conditions were similar but not identical between the MIMS and radionuclide experiments, and, as discussed below, should be taken into account when comparing these two data sets.

**Modeling temperature responses**

The temperature responses of the kinetic parameters were calculated for the equation

\[
\text{Parameter} = k_25 \left( e^{-E_r / R T} \right) (298.15–T_k) /
\]

(8)

where \( k_25 \) is the value of the parameter at 25 °C, \( E_r \) is the Arrhenius activation energy (kJ mol\(^{-1}\)), \( R \) is the molar gas constant (0.008314 kJ mol\(^{-1}\) K\(^{-1}\)), \( T_k \) is the temperature in Kelvin, and the term (298.15–\( T_k \))/298.15 is the scaling factor so that \( k_25 \) may be used as the pre-exponential term. The \( E_r \) and \( k_25 \) values for each Rubisco parameter were calculated by a linear regression of the natural log of the data plotted against \((T_k–298.15)/(T_k)\). such that the y-intercept was equal to the natural log of \( k_25 \) and the slope was equal to \( E_r/(298.15 \times R) \). For comparison, the non-transformed temperature responses are presented in Supplementary Fig. S1 and Supplementary Table S2. Three replicates of \( E_r \) and \( k_25 \) were determined for each parameter, with the exception of radionuclide \( S_{\text{CO}_2} \) where the number of replicates was four. For all MIMS and radionuclide comparisons, other than \( k_{\text{catCO}_2} \), only the curve fitting methods are compared. For simplicity, we exclude the radionuclide single point when comparing ratios of kinetic parameters with MIMS. Differences in the \( k_25 \) and \( E_r \) values were determined by ANOVA, followed by pair-wise comparison (Tukey HSD) with a significance cut-off of \( p<0.05 \) in Statistix 9 (Analytical Software, Tallahassee, FL, USA).

Arrhenius plots for all kinetic parameters were examined for thermal breaks using the package ‘segmented’ in R, which first tests for differences between slopes using the Davies test (Davies, 1987), and then estimates the breakpoints in linear models using maximum likelihood (Muggeo, 2003, 2008; R Core Team, 2013). When breakpoints in the Arrhenius temperature response plots were statistically valid, the \( E_r \) values above and below the break points were compared with other \( E_r \) values as described above; the \( k_25 \) value was held constant when fitting for two \( E_r \) values above and below the breakpoint.

**Equations for reaction mechanisms**

Figure 1 depicts the currently hypothesized reaction mechanism of Rubisco as originally described by Farquhar (1979). The kinetic parameters \( k_{\text{catCO}_2}, k_{\text{catO}_2}, K_C, K_O, \) and \( S_{\text{CO}_2} \) can be described by the individual first-order rate constants (\( k \)) seen in Fig. 1 as follows:

\[
k_{\text{catCO}_2} = \frac{k_h k_h}{k_h + k_d}
\]

(9)

\[
k_{\text{catO}_2} = \frac{k_h k_h}{k_h + k_d}
\]

(10)

\[
K_C = \frac{k_2 + k_0 k_0 + k_{10}}{k_0 + k_0}
\]

(11)

\[
K_O = \frac{k_3 + k_3 + k_{10}}{k_3 + k_3 + k_3 + k_3}
\]

(12)

\[
S_{\text{CO}_2} = \frac{k_6 k_6 + k_6 k_6}{k_6 k_6 + k_6 + k_6 + k_6}
\]

(13)

where the subscript indicates the transition state as numbered in Fig. 1 by the black circles. The approximations in Equations 11–13 are made by assuming that the rates of decarboxylation (\( k_h \)) and deoxygenation (\( k_d \)) are negligible.

These first-order rate constants can be related to temperature using transition state theory and the Eyring equation

\[
k = \frac{k_B T}{h} e^{-\Delta G^\ddagger / RT}
\]

(14)

where \( k_B \) is the Boltzmann constant \( (1.3807 \times 10^{-23} \text{ J K}^{-1}) \), \( h \) is the Planck constant \( (6.6261 \times 10^{-34} \text{ J s}) \), and \( \Delta G^\ddagger \) \( (\text{J mol}^{-1}) \) is the standard free energy difference between the transition state and the substrate (or intermediate). Note that the proportionality constant \( K \), describing the proportion of vibrations that lead to product formation, has been assumed equal to one and left out of the equation. The \( \Delta G^\ddagger \) has components of entropy (\( \Delta S^\ddagger \)) and enthalpy (\( \Delta H^\ddagger \)) as defined by

\[
\Delta G^\ddagger = \Delta H^\ddagger - T \Delta S^\ddagger
\]

(15)

where the double dagger symbol (\( ^{\ddagger} \)) denotes the transition state.

**Modeling rate constants (\( k \)) and \( \Delta G^\ddagger \)**

The proposed Rubisco reaction mechanism (Fig. 1) suggests that \( k_{\text{catCO}_2}, k_{\text{catO}_2}, K_C, K_O, \) and \( S_{\text{CO}_2} \) are described by complex terms made up of two or more elementary reaction rates (Farquhar, 1979; Equations 9–13). The rate constant (\( k \)) is related to the energy barrier for the transition state of the reaction, often referred to as the activation energy (\( \Delta G^\ddagger \)). The relationship between \( k, \Delta G^\ddagger \), and temperature is described by the Eyring equation (Equation 14), where \( \Delta G^\ddagger \) has enthalpic (\( \Delta H^\ddagger \)) and entropic (\( \Delta S^\ddagger \)) components (Equation 15). From Equation 15, a plot of \( \Delta G^\ddagger \) with temperature has a slope of \( \Delta S^\ddagger \) and a y-intercept of \( \Delta H^\ddagger / T \). For the discussion of Rubisco kinetics, all numbering of \( k, \Delta G^\ddagger, \Delta H^\ddagger, \) and \( \Delta S^\ddagger \) refers to reaction steps initially described by Farquhar (1979) and reproduced in Fig. 1. The Eyring equation has been previously used to calculate \( \Delta G^\ddagger \) values for \( k_{\text{catCO}_2}, k_{\text{catO}_2} \) and \( S_{\text{CO}_2} \) (Chen and Spreitzer, 1992; Tcherkez et al., 2006; McNevin et al., 2007; Tcherkez, 2013). Because \( k_{\text{catCO}_2} \) and \( k_{\text{catO}_2} \) are first-order rate constants, they have been represented as

\[
-\ln \left( \frac{k_{\text{catCO}_2}}{k_h k_h} \right) \frac{RT}{k_h} \Delta G^\ddagger = \Delta H^\ddagger / k_{\text{catCO}_2} + T \Delta S^\ddagger
\]

(16)
and because $S_{C/O}$ is the ratio of two first-order rate constants (Equation 13), it has been represented as

$$\ln \left( \frac{k_{catO2}}{k_{catCO2}} \right) RT = \Delta G_{catO2}^\circ = \Delta H_{catO2}^\circ - T \Delta S_{catO2}^\circ$$

(17)

The $\Delta G^\circ$ terms in Equations 16–18 can be calculated directly from measured values, and the $\Delta H^\circ$ and $\Delta S^\circ$ terms would describe a linear fit of $\Delta G^\circ$ to the temperature response, assuming $\Delta H^\circ$ and $\Delta S^\circ$ are constant within the temperature range. However, the use of Equations 16–18 does not provide information regarding an elementary rate constant or a corresponding energy barrier. Modeling to estimate individual rate constants from the measured data is described below.

**Results**

**Breakpoints**

The Davies test indicated significant breakpoints for the $k_{catCO2}$, $k_{catO2}$, and $S_{C/O}$ temperature response for the MIMS data as well as for the radiolabel single point measurement of $k_{catCO2}$ (Table 1; Figs 2, 4). Both the Davies test and the maximum likelihood segmented analysis indicated that the breakpoints in these parameters were near 25 °C (Table 1). All other parameters showed no breakpoints in their temperature responses for either the MIMS or radiolabel data sets (Table 1; Figs 2–4).

**Arrhenius activation energies and modeled value at 25 °C**

The modeled 25 °C values ($k_{35}$) and Arrhenius activation energy ($E_a$) above 25 °C agree with many of the literature values for other C4-type Rubisco, including in *in vitro* and *in vivo* measurements of *A. thaliana* (Flexas et al., 2007; Whitney et al., 2011; Walker et al., 2013; Weise et al., 2015; Galmés et al., 2016). Although, previous reports of Rubisco specificities for CO2 over O2 ($S_{C/O}$) at 25 °C vary widely for C4 species, including for *A. thaliana* which range from below 2125 to above 2655 (Pa$^{-1}$; Flexas et al., 2007; Whitney et al., 2011; Walker et al., 2013; Weise et al., 2015). For the MIMS-derived parameters with breakpoints ($k_{catCO2}$, $k_{catO2}$, and $S_{C/O}$), and the radiolabel single point estimate of $k_{catCO2}$, the lower temperature $E_a$ values were larger than $E_a$ values estimated at higher temperatures (Tables 2, 3). Above 25 °C, the $E_a$ values were similar for all parameters between the radiolabel and MIMS curve fitting methods. The radiolabel $E_a$ for $k_{catCO2}$ determined by curve fitting across all temperatures was intermediate to the two $E_a$ values estimated above and below the breakpoint from the single point radiolabel data. The $k_{35}$ values for $k_{catCO2}$ estimated from radiolabel and MIMS methods were not different from each other, but were larger than the $k_{35}$ for $k_{catCO2}$ determined by MIMS (Table 2). The $E_a$ and $k_{35}$ values for $K_C$ and $K_O$ were not significantly different between methods (Table 3). However, the MIMS $S_{C/O}$ estimated from 10 °C to 25 °C had a lower (more negative) $E_a$ value than the MIMS $S_{C/O}$ $E_a$ value measured from 25 °C to 40 °C and the radiolabel $S_{C/O}$ $E_a$ value (Table 3).
The Ea value for the carboxylation efficiency (katCO2/Kc) below 25 °C was significantly different from zero for the MIMS method, where the carboxylation efficiency increased with temperature; however, above 25 °C, the Ea value was not significantly different from zero (Table 4). The Ea for Kc/Ko was significantly different from zero for both radiolabel and MIMS methods (Table 4).

**Table 1. Testing for thermal breaks for all kinetic parameters**

| Method  | Parameter          | Davies test  | Maximum likelihood |
|---------|--------------------|--------------|--------------------|
|         |                    | Estimated breakpoint (°C) | P-value | Estimated breakpoint (°C) | CI (lower) | CI (upper) |
|         |                    |              |                    |                    |            |            |
| Radiolabel | katCO2             | 26.8         | *                  | 25.1              | 5.3        | 36.9        |
|          | single point       |              |                    |                    |            |            |
|          | katCO2 curve fit   |              |                    |                    |            |            |
|          | Kc                 |              | ns                 |                    |            |            |
|          | Ko                 |              | ns                 |                    |            |            |
|          | Sc/O               |              | ns                 |                    |            |            |
| MIMS     | katCO2             | 25.3         | *                  | 25.3              | 23.1       | 31.5        |
|          | katCO2             | 25.3         | *                  | 25.5              | 24.3       | 32.6        |
|          | Kc                 |              | ns                 |                    |            |            |
|          | Ko                 |              | ns                 |                    |            |            |
|          | Sc/O               |              | ns                 |                    |            |            |

Arrhenius plots were examined using the package “segmented” in R (R Core Team, 2013), which determines the significance of breakpoints in linear models and estimates breakpoint locations as described by Davies (1987). Additionally, breakpoint locations and confidence intervals (CIs, lower and upper) were independently estimated using a maximum likelihood test (Muggeo, 2003, 2008). * indicates a P-value <0.05 for the Davies test and ns is not significant.

**Fig. 2.** The natural log of Rubisco parameters from Arabidopsis thaliana measured using radiolabel (single point, open circle; curve fit, black circle) and MIMS (gray circle) methods are plotted against the inverse of the temperature in Kelvin offset to a y-intercept of 25 °C. The temperature response of catalytic turnover for CO2 (katCO2, s⁻¹, A) and O2 (katO2, s⁻¹, B), and the Michaelis–Menten constant for CO2 (Kc, Pa, C) and O2 (Ko, kPa, D) are shown. The lines represent the model fit to the measured data. The radiolabel katCO2 model in (B) was determined from the relationship with Sc/O described by Equation 4.

The Ea for the carboxylation efficiency (katCO2/Kc) below 25 °C was significantly different from zero for the MIMS method, where the carboxylation efficiency increased with temperature; however, above 25 °C, the Ea value was not significantly different from zero (Table 4). The MIMS Ea for oxygenation efficiency (katO2/Ko) was significantly different from zero above and below 25 °C (Table 4). The Ea for the ratio of catalytic rates (katCO2/katO2) measured by MIMS was only significantly different from zero above 25 °C (Table 4).

**Modeling k and ΔG‡**

Above 25 °C, the ΔG‡ for Sc/O from radiolabel and MIMS (Fig. 5) are similar to previous calculations for C3 species reported by Tcherkez et al. (2006). However, the MIMS entropy difference between O2 and CO2 addition (ΔS‡ CO2/ΔS‡ O2),...
The free energy of activation associated with $k_{\text{catCO}_2}$ ($\Delta G_{k\text{catCO}_2}^\ddagger$) plotted against temperature increased linearly for the radiolabel curve fit method, while the $\Delta G_{k\text{catO}_2}^\ddagger$ calculated from MIMS measurements decreased from 10 °C to 25 °C and then increased from 25 °C to 40 °C (Fig. 6). A similar temperature response was also observed for MIMS $\Delta S_{k\text{catO}_2}^\ddagger$, although the absolute values of $\Delta G_{k\text{catCO}_2}^\ddagger$ are larger than $\Delta G_{k\text{catCO}_2}^\ddagger$ as evident by a lower $k_{\text{catO}_2}$ compared with $k_{\text{catCO}_2}$ at all temperatures (i.e. larger energy barriers result in slower reactions). The slope of $\Delta G_{k\text{catCO}_2}^\ddagger$ values presented in Fig. 6 (equivalent to the entropy term $\Delta S^\ddagger$; see Supplementary Table S4) calculated for radiolabel and MIMS above 25 °C are slightly larger than those reported for Nicotiana tabacum (McNevin et al., 2007). The MIMS $\Delta S_{k\text{catCO}_2}^\ddagger$ and $\Delta S_{k\text{catO}_2}^\ddagger$ showed a sign change above and below the breakpoint (negative slope to positive slope, Fig. 6; Supplementary Table S4).

Temperature responses of the rate constants ($k$) and corresponding energy barriers of the transition states ($\Delta G^\ddagger$) are shown in Fig. 7, while the modeled $\Delta H^\ddagger$ and $\Delta S^\ddagger$ values are presented in Supplementary Table S5. Calculations of elementary rate constants and corresponding $\Delta G^\ddagger$ are similar to previous calculations at 25 °C from Tcherkez (2013, 2016). In order to model breakpoints in the MIMS $k_{\text{catCO}_2}$, $k_{\text{catO}_2}$, and $S_{\text{CO}_2/O}$ parameters, breakpoints are needed in the rate constants for the cleavage ($k_s$ and $k_i$) and for gas addition ($k_p$ and $k_g$). This is required because it was not possible to model a simultaneous change in the rate-limiting step for both the $k_{\text{catCO}_2}$ and $k_{\text{catO}_2}$ parameter (Supplementary Fig. S2). This further required that breakpoints were needed in the rate constants for CO$_2$ and O$_2$ addition ($k_p$ and $k_g$, respectively) to maintain the observed linearity for the $K_C$ and $K_O$ Arrhenius plots (Fig. 2).
Table 2. Comparison of k_{25} and E_a values for k_{cat} measurements from the different methods

| Method     | Temperature (°C) | Parameter    | k_{25} (s^{-1}) | E_a (kJ mol^{-1}) |
|------------|------------------|--------------|-----------------|------------------|
| Radiolabel | 0–25             | k_{catCO2}   | 3.50 ± 0.20 A   | 79.53 ± 2.03 A   |
|            | 25–40            |              | –               | 42.11 ± 3.45 c   |
| Curve fit  | 10–35            |              | 3.10 ± 0.07 A   | 59.64 ± 3.93 b   |
| MIMS       | 10–25            |              | 3.53 ± 0.25 A   | 90.36 ± 1.03 a   |
|            | 25–40            |              | –               | 62.20 ± 2.68 b   |
|            | 10–25            | k_{catCO2}   | 1.38 ± 0.05 B   | 92.95 ± 7.31 a   |
|            | 25–40            |              | –               | 47.11 ± 2.33 b,c |

The k_{25} and E_a values are the mean of 3–4 replicates, calculated from linear regressions of Arrhenius plots. The temperature ranges for each regression were determined by segment analysis. Letters indicate significant differences between groups (Tukey HSD, P<0.05).

Table 3. Comparison of K_{C}, K_{O}, S_{C/O}, parameters k_{25} and E_a resulting from the different methods

| Method     | Temperature range (°C) | Parameter | k_{25} (Pa) | E_a (kJ mol^{-1}) |
|------------|-------------------------|-----------|-------------|------------------|
| Radiolabel | 10–35                   | K_{C}     | 36 ± 2      | 63.09 ± 6.23     |
|            | 10–40                   | K_{O}     | 34 ± 1      | 62.62 ± 3.44     |
| Radiolabel | 15–35                   | S_{C/O}   | 2003 ± 22   | –28.66 ± 0.51 b  |
| MIMS       | 10–40                   |           | 24 400 ± 701| 17.01 ± 2.48     |
|            | 10–25                   |           | 1814 ± 117  | –48.19 ± 4.17    |
|            | 25–40                   |           | –           | –30.51 ± 6.41 b  |

No differences were observed in k_{25} between methods. No differences were observed in E_a values for K_{C} and K_{O} values between methods (ANOVA). The letters next to the E_a values indicate significant differences for the S_{C/O} values (Tukey HSD, P<0.05).

Table 4. The E_a and k_{25} parameters for k_{catCO2}/K_{C}, k_{catO2}/K_{O}, k_{catCO2}/k_{catO2}, and K_{O}/K_{C} ratios

| Method     | Temperature range (°C) | Parameter    | k_{25} (s^{-1} Pa^{-1}) | E_a (kJ mol^{-1}) |
|------------|-------------------------|--------------|-------------------------|------------------|
| Radiolabel | 10–35                   | k_{catCO2}/K_{C} | 0.09 ± 0.00             | –3.45 ± 3.94     |
|            | 10–25                   | (s^{-1} Pa^{-1}) | 0.10 ± 0.01             | 27.75 ± 3.38*    |
|            | 25–40                   |              | –                      | –0.41 ± 6.10     |
| MIMS       | 10–25                   | k_{catO2}/K_{O} | 0.06 ± 0.00             | 75.93 ± 7.41*    |
|            | 25–40                   | (s^{-1} Pa^{-1}) | –                      | 30.09 ± 0.70*    |
| MIMS       | 10–25                   | k_{catCO2}/k_{catO2} | 2.55 ± 0.16             | –2.58 ± 6.73     |
|            | 25–40                   |              | –                      | 15.10 ± 4.92*    |
| Radiolabel | 15–35                   | K_{O}/K_{C}  | 0.65 ± 0.11             | –46.20 ± 8.80*   |
| MIMS       | 10–40                   | (Pa^{-1})    | 0.71 ± 0.01             | –45.60 ± 2.57*   |

The E_a parameters were tested to determine if they were significantly different from zero (t-test), where the * next to the E_a values indicates a P-value <0.05.

Discussion

Radiolabel single point k_{catCO2} breakpoint

The radiolabel single point method reported here utilized a single bicarbonate concentration with temperature (11 mM) and resulted in a thermal breakpoint similar to Björkman and Pearly (1970). Because Björkman and Pearly (1970) suggested that there could be inhibition at low temperature and subsaturating concentrations at high temperature, we plotted the predicted CO2 concentration achieved by 11 mM NaHCO3 at each temperature against the measured and modeled CO2 response of the enzyme determined by both radiolabel and MIMS curve fitting methods (Supplementary Fig. S3). The CO2 concentration provided by the 11 mM NaHCO3 appears saturating at 10 °C and 15 °C, but becomes increasingly less saturating at higher temperatures, as indicated where the shaded area intersects the modeled CO2 response (Supplementary Fig. S3). This suggests that the lower E_a value of the single point method at high temperatures could be caused by subsaturating CO2 concentrations.

MIMS k_{catCO2}, k_{catO2}, and S_{C/O} breakpoints

The non-linearity of Arrhenius plots of k_{catCO2}, k_{catO2}, and S_{C/O} for the MIMS data were interpreted as 25 °C breakpoints. Badger and Collatz (1977) also observed breakpoints in k_{catCO2}, k_{catO2}, and S_{C/O}; however, they observed an additional thermal breakpoint in K_{C}, which was not observed with the MIMS data presented here. As S_{C/O} is a ratio of k_{catCO2}/K_{C}, K_{O}, and k_{catO2} (Equation 4), the differences in S_{C/O} breakpoints between Badger and Collatz (1977) and our MIMS data could suggest different mechanisms driving the thermal response of S_{C/O}. Furthermore, no breakpoint in S_{C/O} has been observed in any study using the [3H]RuBP method.

The breakpoints observed in MIMS k_{catCO2} and k_{catO2} are unlikely to be caused by insufficient or inhibitory CO2 concentrations, as subsaturation or inhibition should be evident in the CO2 response curves (Supplementary Fig. S3). A breakpoint in both k_{catCO2} and k_{catO2} could be caused by deactivation of the enzyme, as was suggested by Kubien et al. (2003). However, deactivation is unlikely to change the k_{catCO2}/k_{catO2} temperature response as was observed in Fig. 3C, because both catalytic rates are expected to be affected in the same way by deactivation. Alternatively, the observed breakpoints in MIMS could be related to methodology as the radiolabel Arrhenius plots presented here for k_{catCO2} and S_{C/O} were sufficiently linear.
The Rubisco kinetic parameters for *A. thaliana* measured with the radiolabel and MIMS curve fitting methods were similar at and above 25 °C, suggesting similar kinetic parameters under these conditions, despite slight differences in plant growth environments, as well as sample extraction and assay conditions. However, at lower temperatures, the observed breakpoints in MIMS and the corresponding linearity of the Radiolabel temperature responses could imply that plant-specific growth differences were important. For example, spinach Rubisco appears to acclimate to growth temperature, with warm-grown Rubisco showing a thermal breakpoint in the carboxylation rate at 15 °C, below which rates are lower than those of a cold-grown enzyme (Yamori et al., 2006). This is similar to the breakpoint evident in the MIMS data set presented here; however, the daytime temperature differential between plants grown for the MIMS (23 °C) and radiolabel (20 °C) plants was much smaller than the 15 °C differential used by Yamori et al. (2006). Further, the MIMS technique had a lower S_C/O than radiolabel parameters at temperatures above 25 °C, and a higher value at temperatures below 25 °C, opposite to what Yamori et al. (2006) observed, suggesting that the kinetic differences between the MIMS and radiolabel measurements were not due to temperature acclimation of Rubisco.

The possibility remains that the differences, particularly at cold temperatures, are due to methodology artifacts arising from differences in buffer composition. However, preparations of Rubisco for MIMS or radiolabel assays both include components known to affect Rubisco stability (i.e. DTT, MgCl₂, and NaHCO₃) albeit at different concentrations. It is also possible that either the MIMS or the radiolabel assays cause erroneous kinetic estimates at low temperatures; however, this uncertainty is difficult to explain given that breakpoints have been observed by different laboratories using varying methods and species (Badger and Collatz, 1977; Sage, 2002, Kubien et al., 2003; Sharwood et al., 2016). Therefore, additional analysis of diverse species with the MIMS system is needed to better understand if this is a technique- or species-specific phenomenon.

Nevertheless, breakpoints have persisted in the Rubisco literature for >40 years without sufficient explanation and warrant further investigations into their underlying causes. Badger and Collatz (1977) suggested that changes in the rate-limiting step of the reaction mechanism were brought about by conformational changes. If the elementary rate constants defining a specific parameter have different temperature responses then this could cause breakpoints if they cross over, causing a change in the rate-limiting step. The discussion below utilizes the currently accepted reaction mechanism of Rubisco (Fig. 1) and transition state theory to explore breakpoints as a function of changes in energy barriers to elementary reactions.

**Rubisco reaction mechanisms and breakpoints**

For the MIMS data, the breakpoints observed in *k_catCO₂* and *k_catO₂* could be due to changes in the rate-limiting step, as suggested by Badger and Collatz (1977). For example, *k_catCO₂* is a function of the rate of cleavage of the carboxylated intermediate (*k₈*) and the rate of RuBP enolization (*k₉*). This would mean that *k₈* and *k₉* have different temperature responses such that they cross over at around the breakpoint observed at 25 °C. However, modeling this change in rate-limiting steps due to different temperature responses cannot simultaneously explain the observed breakpoint in *k_catCO₂* and *k_catO₂*, because the value of *k₈* defining the cleavage of the oxygenated intermediate is lower than *k₉*. This means that *k₈* cannot cross over both *k₉* and *k₉* at 25 °C (Supplementary Fig. S2).

In order to model the reaction mechanism suggested by MIMS measurements, breakpoints in four elementary rate constants (*k₂, k₃, k₅, and k₆*) are needed to describe the breakpoints in *k_catCO₂*, *k_catO₂*, and S_C/O (Fig. 7D, E). While it seems unlikely that such an entropy change could be driven by a conformational change in the enzyme brought about by such minimal changes in temperature, a similar change in entropy...
for $k_{\text{cat CO}_2}$ was observed between wild-type *N. tabacum* and a mutant (L335V) Rubisco (McNevin *et al.*, 2007). This could suggest that the entropy changes proposed here may be possible given enzyme conformational changes with temperature.

The modeling presented here is largely based on isotope exchange studies, which suggest similar energy barriers between enolization ($\Delta G^\ddagger_9$) and cleavage ($\Delta G^\ddagger_8$). However, these measurements have been limited to 25 °C (Van Dyk and Schloss, 1986; Tcherkez *et al.*, 2013), and extension of isotope exchange studies to temperature responses would help constrain how the elementary rate constants vary with temperature. Contrary to the above proposal that the cleavage transition state ($k_8$) undergoes changes above and below 25 °C, is that Rubisco discrimination against $^{13}$CO$_2$ is believed to remain constant with temperature (Christeller and Laing, 1976). If the rate of cleavage ($k_8$) decreases, then the decarboxylation reaction ($k_7$) may increase, or the $k_7/k_8$ ratio could increase, which would change Rubisco discrimination against $^{13}$CO$_2$. Furthermore, the above modeling relies on the assumption that decarboxylation ($k_7$) was negligible at all temperatures; therefore, changes in fractionation with temperature for an enzyme exhibiting breakpoints should help test the validity of these assumptions.

**Conclusion**

The measured temperature responses of Rubisco kinetic parameters were consistent between methods at and above 25 °C; however, there were thermal breakpoints at 25 °C in the MIMS data set for $k_{\text{cat CO}_2}$, $k_{\text{cat O}_2}$, and $S_{C/O}$. Additionally, the radiolabel method

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Fig. 7. A kinetic energy barrier diagram showing the modeled temperature responses of the energy barrier to the transition state ($\Delta G^\ddagger_8$) and the corresponding first-order rate constant $k$. The $\Delta G^\ddagger$ and $k$ are indicated by the numbered step of the reaction following Fig. 1. The assumptions made for this model are stated in the Materials and methods. For steps 3 and 6 (O$_2$ and CO$_2$ addition, respectively), the rate constants were multiplied by ambient concentrations O$_2$ (21 kPa) and CO$_2$ (41 Pa) as a pseudo-first-order approximation for comparison with the other rate constants and to calculate their respective $\Delta G^\ddagger$. For the bottom figure, the left-hand column is modeled on the radiolabel data and the right-hand column on the MIMS data so that comparisons between continuous and breakpoint temperature responses can be made. The values for intermediates were taken from Tcherkez (2013) for (A) and Tcherkez (2016) for (B) and assumed to remain constant with temperature.
using a single bicarbonate concentration showed a breakpoint for $k_{\text{cat CO}_2}$ probably caused by non-saturating CO$_2$ concentrations at higher temperatures. Previous studies suggest that breakpoints are caused by either a change in the rate-limiting step of the reaction mechanism or deactivation of the enzyme at low temperatures. By modeling elementary steps of the reaction mechanism, we showed that neither cause is sufficient to explain simultaneous breakpoints in $k_{\text{cat CO}_2}$, $k_{\text{cat O}_2}$, and $S_{\text{C/O}_2}$. Instead, breakpoints in the elementary rate constants would be needed. Because the modeling presented here is largely based on isotope exchange studies, moving forward, the temperature response of isotopic substitution experiments would advance our understanding of how elementary rate constants change in relation to one another with temperature.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Temperature response of Rubisco parameters from Arabidopsis thaliana measured using radiolabel and MIMS methods.

Fig. S2. Two possible crossover models that result in breakpoints for $k_{\text{cat CO}_2}$ for MIMS data.

Fig. S3. CO$_2$ response curves from 10°C to 40°C showing measured values from the radiolabel and MIMS curve fitting methods.

Table S1. pKa values used in calculations.

Table S2. Average Rubisco kinetic parameters measured at each a temperature with ±SE.

Table S3. The $\Delta H^\theta$ and $\Delta S^\theta$ calculated for the $\Delta G^\theta$ values presented in Fig. 5 using Equation 18.

Table S4. The $\Delta H^\theta$ and $\Delta S^\theta$ calculated for the $\Delta G^\theta$ values presented in Fig. 6 using Equations 16 and 17.

Table S5. The $\Delta H^\theta$ and $\Delta S^\theta$ calculated for the $\Delta G^\theta$ values presented in Fig. 7 using Equations 9–15.

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Author contributions

ABC and DSK proposed the original concept and design for the project; RAB and APC performed the experiments and data analysis; RAB wrote the article with the contributions of all the authors; ABC supervised and complemented the writing.

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