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# Simultaneous determination of Rubisco carboxylase and oxygenase kinetic parameters in *Triticum aestivum* and *Zea mays* using membrane inlet mass spectrometry

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## ABSTRACT

**The lack of complete Rubisco kinetic data for numerous species is partly because of the time consuming nature of the multiple methods needed to assay all of the Rubisco parameters. We have developed a membrane inlet mass spectrometer method that simultaneously determines the rate of Rubisco carboxylation ($v_c$) and oxygenation ($v_o$), and the $CO_2$ and $O_2$ concentrations. Using the collected data, the Michaels-Menten equations for $v_c$ and $v_o$ in response to changing $CO_2$ and $O_2$ concentrations were simultaneously solved for the $CO_2$ ($K_c$) and $O_2$ ($K_o$) constants, the maximum turnover rates of the enzyme for $CO_2$ ($k\text{cat}_{CO2}$) and $O_2$ ($k\text{cat}_{O2}$) and the specificity for $CO_2$ relative to $O_2$ ($S_{c/o}$). In the $C_4$ species *Zea mays* $K_c$ was higher but $K_o$ was lower compared with the $C_3$ species *Triticum aestivum*. The $k\text{cat}_{CO2}$ was higher and the $k\text{cat}_{O2}$ lower in *Z. mays* compared with *T. aestivum* and $S_{c/o}$ was similar in the two species. The $V_{omax}/V_{cmax}$ was lower in *Z. mays* and thus did not correlate with changes in $S_{c/o}$. In conclusion, this mass spectrometer system provides a means of simultaneously determining the important Rubisco kinetic parameters, $K_c$, $K_o$, $k\text{cat}_{CO2}$, $k\text{cat}_{O2}$ and $S_{c/o}$ from the same set of assays.**

*Key-words*: $CO_2$; enzyme kinetics; photosynthesis.

## INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) is the most abundant protein on the earth and is an essential component of the photosynthetic process of fixing inorganic carbon ($CO_2$) into organic carbon (sugars). Rubisco catalyses the $CO_2$ carboxylation reaction of ribulose-1,5-bisphosphate (RuBP) initiating the photosynthetic carbon assimilation pathway. However, this enzyme also catalyses the oxygenation reaction between $O_2$ and RuBP, which initiates the first step in the photorespiratory pathway. Oxygenation competes with photosynthetic carbon assimilation, producing $CO_2$ while consuming ATP and reducing equivalents thus reducing the efficiency of net $CO_2$ assimilation. In fact, the rates of photorespiration are about 20% of the rate of net $CO_2$ assimilation in $C_3$ plants under current ambient $CO_2$ concentrations at 25 °C and thus consume a large portion of the solar energy absorbed by a leaf (Badger 1985; Sharkey 1988; Ort & Baker 2002). In addition to the partial pressure of $CO_2$ and $O_2$ at the active site, the rates of these competing oxygenation and carboxylation reactions are determined by the kinetic properties of Rubisco (Collatz *et al.* 1990; von Caemmerer & Quick 2000; Parry *et al.* 2007).

The mechanistic models of leaf photosynthesis are based on the kinetic properties of Rubisco (von Caemmerer 2000) and the accuracy of these photosynthetic models depends on knowing the Rubisco kinetic parameters of the species in question. It is generally assumed that the Rubisco kinetics in $C_3$ plants are relatively conserved and a general set of kinetic parameters, typically from tobacco, are often used for modelling photosynthesis (Sharkey *et al.* 2007). There is variation in the kinetic parameters reported in the literature and this is likely only a small subset of the natural variation as Rubisco kinetic parameters have been determined for only about 100 of the 300 000 or more plants species (Jordan & Ogren 1981; Brooks & Farquhar 1985; Gutteridge *et al.* 1986; Keys 1986; Parry, Keys & Gutteridge 1989; Kent *et al.* 1992; Lee, Kostov & McFadden 1993; Kane *et al.* 1994; Delgado *et al.* 1995; Kent & Tomany 1995; Balaguer *et al.* 1996; Uemura *et al.* 1997; Beerling & Osborne 2002; Sage 2002; Galmes *et al.* 2005b; Ghannoum *et al.* 2005; Kubien *et al.* 2008). Determining the natural variation in Rubisco kinetics within more plant functional types is essential for providing higher resolution models of global photosynthetic productivity under both current and future climatic conditions (Sage, Way & Kubien 2008). It will also increase the accuracy of prediction of the photosynthetic response of an organism at the leaf, canopy, regional and global scale to climate change. Additionally, comparing the

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natural variation in Rubisco kinetic parameters with the corresponding amino acid sequence will potentially identify residues of importance for human-directed Rubisco engineering (Christin *et al.* 2008).

Although there are a limited number of species in which Rubisco kinetic parameters have been analysed there are even fewer complete data sets describing the five most important parameters, the $CO_2$ ($K_c$) and $O_2$ ($K_o$) Michaelis–Menten constants, the maximum turnover rates of the enzyme for $CO_2$ ($k\text{cat}_{CO2}$) and $O_2$ ($k\text{cat}_{O2}$) and the specificity for $CO_2$ relative to $O_2$ ($S_{c/o}$) of Rubisco. Various methods have been used to determine these kinetic parameters but as of yet there has not been a technique to simultaneously determine all five parameters on a single set of measurements for both the carboxylation and oxygenation reactions. We describe a new technique using a membrane inlet mass spectrometer system developed for simultaneously determining $K_c$, $K_o$, $k\text{cat}_{CO2}$, $k\text{cat}_{O2}$ and $S_{c/o}$ of Rubisco. The system was tested by determining the Rubisco kinetic parameters from both a $C_3$ (*Triticum aestivum*) and $C_4$ (*Zea mays*) species and proved to be robust for crude extract measurements.

## MATERIALS AND METHODS

### Growth conditions

*Triticum aestivum* and *Zea mays* were grown in a glasshouse under natural light conditions (27°C day and 18°C night temperatures) in 2–5 L pots in garden mix with 2.4–4 g Osmocote/L soil (15/4.8/10.8/1.2 N/P/K/Mg + trace elements: B, Cu, Fe, Mn, Mo, Zn, Scotts Australia Pty Ltd., Castle Hill, Australia) and watered daily.

### Sample preparation

Leaves were sampled in the greenhouse and placed into a plastic bag with a wet paper towel just before grinding. For each assay two young fully expanded leaves were sampled from *T. aestivum* and half of a leaf (2 g fresh weight) from *Z. mays*. The leaf material was cut into small pieces and placed in a liquid nitrogen cooled mortar and pestle, and subsequently ground to a fine power. The powder was transferred to a new ice cold mortar containing 3–4 mL of the extraction buffer [50 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.8, 10 mM DTT (dithiothreitol), 20 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM $NaHCO_3$, 1% PVPP(polyvinylpyrrolidone)] + 10 $\mu$L $L^{-1}$ protease inhibitor cocktail (Sigma, St Louis, MO, USA).

Leaf extracts were centrifuged in glass test tubes for 10 min at 4 °C and 15 000 r.p.m. (26 000 *g*) using a SS34 rotor in a Sorvall RC5c centrifuge (Thermo Fischer, Waltham, MA, USA). The top layer of the supernatant was collected to avoid membranes and other particulates. An Econo-Pac 10DG desalting column (BioRad, Hercules, CA, USA) was washed with 10 mL eluent (100 mM Hepes pH 7.95, 25 mM $MgCl_2$, 1 mM EDTA, 10 mM $NaHCO_3$, 0.5 mM DTT) and subsequently loaded with 3 mL of the supernatant. Approximately, 2 mL of green eluent coming off the column was collected for each sample. The entire volume of this eluent was passed through a 0.22 $\mu$m Millex GP Filter (Millipore, Carrigtwahill, Ireland) and placed onto a spin filter (Centricon-30, Millipore) with a 30 000 MW cut-off, and spun in plastic tubes at 5000 RPM in a SS34 rotor for 20 min at 4 °C. The remaining concentrate was placed on ice and immediately used for assays.

Rubisco was activated with 25 mM $MgCl_2$ and 10 mM $NaHCO_3$ in the extraction buffer. To maintain the $MgCl_2$ and $NaHCO_3$ concentration during the desalting procedure the eluent contained 25 mM $MgCl_2$ and 10 mM $NaHCO_3$. The assay buffer contained 25 mM $MgCl_2$ and various levels of $NaHCO_3$ (see further discussion). Rubisco assays at each $CO_2$ and $O_2$ concentration were conducted over a short time period (less than two minutes) to minimize deactivation of the enzyme when assayed at the low $CO_2$ concentrations.

### Rubisco sites quantification

Between 5 and 20 $\mu$L of the cleaned, desalted, filtered and concentrated extract was used to determine the content of Rubisco catalytic sites measured by stoichiometric binding of $^{14}$C-carboxy-arabinitol-$P_2$ (Ruuska *et al.* 1998).

### Mass spectrometer calibration

Enzyme assays were conducted in a water-jacketed temperature controlled cuvette in a 600 $\mu$L reaction volume and all assays were conducted at 25 °C. The cuvette was linked to a mass spectrometer (Isoprime, Micromass, Manchester, UK) through a gas permeable plastic membrane followed by an ethanol/dry ice water trap (Badger & Price 1989; Maxwell, Badger & Osmond 1998). The $O_2$ (mass 32) and $CO_2$ (mass 44) concentrations were monitored continuously during the assay. The mass spectrometer was calibrated each day prior to the assay measurements in order to calculate the $O_2$ and $CO_2$ concentrations and changes in the membrane permeability (Fig. 1).

#### *$CO_2$ calibration*

For the $CO_2$ calibrations, known volumes of 100 mM $NaHCO_3$ were injected with glass syringe (SGE Analytical Science, Melbourne, Australia) into 600 $\mu$L of 0.1 N HCl. The acidic solution converted all the inorganic carbon into $CO_2$ providing a measure of the total inorganic carbon in the $NaHCO_3$ solution. The reaction vial was then flushed with water several times and rinsed with the assay buffer (100 mM Hepes pH 7.95, 25 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT). Known volumes of 100 mM $NaHCO_3$ were injected into the cuvette containing the 600 $\mu$L assay buffer to determine the [$CO_2$] produced in the buffered solution. This allowed us to determine the relationships between [$CO_2$] and total inorganic carbon ($CO_2 + HCO_3^-$) in the assay buffer to infer the carboxylation rate from the $CO_2$ decline. There was no need to know pKa values for this

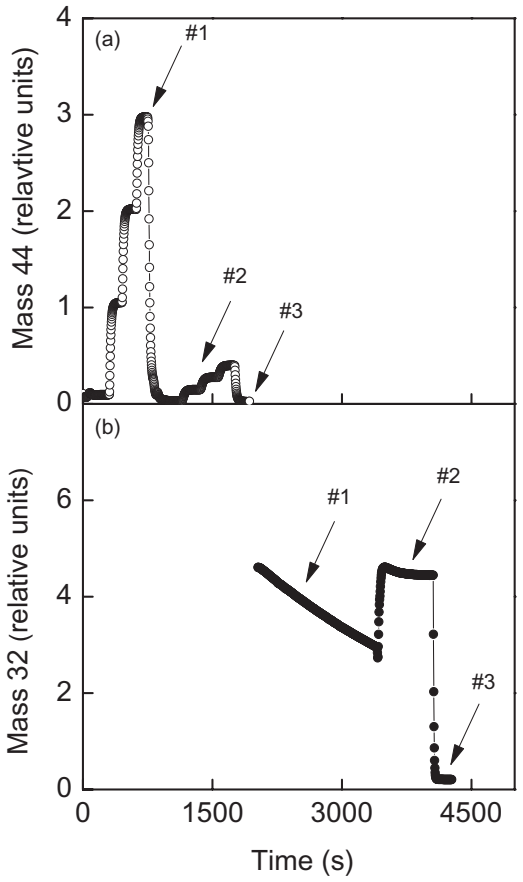


**Figure 1.** $CO_2$ and $O_2$ calibration of the membrane inlet mass spectrometer system. To determine the total $CO_2$ per mass 44 in the 100 mM $HCO_3^-$ solution, three injections of 2 $\mu$L of 100 mM $HCO_3^-$ were introduced into the assay vial that contained 0.1 N HCl (a, arrow #1). The acid solution converted all the $HCO_3^-$ to $CO_2$ injected into the vial. The assay vial was subsequently flushed and filled with the enzyme assay buffer (100 mM Hepes, pH 7.95). Ten microlitres of 100 mM $HCO_3^-$ were introduced into the system three times to determine the $CO_2$ concentration per mass 44 in the assay buffer (a, arrow #2). A zero reading was then determined by injecting 50 $\mu$L of 1 N NaOH, which increased the pH and was assumed to reduce $CO_2$ to zero (a, arrow #3). The rate of $O_2$ consumption by the membrane was determined by the linear change in mass 32 over time (b, arrow #1). The oxygen concentration per mass 32 signal was determined with water, which was in gaseous equilibrium with air, and at 25 °C the dissolved $O_2$ concentration in water was taken to be 250 $\mu$mol/L (b, arrow #2). The zero value for $O_2$ (b, arrow #3) was obtained by isolating the mass spectrometer cuvette from the source inlet line of the mass spectrometer.

purpose. The fact that the mass spectrometer system measures the $CO_2$ and $O_2$ concentration in the gas phase avoids the need to accurately know the $pK_a$ for $HCO_3^-$ of the assay buffer (Yokota & Kitaoka 1985). Subsequently, 50 $\mu$L of 1 N NaOH was injected into the cuvette to convert all the $CO_2$ to $HCO_3^-$ to determine a zero reading for $CO_2$ (Fig. 1).

### *$O_2$ calibration*

The reading for mass 32 (oxygen) with water in full equilibrium with the atmosphere, minus the zero reading, was used to determine the $O_2$ concentration per mass 32 reading, assuming at 25 °C that the dissolved $O_2$ concentration in water is 250 $\mu$mol/L. To correct for the consumption of $O_2$ in the assay buffer by the membrane consumption into the mass spectrometer vacuum, the decline in $O_2$ concentration over time was determined at a range of $O_2$ concentrations (Fig. 1) and was assumed to be linear with respect to $[O_2]$. A correction factor was used to calculate membrane consumption at each $[O_2]$ and was subtracted from total $[O_2]$ uptake rates (in V/s) to give the remaining oxygenase dependent component. It was difficult to maintain measurements at $O_2$ concentration above 75% $O_2$ because of the increase in membrane $O_2$ consumption and $O_2$ leakage out of the cuvette.

### Non-Rubisco consumption of $CO_2$ and $O_2$ in assay buffer and leaf extract

We tested the rates of non-Rubisco $CO_2$ and $O_2$ consumption of the assay buffer and leaf extract, without supplemental RuBP, by adding the assay buffer, $NaHCO_3$, carbonic anhydrase and the Rubisco extract to the reaction vial (Fig. 2, arrows 1, 2 and 3, respectively). Once the rates attained a steady state condition the substrate RuBP was added to the reaction vial to initiate the Rubisco dependent $CO_2$ and $O_2$ uptake (Fig. 2, addition of RuBP indicated by arrow #4).

### Rubisco assay protocol

The rates of Rubisco $CO_2$ and $O_2$ consumption, $v_c$ and $v_o$ respectively, were determined in response to $CO_2$ availability by adding to the assay cuvette 600 $\mu$L of the assay buffer (100 mM Hepes pH 7.95, 25 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT), 0.3 mg $mL^{-1}$ of carbonic anhydrase, various volumes of 100 mM $NaHCO_3$, and 1.2 mM RuBP in that order. A pH of 7.95 was chosen to maximize the ratio of $CO_2$ to $HCO_3^-$ in the assay without compromising the activity of Rubisco with regard to its pH optimum. All assays were conducted at 25 °C. The reaction was initiated with 5 to 10 $\mu$L of fully activated leaf extraction added to the assay. Different volumes of 100 mM $NaHCO_3$ were injected into the assay mixture to achieve approximately 3.3, 0.8, 1.6, 0, 2.5, 0.4 and 0.25 mM in the reaction (Figs 3a & 4a). The rates of $v_c$ and $v_o$ in response to $[O_2]$ were determined by setting the $O_2$ using mass flow controllers to purge the assay buffer for a minimum of 10 min with different percentages of $O_2$ in a nitrogen background, (0, 50, 10, 100 and 70% – Figs 3b & 4b). The equilibrated assay buffer was added to the reaction vial with 0.3 mg $mL^{-1}$ CA, 1.2 mM RuBP, and the reaction was initiated with activated leaf extract. To check the integrity of the membrane inlet system over the course of the measurements and to determine if the Rubisco activity had been compromised during this time period the rates of $v_c$ and $v_o$ were determined again at the end of the assay under the initial measurement conditions (Figs 3 & 4).

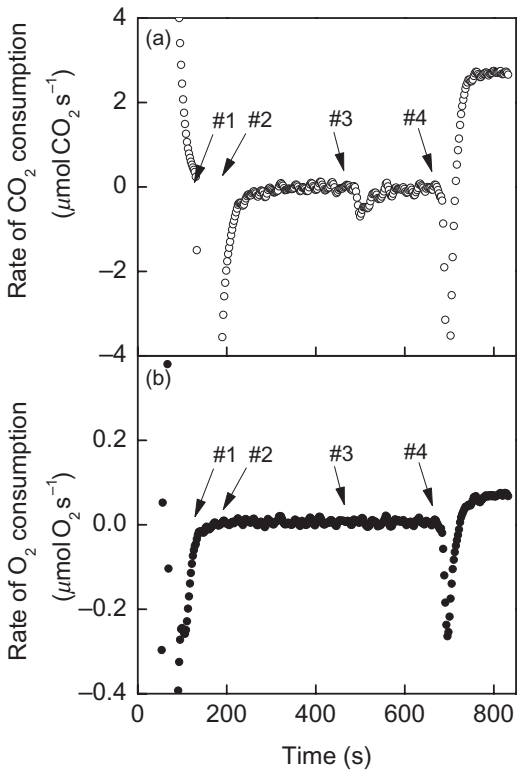


**Figure 2.** Non-ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) $CO_2$ and $O_2$ consumption in the assay buffer and leaf extract. To the assay buffer (pH 7.95) 2.7 mM $HCO_3^-$ was added (arrow #1), followed by 0.3 mg $mL^{-1}$ carbonic anhydrase (arrow #2) and 15 $\mu$L of Rubisco extract (arrow #3). The rates of $CO_2$ (a) and $O_2$ (b) consumption were determined by the changes in mass 44 and mass 32 signals, respectively. Rates of $CO_2$ and $O_2$, corrected for membrane consumption, were insignificant until the Rubisco reaction was initiated with 1.2 mM ribulose-1,5-bisphosphate (arrow #4). Measurements were conducted at 25 °C.

## Data analysis

All values of $v_c$, $v_o$, $CO_2$ and $O_2$ concentrations for both the $CO_2$ and $O_2$ response curve were fitted using a non-linear best fit function in Origin (Origin Lab Corporation, Northampton, MA, USA). The Michaelis–Menten equations for $v_c$ and $v_o$

$$v_c = \frac{CO_2 \cdot V_{cmax}}{CO_2 + K_c(1 + O_2/K_o)} \quad (1)$$

$$v_o = \frac{O_2 \cdot V_{omax}}{O_2 + K_o(1 + CO_2/K_c)} \quad (2)$$

were solved simultaneously for $V_{cmax}$, $V_{omax}$, $K_c$ and $K_o$. The relative specificity factor of Rubisco ($S_{c/o}$) was calculated as:

$$S_{C/O} = \frac{V_{cmax} \cdot K_o}{V_{omax} \cdot K_c} \quad (3)$$

# RESULTS

## Mass spectrometer calibration

The mass spectrometer calibrations for each day prior to the assay measurements are presented in Table 1. At the beginning of the day, $CO_2$ calibrations were conducted to determine the ratio of $CO_2$ to $HCO_3^-$ in the 100 mM $NaHCO_3$ and the concentration of $CO_2$ per mass 44 (nmol $CO_2$/mL/mass 44 [V]). The $CO_2/HCO_3^-$ varied between 29 and 38, and the concentration of $CO_2$ per mass 44 ranged between 7732 and 10976 nmol $CO_2$/mL/mass44 (V) during the 4 months that the experiments were conducted (Table 1). The $O_2$ calibrations for the rate of $O_2$ consumed per concentration of $O_2$ and the concentration of $O_2$ per mass 32 (nmol $O_2$/mL/mass 32 [V]) were also calculated at the beginning of each measurement day (Table 1). The $O_2$ consumption ranged from 0.0003–0.0004 V $O_2$/V $O_2$/s and the concentration of $O_2$ per mass 32 ranged between 43 and 57 nmol $O_2$/mL/mass 32 (V). A representative calibration procedure for both $CO_2$ and $O_2$ is presented in Fig. 1.

## Non-Rubisco consumption of $CO_2$ and $O_2$ in assay buffer and leaf extract

The rates of $CO_2$ and $O_2$ consumption were negligible in the assay mixture without the addition of RuBP (Fig. 2). Once the non-Rubisco rates of $CO_2$ and $O_2$ consumption were determined the Rubisco carboxylation and oxygenation reactions were initiated with the addition of 1.2 mM RuBP at the time point indicated by #4 (Fig. 2). The non-Rubisco consumption of $CO_2$ and $O_2$ was insignificant compared with the Rubisco reaction rates (Fig. 2).

## Rubisco carboxylation and oxygenation rates in response to $CO_2$ and $O_2$ concentrations

The rates of Rubisco carboxylation ($v_c$) and oxygenation ($v_o$) for both *Triticum aestivum* (Fig. 3) and *Zea mays* (Fig. 4) showed a strong $CO_2$ and $O_2$ dependency. In *T. aestivum* the rates of $v_c$ saturated around 50 $\mu$M $CO_2$ (Fig. 3a), whereas in *Z. mays* the $v_c$ rates appeared to saturate closer to 100 $\mu$M $CO_2$ (Fig. 4a). The situation was reversed for $v_o$ where rates did not saturate until approximately 700 $\mu$M $O_2$ in *T. aestivum* and at much lower concentrations (around 400 $\mu$M $O_2$) in *Z. mays*.

## Kinetic parameters

The $K_c$ for *T. aestivum* was similar to a previous publication by Makino, Mae & Ohira (1988); however, the measured $K_o$ values in our study were lower compared with Makino *et al.* (1988) (Table 2). The ratio of $V_{omax}/V_{cmax}$ and $S_{c/o}$ were similar to the Makino *et al.* (1988) report but the $S_{c/o}$ were higher then those reported by Parry *et al.* (1989) & Kane *et al.* (1994). In the $C_4$ plant *Z. mays* the measured $K_c$ values were similar to those reported by Kubien *et al.* (2008) but lower than other reports (Table 2). Additionally, values of $K_o$ were similar to Kubien *et al.* (2008) but lower than the $K_o$ values

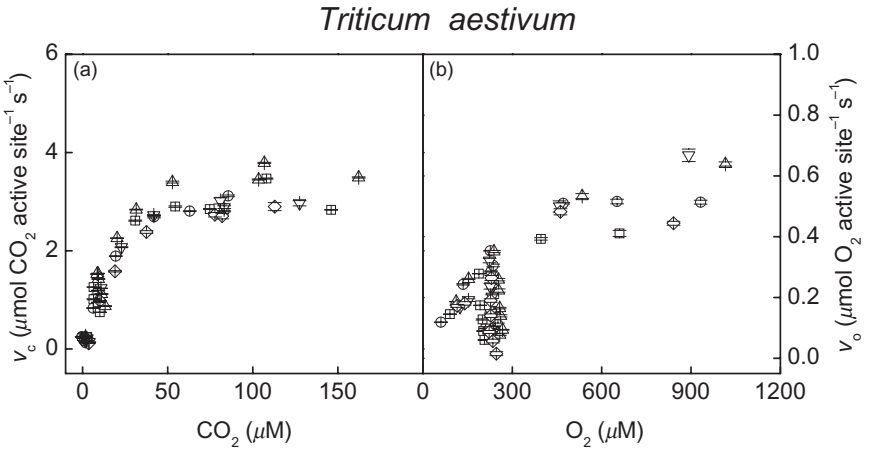


**Figure 3.** Rates of $v_c$ (a) and $v_o$ (b) determined at various $CO_2$ and $O_2$ concentrations for *Triticum aestivum* at 25 °C and pH of 7.95. Shown are the average rates with standard errors over a 20 to 30 second time period at each given $CO_2$ or $O_2$ concentration. The various symbols represent a total of five separate extractions and independent assays. The spread of $v_c$ and $v_o$ around 0 μM $CO_2$ and 300 μM $O_2$, a and b, respectively, is caused by changes in $O_2$ concentration at a constant $CO_2$ concentration or a constant $O_2$ concentration with changes in $CO_2$. Data from both the $CO_2$ and $O_2$ response curve were plotted in both panels and the combined data was used in the fitting routine.

reported by others (Table 2). The $V_{omax}/V_{cmax}$ in *Z. mays* was approximately half of that measured in *T. aestivum* and lower than the value reported by Jordan & Ogren (1981). The $k_{cat}$ values determined by our technique were lower in *T. aestivum* compared with *Z. mays* for both $CO_2$ and $O_2$.

## DISCUSSION

### Concurrent measurements of Rubisco carboxylation and oxygenation

Approximately 12 different combinations of $CO_2$ and $O_2$ concentration were used and all data were fitted to Eqns 1 and 2 simultaneously. This assumes that $CO_2$ and $O_2$ are competitive substrates with $K_c$ equal to $K_i(CO_2)$ and $K_o$ equal to $K_i(O_2)$ as is commonly assumed (Badger & Collatz 1977; Farquhar 1979). The maximum turnover rates of the enzyme for $CO_2$ ($k$cat$_{CO2}$) and $O_2$ ($k$cat$_{O2}$) were determined with an additional measurement of Rubisco site concentration as described in the Materials and Methods. Mass spectrometer calibrations were performed daily and the majority of the calibration parameters were fairly constant over the 4 month time period (Table 1) with subtle differences caused by the changes in the permeability of the particular membrane used for each experiment. Given that the calibrations were conducted daily, the day to day

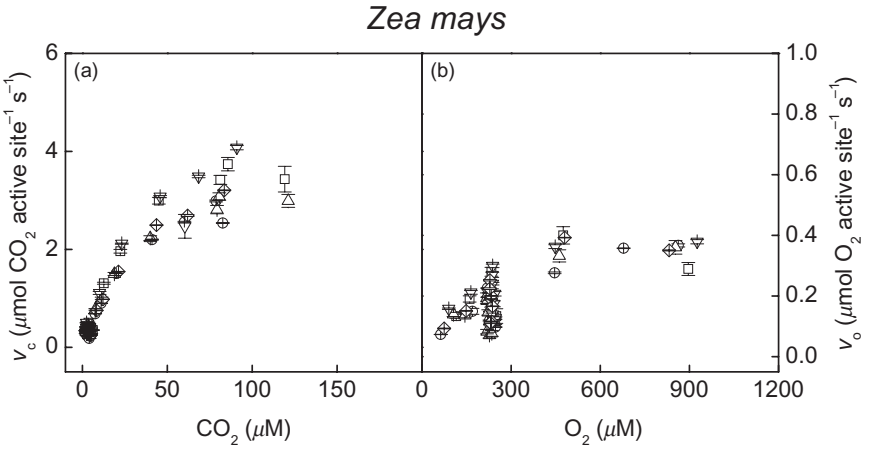


**Figure 4.** Rates of $v_c$ (a) and $v_o$ (b) determined at various $CO_2$ and $O_2$ concentrations for *Zea mays* at 25 °C and pH of 7.95. Shown are the average rates with standard errors over a 20 to 30 second time period at each given $CO_2$ or $O_2$ concentration. The various symbols represent a total of five separate extractions and independent assays. The spread of $v_c$ and $v_o$ around 0 μM $CO_2$ and 300 μM $O_2$, a and b, respectively, is caused by changes in $O_2$ concentration at a constant $CO_2$ concentration or a constant $O_2$ concentration with changes in $CO_2$. Data from both the $CO_2$ and $O_2$ response curve were plotted in both panels and the combined data was used in the fitting routine.

**Table 1.** Calibration parameters

| Parameter | Measurement date | | | | | | |
|---|---|---|---|---|---|---|---|
| | 8/20/07 | 8/21/07 | 10/25/07 | 10/26/07 | 10/29/07 | 10/31/07 | 11/1/07 |
| $CO_2$ calibrations | | | | | | | |
| Mass 44 per $\mu$L of 100 mM $NaHCO_3$ in 0.1 N HCl | 0.52 | 0.59 | 0.53 | 0.52 | 0.53 | 0.65 | 0.64 |
| Assay buffer | 0.018 | 0.019 | 0.014 | 0.014 | 0.014 | 0.019 | 0.018 |
| Assay buffer with 83 mM NaOH | 0.024 | 0.025 | 0.022 | 0.01 | 0.028 | 0.031 | 0.033 |
| Ratio of $CO_2/HCO_3^-$ in 100 mM $NaHCO_3$ | 29 | 31 | 38 | 37 | 38 | 34 | 36 |
| Concentration of $CO_2$ per mass 44 (nmol $CO_2$/mL/mass 44) | 8257 | 7732 | 10949 | 10976 | 10638 | 8079 | 8349 |
| $O_2$ calibrations | | | | | | | |
| $O_2$ consumption per concentration of $O_2$ ($O_2/O_2$/s) | 0.0003 | 0.0003 | 0.0004 | 0.0004 | 0.0003 | 0.0003 | 0.0004 |
| $O_2$ concentration per mass 32 (nmol $O_2$/mL/mass 32) | 57 | 52 | 51 | 53 | 52 | 43 | 44 |
| Plant measured | *T. aestivum* | *T. aestivum* | *T. aestivum* | *T. aestivum* *Z. mays* | *Z. mays* | *Z. mays* *Z. mays* | *T. aestivum* *Z. mays* |

The membrane inlet mass spectrometer systems was calibrated each day to determine the Rubisco-mediated rates of $CO_2$ and $O_2$ consumption, and the $CO_2$ and $O_2$ concentrations. The $CO_2$ calibrations determined the ratio of $CO_2/HCO_3^-$ and the concentration of $CO_2$ per mass 44 signal. The $O_2$ calibrations were used to determine the non-enzyme rate of $O_2$ consumption by the membrane inlet system and the concentration of $O_2$ per mass 32 signal. All measurements were made at 25 °C and the assay buffer had a pH of 7.95. The last row indicates which plant species was measured on each particular day, some times two where run on the same day.

variations in the features of the membrane were accounted for.

All the data for $v_c$ and $v_o$ in response to $CO_2$ and $O_2$ are plotted in both panels a and b (Figs 3 & 4). The apparent clustering of $v_c$ data at low $CO_2$ concentrations is because of the fact that these are rates measured in response to $O_2$ concentration at one low $CO_2$ concentration (Figs 3a & 4a). Similarly, in the plots of $v_o$ versus $O_2$ concentrations (Figs 3b & 4b) rates of $v_o$ appear to cluster just below an $O_2$ concentration of 300 $\mu$M for both species. This variation in $v_o$ is caused by changes in the $CO_2$ concentration at a constant $O_2$ concentration and not associated with errors in the measurements.

## Using crude leaf extracts

We made measurements on freshly prepared leaf extracts, which were desalted, filtered and then concentrated as described in the Materials and Methods section. Concentration of the leaf extract was required especially for the $C_4$ specie so that only a small volume of leaf extract, typically between 5 and 10 $\mu$L containing approximately 0.8 nmol Rubisco sites (~3.5 ng protein) needed to be added to the mass spectrometer cuvette, minimizing perturbations in the assay system. Stability of the enzyme extracts was checked by making a comparative measurement at the beginning and the end of the measurement cycle. The average activity ratios of last and first measurements were 0.94 $v_c$ and 0.93 $v_o$ for *T. aestivum* and 0.89 $v_c$ and 0.93 $v_o$ for *Z. mays*. Using fresh leaf extract raises the concern of the presence of other $O_2$ consuming reactions, but we detected no significant $O_2$ consumption in the leaf extracts in the absence of RuBP (Fig. 2). Indeed, if other significant $O_2$ consuming reactions had been present, this would have lead to reduced $S_{c/o}$ values and there is no evidence for this from the derived parameters (Table 1). The advantage of using fresh leaf extracts is the ease of preparation and the frequent observation that catalytic turnover rates are often greater and more consistent in fresh compared with purified Rubisco preparations (Sharwood *et al.* 2008).

## Kinetic parameters

Comparison of the kinetic constants derived from our measurements in comparison with literature values is shown in Table 2. The Michaelis–Menten constant for $CO_2$ ($K_c$) is one of the most extensively measured Rubisco kinetic parameters among different species (Yeoh, Badger & Watson 1980, 1981; Jordan & Ogren 1983; Kubien *et al.* 2008). These studies established that $C_4$ species have greater values of $K_c$ compared with $C_3$ species and that is also apparent in our measurements (Table 2).

The $K_o$ and $v_o$ parameters have been measured less frequently and are mostly measured as the $K_i$ ($O_2$) of the carboxylase reaction (Jordan & Ogren 1981; Whitney *et al.* 1999; Kubien & Sage 2008) and inferred from the specificity factor. True $K_o$ values determined from oxygenase activity measurements made with $O_2$ electrodes have been reported for *Z. mays* (Badger & Andrews 1974) and *T. aestivum* (Makino *et al.* 1988). There is a fivefold difference in the values reported for *Z. mays* (Table 2), which presumably reflect the difficulty in estimating this parameter. In the mass spectrometer method both the rates of carboxylation and the oxygenation were used in the estimation of $K_o$ and

**Table 2.** Ribulose 1,5-bisphosphate carboxylase/oxygenase kinetic parameters

| Species | $K_c$ (μM) | $K_c$ (μbar) | $K_o$ (μM) | $K_o$ (mbar) | $V_{omax}/V_{cmax}$ | $S_{c/o}$ | $k_{catc}$ | $k_{cato}$ | Reference |
|---|---|---|---|---|---|---|---|---|---|
| *T. aestivum* | 9.7 ± 0.3 | 291 ± 10 | 244 ± 20 | 194 ± 16 | 0.22 ± 0.02 | 114 ± 4[b] (3022 ± 106[c]) | 3.8 ± 0.1 | 0.83 ± 0.09 | This report |
| | 11.2 ± 0.8 | 335 ± 24 | 383 ± 38 | 304 ± 30 | 0.29 ± 0.07 | 120 ± 38 | | | Makino *et al.* (1988) |
| | | | | | | 107 ± 3 | | | Parry *et al.* (1989) |
| | | | | | | 90 ± 1 | | | Kane *et al.* (1994) |
| *Z. mays* | 16.2 ± 1.7 | 485 ± 50 | 183 ± 19 | 146 ± 15 | 0.11 ± 0.01 | 108 ± 6 (2862 ± 160) | 4.7 ± 0.3 | 0.49 ± 0.11 | This report |
| | 21.2 ± 4.1 | 635 ± 123 | 157 ± 3 | 125 ± 2[a] | | 75 ± 1 | 4.1 ± 0.1 | | Kubien *et al.* (2008) |
| | | | | | | 92 ± 7 | | | Parry *et al.* (1987) |
| | 33 | 988 | 550 | 437 | | | | | Badger & Andrews (1974) |
| | 28 | 838 | 610 | 484 | | | | | Badger & Andrews (1974) |
| | 34 | 1018 | 810 | 643[a] | 0.3 | 78 | | | Jordan & Ogren (1981) |
| | | | | | | 79 ± 1 | | | Kane *et al.* (1994) |

The kinetic parameters for *T. aestivum* and *Z. mays* determined with the membrane inlet mass spectrometer system and from previous publications. Current measurements were made at 25 °C and pH of 7.95.

[a]Measured as $K_i(O_2)$.

[b]$S_{c/o}$ solution concentration.

[c]$S_{c/o}$ as gas phase mole fraction.

To convert $K_c$ and $K_o$ values from concentration to partial pressures, solubilities for $CO_2$ of 0.0334 mol (L bar)$^{-1}$ and for $O_2$ of 0.00126 mol (L bar)$^{-1}$ were used (von Caemmerer 2000).

our estimate of 183 ± 2 μM is similar to estimates by Kubien *et al.* (2008) but considerably less than what was reported by Badger & Andrews (1974) and Jordan & Ogren (1981). Reliable estimated of $K_o$ are important for modelling $C_3$ photosynthesis at present atmospheric $O_2$ concentrations as it is the apparent $K_m(CO_2) = K_c \cdot (1 + O_2/K_O)$ that affects estimates of $CO_2$ assimilation (von Caemmerer 2000).

The $V_{omax}/V_{cmax}$ ratio of 0.22 reported here is similar to the values determined for *T. aestivum* (Makino *et al.* 1988) and also similar to estimates on *Spinacia oleracea* (Badger & Andrews 1974) but contrast with the much higher ratios (0.43 to 0.77 ) reported for $C_3$ species by Jordan & Ogren (1981). Our value of 0.11 determined for *Z. mays* is half the value determined for *T. aestivum* and also less than the values of 0.3 previously determined by Jordan & Ogren (1981) (Table 2). However, Jordan & Ogren (1981) also recorded a lower value for *Z. mays* relative to other $C_3$ species measured.

Rubisco $CO_2/O_2$ specificity ($S_{c/o}$) is the most frequently measured Rubisco kinetic parameter, often measured in isolation without determination of other kinetic constants. Species' variations in $S_{c/o}$ were reported by Jordan & Ogren (1981) showing that photosynthetic bacteria and cyanobacteria have low specificity values compared with higher plants. It has been suggested that some of this variation in $S_{c/o}$ and other kinetic parameters of Rubisco have coevolved with the development of photosynthetic $CO_2$ concentration mechanisms in bacteria, cyanobacteria, algae and plants (Badger & Andrews 1987; Badger *et al.* 1998). The variation in $S_{c/o}$ among $C_3$ species has also been observed (Parry *et al.* 1989; Delgado *et al.* 1995; Galmes *et al.* 2005a). A wide variety of methods have been used to measure $S_{c/o}$, for review see Kane *et al.* (1994). Methods that measure oxygenase activity through measurements of $O_2$ exchange with an $O_2$ electrode (Makino *et al.* 1988; Parry *et al.* 1989; Delgado *et al.* 1995; Galmes *et al.* 2005a) report greater values of $S_{c/o}$ than methods that rely on labelled $CO_2$ or RuBP and the analysis of product by HPLC (Jordan & Ogren 1981; Kane *et al.* 1994; Kubien *et al.* 2008). We report very similar values for *T. aestivum* and *Z. mays*, and our values agree with those reported by Delgado *et al.* (1995), but we have no explanation why different values are reported for these various techniques.

## CONCLUSION

In conclusion, the mass spectrometer system provides a robust means of simultaneously determining the $CO_2$ ($K_c$) and $O_2$ ($K_o$) Michaelis–Menten constants, the maximum turnover rates of the enzyme for $CO_2$ ($k$cat$_{CO2}$) and $O_2$ ($k$cat$_{O2}$) and the specificity for $CO_2$ relative to $O_2$ ($S_{c/o}$). This allows these parameters to be determined from a coherent set of measurements on the same leaf extract. Additionally, this system decreases the time requirement per measurement, making it possible to more rapidly screen various types of Rubisco at a range of temperatures and to compare

the properties of Rubisco isolated from species native to different environments.

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