Differences in Carbon Isotope Discrimination of Three Variants of D-Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Reflect Differences in Their Catalytic Mechanisms

The carboxylation kinetic (stable carbon) isotope effect was measured for purified D-ribulose-1,5-bisphosphate carboxylases/oxygenases (Rubisco) with aqueous CO$_2$ as substrate by monitoring Rayleigh fractionation using membrane inlet mass spectrometry. This resulted in discriminations ($\Delta$) of 27.4 ± 0.9‰ for wild-type tobacco Rubisco, 22.2 ± 2.1‰ for Rhodospirillum rubrum Rubisco, and 11.2 ± 1.6‰ for a large subunit mutant of tobacco Rubisco in which Leu$^{335}$ is mutated to valine (L335V). These $\Delta$ values are consistent with the photosynthetic discrimination determined for wild-type tobacco and transplastomic tobacco lines that exclusively produce R. rubrum or L335V Rubisco. The $\Delta$ values are indicative of the potential evolutionary variability of $\Delta$ values for a range of Rubiscos from different species: Form I Rubisco from higher plants; prokaryotic Rubiscos, including Form II; and the L335V mutant. We explore the implications of these $\Delta$ values for the Rubisco catalytic mechanism and suggest that Rubiscos that are associated with a lower $\Delta$ value have a less product-like carboxylation transition state and/or allow a decarboxylation step that evolution has excluded in higher plants.

Measurement of the enzymatic depletion of the stable carbon isotope $^{13}$C with respect to the relatively abundant $^{12}$C is a valuable technique for understanding kinetic reaction mechanisms. Plant enzymes that have been analyzed in this way include carboxylic anhydrase (1), phosphoenolpyruvate carboxylase (EC 4.1.1.31) (2–4), and D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); EC 4.1.1.39) (5–7). Models of carbon isotope discrimination in C$_3$ plants (8, 9), C$_4$ plants (10), C$_3$–C$_4$ intermediates (11), and, more recently, Crassulacean acid metabolism plants (12–14) also form the basis for explaining $^{13}$C depletion in plant material. This depletion is expressed in terms of the carbon isotope ratio ($R$) of a substance defined as in Equation 1 (15, 16).

$$ R = \frac{[^{13}C]}{[^{12}C]} \quad \text{(Eq. 1)} $$

The isotope composition of a substance, $\delta^{13}$C or $\delta$, is given by Equation 2 (17, 18),

$$ \delta^{13}C = \delta = \frac{R}{R_{std}} - 1 \quad \text{(Eq. 2)} $$

where $R_{std}$ is the isotope ratio for a given standard (marine limestone from the Pee Dee Cretaceous belemnite formation in South Carolina or an artificial version from Vienna (referred to as VPDB)). The units for $\delta$ are usually expressed in terms of parts/thousand or “parts/mil” (‰). For a process that transforms a substrate or source from one isotope composition ($R_s$) to a product with composition $R_p$, the isotope effect or fractionation factor ($\alpha$) is as in Equation 3 (19).

$$ \alpha = \frac{[^{13}C_P]/[^{13}C_S]}{[^{12}C_P]/[^{12}C_S]} = \frac{R_p}{R_s} = \frac{R_p/R_{std}}{R_s/R_{std}} = 1 + \delta_p - 1 = \frac{1 + \delta_p}{1 + \delta_p} \quad \text{(Eq. 3)} $$

The isotope fractionation or discrimination ($\Delta$) associated with this process is then as in Equation 4 (15).

$$ \Delta = \alpha - 1 = \frac{1 + \delta_p - 1 = \delta_s - \delta_p}{1 + \delta_p} = \frac{\delta_s - \delta_p}{1 + \delta_p} \quad \text{(Eq. 4)} $$

A kinetic isotope effect occurs when a substrate or source is continuously converted into a product with fractionation against the heavier isotope. For Rubisco, where $^{12}$C and $^{13}$C are competing for the same active site, the isotope effect associated with CO$_2$ fixation can be shown to be as in Equation 5 (8, 20).

$$ \alpha_{enzyme} = \frac{k_{cat}^{12}}{k_{cat}^{13}} \frac{K_{M}^{12}}{K_{M}^{13}} \quad \text{(Eq. 5)} $$

where $k_{cat}^{12}$ and $k_{cat}^{13}$ are the turnover numbers for $^{12}$C and $^{13}$C, respectively, and $K_{M}^{12}$ and $K_{M}^{13}$ are the apparent Michaelis-Menten (half-saturation) constants for $^{12}$C and $^{13}$C, respectively. In this case, enrichment and discrimination are independent of the concentration of either isotope, a fact supported by...
Kinetic Isotope Effect for Three Rubiscos

We then have two possible mechanisms by which the kinetic isotope effect may differ between Rubiscos. First, the intrinsic isotope effect ($k_{12}/k_{13}$) may vary according to the carboxylation transition state structure with a more product-like transition state having a higher fractionation factor. Alternatively, the fractionation factor will be reduced as the decarboxylation rate increases or as the commitment of the carboxylated intermediate (CKABP) to hydrolysis and cleavage is reduced. This has the effect of replenishing free $^{12}\text{CO}_2$ that has been depleted by carboxylation.

Rubisco occurs as a so-called Form I enzyme in higher plants, cyanobacteria, and proteobacteria (comprising a hexadecamer of eight large and eight small subunits) and as a simpler Form II enzyme (comprising one more dimers of large subunits) in proteobacteria and some dinoflagellates (23, 30, 31). In this study, we compare the kinetic isotope effects (in terms of discrimination ($\Delta$)) for Rubisco from a higher plant (tobacco, Form I) and from $\alpha$-proteobacterium (Rhodospirillum rubrum, Form II) and a large subunit mutant of tobacco Rubisco in which $^{13}\text{C}$ is mutuated to valine (L335V) (32). $^{13}\text{C}$ sits on loop 6 of the $\alpha/\beta$-barrel at the carboxyl-terminal end of the Rubisco large subunit (32). This loop closes over the substrate RuBP as it is bound (31, 33, 34). We speculate that this residue is involved in stabilization of the transition state for either the hydrolysis or cleavage step (or both).

We have measured $\Delta$, based on Rayleigh fractionation, by continuous real-time measurement of individual carbon isotope concentrations via membrane inlet mass spectrometry (35). We show that the $\Delta$ values for $R$. rubrum and L335V tobacco Rubiscos are consistent with photosynthetic discrimination by transgenic tobacco plants in which native Rubisco has been replaced with Form II or a mutant variant. We examine the implications of the differences in $\Delta$ values for the catalytic mechanism of the variant enzymes.

Experimental Procedures

Wild-type and Transgenic Tobacco Plants—Tobacco (Nicotiana tabacum) grown for Rubisco harvesting and leaf tissue carbon isotope measurements included wild-type (WT) plants and two transplastomic mutants: one producing mutant tobacco Rubisco containing the large subunit mutation L335V (32) and one producing $R$. rubrum Rubisco (referred to as tobacco-rubrum) (36). As the mutants are unable to grow in air, all plants were germinated from seed in 5-liter pots of soil in a growth chamber equilibrated with air supplemented with 1% (v/v) $^{12}\text{CO}_2$. The air temperature was 25 °C with a 14-h photoperiod (400 $\mu$mol quanta m$^{-2}$ s$^{-1}$) and 60% relative humidity. The WT plants were also grown in air in an air-conditioned glasshouse as a control.

Rubp, 2-Carboxyerythritol 1,5-Bisphosphate, and Rubisco Purification—Pure Rubp was prepared according to Kane et al. (37). WT Rubisco was purified from tobacco leaves by polyethylene glycol precipitation followed by crystallization via dialysis as described by Servaites (38). L335V tobacco Rubisco was purified by polyethylene glycol precipitation followed by anion-exchange chromatography (AKTA Tm Explorer, Amersham Biosciences AB, Upppsala, Sweden) on a Waters Protein-Pak Tm Q
column as described by Edmondson et al. (39) but omitting the
final gel filtration step. R. rubrum Rubisco production in XL1-
Blue cells transformed with plasmid pRR1 (40) was induced with
1 mM isopropyl β-D-thiogalactopyranoside at 37 °C for 6 h,
and the enzyme was purified according to Andrews and Kane
(41). All enzymes were >95% pure as determined by SDS-
PAGE. They were snap-frozen in liquid nitrogen and stored at
−80 °C in storage buffer (25 mM EPPS-NaOH, pH 8.0, 50 mM
NaCl, 1 mM EDTA, and 20% (v/v) glycerol). The active-site con-
centration for each enzyme was determined by the 2-[14C]car-
oxyl-d-arabinitol 1,5-bisphosphate binding method of Butz and
Sharkey (42) as modified by Ruuska et al. (43) using 2-[14C]carboxybenzil 1,5-bisphosphate (an isomeric mix-
ture of 2-[14C]carboxy-d-arabinitol 1,5-bisphosphate and
2-[14C]carboxyribitol 1,5-bisphosphate) that was synthesized
according to Pierce et al. (44).
Mass Spectrometry Isotope Discrimination Analyses—CO2
uptake in the carboxylation reactions facilitated by purified
Rubiscos was monitored by membrane inlet mass spectrometry
as described (35). The signals for each isotope were corrected
for their zero offsets, and the corrected signals were then used to
obtain a raw discrimination (Δtotal) with respect to aqueous
CO2 according to Equation 8,

\[ 1 + \Delta_{\text{total}} = \alpha_{\text{total}} = \frac{d(\ln[13C])}{d(\ln[12C])} \]  
(Eq. 8)

where \( \alpha_{\text{total}} \) is the slope of a line of best fit through a plot of
points (\( \ln[13C] \) and \( \ln[12C] \)). An enzyme discrimination was
then calculated for each reaction according to Equation 9
(35),

\[ \Delta_{\text{Rubisco}} = \frac{1 + \Delta_{\text{total}}}{1 + \Delta_{\text{part}}} - 1 \]  
(Eq. 9)

where \( \Delta_{\text{part}} \) is the discrimination due to partitioning of inor-
ganic carbon between CO2 and HCO3⁻ in solution given by
Equation 10 (35).

\[ \Delta_{\text{part}} = \frac{-\Delta_{\text{eq}}}{1 + (1 + \Delta_{\text{eq}}) 10^{\frac{pK_{\text{eq}}-\text{pH}}{2}}} + \Delta_{\text{eq}} \]  
(Eq. 10)

Here, \( \Delta_{\text{eq}} \) is the discrimination due to the equilibrium isotope
effect between CO2 (aq) and HCO3⁻ (aq) (relative to CO2) equal
to −8.9‰ (45), and pKₐₐ (aq) is the equilibrium dissociation constant
for CO2 (aq)/HCO3⁻ (aq) equal to 6.25 at 25 °C.

Rubisco Activity—Purified Rubisco was used to measure sub-
strate-saturated carboxylase activities at 15, 20, 25, 30, and
35 °C using a 14CO2 fixation assay based on that of Andrews
(46). Reactions (200 μl) were prepared in 7-ml scintillation vials
and were buffered with 80 mM EPPS, pH 8.0, 20 mM MgCl₂, 1
mM EDTA, and 0.1 mg/ml carbonic anhydrase (Sigma). The
final concentrations of purified Rubisco and NaHCO₃ varied as
follows: 0.1 mg/ml WT tobacco enzyme (1.6 μM active sites)
with 20 mM NaHCO₃, 0.05 mg/ml R. rubrum enzyme (0.9 μM
active sites) with 40 mM NaHCO₃, and 0.15 mg/ml L335V
tobacco enzyme (2.2 μM active sites) with 20 mM NaHCO₃. One
microliter of 74 MBq/ml NaH¹⁴CO₃ was added to the reac-
tions, resulting in specific activities of 15 (WT tobacco), 7 (R.
rubrum), and 15 (L335V tobacco) Bq/nmol. The reactions were
incubated for 15 min at the different temperatures before initi-
atating catalysis by adding RuBP to a final concentration of 1 mM.
After 1 min, the reactions were quenched with 100 μl of 25%
(v/v) formic acid and heated to dryness at 80 °C. The residue
was dissolved in 0.5 ml of water and then mixed with 1 ml of
liquid scintillant (Ultima Gold™ XR, PerkinElmer Life Sciences),
and acid-stable 14C was measured in a Searle Delta 300
scintillation counter. The turnover number (kcat) was calcu-
lated by dividing the carboxylation rate by the active-site con-
tent quantified by 2-[14C]carboxy-d-arabinitol 1,5-bisphos-
phate binding (see above).

Isotope Composition of Tobacco Leaf Tissue and Air from the
Growth Environment—The isotope composition of whole
leaves from tobacco plants (three each of WT, tobacco- rubrum,
and L335V) was determined according to Wright et al. (47).
The third and fourth leaves of each plant were harvested after
63 days (WT; ambient air), 37 days (WT; 1% CO2), and 51 days
(L335V and R. rubrum mutants; 1% CO2). The mid-vein from
each leaf was removed, and the leaves were then dried at 80 °C
before grinding in a ball mill (Retsch GmbH, Haan, Germany).
Approximately 1 mg of each dried and ground sample was
dropped into an EA1110 elemental analyzer (Fisons Instru-
ments S.p.A., Milan, Italy) in a helium carrier and combusted in
a pulse of oxygen. The oxidized products were then reduced to
CO2, N2, and water before separation in a Porapak QS packed
column (Alltech Biotechnology). The pulses of pure CO2 then
passed into an IsoChrom continuous flow stable isotope ratio
mass spectrometer (Micromass UK Ltd.) in the helium carri-
er, and the ratios of masses 45:44 and 46:44 were determined.
Calibration was performed against similarly analyzed beet sucrose
internal standard (δ¹³C = −24.62‰ VPDB) and Australian
National University sucrose (δ¹³C = −10.45‰ VPDB). Meas-
urements had a Craig correction applied to compensate for
contributions to mass 45 from other isotopic combinations (e.g.
¹²C¹⁷O¹⁶O rather than ¹³C¹⁶O¹⁶O).

The carbon isotope composition in the air from the two
growth environments (air in the glasshouse and air plus 1%
(v/v) CO₂ in the growth chamber) was also sampled at least
three times into a vacuum-evacuated 1-liter stainless steel cy-
linder. The sampled air was drawn at low pressure (<10 milli-
bars) into a vacuum line through a dry ice/ethanol bath (to
condense water vapor) and then a liquid nitrogen trap (to
condense CO₂ but not O₂). The condensed CO₂ was subjected
to high vacuum (<10⁻² millibars) to evacuate the trap of all but
CO₂. The liquid nitrogen bath was replaced with a dry ice/ethanol
bath so that CO₂ was evaporated from the trap and recondensed
in a connected glass tube immersed in liquid nitrogen. The tube was
sealed under vacuum with an oxyacetylene torch. The carbon iso-
tope ratios of these samples were then determined by analysis
on a GV Instruments IsoPrime stable isotope ratio mass spec-
trometer fitted with a dual inlet. The glass tubes containing
the sample CO₂ were broken in an evacuated tube cracker, expand-
ing the gas into the bellows of the dual inlet. The bellows were
then compressed to produce a beam of ~8 nA for beam 1 (mass
44). The reference bellows were filled with a laboratory internal
standard CO₂ (δ¹³C = −25.54‰ VPDB), and the two gases
The discrimination with respect to gaseous CO₂ (parameter \( b \)) is \( 1\‰ \) greater than the values shown here. In the first column, \( C_3 \) and \( C_4 \) refer to the photosynthetic pathways, and CCM refers to an aquatic CO₂-concentrating mechanism. The probable CO₂ and O₂ environment during Rubisco catalysis is indicated in the fourth column.

TABLE 1
Discrimination for Rubiscos from a number of different species with aqueous CO₂ as substrate and with errors as reported

| Rubisco form | Species | Common name | \( \text{CO}_2 \) and \( \text{O}_2 \) environment | \( \Delta \) | Conditions | Source |
|--------------|---------|-------------|-----------------------------------------------|---------|------------|--------|
| Form IA      | S. velum symbiont | Bivalve clam symbiont | Low-to-medium \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 23.2–25.6 (95) | 24 °C, pH 8.5 | Ref. 62 |
| Form IB, C₄ | S. bicolor | Sorghum | Medium \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 33.7 ± 6.6 | 24 °C, pH 8.2 | Ref. 2 |
| Form IB, C₃ | Sorghum | Medium \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 18.3 | 37 °C, pH 8.2 | Ref. 2 |
| Form IB, C₃ | G. max | Soybean | Low \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 28.3 ± 1.5 | 15–35 °C, 2–50 mM HCO₃⁻ | Ref. 5 |
| Form IB, C₃ | S. oleracea | Spinach | Low \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 27.1 ± 3.5 | 35 °C, pH 7.5 | Ref. 63 |
| Form IB, C₃ | S. oleracea | Spinach | Low \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 29.7 ± 0.8 | 25 °C, pH 7.0 | Ref. 6 |
| Form IB, C₃ | S. oleracea | Spinach | Low \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 29 ± 1 | 25 °C, pH 8.0 | Ref. 6 |
| Form IB, C₃ | S. oleracea | Spinach | Low \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 26.4 ± 0.6 | 25 °C, pH 9.0 | Ref. 6 |
| Form IB, C₃ | S. oleracea | Spinach | Low \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 29.0 ± 0.3 | pH 7.6 | Ref. 7 |
| Form IB, C₃ | S. oleracea | Spinach | Low \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 30.3 ± 0.8 | pH 8.5 | Ref. 7 |
| Form IB, C₃ | S. oleracea | Spinach | Low \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 26.2–29.8 (95) | 24 °C, pH 8.5 | Ref. 62 |
| Form IB, C₃ | Anacystis nidulans | | High \( \text{CO}_2 \), 21% \( \text{O}_2 \) | 22.0 ± 0.2 | pH 8.1 | Ref. 7 |
| Form IB | R. rubrum | | High \( \text{CO}_2 \), low \( \text{O}_2 \) | 17.8 ± 0.8 | 25 °C, pH 7.8 | Ref. 50 |
| Form IB | R. rubrum | | High \( \text{CO}_2 \), low \( \text{O}_2 \) | 19–24 | pH 7.9, 2–25 mM Mg²⁺ | Ref. 7 |
| Form IB | R. pachyptila endosymbiont | | High \( \text{CO}_2 \), low \( \text{O}_2 \) | 19.5 ± 1.0 | 30 °C, pH 8.0 | Ref. 64 |

FIGURE 1. Discrimination (\( \Delta \)) with respect to aqueous CO₂ for purified Rubiscos from WT tobacco (\( n = 5 \)), R. rubrum (\( n = 12 \)), and L335V tobacco (\( n = 4 \)) measured by us in this study (dark gray bars with S.D.) together with discrimination for Rubiscos from spinach and Synechococcus measured by us previously (light gray bars with S.D.) (35) and discrimination for Rubiscos from other species measured by other workers (white bars with errors as reported) (Table 1). The discrimination with respect to gaseous CO₂ (parameter \( b \)) is \( 1\‰ \) greater than the values shown here.
Kinetic Isotope Effect for Three Rubiscos

The activation energy and standard entropy and enthalpy differences obtained from Fig. 2 are shown for each of the purified enzymes in Fig. 3, together with the free energy difference at 25 °C obtained from Equation 14.

\[ \Delta G^\ddagger = \Delta H^\ddagger - T \Delta S^\ddagger = \Delta H^\ddagger - (298 \text{ K})\Delta S^\ddagger \]  

(Eq. 14)

Both \( \Delta H^\ddagger \) and \( \Delta S^\ddagger \) depend on temperature, but in the temperature range investigated, their variation is clearly negligible, so they are considered constant. Furthermore, the entropic component of the free energy difference \( (T\Delta S^\ddagger) \) does not vary significantly between 15 and 35 °C, and hence, the total free energy difference \( \Delta G^\ddagger \) is relatively constant in this temperature range.

The isotope compositions of leaf tissues (\( \delta_{\text{leaf}} \)) from the tobacco plants with WT, \( R. \text{rubrum} \), and L335V Rubiscos are shown in Fig. 4, together with the isotope compositions of the air in the growth environment (\( \delta_{\text{air}} \)) in each case. The air in the high CO₂ environment was more depleted in \(^{13}\text{C} \) (\( \delta_{\text{air}} \approx -18\% \)) compared with the ambient air (\( \delta_{\text{air}} \approx -10\% \)), which accounts in part for the fact that the leaf tissue from WT tobacco grown in high CO₂ was more depleted (\( \delta_{\text{leaf}} \approx -44\% \)) than the leaf tissue from WT tobacco grown in ambient air (\( \delta_{\text{leaf}} \approx -27\% \)). The discrimination due to photosynthetic growth for each plant, this time with respect to gaseous CO₂ as the source, is given by application of Equation 4 as in Equation 15.

\[ \Delta = \frac{\delta_{\text{air}} - \delta_{\text{leaf}}}{1 + \delta_{\text{leaf}}} \approx \frac{\delta_{\text{air}} - \delta_{\text{leaf}}}{1} \]  

(Eq. 15)

Hence, for WT tobacco, \( \Delta \approx 17\% \) for growth in ambient air and \( \Delta \approx 26\% \) for growth in 1% CO₂. These numbers are different chiefly because discrimination is sensitive to the ratio of the...
Kinetic Isotope Effect for Three Rubiscos

The partial pressure of CO₂ at the site of carboxylation in the leaf ($p_c$) to the partial pressure of CO₂ in the atmosphere ($p_a$) according to Equation 16 (8, 48, 49),

$$\Delta = \bar{a} + (b - \bar{a}) \frac{p_c}{p_a}$$  \hspace{1cm} (Eq. 16)

where $\bar{a}$ is a weighted overall fractionation due to diffusion, and $b$ is the net discrimination due to carboxylation. At 1% CO₂, $p_c/p_a$ will be greater than at ambient CO₂ concentrations and should approach unity. This is because when the CO₂ concentration is very large, the one-way back-leakage from the sites of carboxylation is much greater than the rate of CO₂ fixation. For two different plants (1 and 2) grown at 1% CO₂ and varying only in Rubisco, Equation 17 follows.

$$\Delta_1 - \Delta_2 = b_1 - b_2$$  \hspace{1cm} (Eq. 17)

Fig. 4 also shows $\Delta - \Delta_{WT}$ for R. rubrum and L335V Rubiscos for both discrimination due to photosynthesis by the two mutant tobacco plants in 1% CO₂ (derived from leaf tissue) and discrimination of the purified enzymes ($\Delta_{Rubisco}$). For the tobacco-rubrum plant, $\Delta_{R.\,rubrum} - \Delta_{WT} \approx -6\%$ for both leaf tissue and purified R. rubrum enzyme. For the L335V transgenic plant, $\Delta_{1,335V} - \Delta_{WT} \approx -14\%$ for both leaf tissue and purified enzyme.

**DISCUSSION**

$\Delta$ for Purified Rubisco Can Show a Range of Values—Based on the available discrimination values for variant Rubiscos, the different enzymes can be clustered into three distinct classes according to $\Delta$ (Fig. 1). One class comprises WT tobacco Rubisco, the measured discrimination of which matches that of the other Form I Rubiscos from other higher plants, including spinach (Spinacia oleracea), sorghum (Sorghum bicolor), and soybean (Glycine max). A second class includes R. rubrum Rubisco, the measured discrimination of which matches that determined previously for this enzyme (with the possible exception of that measured by Roeske and O’Leary (50)). Within this class are included other Form II Rubiscos, such as that from a bacterial endosymbiont of the hydrothermal vent tubeworm Riftia pachyptila, and the cyanobacterial Form I Rubisco from Synechococcus, which all show comparable discrimination values. Because of its uniquely low discrimination value, L335V tobacco Rubisco constitutes a class of its own.

The two previous measurements of $\Delta_{Rubisco}$ for R. rubrum are notably different despite being measured under comparable experimental conditions (Table 1). Our measured value of 22.2 ± 2.1‰ is consistent with that of Guy et al. (7), who also used Rayleigh fractionation, whereas the lower $\Delta_{Rubisco}$ measured by Roeske and O’Leary (50) was determined by measuring $\delta^{13}\text{C}$ for substrate (CO₂) and product (3-phosphoglycerate) according to Equation 4.

Tcherkez et al. (29) demonstrated that the specificity for CO₂ over O₂ ($s_{\text{C/O}}$) and the half-saturation constant for carboxylation ($K_{s}$) are related to $\Delta_{Rubisco}$ with lower $K_{s}$ and higher $s_{\text{C/O}}$ correlated with increased $\Delta$ values. In this context, it is apparent from these results and Table 1 that $\Delta_{Rubisco}$ has been measured in a relatively restricted range of Rubiscos from autotrophic organisms that show variation in these parameters. For example, Form ID Rubisco from red algae and non-green algae can show a wide range of $K_{s}$ values, and Form IC Rubiscos from proteobacteria generally show intermediate $K_{s}$ and $s_{\text{C/O}}$ values (51). In addition, there are two distinct Form IA enzymes that are either carboxysome- or non-carboxysome-associated, with the non-carboxysome form (represented by the Solemya velum symbiont in Table 1) having a lower $K_{s}$ value (52). It has also been clearly established that the $K_{s}$ of Rubisco, even within a single form, is able to adapt to the presence or absence of a CO₂-concentrating mechanism, with Rubisco from CO₂-concentrating mechanism-containing organisms having increased $K_{s}$ values (51). None of this variation is represented in Table 1 or the literature, so general interpretations about how variable $\Delta_{Rubisco}$ is in nature must be tempered by the fact that not all relevant forms and variants of Rubisco have been sampled.

$\Delta$ Values for Purified Enzyme Are Consistent with $\Delta$ Values for Photosynthetic Growth—As an additional check on our measured values of discrimination for R. rubrum and L335V Rubiscos, we compared our values for $\Delta_{Rubisco} - \Delta_{WT}$ Rubisco for purified enzyme with those for $\Delta - \Delta_{WT}$ due to photosynthetic growth. Because genes for these enzymes were transplanted into the chloroplasts of tobacco, we are able to compare discrimination for the tobacco mutants against that for WT tobacco and neglect species-specific differences not due to Rubisco (assuming there are no pleiotropic effects, such as a change in internal conductance). For growth at high CO₂ concentration, for which the possible conductance effects become negligible, we have, from application of Equation 17, what follows in Equation 18.

$$\Delta - \Delta_{WT} \approx b - b_{WT}$$  \hspace{1cm} (Eq. 18)

The value of $b$ represents net discrimination due to carboxylation and could include small contributions from phosphoenolpyruvate carboxylase (9, 48, 49). Even so, Fig. 4 shows that, for both R. rubrum and L335V Rubiscos, $\Delta_{Rubisco} - \Delta_{WT}$ Rubisco (determined from purified enzyme) does not differ significantly from $\Delta - \Delta_{WT}$ (determined from leaf tissue). Furthermore, the values do differ significantly between R. rubrum and L335V Rubiscos. Our discrimination values for purified enzyme are thus consistent with our discrimination values for leaf tissue where those enzymes have been transplanted into tobacco plants.

$\Delta$ Values Provide Insight into the Reaction Mechanism—Both our measurements and those of other workers (Fig. 1) show that the carbon isotope discrimination by Rubisco ($\Delta_{Rubisco}$) is greater for higher plants than for Synechococcus (a cyanobacterium), R. rubrum (a purple, non-sulfur bacterium), and other symbiotic bacteria. As described by Tcherkez and Farquhar (20), there are two possible reasons for this: either the intrinsic isotope effect ($k_{\text{carb}}/k_{\text{oxo}}$) may be lower for prokaryotic Rubiscos, reflecting a carboxylation transition state that is less product-like than for higher plants, or the carbonylated intermediate (CKABP) may be less committed to product formation in prokaryotic Rubiscos, resulting in a greater decarboxylation rate.

The first possibility is theoretically possible. Tcherkez et al. (29) pointed out that the more the carboxylation transition
Kinetic Isotope Effect for Three Rubiscos

state resembles its product, the shorter the O₄C–C bond of the joining CO₂ will be and thus the higher its energy and vibrational frequency. The theoretical relationship between this frequency and the kinetic isotope effect for the formation of this bond predicts that the intrinsic isotope effect ($k_{\text{decarb}}/k_{\text{cat}}$) for CO₂ addition should increase as the transition state becomes more product-like. There is some support for this position from Buncel and Lee (53), who studied isotope effects resulting from the addition of OH⁻ during the rate-determining step in the hydrolysis of esters. Comparison of isotope effects could reveal the extent to which evolution has selected the Rubisco of a particular genotype for specificity (for CO₂ over O₂) over maximum catalytic rate (29).

There is also evidence for the second possibility. The fractionation factor for Rubisco is given by Equation 6 and has a maximum value when $k_{\text{decarb}}/k_{\text{cat}}$ is minimized (1, 20). A lower decarboxylation rate and/or higher turnover rate would increase the specificity of Rubisco for CO₂ over O₂ if the corresponding oxygenation process, associated with photorespiration, were not similarly affected (23, 29). We know that higher plants have a lower turnover number ($k_{\text{cat}}$) compared with R. rubrum or Synechococcus (40, 54, 55). Therefore, it may be a lower decarboxylation rate constant that results in higher $\Delta_{\text{Rubisco}}$ for higher plants. Indeed, Pierce et al. (56) found that partitioning of the CKABP intermediate toward hydrolysis over decarboxylation was less (93–99%) for Rubisco derived from R. rubrum and Synechococcus than that for Rubisco from spinach (100%). For 100% partitioning toward products, $k_{\text{decarb}}/k_{\text{cat}} = 0$. For 95% partitioning, $k_{\text{decarb}}/k_{\text{cat}} ≈ 0.05$. Thus, it is possible (for maximum discrimination during decarboxylation) that the decarboxylation rate constant ($k_{\text{decarb}}$) may be ~5% of the value of $k_{\text{cat}}$ for prokaryotic Rubiscos. Jaworowski and Rose (57) quenched a reaction mixture of R. rubrum Rubisco and labeled RuBP and found that as much as 70% of the bound intermediate was returned as RuBP.

The partitioning constant for the carboxylated intermediate ($p$) may be defined as in Equation 19 (56).

$$ p = \frac{1}{1 + \frac{k_{\text{decarb}}}{k_{\text{cat}}}} = \frac{k_{\text{cat}}}{k_{\text{cat}} + k_{\text{decarb}}} \quad (\text{Eq. 19}) $$

The fractionation factor for Rubisco can then be expressed as in Equation 20,

$$ \alpha_{\text{Rubisco}} = \frac{k_{\text{12}}^\text{carb}}{k_{\text{13}}^\text{carb}} \left( p + \frac{1 - p}{k_{\text{decarb}}/k_{\text{cat}}} \right) = \frac{\alpha_{\text{carb}}}{\alpha_{\text{decarb}}} \left( p + \frac{1 - p}{\alpha_{\text{decarb}}} \right) \quad (\text{Eq. 20}) $$

where $\alpha_{\text{carb}}$ and $\alpha_{\text{decarb}}$ are the intrinsic isotope effects for carboxylation and decarboxylation, respectively. If $\Delta_{\text{Rubisco}} ≈ 30\%$ represents an upper limit (equivalent to the intrinsic isotope effect ($\alpha_{\text{cat}}$) for carboxylation, as suggested by Tcherkez and Farquhar (20)), then for prokaryotic Rubiscos, Equation 21 follows.

$$ \alpha_{\text{Rubisco}} = 1.030 \left( p + \frac{1 - p}{\alpha_{\text{decarb}}} \right) \quad (\text{Eq. 21}) $$

Maximum intrinsic isotope effects are generally proportional to the mass difference between isotopes, which is 8% for $^{13}$C and $^{12}$C. Indeed, intrinsic isotope effects associated with decarboxylation are generally ~1.065 (58). If the isotope effect for decarboxylation were near this value for prokaryotic Rubiscos and if partitioning is in the 93–99% range as found for R. rubrum (56), then we might expect to see discrimination reduced to 25–29% as a result of decarboxylation alone, even <25% if we account for the partitioning found by Jaworowski and Rose (57). Hence, prokaryotic Rubiscos (typified by R. rubrum) may exhibit less discrimination than higher plant Rubiscos (typified by WT tobacco) because they allow a greater degree of decarboxylation. This is consistent with the fact that they do not bind the carboxylated intermediate as tightly as higher plant Rubiscos, thus providing a smaller energy barrier to decarboxylation. If, however, the intrinsic isotope effect for decarboxylation is closer to the overall observed isotope effect for Rubisco-mediated carboxylation in higher plants (~30%), then decarboxylation alone cannot explain the lower fractionalization values in R. rubrum.

Fig. 3 (derived from Fig. 2) shows that the enthalpic component of the free energy barrier ($\Delta H^\ddagger$) and the associated activation energy ($E_a$) to the hydrolysis/cleavage step vary much more between enzymes than does the total free energy barrier ($\Delta G^\ddagger$), which determines the rate of hydrolysis/cleavage ($k_{\text{cat}}$). This is because of the different contributions to free energy from the entropic components, which, in common with many solvated protein processes, results in entropy/enthalpy compensation at ambient temperatures (59).

Fig. 3 also shows the differences in entropy between the CKABP intermediate and the transition state associated with hydrolysis and cleavage ($\Delta S^\ddagger = S^\ddagger - S_{\text{CKABP}}$). Although WT tobacco undergoes a relatively small change in entropy between the enzyme-bound CKABP intermediate and the transition state, R. rubrum undergoes a significant decrease in entropy, whereas L335V tobacco undergoes an increase in entropy. Because the entropy change is associated with the degrees of translational and rotational freedom of the transition state (60), it might appear that Rubisco from R. rubrum has a more highly constrained transition state (compared with the carboxylated intermediate, CKABP) than that from WT tobacco. Rather, we suggest that the small $\Delta S^\ddagger$ value in WT tobacco Rubisco is consistent with the assumption of a tightly bound CKABP intermediate. Thus, the binding constant of the latter is closer to that of a transition state rather than a substrate. As a result, there is no important difference in the number of degrees of freedom between WT tobacco CKABP and the transition state for hydrolysis/cleavage. By contrast, in the R. rubrum enzyme, the CKABP molecule is much less tightly bound, and there is a large difference in the degrees of freedom between CKABP and the transition state. In addition, because R. rubrum Rubisco has a significantly lower activation energy compared with WT tobacco, it results overall in a lower total free energy change for hydrolysis/cleavage (and hence a faster $k_{\text{cat}}$).

On the other hand, L335V tobacco Rubisco has a higher activation energy compared with WT tobacco Rubisco and a higher total free energy change (and hence a slower $k_{\text{cat}}$). We also note that the entropic component associated with hydration/cleavage is positive; this indicates that the restriction of the degrees of freedom is very weak at the transition state level compared with the CKABP molecule, which is tightly bound (see above).
Kinetic Isotope Effect for Three Rubiscos

In other words, this would agree with a very "loose" transition state for hydrolysis/cleavage in the L335V enzyme. This is then accompanied by a loss of catalytic efficiency, as revealed by the slower rate and the higher activation energy than compared with WT tobacco (Fig. 3).

It appears likely then that the L335V mutant Rubisco exhibits a lower fractionation because of its crippled hydrolysis/cleavage rate, which would result in a higher energy barrier for hydrolysis/cleavage and a greater degree of decarboxylation. The discrimination value of the L335V enzyme may be explained by a partitioning factor of ~0.7 and a decarboxylation fractionation factor of 1.065. Such parameter values result from a $k_{cat}$ that is ~10 times less than that of WT Rubisco, consistent with our measured $k_{cat}$ values (Fig. 2). In other words, the commitment of the carboxylated intermediate to hydrolysis/cleavage is diminished, even though this intermediate is bound just as tightly by L335V Rubisco as it is by WT tobacco Rubisco (61). Given the relationship between the transition state and the fractionation as argued by Tcherkez et al. (29), it is unlikely then that L335V tobacco Rubisco has a lower intrinsic isotope effect associated with the CO$_2$ addition step (and hence a less product-like transition state for this step). The case of the L335V mutant tobacco differs fundamentally from prokaryotic and higher plant Rubiscos, as it is unlikely to be on the locus of evolutionary optimization. However, it suggests that decarboxylation of enolized RuBP is physically possible.

The two explanations for differences in fractionation between Rubiscos are thus not mutually exclusive: both a more product-like transition state associated with carboxylation (which may cause a stabilization of the CKABP intermediate) and a reduction in the rate of decarboxylation may cause increases in the carbon isotope fractionation of higher plants. The impact of decarboxylation will be appreciable only if the intrinsic isotope effect for decarboxylation ($\alpha_{\text{decarb}}$) is significantly greater than that for carboxylation. It is likely then that the major origin of the larger $^{12}$C/$^{13}$C discrimination of the higher plant enzyme is a more advanced transition state for carboxylation. On the other hand, the L335V mutant has a lower fractionation mainly because of the enhancement of decarboxylation.

Although *R. rubrum* Rubisco has a higher $k_{cat}$ than does WT tobacco Rubisco, it may not be enough to prevent an increase in decarboxylation over the WT enzyme, possibly reflected in a higher energy hydrolysis/cleavage transition state than that for WT enzyme. The L335V mutation in tobacco results in a higher free energy for the transition state, but with a decrease, rather than an increase, in $k_{cat}$ (due to its CKABP intermediate being more tightly bound than in prokaryotes). Thus, L335V may suffer the double inefficiency of a reduced $k_{cat}$ and increased decarboxylation. Its $\Delta$ value is even less than that for *R. rubrum*.

Under the selective pressure of low [CO$_2$], high [O$_2$] terrestrial environments, Rubiscos from higher plants with the C$_3$ photosynthetic pathway appear to have eliminated the decarboxylation reaction, which may exist for enzymes of prokaryotes of high [CO$_2$], low [O$_2$] environments. Such an evolutionary trend probably proceeded by modifying the carboxylation transition state toward a more advanced geometry as a response to the need to reduce the oxygenation reaction (29). Efforts to artificially improve Rubisco for higher plants may therefore be restricted by the trade-off between increasing the turnover number ($k_{cat}$) on the one hand and elevating the enzyme specificity ($s_{C/O}$) and affinity (lower $K_{m}$) for CO$_2$ on the other. Thus, the potential for genetically modifying the enzyme may necessitate the manipulation of residues that have an impact on the carboxylation and hydrolysis/cleavage transition states. It should be possible to address these hypotheses and to identify mechanisms underlying the natural kinetic variability of Rubiscos by further screening the carbon isotope discrimination and the temperature dependences of $k_{cat}$ and $s_{C/O}$ for isolated Rubisco from a broader range of photosynthetic organisms.

Acknowledgments—We thank Hilary Stuart-Williams and Sue Wood for carbon isotope analysis of air and leaf samples and Chin Wong for help with CO$_2$ trapping from air.
Kinetic Isotope Effect for Three Rubiscos

REFERENCES

1. Paneth, P., and O’Leary, M. H. (1985) Biochemistry 24, 5143–5147
2. Whelan, T., Sackett, W. M., and Benedict, C. R. (1973) Plant Physiol. 51, 1051–1054
3. Reibach, P. H., and Benedict, C. R. (1977) Plant Physiol. 59, 564–568
4. O’Leary, M. H., Rife, J. E., and Slater, J. D. (1981) Biochemistry 20, 7308–7314
5. Christeller, J. T., Laing, W. A., and Troughton, J. H. (1976) Plant Physiol. 57, 580–582
6. Roeseke, C. A., and O’Leary, M. H. (1984) Biochemistry 23, 6275–6284
7. Guy, R. D., Fogel, M. L., and Berry, J. A. (1993) Plant Physiol. 101, 37–47
8. Farquhar, G. D., O’Leary, M. H., and Berry, J. A. (1982) Aust. J. Plant Physiol. 9, 121–137
9. Farquhar, G. D., and Richards, R. A. (1984) Aust. J. Plant Physiol. 11, 539–552
10. Farquhar, G. D. (1983) Aust. J. Plant Physiol. 10, 205–226
11. von Caemmerer, S. (1989) Planta 178, 463–474
12. Griffiths, H., Broadmeadow, M. S. J., Borland, A. M., and Hetherington, C. S. (1990) Planta 181, 604–610
13. Griffiths, H. (1992) Plant Cell Environ. 15, 1051–1062
14. Griffiths, H., Cousins, A. B., Badger, M. R., and von Caemmerer, S. (2007) Plant Physiol. 143, 1055–1067
15. O’Leary, M. H. (1981) Photochemistry 20, 553–567
16. O’Leary, M. H., Madhavan, S., and Paneth, P. (1992) Plant Cell Environ. 15, 1099–1104
17. Craig, H. (1957) Geochim. Cosmochim. Acta 12, 133–149
18. Kroopnick, P., and Craig, H. (1976) Earth Planet. Sci. Lett. 32, 375–388
19. Lane, G. A., and Dole, M. (1956) Science 123, 574–576
20. Tcherkez, G., and Farquhar, G. D. (2005) Funct. Plant Biol. 32, 277–291
21. Laing, W. A., and Christeller, J. T. (1976) Biochem. J. 159, 563–570
22. Badger, M. R., and Collatz, G. J. (1977) Carnegie Inst. Wash. Year Book 76, 355–361
23. Badger, T. J., and Beck, E. J. (2008) J. Exp. Bot. in press
24. Buncel, E., and Lee, C. C. (1977) Carbon-13 in Organic Chemistry, Elsevier Science Publishing Co., Inc., New York
25. Andrews, T. J., and Loomer, G. H. (1985) J. Biol. Chem. 260, 4632–4636
26. Morell, M. K., Paul, K., Oshea, N. J., Kane, H. J., and Andrews, T. J. (1994) J. Biol. Chem. 269, 8091–8098
27. Pierce, J., Andrews, T. J., and Loomer, G. H. (1986) J. Biol. Chem. 261, 248–256
28. Anderson, I., and Taylor, C. T. (2003) Arch. Biochem. Biophys. 414, 130–140
29. Whitney, S. M., von Caemmerer, S., Hudson, G. S., and Andrews, T. J. (1999) Plant Physiol. 121, 579–588
30. Gutteridge, S., and Gatenby, A. A. (1995) Plant Cell 7, 809–819
31. Taylor, T. C., and Andersson, I. (1996) Nat. Struct. Biol. 3, 95–101
32. McNevin, D., Badger, M. R., Kane, H. J., and Farquhar, G. D. (2006) Funct. Plant Biol. 33, 1115–1128
33. Whitney, S. M., and Andrews, T. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14738–14743
34. Kane, H. J., Wilkin, J. M., Portis, A. R., and Andrews, T. J. (1998) Plant Physiol. 117, 1059–1069
35. Servaites, J. C. (1985) Arch. Biochem. Biophys. 238, 154–160
36. Edmondson, D. L., Badger, M. R., and Andrews, T. J. (1990) Plant Physiol. 93, 1376–1382
37. Morell, M. K., Kane, H. J., and Andrews, T. J. (1990) FEBS Lett. 265, 41–45
38. Andrews, T. J., and Kane, H. J. (1991) J. Biol. Chem. 266, 9447–9452
39. Butz, N. D., and Sharkey, T. D. (1989) Plant Physiol. 95, 735–739
40. Ruuska, S., Andrews, T. J., Badger, M. R., Hudson, G. S., Laik, A., Price, G. D., and von Caemmerer, S. (1998) Aust. J. Plant Physiol. 25, 859–870
41. Pierce, J., Tolbert, N. E., and Barker, R. (1980) Biochemistry 19, 934–942
42. Mook, W. G., Bommer, J. C., and Staverman, W. H. (1974) Earth Planet. Sci. Lett. 22, 169–176
43. Andrews, T. J. (1988) J. Biol. Chem. 263, 12213–12219
44. Wright, G. C., Hubick, K. T., and Farquhar, G. D. (1988) Aust. J. Plant Physiol. 15, 815–825
45. Farquhar, G. D., Ehleringer, J. R., and Hubick, K. T. (1989) Annu. Rev. Plant Physiol. 40, 503–537
46. Brungnoli, E., and Farquhar, G. D. (2000) in Photosynthesis: Physiology and Metabolism (Leegood, R. C., Sharkey, T. D., and von Caemmerer, S., eds) pp. 399–434, Kluwer Academic Publishers, Norwell, MA
47. Roeseke, C. A., and O’Leary, M. H. (1985) Biochemistry 24, 1603–1607
48. Badger, M. R., Andrews, T. J., Whitney, S. M., Ludwig, M., Yellowlees, D. C., Leggat, W., and Price, G. D. (1998) Can. J. Bot. 76, 1052–1071
49. Badger, M. R., and Bek, J. E. (2008) J. Exp. Bot. in press
50. Buncel, E., and Lee, C. C. (1977) Carbon-13 in Organic Chemistry, Elsevier Science Publishing Co., Inc., New York
51. Andrews, T. J., and Loomer, G. H. (1985) J. Biol. Chem. 260, 4632–4636
52. Morell, M. K., Paul, K., Oshea, N. J., Kane, H. J., and Andrews, T. J. (1994) J. Biol. Chem. 269, 8091–8098
53. Pierce, J., Andrews, T. J., and Loomer, G. H. (1986) J. Biol. Chem. 261, 248–256
54. Powarowski, A., and Rose, I. A. (1985) J. Biol. Chem. 260, 944–948
55. O’Leary, M. H. (1988) Acc. Chem. Res. 21, 450–455
56. Cooper, A. (1999) Curr. Opin. Chem. Biol. 3, 557–563
57. Moore, W. J. (1983) Basic Physical Chemistry, Prentice-Hall International, London
58. Pearce, F. G., and Andrews, T. J. (2003) J. Biol. Chem. 278, 32526–32536
59. Scott, K. M., Schwedock, J., Schrag, D. P., and Cavanaugh, C. M. (2004) Environ. Microbiol. 6, 1210–1219
60. Wong, W. W., Benedict, C. R., and Kohel, R. J. (1979) Plant Physiol. 63, 852–856
61. Robinson, J. J., Scott, K. M., Swanson, S. T., O’Leary, M. H., Horken, K., Tabita, F. R., and Cavanaugh, C. M. (2003) Limnol. Oceanogr. 48, 48–54
62. Farquhar, G. D. (1979) Arch. Biochem. Biophys. 193, 456–468
63. von Caemmerer, S. (2000) Biochemical Models of Leaf Photosynthesis, CSIRO Publishing, Collingwood, Victoria, Australia