

1 **Title: Temperature response of Rubisco kinetics in *Arabidopsis thaliana*: thermal**
2 **breakpoints and implications for reaction mechanisms.**

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4 Running title: **Rubisco kinetic thermal breakpoints and reaction mechanisms**

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20 ABSTRACT

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

35

36 Keywords: Rubisco, Temperature, Kinetic breakpoints, Membrane inlet mass spectrometry,

37 *Arabidopsis*, reaction mechanisms

38

39 INTRODUCTION

40 The enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the
41 reaction of CO₂ or O₂ with Ribulose-1,5-bisphosphate (RuBP) initiating the photosynthetic
42 carbon reduction cycle or photorespiratory cycle, respectively (Bowes *et al.*, 1971; Andrews *et*
43 *al.*, 1973). Kinetic studies on Rubisco typically report the Michaelis-Menten constants for
44 carboxylation (K_C) and oxygenation (K_O), the catalytic rate of carboxylation (k_{catCO_2}) and
45 oxygenation (k_{catO_2}), and the specificity of the enzyme for CO₂ over O₂ ($S_{C/O}$) as these parameters
46 are used for modeling leaf gas exchange (von Caemmerer, 2000). Each of the above Michaelis-
47 Menten parameters is a combination of elementary rate constants that describe the reaction
48 mechanism; however, the rate constants are less well studied (Tcherkez, 2013; Tcherkez, 2016).
49 Optimization of Rubisco kinetics for enhanced CO₂ reduction has been proposed (Spreitzer and
50 Salvucci, 2002), but this effort is limited by our current understanding of the reaction mechanism
51 (Tcherkez *et al.*, 2006; Tcherkez, 2013).

52 The carboxylation and oxygenation reaction mechanisms can be separated into
53 elementary rate constant as originally proposed by Farquhar (1979), reviewed by Tcherkez
54 (2013) and reproduced in Figure 1. Since the initial description of the reaction mechanism
55 (Hurwitz *et al.* 1956) there has been slow progress in defining rate constants due to experimental
56 difficulties in isolating their individual effects. However, the use of membrane inlet mass
57 spectrometry (MIMS) to study Rubisco kinetics may hold promise. The traditional Radiolabel
58 method used in most Rubisco publications relies on ¹⁴C assays to determine k_{catCO_2} , K_C , K_O , a
59 separate ³H assay to determine $S_{C/O}$, leaving k_{catO_2} to be calculated. Alternatively, the MIMS
60 assay simultaneously measures changing concentrations of CO₂ and O₂ and can therefore
61 determine all kinetic parameters with a single assay (Cousins *et al.*, 2010; Boyd *et al.*, 2015). An

62 advantage of the MIMS method is that in addition to the abundant isotopologues of CO₂ (¹²CO₂)
63 and O₂ (¹⁶O₂) the system can monitor less abundant stable isotopologues such as ¹³CO₂ and
64 ¹⁶O¹⁸O. Measurements of primary kinetic isotope effects have been useful in defining enzyme
65 reaction mechanisms (O’Leary *et al.*, 1992); therefore, the MIMS system may provide new
66 information regarding the individual rate constants. At 25 °C the MIMS method has been used
67 for determining both Rubisco carbon fractionation (McNevin *et al.*, 2006; McNevin *et al.*, 2007;
68 Tcherkez *et al.*, 2013), and Michaelis-Menten constants of the carboxylation (v_c) and
69 oxygenation (v_o) reactions (Cousins *et al.*, 2010). Additionally, it was used to determine the
70 temperature dependencies of the Rubisco kinetic parameters in the C₄ species *Setaria viridis*
71 (Boyd *et al.*, 2015). However, previous work using the Radiolabel method suggest lower E_a
72 values for V_{cmax} in C₄ species than that measured by Boyd *et al.* (2015; Sharwood *et al.*, 2016;
73 Sage, 2002; Kubien *et al.*, 2003; Perdomo *et al.*, 2015), suggesting comparisons between the
74 MIMS E_a values and the traditional Radiolabel method are needed.

75 Here we measured the temperature response of Rubisco kinetic parameters from
76 *Arabidopsis thaliana* using two methods. First, we used the traditional method involving the use
77 of radiolabeled substrate and analysis of labeled products following the reaction in known
78 concentrations of CO₂ and O₂ (Jordan and Ogren, 1981), which we referred to as the Radiolabel
79 method. Secondly, we used the MIMS method following the simultaneous consumption of CO₂
80 and O₂ over time, giving a direct measure of v_c , v_o , CO₂, and O₂ leading to simultaneous
81 determination of k_{catCO_2} , k_{catO_2} , K_C , K_O , and $S_{C/O}$ (Cousins *et al.*, 2010; Boyd *et al.*, 2015).
82 Additionally, for the Radiolabel method we compared curve fitting CO₂ responses to determine
83 K_C and k_{catCO_2} simultaneously in an O₂ free buffer, and k_{catCO_2} determined at a single bicarbonate
84 concentration at all temperatures in open air. The later is a common practice for determining

85 k_{catCO_2} temperature responses (Tieszen and Sigurdson, 1973; Sage *et al.*, 1995; Crafts-Brander
86 and Salvucci, 2000; Pittermann and Sage, 2000; Sage, 2002; Kubien *et al.*, 2003; Perdomo *et al.*,
87 2015).

88 Recently, the existence of breakpoints in the k_{catCO_2} temperature response was highlighted
89 as a source of variability in the Rubisco temperature response literature (Sharwood *et al.*, 2016).
90 Initial observations of breakpoints in V_{cmax} temperature responses were determined to be a
91 methodological artifact due to the use of a single bicarbonate concentration at all temperatures
92 and were corrected by varying bicarbonate concentration with temperature (Björkman and
93 Percy, 1970). However, breakpoints were later observed for k_{catCO_2} , k_{catO_2} , and K_C at 15 °C using
94 a curve fitting method (Badger and Collatz, 1977). It was suggested that these breakpoints could
95 be due to changes in rate limiting steps of the reaction mechanism caused by changes in enzyme
96 conformation (Badger and Collatz, 1977). An additional breakpoint was reported in the k_{catCO_2} of
97 *Oryza sativa* at 22 °C (Sage, 2002) and Kubien *et al.* (2003) observed different temperature
98 responses when k_{catCO_2} was measured from 0 to 12 °C compared to 18 to 42 °C in *Flaveria*
99 *bidentis*. Most recently, Sharwood *et al.* (2016) observed breakpoints in k_{catCO_2} at 25 °C for
100 Panicoid grasses when using a curve fitting method. Inconsistencies are evident between studies,
101 and it is unclear if breakpoints are universal to all temperature response studies of plant Rubisco.
102 Here, we discuss the possible causes of breakpoints, focusing on the three previously proposed
103 causes of breakpoints: erroneous bicarbonate concentrations, changes in rate limiting step of the
104 reaction mechanism, and deactivation of Rubisco at low temperature, using the Radiolabel and
105 MIMS data sets reported here.

106

107

108 MATERIALS AND METHODS

109

110 **Plant Growth**

111 Plants for the Radiolabel method were grown and assayed at the University of New
112 Brunswick, Fredericton, Canada. *Arabidopsis thaliana* (Col-0) seeds were stratified for 3 days at
113 4 °C on Promix (Plant Products, Brampton, Canada), transferred to a growth chamber (E-15,
114 Conviron, Winnipeg, Manitoba, Canada) and grown under photoperiod conditions of 10 h
115 light/14 h dark, 20/18 °C, and a photosynthetic photon flux density (PPFD) of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$.
116 Plants were watered with modified Hoagland's solution as needed.

117 Plants for MIMS were grown and assayed at Washington State University, Pullman,
118 Washington, U.S.A. Seeds of *A. thaliana*, ecotype Col-0, were placed in 2 L pots containing
119 commercial soil (LC1 Sunshine Mix, Sun Gro Horticulture, Vancouver, Canada) and grown in
120 an environmental growth chamber (Biochambers GC-16, Winnipeg, Manitoba, Canada) at a
121 PPFD of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at plant height, relative humidity was not controlled, air/night
122 temperature of 23/18 °C, with a 14 hour photoperiod and 10 hours of dark. Plants were fertilized
123 weekly (Peters 20-20- 20, Allentown, PA, USA) and watered as needed.

124

125 **Sampling for Radiolabel Analysis**

126 Leaf punches were obtained at mid-day, flash frozen in liquid nitrogen and stored at -80
127 °C until extraction. Leaf tissue was ground (1.1 cm^2 disks, ca. 20 mg) in a Tenbroeck glass tissue
128 homogenizer containing 3 mL of ice-cold extraction buffer (100 mM HEPES pH 7.6, 2 mM Na-
129 EDTA, 5 mM MgCl_2 , 5 mM dithiothreitol [DTT], 10 mg ml^{-1} polyvinyl polypyrrolidone, 2%
130 (vol/vol) Tween-80, 2 mM NaH_2PO_4 , 12 mM amino-*n*-capronic acid, and 2 mM benzamidine)

131 and 50 μ L Protease Inhibitor Cocktail (Sigma). This leaf homogenate was centrifuged at 16000x
132 g at 4 °C for 60 seconds. The resulting supernatant was then desalted and concentrated as
133 described by Cousins *et al.* (2010), and aliquots were incubated with 20 mM MgCl₂ and 10 mM
134 NaHCO₃ at 30 °C for 20 min to fully carbamylate Rubisco. Rubisco content was quantified using
135 [¹⁴C]carboxy-arabinitol bisphosphate (¹⁴CABP)-binding assay (Ruuska *et al.*, 1998, Kubien *et*
136 *al.*, 2011).

137

138 **Sampling for MIMS Analysis**

139 The youngest fully expanded leaves of plants 30 to 40 days after planting were sampled
140 for Rubisco extraction. The mid vein was removed and approximately 2 g of leaf tissue was
141 ground in 2 mL of ice-cold extraction buffer (100 mM HEPES pH 7.8, 10 mM DTT, 25 mM
142 MgCl₂, 1 mM EDTA, 10 mM NaHCO₃, 1% (g/mL) PVPP, 0.5% (v/v) 100x Triton) with a
143 mortar and pestle on ice. 67 μ L of protease inhibitor cocktail (P9599, Sigma-Aldrich, St. Louis,
144 Missouri) to 2 g of fresh leaf tissue was added to the extraction buffer before grinding. The
145 homogenized extract was centrifuged at 4 °C, for 10 min, at 17000x g. The supernatant was
146 collected and desalted using an Econo Pac 10DG column (Bio-Rad), filtered through a Millex
147 GP 33-mm syringe-driven filter unit (Millipore), and then centrifuged using Amicon Ultra
148 Ultracel 30K centrifugal filters (Millipore) at 4 °C for 1 hour at 3000x g. The layer maintained
149 above the filter unit was collected, brought to 20% (v/v) glycerol, flash frozen in liquid nitrogen,
150 and stored at -80 °C until measured. Rubisco content was determined as described above.

151

152 **Radiolabel Measurement of Rubisco Kinetic Parameters**

153 The maximum carboxylation rate of fully activated Rubisco (V_{cmax}) was measured
154 following the methods of Kubien *et al.* (2011) from 0 to 35 °C, by the incorporation of ^{14}C into
155 acid-stable products. This method is later referred to as the ‘single point’ method. Assays were
156 initiated by the addition of 50 μL of activated extract (as described above) to 250 μL assay buffer
157 (100 mM Bicine-NaOH (pH 8.2), 1 mM Na-EDTA, 20 mM MgCl_2 , 5 mM DTT, 400 μM RuBP,
158 and 11 mM $\text{NaH}^{14}\text{CO}_3$ [~ 700 Bq nmol^{-1}]), and stopped after 30 to 60 seconds by adding 250 μL
159 of 1 M formic acid. Samples were dried at 90 °C, and ^{14}C acid stable products were counted
160 using a scintillation counter (LS-6500, Beckman-Coulter). Michaelis-Menten parameters for CO_2
161 (K_C), and apparent K_C at 21% O_2 ($K_C(21\% \text{O}_2)$) were determined by assaying Rubisco activity in 7
162 mL septum-sealed, N_2 -sparged vials over a range of seven $\text{NaH}^{14}\text{CO}_3$ concentrations (Paul *et al.*,
163 1991; Kubien *et al.* 2008. This analysis, referred to as the ‘curve fitting’ method, gave a separate
164 temperature response of k_{catCO_2} from the single point method described above. Rubisco $S_{\text{C/O}}$ was
165 determined following the method described by Kane *et al.* (1994). Additional details for these
166 assays are presented in the supplemental files.

167

168 **MIMS Measurement of Rubisco Kinetic Parameters**

169 Rubisco assays were conducted in a 600 μL temperature controlled cuvette linked to an
170 isotope ratio mass spectrometer (Thermo-Fischer Delta V) and calibrated as previously described
171 (Cousins *et al.*, 2010; Boyd *et al.*, 2015). Samples were measured similar to Boyd *et al.* (2015);
172 four oxygen concentrations ranging from 40 to 1600 μM , and five CO_2 concentrations ranging
173 from 10 to 200 μM at each oxygen level were made. Measurements were made in 5 °C intervals
174 from 10 °C to 40 °C, and the same three replicates were measured at each temperature. The assay
175 buffer contained 200 mM HEPES (pH 7.7 at each measurement temperature), 20 mM MgCl_2 , 0.1

176 mM α -hydroxypyridinemethanesulfonic acid (α -HPMS), 8 mg mL⁻¹ CA (Sigma), and 0.6 mM
177 RuBP. 10 μ L of extract was added per measurement. Rubisco was activated by leaving the
178 extract at room temperature for 10 minutes prior to returning to ice before measurement.
179 Additional details for these measurements are presented in the supplemental files.

180

181 **Modeling Temperature Responses**

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

$$183 \text{parameter} = k_{25} e^{(-E_a/R T_K)(298.15-T_K)/(298.15)} \quad \text{Eq. 1}$$

184 where k_{25} is the value of the parameter at 25 °C, E_a is the Arrhenius activation energy (kJ mol⁻¹),
185 R is the molar gas constant (0.008314 kJ mol⁻¹ K⁻¹), T_K is the temperature in Kelvin, and the term
186 $(298.15-T_K)/298.15$ is the scaling factor so that k_{25} may be used as the pre-exponential term. The
187 E_a and k_{25} values for each Rubisco parameter were calculated by a linear regression of the natural
188 log of the data plotted against $(T_K-298.15)/(T_K)$, such that the y-intercept was equal to natural log
189 of k_{25} and the slope was equal to $E_a/(298.15 R)$. For comparison the non-transformed
190 temperature response are presented in Supp. Fig. 3. Three replicates of E_a and k_{25} were
191 determined for each parameter, with the exception of Radiolabel $S_{C/O}$ where the replication was
192 four. For all MIMS and Radiolabel comparisons, other than k_{catCO_2} , only the curve fitting
193 methods are compared. For simplicity we exclude the Radiolabel single point when comparing
194 ratios of kinetic parameters to MIMS. Differences in the k_{25} and E_a values were determined by
195 ANOVA, followed by pair-wise comparison (Tukey HSD) with a significance cutoff of $p < 0.05$
196 in Statistix 9 (Analytical Software, Tallahassee, USA).

197 Arrhenius plots for all kinetic parameters were examined for thermal breaks using the
198 package ‘segmented’ in R, which first tests for differences between slopes using the Davies test

199 (Davies 1987), and then estimates the breakpoints in linear models using maximum likelihood
200 (Muggeo 2003; Muggeo 2008; R Core Team, 2013, <http://www.R-project.org/>). When
201 breakpoints in the Arrhenius temperature response plots were statistically valid, the E_a values
202 above and below the break points were compared to other E_a values as described above, the k_{25}
203 value was held constant when fitting for two E_a values above and below the breakpoint.

204

205 Equations for Reaction Mechanisms

206 Figure 1 depicts the currently hypothesized reaction mechanism of Rubisco as originally
207 described by Farquhar (1979). The kinetic parameters k_{catCO_2} , k_{catO_2} , K_C , K_O , and $S_{C/O}$ can be
208 described by the individual first order rate constants (k) seen in Figure 1 as follows:

$$209 \quad k_{catCO_2} = \frac{k_8 k_9}{k_8 + k_9} \quad \text{Eq. 2}$$

$$210 \quad k_{catO_2} = \frac{k_5 k_9}{k_5 + k_9} \quad \text{Eq. 3}$$

$$211 \quad K_C = \frac{k_7 + k_8}{k_6} \frac{k_9 + k_{10}}{k_8 + k_9} \approx k_{catCO_2} \frac{k_9 + k_{10}}{k_9 k_6} \quad \text{Eq. 4}$$

$$212 \quad K_O = \frac{k_4 + k_5}{k_3} \frac{k_9 + k_{10}}{k_5 + k_9} \approx k_{catO_2} \frac{k_9 + k_{10}}{k_9 k_3} \quad \text{Eq. 5}$$

$$213 \quad S_{C/O} = \frac{k_6}{k_3} \frac{k_4 + k_5}{k_7 + k_8} \frac{k_8}{k_5} \approx \frac{k_6}{k_3} \quad \text{Eq. 6}$$

214 where the subscript indicates the transition state as numbered in Figure 1 by the black circles.

215 The approximations in Equations 4, 5, and 6 are made by assuming the rates of decarboxylation
216 (k_7) and deoxygenation (k_4) are negligible.

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

$$219 \quad k = \frac{k_B T}{h} e^{-\Delta G^\ddagger / RT} \quad \text{Eq. 7}$$

220 where k_B is the Boltzmann constant ($1.3807 \cdot 10^{-23}$ J K⁻¹), h is the Planck constant ($6.6261 \cdot 10^{-34}$ J
221 s), ΔG^\ddagger (J mol⁻¹) is the standard free energy difference between the transition state and the
222 substrate (or intermediate). Note the proportionality constant κ , describing the proportion of
223 vibrations that lead to product formation, has been assumed equal to one and left out of the
224 equation. The ΔG^\ddagger has components of entropy (ΔS^\ddagger) and enthalpy (ΔH^\ddagger) as defined by
225
$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger \quad \text{Eq. 8}$$

226 where the double dagger symbol (‡) denotes the transition state.

227

228 **Modeling k and ΔG^\ddagger**

229 The proposed Rubisco reaction mechanism (Fig. 1) suggests k_{catCO_2} , k_{catO_2} , K_C , K_O , and
230 $S_{C/O}$ are described by complex terms made up of two or more elementary reaction rates
231 (Farquhar, 1979; Equations 2 through 6). The rate of an elementary reaction (k) is related to the
232 energy barrier for the transition state of the reaction, often referred to as the activation energy
233 (ΔG^\ddagger). The relationship between k , ΔG^\ddagger , and temperature is described by the Eyring equation
234 (Equation 7), where ΔG^\ddagger has enthalpic (ΔH^\ddagger) and entropic (ΔS^\ddagger) components (Equation 8). From
235 Equation 8, a plot of ΔG^\ddagger with temperature has a slope of ΔS^\ddagger and a y-intercept of ΔH^\ddagger . For the
236 discussion of Rubisco kinetics all numbering of k , ΔG^\ddagger , ΔH^\ddagger , ΔS^\ddagger refer to reaction steps initially
237 described by Farquhar (1979) and reproduced in Figure 1. The Eyring equation has been
238 previously used to calculate ΔG^\ddagger values for k_{catCO_2} , k_{catO_2} , and $S_{C/O}$ (Chen and Spreitzer, 1992;
239 Tcherkez *et al.*, 2006; McNevin *et al.*, 2007; Tcherkez, 2013). Because k_{catCO_2} and k_{catO_2} are first
240 order rate constants they have been represented as

$$241 \quad -\ln\left(k_{\text{catCO}_2} \frac{h}{k_B T}\right) RT = \Delta G_{\text{catCO}_2}^\ddagger = \Delta H_{\text{catCO}_2}^\ddagger - T\Delta S_{\text{catCO}_2}^\ddagger \quad \text{Eq. 9}$$

242 and

243
$$-\ln\left(k_{catO2} \frac{h}{k_B T}\right) RT = \Delta G_{kcatO2}^\ddagger = \Delta H_{kcatO2}^\ddagger - T \Delta S_{kcatO2}^\ddagger, \quad \text{Eq. 10}$$

244 and because $S_{C/O}$ is the ratio of two first order rate constants (Equation 6) it has been represented
245 as

246
$$\ln(S_{C/O}) RT = \Delta G_3^\ddagger - \Delta G_6^\ddagger = (\Delta H_3^\ddagger - \Delta H_6^\ddagger) - T(\Delta S_3^\ddagger - \Delta S_6^\ddagger). \quad \text{Eq. 11}$$

247 The ΔG^\ddagger terms in Equations 9, 10, and 11 can be calculated directly from measured values, and
248 the ΔH^\ddagger and ΔS^\ddagger terms would describe a linear fit to the temperature response, assuming ΔH^\ddagger and
249 ΔS^\ddagger are constant within the temperature range. However, the use of Equations 9, 10, and 11 do
250 not provide information regarding an elementary rate constant or a corresponding energy barrier.
251 Further modeling to estimate individual rate constants from the measured data is described
252 below.

253

254 *Modeling of Radiolabel Data*

255 Each of the rate constants (k) in Figure 1 has a corresponding energy of activation (ΔG^\ddagger)
256 from Equation 7), which has a corresponding enthalpic and entropic component (ΔH^\ddagger and ΔS^\ddagger)
257 from Equation 8). We assumed that the values of ΔH^\ddagger and ΔS^\ddagger are constant within the
258 temperature range. Therefore, we fit Michaelis-Menten parameters calculated from elementary
259 rate constants using Equations 2 through 6 to the measured Michaelis-Menten parameters by
260 varying the corresponding ΔH^\ddagger and ΔS^\ddagger values. All modeling used the solver function in Excel
261 (2016, Microsoft, Redmon, WA, USA) to minimize the sum of the differences squared between
262 modeled and measured parameters.

263 The rate constants k_8 (cleavage of carboxylated intermediate) and k_9 (enolization of
264 RuBP) were calculated from measured k_{catCO2} values following the calculations of Tcherkez *et al.*
265 (2013) such that k_8/k_9 is 0.83 at 25 °C. The rate constant k_{10} (de-enolization) was modeled

266 assuming k_9/k_{10} is 0.43 at 25 °C following the calculations of Tcherkez *et al.* (2013), we further
267 assumed that this ratio remained constant with temperature. This allowed for calculation of the
268 rate of k_6 (CO₂ addition) as the only remaining unknown when fitting measured values of K_C
269 with Equation 4 assuming k_7 (de-carboxylation) was negligible. After calculating k_6 , k_3 (O₂
270 addition) was modeled from measured $S_{C/O}$ values and Equation 6, assuming rate constants k_7
271 (decarboxylation) and k_4 (deoxygenation) are negligible. Finally, the rate constant k_5 (cleavage of
272 the oxygenated intermediate) was calculated from measured K_O values and Equation 7, again
273 assuming k_4 (deoxygenation) was negligible. This process allowed for estimation of the
274 temperature response for k and ΔG^\ddagger values for each step of the reaction mechanism listed in
275 Equations 2 through 6, with the exception of the decarboxylation and deoxygenation steps that
276 were assumed negligible (Tcherkez *et al.*, 2013; Tcherkez, 2013; Tcherkez, 2016).

277

278 *Modeling of MIMS Data*

279 For the MIMS data, where measurements of k_{catO_2} were available and non-linearity of
280 Arrhenius plots were observed, the rate constants and corresponding ΔG^\ddagger , ΔH^\ddagger , and ΔS^\ddagger values
281 were determined differently. The ΔH^\ddagger and ΔS^\ddagger values corresponding to the rate constants for k_8
282 (cleavage of carboxylated intermediate), k_5 (cleavage of oxygenated intermediate), and k_9 (RuBP
283 enolization) were determined by fitting to measured k_{catCO_2} and k_{catO_2} values, assuming k_8/k_9 was
284 0.83 at 25 °C, and using Equations 2 and 3. Because k_{catCO_2} showed a breakpoint, it is possible
285 that k_8 and k_9 have different temperature responses, with a crossover at approximately 25 °C.
286 However, k_{catO_2} also showed a breakpoint at 25 °C and the carboxylated intermediate cleavage rate
287 (k_8) is much greater than the oxygenated cleavage rate (k_5) because measured k_{catCO_2} values are
288 greater than measured k_{catO_2} . Therefore, a crossover of k_5 , k_8 , and k_9 at a single temperature is not

289 possible and a breakpoint in k_{catCO_2} and k_{catO_2} co-occurring at a single temperature cannot be
290 modeled as a changing rate limiting step. Therefore, we modeled the breakpoint in k_{catO_2} by
291 allowing k_5 to have separate ΔH^\ddagger and ΔS^\ddagger values above and below the breakpoint, and assuming
292 k_9 had the same values of ΔH^\ddagger and ΔS^\ddagger above and below the breakpoint. Because k_9 (rate
293 constant of RuBP enolization) temperature response was assumed constant for models of k_{catO_2} , it
294 was also assumed constant when modeling k_{catCO_2} . Therefore, k_8 was allowed to have separate
295 values of ΔH^\ddagger and ΔS^\ddagger above and below the breakpoint. The k_{10} (rate constant of de-eneolization)
296 was subsequently calculated assuming the ratio k_9/k_{10} was 0.43 and constant with temperature.
297 The value k_6 (rate constant of CO_2 addition) was then calculated from measured K_C and the
298 approximation of Equation 4 assuming decarboxylation is negligible. This was also done for k_3
299 (rate constant for O_2 addition) using K_O and the approximation of Equation 5 assuming de-
300 oxygenation (k_4) was negligible. It was required that k_6 and k_3 have separate ΔH^\ddagger and ΔS^\ddagger values
301 above and below the breakpoint to model linear Arrhenius plots of K_C and K_O . This process
302 provided estimates of the temperature response for k and ΔG^\ddagger values for each step of the reaction
303 mechanisms making up the measured Michaelis-Menten parameters (Equations 2 through 6),
304 with the exception of the decarboxylation and deoxygenation steps, which were assumed
305 negligible.

306

307 RESULTS

308 **Breakpoints**

309 The Davies test indicated significant breakpoints for the k_{catCO_2} , k_{catO_2} , and $S_{\text{C/O}}$
310 temperature response for the MIMS data as well as for the Radiolabel single point measurement
311 of k_{catCO_2} (Table 1, Figures 2 and 4). Both the Davies test and the maximum likelihood
312 segmented analysis indicated that the breakpoints in these parameters were near 25 °C (Table1).
313 All other parameters showed no breakpoints in their temperature responses for either the MIMS
314 or Radiolabel data sets (Table1, Figures 2, 3 and 4).

315

316 **Arrhenius Activation Energies and Modeled Value at 25 °C**

317 The E_a , and k_{25} for k_{catCO_2} , k_{catO_2} (Table 2), K_C , K_O , $S_{\text{C/O}}$ (Table 3) and ratios of interest
318 (Table 4) were calculated from the linear regressions shown in Figures 2 through 4. For the
319 MIMS derived parameters with breakpoints (k_{catCO_2} , k_{catO_2} , $S_{\text{C/O}}$), and the Radiolabel single point
320 estimate of k_{catCO_2} the lower temperatures E_a values were larger than E_a values estimated at
321 higher temperatures (Table 2 and 3). Above 25 °C, the E_a values were similar for all parameters
322 between the Radiolabel and MIMS curve fitting methods. The Radiolabel E_a for k_{catCO_2}
323 determined by curve fitting across all temperatures was intermediate to the two E_a values
324 estimated above and below the breakpoint from the single point Radiolabel data. The k_{25} values
325 for k_{catCO_2} estimated from Radiolabel and MIMS methods were not different from each other, but
326 were larger than the k_{25} for k_{catO_2} determined by MIMS (Table 2). The E_a and k_{25} values for K_C
327 and K_O were not significantly different between methods (Table 3). However, the MIMS $S_{\text{C/O}}$
328 measured from 10 to 25 °C had a lower (more negative) E_a value than the MIMS $S_{\text{C/O}}$ E_a value
329 measured from 25 to 40 °C and the Radiolabel $S_{\text{C/O}}$ E_a value (Table 3).

330 The E_a value for the carboxylation efficiency (k_{catCO_2}/K_C) below 25 °C was significantly
331 different from zero for the MIMS method, where the carboxylation efficiency increased with
332 temperature; however, above 25 °C the E_a was not significantly different from zero (Table 4).
333 The MIMS E_a for oxygenation efficiency (k_{catO_2}/K_O) was significantly different from zero above
334 and below 25 °C (Table 4). The E_a for the ratio of catalytic rates ($k_{\text{catCO}_2}/k_{\text{catO}_2}$) measured by
335 MIMS was only significantly different than zero above 25 °C (Table 4). The E_a for K_O/K_C was
336 significantly different from zero for both Radiolabel and MIMS methods (Table 4).

337

338 **Modeling k and ΔG^\ddagger**

339 Above 25 °C the $\Delta G_3^\ddagger - \Delta G_6^\ddagger$ for $S_{C/O}$ from Radiolabel and MIMS (Fig. 5) are similar to
340 previous calculations for C_3 species reported by Tcherkez *et al.* (2006). However, the MIMS
341 entropy difference between O_2 and CO_2 addition ($\Delta S_3^\ddagger - \Delta S_6^\ddagger$, slope of line in Fig. 5, see Eq. 11,
342 Supp. Table 1), from data collected below 25 °C appear more similar to the $\Delta S_3^\ddagger - \Delta S_6^\ddagger$ of red algae
343 rather than higher plants, when compared to data presented in Tcherkez *et al.* (2006).

344 The free energy of activation associated with k_{catCO_2} ($\Delta G_{\text{kcatCO}_2}^\ddagger$) plotted against
345 temperature, increased linearly for the Radiolabel curve fit method, while the $\Delta G_{\text{kcatCO}_2}^\ddagger$
346 calculated from MIMS measurements decreased from 10 to 25 °C and then increased from 25 to
347 40 °C (Fig. 6). A similar temperature response was also observed for MIMS $\Delta G_{\text{kcatO}_2}^\ddagger$, although
348 the absolute values of $\Delta G_{\text{kcatO}_2}^\ddagger$ are larger than $\Delta G_{\text{kcatCO}_2}^\ddagger$ as evident by a lower k_{catO_2} compared
349 to k_{catCO_2} at all temperatures (i.e. larger energy barriers result in slower reactions). The slope of
350 $\Delta G_{\text{kcatCO}_2}^\ddagger$ values presented in Figure 6 (equivalent to the entropy term $\Delta S_{\text{kcatCO}_2}^\ddagger$, Supp. Table 2)
351 calculated for Radiolabel and MIMS above 25 °C are slightly larger than those reported for

352 *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
353 change above and below the breakpoint (negative slope to positive slope, Fig. 6, Supp. Table 2).

354 Temperature responses of the rate constants (k) and corresponding energy barriers of the
355 transition states (ΔG^\ddagger) are shown in Figure 7, while the modeled ΔH^\ddagger and ΔS^\ddagger values are
356 presented in Supplemental Table 3. Calculations of elementary rate constants and corresponding
357 ΔG^\ddagger are similar to previous calculations at 25 °C from Tcherkez (2013) and Tcherkez (2016). In
358 order to model breakpoints in the MIMS k_{catCO_2} , k_{catO_2} , and $S_{\text{C/O}}$ parameters, breakpoints are
359 needed in the rate constants for the cleavage (k_8 and k_5) and for gas addition (k_6 and k_3). This is
360 required because it was not possible to model a simultaneous change in the rate limiting step for
361 both the k_{catCO_2} and k_{catO_2} parameter (Supp. Fig. 2). This further required that breakpoints were
362 needed in the rate constants for CO₂ and O₂ addition (k_6 and k_3 , respectively) to maintain the
363 observed linearity for K_C and K_O Arrhenius plots (Fig. 2).

364

365 DISCUSSION

366 **Temperature Responses of Rubisco Michaelis-Menten Kinetic Parameters**

367 The Rubisco kinetic parameters for *Arabidopsis thaliana* measured with the Radiolabel
368 and MIMS curve fitting methods were similar at and above 25 °C. Additionally, the modeled 25
369 °C values (k_{25}) and Arrhenius activation energy (E_a) above 25 °C agree with many of the
370 literature values for other C₃-type Rubiscos, including *in vitro* and *in vivo* measurements of *A.*
371 *thaliana* (Flexas *et al.*, 2007; Whitney *et al.*, 2011; Walker *et al.*, 2013; Weise *et al.*, 2015;
372 Galmés *et al.*, 2016). Although, previous reports of Rubisco specificities for CO₂ over O₂ ($S_{C/O}$)
373 at 25 °C vary widely for C₃ species, including for *A. thaliana* which range from below 2125 to
374 above 2655 (Pa Pa⁻¹; Flexas *et al.*, 2007; Whitney *et al.*, 2011; Walker *et al.*, 2013; Weise *et al.*,
375 2015).

376 Galmés *et al.* (2016) highlighted contradictory trends in the temperature response of K_O
377 when measured by *in vitro* assay; either increasing or decreasing with temperature (when
378 expressed in units of molarity; converting between molarity and partial pressure changes the
379 temperature response because the solubility of O₂ decreases with temperature). Here, both the
380 Radiolabel and MIMS method show increases in K_O with temperature, with lower E_a values
381 compared to K_C . The two data sets presented here confirm trends from the growing literature on
382 C₃ Rubisco temperature responses, at least for values measured above 25 °C.

383 Alternatively, below 25 °C the Radiolabel and MIMS derived parameters had different
384 temperature responses where the Arrhenius plots of MIMS determined k_{catCO_2} , k_{catO_2} , and $S_{C/O}$
385 were non-linear (Fig. 2, Fig. 4). Different temperature responses at high and low temperatures
386 were interpreted as breakpoints for these kinetic parameters at 25 °C (Fig. 2). However, for the
387 Radiolabel curve fit data all kinetic parameters appeared sufficiently linear. This could suggest

388 methodology artifacts; however, it is difficult to identify methodological errors that may give rise
389 to breakpoints given that they have also been observed by different laboratories using varying
390 methods and species (Badger and Collatz, 1977; Sage 2002, Kubien *et al.*, 2003; Sharwood *et*
391 *al.*, 2016).

392

393 **Evidence for Breakpoints in the Literature**

394 Björkman and Pearcy (1970) first identified a thermal breakpoint occurring in the
395 temperature response of V_{cmax} from two *Atriplex* species. However, in the same publication they
396 determined that the apparent breakpoints were caused by non-saturating or inhibitory bicarbonate
397 concentrations at varying temperatures and, when corrected, they obtained sufficiently linear
398 Arrhenius plots. Subsequently, Badger and Collatz (1977) identified breakpoints in k_{catCO_2} , k_{catO_2} ,
399 and K_C occurring at 15 °C, with sufficiently linear Arrhenius plots of K_O . While Badger and
400 Collatz (1977) did not discuss $S_{\text{C/O}}$, using Equation S6 with their data suggests a breakpoint in
401 $S_{\text{C/O}}$. Badger and Collatz (1977) hypothesized that breakpoints were the result of possible
402 changes in enzyme conformation which change the rate limiting step of the reaction mechanism.
403 Sage (2002) identified breakpoints in k_{catCO_2} at 22 °C for *Oryza sativa*, but observed linear
404 Arrhenius plots for other species. Furthermore, Kubien *et al.* (2003) also observed a breakpoint
405 in k_{catCO_2} between 12 and 18 °C for *Flaveria bidentis*, and suggested it was caused by
406 deactivation of the enzyme at low temperature, possibly by dissociation of the haloenzyme.
407 These analyses generally identified E_a values above the breakpoint similar to what is often
408 reported in the literature for the temperature response of Rubisco (E_a for k_{catCO_2} ~60 kJ mol⁻¹),
409 with larger E_a values at low temperatures.

410 Recently, Sharwood *et al.* (2016) suggested breakpoints occurring at 25 °C for k_{catCO_2} in
411 eleven Panicoid grasses and tobacco. While E_a values at lower temperatures remain larger than
412 the E_a values at higher temperatures, Sharwood *et al.*'s (2016) findings differs from the previous
413 breakpoint publications, because the E_a below the breakpoint is around the expected value (~60
414 kJ mol⁻¹) and the E_a above the breakpoint is lower than expected (~30 kJ mol⁻¹). Sharwood *et al.*
415 (2016) did not observe breakpoints in S_{CO} , but it is worth noting that they calculated S_{CO} from a
416 separate assay from k_{catCO_2} using the ratio of ³H-glycerate to ³H-glycolate as described here for
417 the Radiolabel method. They also did not measure the temperature responses of K_C , K_O or k_{catO_2}
418 limiting direct comparisons to the data presented here.

419

420 **Radiolabel Single Point k_{catCO_2} Breakpoint**

421 The Radiolabel single point method reported here utilized a single bicarbonate
422 concentration with temperature (11 mM). This method resulted in a breakpoint, having an E_a
423 value of 79.5 kJ mol⁻¹ at low temperatures, and 42.1 kJ mol⁻¹ at higher temperatures (Fig. 2,
424 Table 2). Similar to Björkman and Pearcy (1970), the linear Arrhenius plot (Radiolabel curve fit)
425 has an E_a value intermediate to the two E_a values determined when using a single bicarbonate
426 concentration (~59.6 kJ mol⁻¹, Fig. 2, Table 2). Because Björkman and Pearcy (1970) suggested
427 that there could be inhibition at low temperature and sub-saturating concentrations at high
428 temperature, we plotted the predicted CO₂ concentration achieved by 11 mM NaHCO₃ at each
429 temperature against the measured and modeled CO₂ response of the enzyme determined by both
430 Radiolabel and MIMS curve fitting methods (Supp. Fig. 1). The CO₂ concentration provided by
431 the 11 mM NaHCO₃ is less saturating at higher temperatures because the K_C of Rubisco

432 increases with temperature and the pK_a temperature response favors HCO_3^- at higher
433 temperatures (Supp. Fig. 1).

434 From the data presented here, the CO_2 concentration appears saturating at 10 and 15 °C,
435 but becomes increasingly less saturating at higher temperatures, as indicated where the shaded
436 area intersects the modeled CO_2 response. This suggest the lower E_a value of the single point
437 method at high temperatures could be caused by sub-saturating CO_2 concentrations. The sub-
438 saturating CO_2 concentrations is likely due to both an increase in K_C with temperature and the
439 predicted concentration of CO_2 decreases with temperature given the temperature response of the
440 pK_a (Harned and Bonner, 1945). Alternatively, an inhibitory concentrations of CO_2 was not
441 observed under any of the measurement conditions.

442

443 **MIMS k_{catCO_2} , k_{catO_2} , and $S_{C/O}$ Breakpoints**

444 The non-linearity of Arrhenius plots of k_{catCO_2} , k_{catO_2} , and $S_{C/O}$ for the MIMS data were
445 interpreted as 25 °C breakpoints. Badger and Collatz (1977) also observed breakpoints in k_{catCO_2} ,
446 k_{catO_2} , and $S_{C/O}$; however, they observed an additional thermal breakpoint in K_C , which was not
447 observed with the MIMS data presented here. As $S_{C/O}$ is a ratio of k_{catCO_2} , K_C , K_O , and k_{catO_2} (Eq.
448 S6), the differences in $S_{C/O}$ breakpoints between Badger and Collatz (1977) and our MIMS data
449 could suggest different mechanisms driving the thermal response of $S_{C/O}$. Furthermore, no
450 breakpoint in $S_{C/O}$ has been observed in any study using the 3H -RuBP method.

451 The breakpoints observed in MIMS k_{catCO_2} and k_{catO_2} are unlikely to be caused by
452 insufficient or inhibitory CO_2 concentrations, as is possible for the breakpoint observed in the
453 Radiolabel single point k_{catCO_2} measurement, as sub-saturation or inhibition should be evident in
454 the CO_2 response curves (Supp. Fig. 1). A breakpoint in both k_{catCO_2} and k_{catO_2} could be caused

455 by deactivation of the enzyme as was suggested by Kubien *et al.* (2003). However, deactivation
456 is unlikely to change the $k_{\text{catCO}_2}/k_{\text{catO}_2}$ temperature response as was observed in Figure 3C,
457 because both catalytic rates are expected to be affected in the same way by deactivation.
458 Alternatively, the observed breakpoints in MIMS could be related to methodology as the
459 Radiolabel Arrhenius plots presented here for k_{catCO_2} and $S_{\text{C/O}}$ were sufficiently linear.

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

468

469 **Rubisco Reaction Mechanisms and Breakpoints**

470 *Radiolabel modelling*

471 For the Radiolabel data, where all Arrhenius plots were sufficiently linear, a model of
472 how the energy barriers for the Rubisco reaction mechanism change with temperature is
473 presented in Figure 7, Panel C and E, and depicted as a kinetic energy barrier diagram in Panel A
474 and B. As previously suggested by Tcherkez (2013) the k_{catCO_2} and k_{catO_2} values can be modeled
475 assuming identical temperature responses for the rate of enolization (k_9), and cleavage for the
476 carboxylated intermediate (k_8) and oxygenated-intermediate (k_5). Interestingly, the modeled
477 addition of CO_2 (ΔG_6^\ddagger) had high entropic cost leading to a decreasing temperature response for

478 the rate of CO₂ addition (k_6), suggesting the reaction becomes slower with increasing
479 temperature. Additionally, the increase in the energy barrier for CO₂ addition (ΔG_6^\ddagger) is greater
480 than that for O₂ addition (ΔG_3^\ddagger) such that the ratio k_6/k_3 decreased with temperature. This fits
481 with the observation that $\Delta G_3^\ddagger - \Delta G_6^\ddagger$ decreases with temperature (Fig. 5). While our model for
482 both CO₂ and O₂ addition has positive entropy of the transition states, the greater entropic cost
483 for CO₂ addition could be the cause of S_{CO} decreases with temperature, more than would be
484 assumed if $\Delta G_3^\ddagger - \Delta G_6^\ddagger$ remained constant with temperature.

485

486 *MIMS modeling*

487 For the MIMS data, the breakpoints observed in k_{catCO_2} and k_{catO_2} could be due to changes
488 in rate limiting step as suggested by Badger and Collatz (1977). For example, k_{catCO_2} is a function
489 of the rate of cleavage of the carboxylated-intermediate (k_8) and the rate of RuBP enolization
490 (k_9). This would mean that k_8 and k_9 have different temperature response such that they crossover
491 around the breakpoint observed at 25 °C. However, modeling this change in rate limiting steps
492 due to different temperature responses cannot simultaneously explain the observed breakpoint in
493 k_{catCO_2} and k_{catO_2} , because the value of k_5 defining the cleavage of the oxygenated intermediate is
494 lower than k_8 . This means that k_9 cannot crossover both k_8 and k_5 at 25 °C (Supp. Fig. 2).

495 Therefore, we proposed that rather than a crossover between elementary rate constants, a
496 conformation change in the enzyme could change the temperature response for the cleavage
497 reactions for both carboxylated (k_8) and oxygenated (k_5) intermediates (Fig. 7). The needed
498 change in cleavage reactions (k_8 and k_5) to model a breakpoint suggests a positive entropy for the
499 transition state below the breakpoint (decreasing ΔG_5^\ddagger with temperature) and a negative entropy
500 of the transitions state above the breakpoint (increasing ΔG_5^\ddagger with temperature). While it seems

501 unlikely that such an entropy change could be driven by a conformation change in the enzyme
502 brought about by such minimal changes in temperature, a similar change in entropy for k_{catCO_2}
503 was observed between wild type *Nicotiana tabacum* and a mutant (L335V) Rubisco (McNevin *et*
504 *al.*, 2007). The amino acid substitution in the mutant was suggested to affect the loop that closes
505 over RuBP as it is bound. This could suggest that the entropy changes to the cleavage reactions
506 (k_5 and k_8) proposed here maybe possible given enzyme conformational changes with
507 temperature.

508 The MIMS data also indicates a breakpoint in $S_{\text{C/O}}$ suggesting larger E_a values at low
509 temperatures compared to higher temperatures, therefor the term $\Delta G_3^\ddagger - \Delta G_6^\ddagger$ was modeled with a
510 non-linear temperature response (Fig. 5). As $S_{\text{C/O}}$ can be approximated as k_6/k_3 , this could
511 suggest a breakpoint in the temperature response of CO₂ addition (k_6), O₂ addition (k_3), or both.
512 The individual values for k_6 and k_3 cannot be derived from $S_{\text{C/O}}$ measurements; however, in order
513 for the observed constant temperature response of K_C and K_O to remain constant with
514 temperature the cleavage reactions discussed above need to be offset by breakpoints in both k_6
515 and k_3 (Fig. 7F). Therefore, to model the reaction mechanism suggested by MIMS
516 measurements, breakpoints in four elementary rate constants are needed to describe the
517 breakpoints in k_{catCO_2} , k_{catO_2} , and $S_{\text{C/O}}$ but not in K_C or K_O .

518 The modeling presented here is largely based on isotope exchange studies, which suggest
519 similar energy barriers between enolization (ΔG_9^\ddagger) and cleavage (ΔG_8^\ddagger). However, these
520 measurements have been limited to 25 °C (Van Dyk and Schloss, 1986; Tcherkez *et al.*, 2013)
521 and extension of isotope exchange studies to temperature responses would help constrain how
522 the elementary rate constants vary with temperature. Contrary to the above proposal that the
523 cleavage transition state (k_8) undergoes changes above and below 25 °C, is that Rubisco

524 discrimination against $^{13}\text{CO}_2$ is believed to remain constant with temperature (Christeller *et al.*,
525 1976). If the rate of cleavage (k_8) decreases, then the decarboxylation reaction (k_7) may increase,
526 or the ratio k_7/k_8 could increase, which would change Rubisco discrimination against $^{13}\text{CO}_2$.
527 Furthermore, the above modeling relies on the assumption that decarboxylation (k_7) was
528 negligible at all temperatures; therefore, changes in fractionation with temperature for an enzyme
529 exhibiting breakpoints should help test the validity of these assumptions.

530

531 CONCLUSION

532 The measured temperature responses of Rubisco kinetic parameters were consistent
533 between methods at and above 25 °C; however, there were thermal breakpoints at ~ 25 °C in the
534 MIMS dataset for k_{catCO_2} , k_{catO_2} , and $S_{\text{C/O}}$. Additionally, the Radiolabel method using a single
535 bicarbonate concentration showed a breakpoint for k_{catCO_2} at 25 °C but the curve fitting did not,
536 suggesting this breakpoint was caused in part by non-saturating CO_2 concentrations at higher
537 temperatures. Previous studies suggest that breakpoints are caused by either a change in the rate
538 limiting step of the reaction mechanism or deactivation of the enzyme at low temperatures. By
539 modeling elementary steps of the reaction mechanism, we showed that a simple change in rate
540 limiting step is not sufficient to explain simultaneous breakpoints in both k_{catCO_2} and k_{catO_2} , and
541 that breakpoints in the elementary rate constants are likely needed. Additionally, it is unclear
542 how deactivation would cause the observed breakpoint in $S_{\text{C/O}}$. Moving forward, the temperature
543 response of isotopic substitutions experiments would advance our understanding of how
544 elementary rate constants change in relation to one another with temperature.

545

546

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558

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Table 1. Testing for thermal breaks for all kinetic parameters. Arrhenius plots were examined using the package ‘segmented’ in R (R Core Team, 2013, <http://www.R-project.org/>), which determines significance of breakpoints in linear models and estimates breakpoint locations as described by Davies (1987). Additionally, breakpoint locations and confidence intervals (CI, lower and upper) were independently estimated using a maximum likelihood test (Muggeo 2003; Muggeo 2008). Where * indicates a p-value < 0.05 for the Davies test and ns = not significant.

Method	Parameter	Davies Test		Maximum likelihood		
		Estimated Breakpoint (°C)	p-value	Estimated Breakpoint (°C)	CI (lower)	CI (upper)
Radiolabel						
	k_{catCO_2} single point	26.8	*	25.1	5.3	36.9
	k_{catCO_2} curve fit	-	ns			
	k_{catO_2}	-	-			
	K_C	-	ns			
	K_O	-	ns			
	$S_{C/O}$	-	ns			
MIMS						
	k_{catCO_2}	25.3	*	25.3	23.1	31.5
	k_{catO_2}	25.3	*	25.5	24.3	32.6
	K_C	-	ns			
	K_O	-	ns			
	$S_{C/O}$	25.4	*	25.2	15.0	27.6

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Table 2. Comparison of k_{25} and E_a values for k_{cat} measurements from the different methods.

The k_{25} and E_a values are the mean of three to four replicates, calculated from linear regressions of Arrhenius plots. The temperature ranges for each regression were determined by segment analysis. Superscripts indicate significant differences between groups (Tukey HSD, $p < 0.05$).

Method	Temperature (°C)	Parameter	k_{25}	E_a
Radiolabel				
single point	0 - 25	k_{catCO_2} (s^{-1})	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_{catO_2} (s^{-1})	1.38 ±0.05 ^B	92.95 ±7.31 ^a
	25 - 40		-- --	47.11 ±2.33 ^{bc}

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Table 3. Comparison of K_C , K_O , $S_{C/O}$ parameters k_{25} and E_a resulting from the different methods are shown. 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 superscripts next to the E_a values indicate significant differences for the $S_{C/O}$ values (Tukey HSD, $p < 0.05$).

Method	Temperature Range (°C)	Parameter	k_{25} (Pa)		E_a (kJ mol ⁻¹)	
Radiolabel	10 - 35	K_C	36	±2	63.09	±6.23
MIMS	10 - 40		34	±1	62.62	±3.44
Radiolabel	15 - 35	K_O	23100	±3430	16.89	±2.59
MIMS	10 - 40		24400	±701	17.01	±2.48
Radiolabel	5 - 40	$S_{C/O}$	2003	±22	-28.66	±0.51 ^b
MIMS	10 - 25		1814	±117	-48.19	±4.17 ^a
	25 - 40		--	--	-30.51	±6.41 ^b

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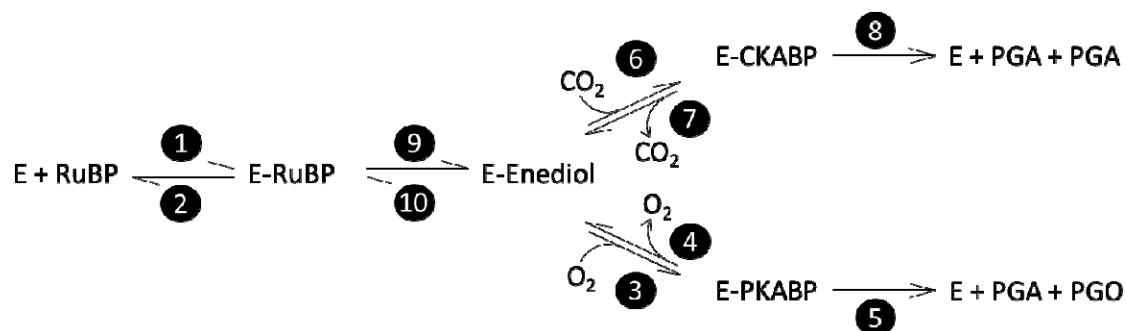
Table 4. The E_a and k_{25} parameters for k_{catCO_2}/K_C , k_{catO_2}/K_O , $k_{\text{catCO}_2}/k_{\text{catO}_2}$, and K_O/K_C ratios. The E_a parameters were tested to determine if they were significantly different than zero (t-test), where the * next to the E_a values indicates a p-value below 0.05.

Method	Temperature Range (°C)	Parameter	k_{25}		E_a	
Radiolabel	10 - 35	k_{catCO_2}/K_C	0.09	±0.00	-3.45	±3.94
MIMS	10 - 25	($\text{s}^{-1} \text{Pa}^{-1}$)	0.10	±0.01	27.75	±3.38*
	25 - 40		--	--	-0.41	±6.10
MIMS	10 - 25	k_{catO_2}/K_O	0.06	±0.00	75.93	±7.41*
	25 - 40	($\text{s}^{-1} \text{kPa}^{-1}$)	--	--	30.09	±0.70*
MIMS	10 - 25	$k_{\text{catCO}_2}/k_{\text{catO}_2}$	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	(kPa Pa^{-1})	0.71	±0.01	-45.60	±2.57*

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745 **Figure 1.** Elementary reactions of Rubisco-catalyzed carboxylation and oxygenation reproduced

746 from Farquhar (1979). Each reaction, forward and reverse, is numbered in a filled circle

747 following the numbering from Farquhar (1979). Steps 8 and 5 are written as irreversible

748 reactions. Step 8 includes cleavage, hydration, and reprotonation as a single step. Step 5 includes

749 cleavage and hydration as a single step. Each step is associated with a rate constant (k) and

750 energy of activation (ΔG^\ddagger) following the same numbering as shown in filled circles.

751 Abbreviations as follows: E, free activated enzyme; RuBP, D-ribulose-1,5-bisphosphate; E-

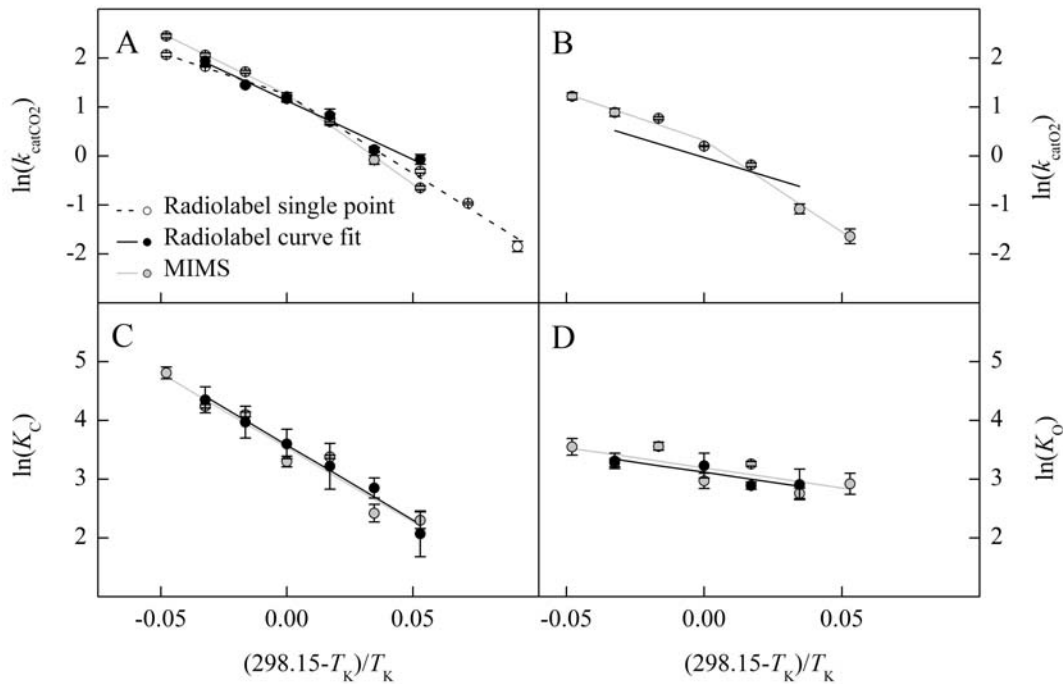
752 RuBP; enzyme bound RuBP; E-Enediol, enzyme bound 2,3-enediolate form of RuBP; CO₂,

753 carbon dioxide; E-CKABP, enzyme bound carboxyketone intermediate; PGA, 3-phospho-D-

754 glycerate; O₂, oxygen; E-PKABP, peroxo intermediate; PGO, 2-phospho-glycolate.

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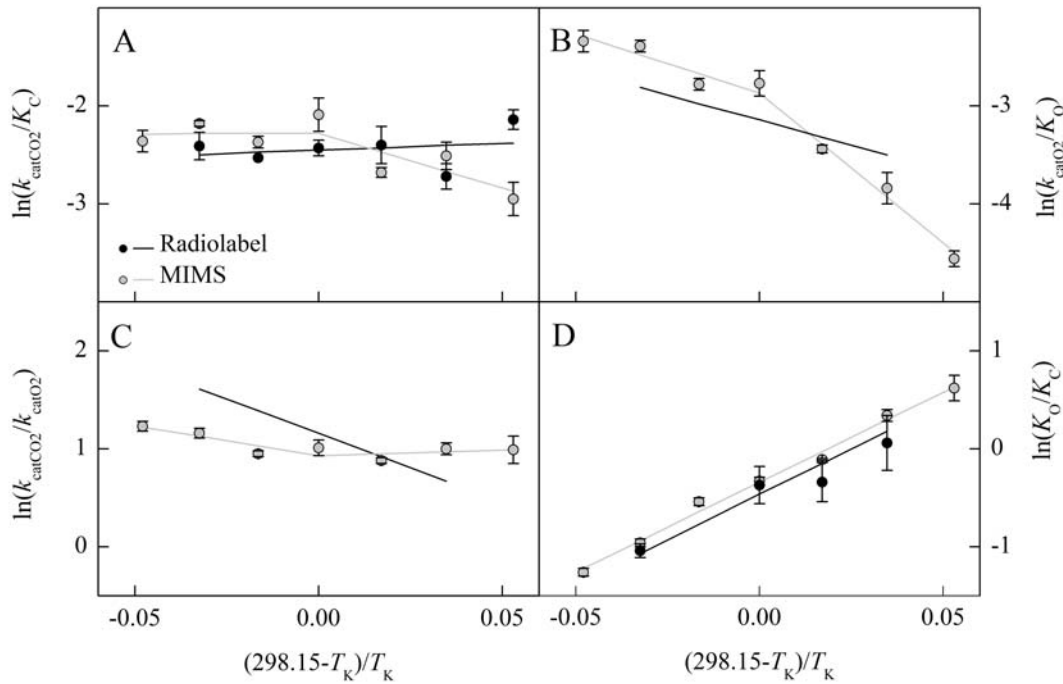


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758 **Figure 2.** The natural log of Rubisco parameters from *Arabidopsis thaliana* measured using
759 Radiolabel (single point, open circle; curve fit, black circle) and MIMS (grey circle) methods are
760 plotted against the inverse of the temperature in Kelvin offset to a y-intercept of 25 °C. The
761 temperature response of catalytic turnover for CO₂ (k_{catCO_2} , s⁻¹, Panel A), and O₂ (k_{catO_2} , s⁻¹, Panel
762 B), the Michaelis-Menten constant for CO₂ (K_C , Pa, Panel C), and O₂ (K_O , kPa, Panel D) are
763 shown. The lines represent the model fit to the measured data. The Radiolabel k_{catO_2} model in
764 panel B was determined from the relationship with $S_{C/O}$ described by Equation S6.

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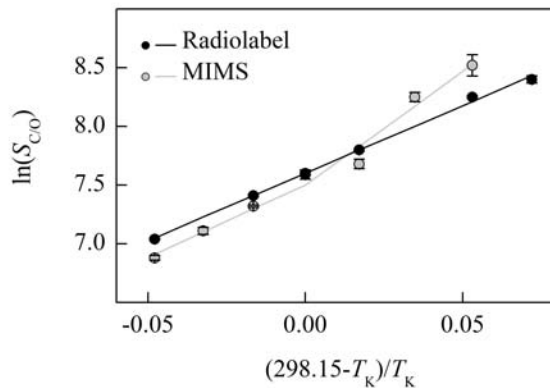


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768 **Figure 3.** The natural log of the Rubisco parameter ratios from *Arabidopsis thaliana* measured
 769 using Radiolabel (black circle) and MIMS (grey circle) are plotted against the inverse of the
 770 temperature in Kelvin offset to a y-intercept of 25 °C. The temperature response of the catalytic
 771 efficiency of the carboxylation (k_{catCO_2} / K_C , Panel A) and oxygenation (k_{catO_2} / K_O , Panel B)
 772 reactions, catalytic turnover ratio for CO₂ over O₂ ($k_{\text{catCO}_2} / k_{\text{catO}_2}$, Panel C), and the Michaelis-
 773 Menten constant ratio for O₂ over CO₂ (K_O / K_C , Panel D) are shown. Lines represent the
 774 combination of models represented in Figure 2 and are not the result of linear regressions to the
 775 ratios.

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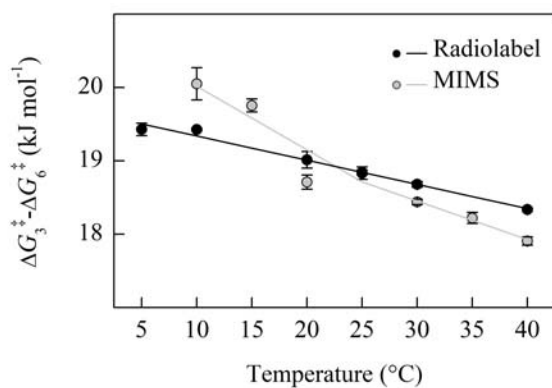


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779 **Figure 4.** The natural log of Rubisco specificity for CO₂ over O₂ ($S_{C/O}$) from *Arabidopsis*
780 *thaliana* measured using Radiolabel (black circle) and MIMS (grey circle) methods are plotted
781 against the inverse of the temperature in Kelvin offset to a y-intercept of 25 °C. The black line
782 represents the model fit to the measured Radiolabel values. The grey line was determined from
783 the relationship of $S_{C/O}$ to the parameters presented in Figure 2, described by Equation S6.

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787 **Figure 5.** The temperature response of $\Delta G_3^\ddagger - \Delta G_6^\ddagger$ calculated from the data presented in Figure 4.

788 Both measurement methods show a decrease with temperature. Solid black circles are the mean

789 of four replicates measured using Radiolabel, filled grey circles are the means from three

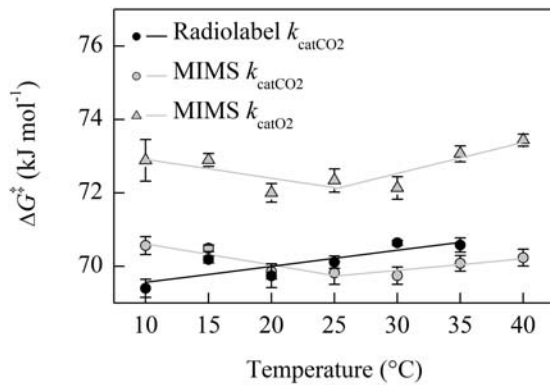
790 replicates using MIMS, standard error is shown. The solid lines indicate the linear regression fit

791 to calculated values.

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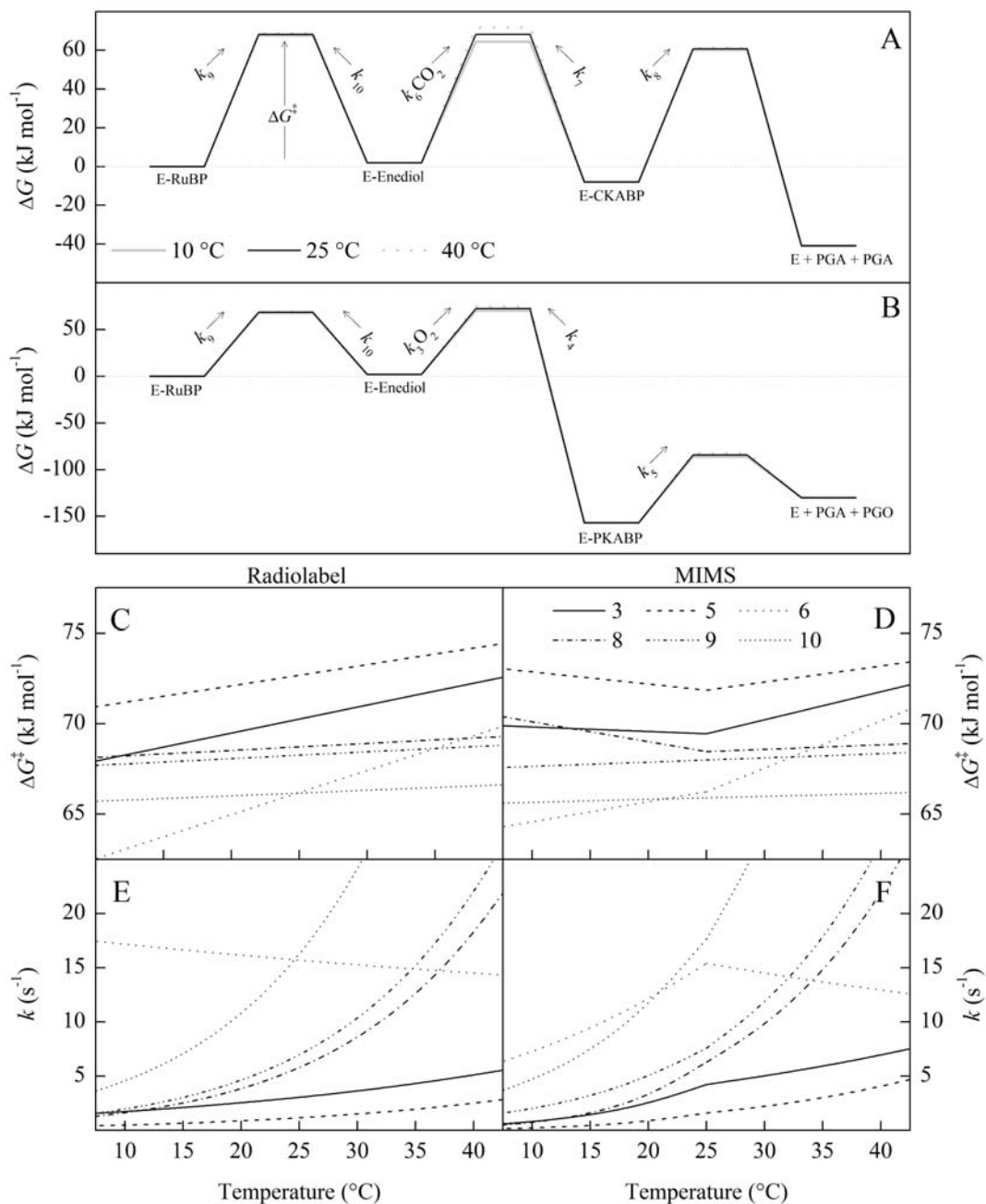


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796 **Figure 6.** The temperature response of $\Delta G_{kcatCO_2}^\ddagger$ for MIMS and Radiolabel methods, and
797 $\Delta G_{kcatCO_2}^\ddagger$ for MIMS calculated from the data presented in Figure 2. Two regressions were fit to
798 the MIMS data on either side of the 25 °C breakpoint, a single regression is fit to the Radiolabel
799 data. Solid black circles are the mean of three replicates measured using Radiolabel, filled grey
800 circles are the means from three replicates using MIMS, standard error is shown.

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 805 **Figure 7.** A kinetic energy barrier diagram showing the modeled temperature responses of the
 806 energy barrier to the transition state (ΔG^\ddagger) and the corresponding first order rate constant k . The
 807 ΔG^\ddagger and k are indicated by the numbered step of the reaction following Figure 1. The
 808 assumptions made for this model are stated in the methods section. For steps 3 and 6 (O_2 and

809 CO₂ addition, respectively), the rate constants were multiplied by ambient concentrations O₂ (21
810 kPa) and CO₂ (41 Pa) as a pseudo-first order approximation for comparison to the other rate
811 constants and to calculate their respective ΔG^\ddagger . For the bottom figure, the left-hand column is
812 modeled on the Radiolabel data and the right-hand column on the MIMS data so that
813 comparisons between continuous and breakpoint temperature responses can be made. The values
814 for intermediates were taken from Tcherkez (2013) for Panel A and Tcherkez (2016) for Panel B
815 and assumed to remain constant with temperature.
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