The biochemistry of Rubisco in *Flaveria*

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Abstract

C4 plants have been reported to have Rubiscos with higher maximum carboxylation rates (kcatCO2) and Michaelis–Menten constants (Km) for CO2 (Kc) than the enzyme from C3 species, but variation in other kinetic parameters between the two photosynthetic pathways has not been extensively examined. The CO2/O2 specificity (SC/O), kcatCO2, Kc, and the Km for O2 (Ko) and RuBP (Km-RuBP), were measured at 25 °C, in Rubisco purified from 16 species of *Flaveria* (Asteraceae). Our analysis included two C3 species of *Flaveria*, four C4 species, and ten C3-C4 or C4-like species, in addition to other C4 (*Zea mays* and *Amaranthus edulis*) and C3 (*Spinacea oleracea* and *Chenopodium album*) plants. The SC/O of the C4 *Flaveria* species was about 77 mol mol⁻¹, which was approximately 5% lower than the corresponding value in the C3 species. For Rubisco from the C4 *Flaveria* species kcatCO2 and Kc were 23% and 45% higher, respectively, than for Rubisco from the C3 plants. Interestingly, it was found that the Ko for Rubisco from the C4 species *F. bidentis* and *F. trinervia* were similar to the C3 *Flaveria* Rubiscos (~650 μM) while the Ko for Rubisco in the C4 species *F. kochiana*, *F. australasica*, *Z. mays*, and *A. edulis* was reduced more than 2-fold. There were no pathway-related differences in Km-RuBP. In the C3-C4 species kcatCO2, Kc were generally similar to the C3 Rubiscos, but the Ko values were more variable. The typical negative relationships were observed between SC/O and both kcatCO2 and Kc, and a strongly positive relationship was observed between kcatCO2 and Kc. However, the statistical significance of these relationships was influenced by the phylogenetic relatedness of the species.

Key words: C3, C4, *Flaveria*, kinetics, Michaelis–Menten, phylogeny, Rubisco, specificity.

Introduction

The most abundant enzyme on Earth is Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39; Ellis, 1979), which fixes most of the carbon entering the biosphere. Rubisco catalyses the addition of CO2 to ribulose-1,5-bisphosphate (RuBP), producing two molecules of 3-phosphoglycerate (3-PGA). The energy required to drive CO2 fixation in eukaryotic phototrophs is delivered by the light-harvesting reactions of the thylakoid; the associated water-splitting biochemistry has produced today’s aerobic atmosphere. This atmosphere greatly reduces the efficiency of photosynthesis because Rubisco is unable to distinguish completely between CO2 and O2 as substrates for fixation to RuBP. The oxygenation reaction produces a 3-PGA and a 2-phosphoglycolate (2-PGO). The recovery of carbon from 2-PGO via the photorespiratory pathway is energetically expensive, and greatly reduces net CO2 fixation. This bi-functionality, coupled with slow catalysis, leads to Rubisco frequently being the principal determinant of the efficiency with which autotrophs use CO2, light, water, and mineral resources (Tcherkez et al., 2006).

The efficiency of photosynthesis is strongly dependent on the relative specificity of Rubisco for CO2 versus O2 (SC/O), which is simply the ratio of the specificities for CO2 and O2:

\[ S_{C/O} = \frac{k_{catCO2}K_o}{k_{catO2}K_c} \]  

where kcatCO2 and kcatO2 are the maximal turnover rates for the carboxylation and oxygenation reactions, and Kc
and $K_o$ are the Michaelis–Menten constants for CO$_2$ and O$_2$, respectively (Laing et al., 1974; Kane et al., 1994). The specificity values denote the proportional efficiencies of carboxylation ($k_{catCO_2}/K_C$) to oxygenation ($k_{catO_2}/K_O$) of a Rubisco in the presence of equal amounts of CO$_2$ and O$_2$. While Form I Rubiscos (e.g. from plants and algae) have $S_{CO_2}$ values ranging from 75–230 mol mol$^{-1}$ (Jordan and Ogren, 1981; Kane et al., 1994; Tcherkez et al., 2006), which greatly favour CO$_2$ fixation, the enzyme falls far short of the near perfect selectivity shown by other enzymes with alternative substrates (Kane et al., 1994). Moreover, the relative level of oxygen is about 500 times greater than that of CO$_2$ in the modern atmosphere, meaning that carboxylation proceeds at only about four times the rate of oxygenation in C$_3$ plants under optimal physiological conditions. A range of evidence indicates that modifying higher plant Rubiscos to emulate the high $S_{CO_2}$ of the enzyme from some of the non-green algae could result in substantial increases in crop yield (Andrews and Whitney, 2003; Zhu et al., 2004). However, the folding and assembly requirements of the Rubiscos from non-green algae are not met by higher plant chloroplasts (Whitney et al., 2001), and our understanding of the subtle structural variations that afford the kinetic variability found amongst Rubiscos is still in its infancy. Consequently, improving yield by increasing $S_{CO_2}$ is beyond our present capabilities.

An interesting feature of Rubisco is that forms with higher $S_{CO_2}$ have slower $k_{catCO_2}$, so that as the fraction of oxygenation reactions declines fewer reactions occur overall (Bainbridge et al., 1995; Tcherkez et al., 2006). The molecular basis for this trade-off is uncertain, although it appears to be a consequence of the Rubisco reaction mechanism (Tcherkez et al., 2006). There is measurable habitat-dependent variation in $S_{CO_2}$ between C$_3$ species, with species from arid habitats having more specific Rubiscos (Delgado et al., 1995; Galmés et al., 2005). However, much of the kinetic variability between Rubiscos is associated with the presence or absence of CO$_2$-concentrating mechanisms (Badger and Andrews, 1987; Badger et al., 1998). Under high CO$_2$ conditions, maximum rates of carboxylation are primarily determined by $k_{catCO_2}$ (Badger and Andrews, 1987; Sage, 2002). For example, in C$_4$ leaves Rubisco is exposed to CO$_2$ concentrations several times above ambient (von Caemmerer and Furbank, 1999); Rubiscos from C$_4$ plants have 25–50% higher $k_{cat}$ than the Rubiscos from C$_3$ species (Wessinger et al., 1989; Sage, 2002). Associated with their faster turnover, the $K_c$ values of C$_4$ Rubiscos are 1.5–3 times higher than that of the C$_3$ enzymes (Yeoh et al., 1980, 1981; Seemann et al., 1984; Wessinger et al., 1989). Despite these kinetic variations, the few $S_{CO_2}$ measurements made in C$_4$ Rubiscos appear comparable with the many values determined in C$_3$ plants (Jordan and Ogren, 1981; Kane et al., 1994). There is also a lack of measured $K_o$ values reported for Rubisco from C$_4$ plants (Badger et al., 1974; Jordan and Ogren, 1981), which impedes efforts to model accurately C$_4$ photosynthesis (von Caemmerer and Furbank, 1999). Knowledge of $K_o$ is requisite for comparing the Rubiscos of C$_3$ and C$_4$ plants.

In order to examine the functional differences in Rubisco from C$_3$ and C$_4$ plants it is necessary to have plants that are closely related phylogenetically. *Flaveria* (Asteraceae) has long been recognized as a powerful system in which to examine the evolution of C$_4$ photosynthesis, particularly because of the presence of C$_3$-C$_4$ intermediate species. Recent molecular evidence suggests that C$_4$ photosynthesis may have arisen between two and four times within *Flaveria* (McKown et al., 2005; Sudderth et al., 2007). The $k_{catCO_2}$ and $K_c$ of Rubisco from 11 species of *Flaveria* have been determined previously (Wessinger et al., 1989). However, the $S_{CO_2}$ and $K_o$ parameters have not been examined, and there is no clear understanding of what, if any, changes to $K_o$ might occur during the transition from C$_3$ to C$_4$ photosynthesis.

The goal of this study was comprehensively to compare the kinetics of Rubisco from C$_3$, C$_4$, and C$_3$-C$_4$ intermediate plant species and to clarify whether or not there is variation in $S_{CO_2}$ and $K_o$ between the two groups. $S_{CO_2}$, $k_{catCO_2}$, and the Michaelis–Menten constants for CO$_2$, O$_2$, and RuBP were measured in 16 species of *Flaveria*. Our analysis includes two C$_3$ species (F. *pringlei* and F. *cronquistii*), and four C$_4$ plants (*F. australasica*, F. *bidentis*, F. *kochiana*, and F. *trinervia*), as well as 10 photosynthetically intermediate *Flaveria* species. For comparison, the kinetics of Rubisco from the C$_3$ plants *Spinacia oleracea* and *Chenopodium album*, and the C$_4$ species *Zea mays* and *Amaranthus edulis*, were also measured. The existing molecular phylogeny of *Flaveria* was used to test for differences in the kinetics of Rubisco between photosynthetic pathways, after correcting for the evolutionary relationships between species.

**Materials and methods**

**Plant material and growth**

Seed or cuttings of 16 *Flaveria* species were obtained from Professor Rowan Sage (University of Toronto). Seed was germinated in soil, and cuttings were rooted in vermiculite on a misting bench. Once seedlings were sufficiently robust, or cuttings had well-developed roots, plants were transplanted in 10 1 pots containing 80% Promix (Plant Products, Brampton, Canada) and 20% sand. Plants were grown in a naturally illuminated glasshouse at the University of New Brunswick (45°56’ N, 66°33’ W). Daytime temperatures in the greenhouse were typically 27–33 °C. Plants were watered as needed, and fertilized monthly.

**Rubisco extraction**

Leaves were harvested when plants were 3–4-months-old. Actively photosynthesizing leaves (approximately 100 g FW) from four or
five plants of each species were detached, pooled, frozen in LN2, and stored at −80 °C. Rubisco extraction was based on the procedures described by Paech and Dying (1986) and Wessinger et al. (1991). Leaf tissue was ground for 60 s in a blender containing 500 ml of ice-cold buffer (20 mM Na2HPO4, 5 mM MgCl2, 1 mM Na2-EDTA, 40 mM e-amino caproic acid, 8 mM benzamide, 100 mM β-mercaptoethanol, 40 mg ml−1 PVP, and 4 mg ml−1 BSA, pH 6.1). The homogenate was filtered through three layers of cheese-cloth and three layers of Miracloth. The sample was made to 5 mM ATP (from a 500 mM, pH 7 stock), heated to 58 °C in a microwave (approximately 130 s), held at that temperature in a water bath for 9 min, and then rapidly cooled to 4 °C (8–10 min in a −80 °C freezer). The extract was centrifuged for 25 min at 15 300 g at 4 °C, and the supernatant was slowly mixed with an equal volume of ice-cold saturated (NH4)2SO4 (pH 7) at 4 °C. After 20 min the sample was centrifuged as above, and the resulting pellet containing Rubisco was dissolved in a minimal amount of a storage buffer (10 mM KH2PO4, 50 mM NaCl, 1 mM Na2-EDTA, 5 mM DTT, pH 7.6), made to 20% glycerol (v/v), and stored at −80 °C.

**CO2/O2 specificity assay**

The purified Rubiscos were used to measure Sc/O2, using the method of Kane et al. (1994), at 25 °C in an atmosphere of 500 ppm CO2 in O2, controlled by a set of three Wösthoff precision gas-mixing pumps. The reaction was initiated by the addition of 1 mMole of 1-3H-RuBP (final assay volume 1 ml), and was terminated after 60 min by the addition of alkaline phosphatase. The 3H-glycerate and 3H-glycolate were separated on a HPX-87H column (Bio-Rad, Gladesville, NSW, Australia) using HPLC (Prominance, Shimadzu, Rydalmere, NSW), and their ratio quantified using an on-line, continuous flow scintillation analyser (505TR, Perkin-Elmer, Melbourne, Vic., Australia).

**Determination of kcat**

The kcat of carboxylation (kcat CO2) was determined on leaf protein extracts, following Kubien et al. (2003). Leaf samples (1.0–1.6 cm2) were harvested from photosynthesizing leaves, frozen in LN2, and stored at −80 °C until being assayed (<72 h). Samples were ground on ice in 2.0–2.5 ml of extraction buffer [100 mM HEPES-KOH, 1 mM Na2-EDTA, 20 mM MgCl2, pH 8, 5 mM DTT, 12 mM e-amino caproic acid, 2.4 mM benzamide, 10 mg ml−1 PVPP, 2 mg ml−1 BSA, 2 mg ml−1 PEG, 2% (v/v) Tween-80, 2 mM NaH12PO4, and 2% (v/v) protease inhibitor cocktail (Sigma, St Louis, MO, USA)]. The extracts were briefly centrifuged, 900 μl of the supernatant was added to 100 μl of an activating solution (100 mM Bicine–NaOH, 200 mM MgCl2, 100 mM NaHCO3, pH 8), and incubated at 25 °C for 30 min to carbamylate Rubisco. The activity of the enzyme at 25 °C was determined by the incorporation of 14C into acid-stable products, as described by Kubien et al. (2003). The concentration of Rubisco catalytic sites was determined by the 14CABP binding assay (Ruuska et al., 1998).

**Determination of Michaelis–Menten constants**

Measurements of $K_c$ and $K_o$ were conducted as described by Paul et al. (1991), and $K_c$ was determined by measuring $K_c$ at 0%, 10%, 20% or 30% O2 (mixed v/v with N2, using a Wösthoff pump). All assays were conducted at 25 °C and used highly purified RuBP, synthesized as described by Kane et al. (1998). Seven RuBP concentrations (0–100 μM) were used to measure $K_o$ at 10 mM NaH14CO3. Measurements of $K_c$ and $K_o$ were carried out in septum-sealed, N2-sparged vials containing assay buffer (100 mM Bicine, 10 mM MgCl2, 1 mM Na2-EDTA, pH 8.1), 500 μM RuBP and 10 μg ml−1 carbonic anhydrase; seven different concentrations of NaH14CO3 (0.15–6.75 mM) were used for each $K_c$ measurement. A pH of 6.25 was used for the CO2/HCO3 equilibrium (Tcherkez et al., 2006). Purified Rubisco was diluted to 1 μM catalytic sites with assay buffer containing 10 mM NaH12CO3, and activated for 30 min at 30 °C before adding 20 μl of the fully-activated enzyme to initiate the assays (final assay volume 500 μl). After 30 s the assays were terminated with 250 μl 20% (v/v) HCOOH.

**Statistical analysis**

To calculate $K_c$ and $K_o$, CO2 or RuBP response curves were fit to the Michaelis–Menten first-order rate equation. To calculate $K_o$ the apparent $K_c$, measured at a range of different oxygen mixtures, was regressed against oxygen; the regression was forced through the ordinate at $K_c$. The regression was used to calculate $K_c$ at 20% O2, from which $K_o$ was calculated:

$$K' = K_c \left(1 + \frac{Q}{K_o}\right)$$

Because closely related species may share similar trait values, they probably do not represent statistically independent data points, and thus violate the assumptions of conventional statistical methods (Felsenstein, 1985). To examine this possibility, we tested the effect of the photosynthetic pathway on Rubisco biochemistry without any phylogenetic correction, treating each species as independent (TIPs analysis), and using equivalent phylogenetically independent contrasts (PICs; Felsenstein, 1985) based on a three-gene consensus phylogeny (i.e. Fig. 5 in McKown et al., 2005). In this more conservative approach the difference in trait values between the descendant species is calculated for each node of the phylogeny, resulting in $n$ contrasts where $n$ is the number of species. PIC analyses assume a Brownian model of character evolution, and weights differences by the length of branches between descendant values. As a result, most of the observed variation is allocated to phylogenetic, rather than physiological, processes (Westoby et al., 1995). The statistical adequacy of four types of branch-lengths was examined: branch lengths from the Flaveria phylogeny (AD McKown, personal communication), branch lengths set to unity, and the branch lengths in Grafen (1989) and Pagel (1992). Based on the plots of absolute values of standardized contrasts versus their standard deviation, uniform branch lengths were adequate and were used for the PIC analysis.

To test for correlations between the continuous biochemical characters ($S_{CO2}, K_c, kcat, and K_o$) Pearson’s correlations (TIPs analysis) and Felsenstein’s (1985) independent contrasts (PICs analysis) were used, as implemented in the PDAP module (Midford et al., 2005) of Mesquite version 1.12 (Maddison and Maddison, 2006). We examined whether each biochemical character varied between the continuous characters and photosynthetic pathway ($C_3, C_4, C_3/C_4$ intermediate, or $C_3$-like) using analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used to test for differences in the slopes of the relationship between $S_{CO2}$ and the other biochemical characters, and between $K_c$ and $kcat$, among the different pathways. For the TIPs analysis, differences between $C_3$ and $C_4$ species were examined using Tukey’s test. For the PICs analyses, $P$-values returned from the ANOVA or ANCOVA were adjusted using simulations of random-walk evolution of each character state, as described below. Both TIPs and PICs ANOVAs and ANCOVAs were conducted using the module PDSINGLE in PDAP version 6.0 (Garland et al., 2005a). PDSINGLE returns values from a conventional analysis of variance or covariance, which may inflate type I error if there is strong phylogenetic signal. For the PICs analysis, the $F$-statistic of each ANOVA or ANCOVA was compared to a null distribution of
F-statistics generated by computer simulation. The module PDSI-MUL in PDAP version 6.0 was used to generate 1000 data sets of simulated trait evolution along the Flaveria phylogeny, assuming a Brownian model of character evolution, and the module PDANOVA to conduct ANOVA and ANCOVA on these simulated datasets. If the F-value of the real data set was greater than the 95% percentile of the distribution of the simulated datasets it was considered that photosynthetic pathway had a significant effect on either the parameter (for ANOVA), or on the slope between two parameters (for ANCOVA).

Results

$S_{CO}$ and $k_{cat}$

The C$_3$ species of Flaveria (F. pringlei and F. cronquistii) had $S_{CO}$ values near 81 mol mol$^{-1}$ (Fig. 1). Slightly lower values were measured in the other C$_3$ species spinach (79.8 mol mol$^{-1}$) and Chenopodium album (78.7 mol mol$^{-1}$). By contrast, in the C$_4$ Flaveria species $S_{CO}$ was between 75.5 mol mol$^{-1}$ and 77.2 mol mol$^{-1}$, similar to the specificity measured for Rubisco from corn (74.9 mol mol$^{-1}$) and Amaranthus edulis (77.5 mol mol$^{-1}$), but about 5% lower than $S_{CO}$ in the C$_3$ Flaveria (TIPs, P <0.001; Table 1). The C$_3$-C$_4$ intermediate and C$_4$-like Flaveria species had $S_{CO}$ ranging between 84.5 mol mol$^{-1}$ (F. floridana) and 77.9 mol mol$^{-1}$ (F. anamola).

Rubisco from the two C$_3$ species of Flaveria had carboxylation turnover rates ($k_{catCO_2}$) of 3.1 s$^{-1}$, which were comparable to that measured for the spinach and C. album Rubiscos (Fig. 1). The $k_{catCO_2}$ of the C$_4$ Flaveria Rubiscos ranged between 4.4 s$^{-1}$ (F. trinervia) and 3.7 s$^{-1}$ (F. kochiana), nearly 25% higher than the C$_3$ species (TIPs, P <0.05; Table 1), but similar to the values measured for the Z. mays and A. edulis enzymes. The C$_3$-C$_4$ intermediate and C$_4$-like Flaveria species showed high variation in $k_{catCO_2}$. Rubisco from F. vagina (C$_4$-like) and F. anamola (C$_3$-C$_4$) had $k_{catCO_2}$ within the range of the C$_4$ Flaveria Rubiscos (Fig. 1). By contrast, Rubisco from F. brownii (C$_4$-like) and F. ramosissima (C$_3$-C$_4$) had $k_{catCO_2}$ of 2.6 s$^{-1}$ and 2.7 s$^{-1}$, respectively, which were nearly 15% lower than for the enzyme from the C$_3$ Flaverias.

**Fig. 1.** Biochemical characteristics of Rubisco from 16 species of Flaveria and four ‘control’ species. All measurements were made at 25 °C. The phylogeny is a consensus tree based on three genes (McKown et al., 2005); ‘A’ and ‘B’ represent the two well-supported clades within the genus. The classification of photosynthetic pathways follows McKown et al. (2005) except that F. kochiana is considered to be a C$_4$ species (McKown and Dengler, 2007; Sudderth et al., 2007). Numbers in brackets indicate the standard error of repeat assays on the same purified enzyme sample ($n$=3–5, except for $k_{catCO_2}$ where $n$=5–7 different leaf samples). Also shown is the apparent Michaelis–Menten constant for CO$_2$ (i.e. $K_{c} = K_{c}(1 + O/K_{o})$ at 210 mbar O$_2$ used to model the RuBP-saturated photosynthetic rate of C$_3$ and C$_4$ photosynthesis (von Caemmerer, 2000). Solubilities of 0.0334 mol (L bar)$^{-1}$ and 0.00126 mol (L bar)$^{-1}$ are assumed for CO$_2$ and O$_2$, respectively (von Caemmerer, 2000).
There was no overall effect of photosynthetic type on either $S_{\text{C/O}}$ or $k_{\text{cat}}$, when all four photosynthetic types were considered and the phylogenetic relationships between the species were accounted for (e.g. PICs; Table 2). There was a significant negative relationship between $S_{\text{C/O}}$ and $k_{\text{cat}}\text{CO}_2$ (Fig. 2a; Table 3). The slope of this relationship was not significantly different between the four different photosynthetic types ($P >0.9$, ANCOVA; Table 4).

**Michaelis–Menten constants**

In *Flaveria*, $K_c$ was highest for the enzyme from the C4 species and the C4-like plant *F. vaginata*, with values exceeding 20 $\mu$M in most cases (Fig. 1; Table 1). Similarly, high $K_c$ were measured for Rubisco from *Z. mays* and *A. edulis*. By contrast, Rubisco from the C3, C3-C4, and other C4-like *Flaverias* had $K_c$ values between 13.5 $\mu$M (*F. palmeri*) and 10.2 $\mu$M (*F. sonorensis*), which were consistent with those determined in spinach (12.1 $\mu$M) and *C. album* (11.2 $\mu$M) Rubiscos. Statistically, the $K_c$ for Rubiscos from C4 species of *Flaveria* were significantly higher than for the enzyme from the C3 species ($P <0.001$; Tables 1, 2). There was a strong negative relationship between $K_c$ and $S_{\text{C/O}}$ in *Flaveria* (Fig. 2b; Table 3). However, the correlation between $K_c$ and $S_{\text{C/O}}$ was not significant when analysed by independent contrasts (Table 3), probably because the slopes differed between photosynthetic pathway (Table 4).

Unlike $K_c$, there was considerable variation in $K_o$ for the different Rubiscos both within and between photosynthetic pathway, and no significant differences were detected (Fig. 1; Tables 1, 2). For Rubiscos from the C4 species of *Flaveria*, $K_o$ was about 660 $\mu$M, which was 13–37% higher than the values determined for the enzyme from spinach and *C. album*. In Rubisco isolated from the C4 plants *F. bidentis* and *F. trinervia*, $K_o$ was the same as the enzyme from the C3 *Flaverias*. By contrast, Rubisco from the C4 species *F. kochiana* and *F. australasica* had $K_o$ values of 150 $\mu$M and 309 $\mu$M, respectively, similar to the low $K_o$ values for the Rubiscos from the C4 plants *Z. mays* (157 $\mu$M) and *A. edulis* (289 $\mu$M). Notably there was considerable variation in the $K_o$ of Rubisco from C3-C4 and C4-like *Flaveria* species, even between species that were on adjacent branches of the phylogeny. For example, in clade A the C4-like *F. palmeri* had a Rubisco with a $K_o$ of 193 $\mu$M, while the enzyme from the C3-C4 intermediate *F. ramosissima* had a $K_o$ of 722 $\mu$M. By contrast, there was less variability in $K_o$ in Rubisco from clade B C3-C4 and C4-like species (Fig. 1). No significant relationship was detected between $S_{\text{C/O}}$ and $K_o$ overall (Fig. 2c; Tables 3, 4), although it was noted that the $K_o$ values from *F. brownii* and *F. palmeri* Rubiscos have a strong effect on the analysis.

No consistent relationship was detected between the photosynthetic pathway and the Michaelis–Menten constant for RuBP ($K_m\text{-RuBP}$) (Fig. 1). For the *Flaveria* Rubiscos the $K_m\text{-RuBP}$ ranged between 9–23 $\mu$M, while

#### Table 1. Biochemical characteristics of Rubisco from two C3 and four C4 species of Flaveria

The $k_{\text{cat}}\text{O}_2$ was determined from equation (1). Standard errors are in parentheses. Significant differences were detected by Tukey’s test without any phylogenetic correction, based on the ANOVA of Table 2 (Asterisks denotes that the two groups are significantly different, *$P <0.05$; **$P <0.001$). Note that $k_{\text{cat}}\text{O}_2$ was not examined statistically, as it was not measured independently.

<table>
<thead>
<tr>
<th>Species</th>
<th>$S_{\text{C/O}}$ ($\text{mol} \cdot \text{mol}^{-1}$)</th>
<th>$K_c$ ($\mu$M)</th>
<th>$K_o$ ($\mu$M)</th>
<th>$k_{\text{cat}}\text{CO}_2$ ($s^{-1}$)</th>
<th>$k_{\text{cat}}\text{O}_2$ ($s^{-1}$)</th>
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<tr>
<td>C3</td>
<td>80.9 (0.10)</td>
<td>11.4 (0.59)</td>
<td>659 (7)</td>
<td>3.12 (0.01)</td>
<td>2.23 (0.10)</td>
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<td>C4</td>
<td>76.7 (0.39)</td>
<td>20.7 (1.06)</td>
<td>442 (127)</td>
<td>4.03 (0.17)</td>
<td>1.23 (0.43)</td>
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</table>

#### Table 2. Conventional (TIPs) and phylogenetically-corrected (PIC) analysis of variance between Rubisco biochemical parameters and photosynthetic pathway in Flaveria

Classification of photosynthetic pathway (C3, C3-C4, C4-like, or C4) and the phylogenetic relationships follows McKown et al. (2005).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>TIPs $F_{\text{crit}}$</th>
<th>TIPs $P$</th>
<th>PIC $F_{\text{crit}}$</th>
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<td>66.2</td>
<td>5.5</td>
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<td>$k_{\text{cat}}\text{CO}_2$</td>
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<tr>
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<td>Among groups</td>
<td>3</td>
<td>1.5e4</td>
<td>5.03e4</td>
<td>1.03</td>
<td>0.41</td>
<td>6.03</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Within groups</td>
<td>11</td>
<td>5.3e4</td>
<td>4.8e4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>14</td>
<td>6.9e4</td>
<td>4.9e4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
the enzyme from the C3 and C4 control species had $K_{m}$-RuBP 7–26 μM.

**Discussion**

This study presents an analysis of the biochemical characteristics of Rubisco from closely related angiosperm taxa with differing photosynthetic pathways. The data include the most extensive measurements of the Michaelis–Menten constant for oxygen ($K_o$) for C4 species published to date, and highlights that the oxygenase function of Rubisco may be more variable than previously realized.

The expected negative relationship was observed between the enzyme’s $S_{C/O}$ and $k_{catCO_2}$, but this is not significantly altered by photosynthetic pathway. By contrast, the negative correlation between $S_{C/O}$ and $K_c$ was affected by the photosynthetic pathway and phylogenetic relationship between species. Our data are consistent with previous studies that have shown the kinetics of Rubisco to differ between C3 and C4 plants, and it is suggested that this represents an evolutionary reversion of the enzyme’s efficiency. Last, it is shown that conclusions drawn from observed variation in the kinetic properties of Rubisco from distinct photosynthetic pathways may change if the phylogenetic relatedness of the species is considered.

$K_o$ of Rubisco from C4 plants

The Michaelis–Menten constant for oxygenation ($K_o$) is clearly the least reported Rubisco kinetic parameter, and the technical complexity associated with its determination has resulted in considerable variation in the $K_o$ values reported in the literature. In C3 species generally, $K_o$ values between 200 and 650 μM have been reported (see von Caemmerer, 2000), with values of 500 μM (Jordan and Ogren, 1984) and 354 μM (Badger and Andrews, 1974) being reported for spinach Rubisco. We obtained slightly higher $K_o$ results for Rubisco from the C4 species Flaveria bidentis and F. trinervia, but across all of the C4 species examined here $K_o$ varied more than 4-fold, and in many cases was considerably lower than was observed in C3 species. The effect of $O_2$ on the measured $K_c$ for Rubisco from four different Flaveria species is shown in Fig. 3. Consistent with $O_2$ being a competitive inhibitor of $CO_2$ there was a linear

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**Figure 2.** Relationships between $S_{C/O}$ and (a) $k_{catCO_2}$, (b) $K_c$, and (c) $K_o$ in Rubisco from C3 (filled squares), C3-C4 intermediate (grey squares), C4-like (striped squares), and C4 (open squares) species of *Flaveria*. Dashed lines indicate significant linear relationships, with Pearson’s coefficient indicated. Each point represents the mean value for each species, shown in Fig. 1. The C4-like species *F. vaginata* (Fv), *F. brownii* (Fb), and *F. palmeri* (Fp) are indicated.

**Table 3.** Pairwise correlations of biochemical characters based on non-phylogenetic (TIPs) and phylogenetic (independent contrasts; PICs) analyses

<table>
<thead>
<tr>
<th>Variable</th>
<th>TIPs r</th>
<th>TIPs p</th>
<th>PIC r</th>
<th>PIC p</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{C/O}/k_{catCO_2}$</td>
<td>-0.76</td>
<td>0.01</td>
<td>-0.54</td>
<td>0.05</td>
</tr>
<tr>
<td>$K_c/k_{catCO_2}$</td>
<td>0.65</td>
<td>0.01</td>
<td>0.06</td>
<td>0.82</td>
</tr>
<tr>
<td>$S_{C/O}/K_c$</td>
<td>-0.64</td>
<td>0.01</td>
<td>-0.27</td>
<td>0.32</td>
</tr>
<tr>
<td>$S_{C/O}/K_o$</td>
<td>0.09</td>
<td>0.74</td>
<td>0.05</td>
<td>0.86</td>
</tr>
</tbody>
</table>
Table 4. Analysis of covariance testing for homogeneity of slopes between Rubisco biochemical parameters and photosynthetic pathway in Flaveria, using on non-phylogenetic (TIPs) and phylogenetic (PICs) analyses

<table>
<thead>
<tr>
<th>Variable</th>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>TIPs F</th>
<th>TIPs P</th>
<th>PIC F_cat</th>
<th>PIC P</th>
</tr>
</thead>
<tbody>
<tr>
<td>S_{CO_2}/kcatCO_2</td>
<td>Among groups</td>
<td>3</td>
<td>0.09</td>
<td>0.03</td>
<td>0.11</td>
<td>0.09</td>
<td>3.84</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>8</td>
<td>2.29</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K_c/kcatCO_2</td>
<td>Among groups</td>
<td>3</td>
<td>1.48</td>
<td>0.49</td>
<td>2.48</td>
<td>0.13</td>
<td>4.70</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>8</td>
<td>1.60</td>
<td>0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_{CO_2}/K_c</td>
<td>Among groups</td>
<td>3</td>
<td>38.42</td>
<td>12.81</td>
<td>4.83</td>
<td>0.03</td>
<td>4.65</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>8</td>
<td>21.21</td>
<td>2.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_{CO_2}/k</td>
<td>Among groups</td>
<td>3</td>
<td>3.18e^5</td>
<td>1.06e^5</td>
<td>3.67</td>
<td>0.07</td>
<td>4.58</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>7</td>
<td>2.03e^5</td>
<td>2.89^5</td>
<td></td>
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</tbody>
</table>

An explanation of the table, as well as discussions on the results, have been removed to maintain the natural text format.
investigated the Rubisco large (L) subunit sequences from pairs of C₃ and C₄ species of *Flaveria*, *Airples*, and *Neurachne*. Only one conserved amino acid difference (Met-309 to Ile) occurred across all three comparisons. However, because this position is occupied by Ile in other C₃ Rubiscos (e.g. *Nicotiana tabacum*, *Chlamydomonas reinhardtii*), Hudson et al. (1990) concluded that this could not explain the altered kinetic phenotype, at least by itself. The comparison of C₃ (*F. pringlei*) and C₄ (*F. trimervia* and *F. bidentis*) L-subunit sequences identified three amino acid differences in total, none of which were suitability positioned to effect the observed variation in kinetic traits (Hudson et al., 1990). Curiously, the Rubisco small (S) units from *F. pringlei* (Genbank AAB67851) and *F. bidentis* (AAP31054) show significant (>93%) homology, so it is difficult to conclude the extent to which the kinetic differences in *Flaveria* Rubiscos are influenced by the S-subunit. Notably, although the precise role of the S-subunit is uncertain (Spreitzer, 2003) it is essential for maximum catalytic activity (Andrews and Ballment, 1983) and sequence modification to S-subunit residues, far from the catalytic sites on the L-subunit, have a pervasive effect on the kinetic properties of the enzyme (Spreitzer, 2003). The influence of the S-subunit on Rubisco catalysis suggests that further study on the multiple nuclear-encoded *rbcS* sequences may identify residues, or structural motifs, that influence kinetic variability amongst Rubiscos, particularly in closely related species where the L-subunit is almost totally conserved.

**Rubisco’s biochemical trade-offs**

It can be shown mathematically that $S_{C/O}$ is a function of the rate constants for the addition of CO₂ or O₂ to the 2,3-benediol of RuBP, and that the $k_{cat}$s are not a principal determinant (Farquhar, 1979; Tcherkez et al., 2006). However, in all cases examined there is an inverse relationship between $S_{C/O}$ and $k_{cat}CO₂$ (Bainbridge et al., 1995; Badger et al., 1998; Tcherkez et al., 2006). Tcherkez et al. (2006) showed that $S_{C/O}$ is also inversely related to $K_c$ across an evolutionarily broad range of Rubiscos. Our data showed similar relationships between $S_{C/O}$ and $k_{cat}CO₂$, where the slope of the relationship was not affected by photosynthetic pathway, and between $S_{C/O}$ versus $K_c$, where a pathway-related difference in the slope was detected (Fig. 2b; Table 4). Rubisco from the C₄ plants (including ‘control’ species) and *F. vaginata* had a much different relationship between $S_{C/O}$ and $K_c$ than the other enzymes examined (Table 4).

To explore the evolution of Rubisco further, we plotted the efficiencies of carboxylation (i.e. $k_{cat}CO₂/K_c$) versus oxygenation ($k_{cat}O₂/K_o$) for the *Flaveria* Rubiscos, and others from the literature (Fig. 4). The slope of this plot is equal to $1/S_{C/O}$ (Badger and Andrews, 1987). Two strategies have been proposed for the evolution of Rubisco in response to the decline in atmospheric CO₂ levels that has occurred since the enzyme evolved (Badger and Andrews, 1987). First, the specificity for CO₂ can increase relative to that for O₂, increasing the rate of carboxylation relative to oxygenation. Second, the two $k_{cat}/K_m$ terms can increase in proportion, leaving $S_{C/O}$ unchanged but allowing a constant rate of carboxylation in the falling CO₂. These strategies are indicated in the inset to Fig. 4. In the *Flaveria* and ‘control’ species measured here there is a strong linear relationship between $k_{cat}CO₂/K_c$ and $k_{cat}O₂/K_o$ (Fig. 4). The enzyme from higher plant sources, including *Flaveria*, tend to cluster and show rather little variation in $S_{C/O}$, when compared to Rubisco from wide evolutionary lineages. Our data support the conclusion of Badger and Andrews (1987) that higher plants have employed strategy 1 in response to declining ratios of CO₂/O₂ in the atmosphere; the higher plant Rubiscos are clearly on a lower slope than the Form II enzyme of *Rhodospirillum rubrum* (Rr) or the Form I Rubisco of the *Chromatium vinosum* (Cv). However, during the transition from C₃ to C₄ plants it appears that Rubisco underwent what amounts to an evolutionary reversion, where the specificities for both CO₂ and O₂ declined as the CO₂ experienced by the enzyme increases. This can be seen by comparing the C₃ (black squares)
with the C₄ enzyme (white squares) in Fig. 4, and is essentially the second strategy proposed by Badger and Andrews (1987).

Sage (2004) suggested that ‘optimization’ of enzyme characteristics is the final step in the evolution of completely expressed C₄ photosynthesis. However, these data suggest that this might not be a universal pattern, as F. vaginata clearly has a Rubisco that kinetically resembles the enzyme from the recognized C₄ Flaverias (Figs 1, 2). We are not suggesting that F. vaginata is a C₄ species; although the leaf anatomy is very similar to the C₄ Flaverias (McKown and Dengler, 2007) the O₂-sensitivity of photosynthesis in considerably higher than the other C₄ plants (Ku et al., 1991). Flaveria vaginata may represent a case where the evolution of a C₄-type Rubisco preceded the development of full C₄ photosynthesis. von Caemmerer and Quick (2000) suggested that if C₄ species evolved from C₃ ancestors then high CO₂ affinity must be a readily labile characteristic of Rubisco. Significant changes to the biochemistry of Rubisco are readily apparent in C₄ species, and high CO₂ affinity must be a labile trait for this to have happened in the relatively short time since the C₄ and C₃ Flaverias diverged. Similarly, differences in Sₑ/Cₒ between Limonium sp. (C₃) may be the result of rapid selection on Rubisco kinetics to maximize fitness in arid habitats (Galmés et al., 2005).

Phylogenetic analysis of continuous enzymatic properties

Previous studies have concluded that there is little variation in Sₑ/Cₒ between C₃ and C₄ plants (Jordan and Ogren, 1981; Kane et al., 1994). If analysed without consideration of phylogeny it was possible to detect a small but significant reduction in the Sₑ/Cₒ of Rubiscos from C₄ Flaverias compared with the enzyme from the C₃ species, and our measurements on four non-Flaveria species are consistent with this. If most of the variation in Sₑ/Cₒ is assumed to be due to phylogeny rather than photosynthetic pathway (e.g. PIC analysis) then the differences between pathways are non-significant. Differences in the results between PICs and TIPs analyses may indicate a loss of statistical power, as closely related taxa were likely to have similar photosynthetic pathways. Standardized phylogenetically independent contrasts are calculated as the difference in phenotypic values between sister taxa or node for the character of interest, divided by the total branch length between the taxa, and assumes a certain model of trait evolution (generally Brownian). Thus, these analyses are sensitive to the topology of the phylogeny, the assumption of branch lengths, and the model of character evolution (Felsenstein, 1985; Donoghue and Ackerly, 1996; Garland et al., 2005b).

Statistical issues aside, differences in analyses using TIPs versus PIC may provide some indication of the evolutionary lability of the traits of interest. For example, large changes that occur early in an adaptive radiation will show the greatest difference between the two analyses (Price, 1997). The severe structural, anatomical, and biochemical modifications necessary for transitions between C₃ and C₄ photosynthesis suggest strong stabilizing selection for the maintenance of certain adaptations, such as the localization of glycine decarboxylase to the bundle sheath, that seem to be necessary for the evolution of C₄ photosynthesis (Monson, 1999; Sage, 2004). Indeed, the occurrence of one documented case of a reversion from C₄ to C₃ (Ellis, 1984) suggest that fully-expressed C₄ photosynthesis is strongly maintained, and represents an essentially irreversible evolutionary transition (sensu Bull and Charnov, 1985). Because the evolutionary transitions between C₃ and C₄ photosynthesis are highly constrained, and because few genera contain both pathways, the problems associated with using phylogenetically-independent contrasts will be found in studies of other groups below the family level. Larger analyses of photosynthetic pathway and biochemical traits across families may help resolve some of these issues.

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References


