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Possible explanation of the disparity between the *in vitro* and *in vivo* measurements of Rubisco activity: a study in loblolly pine grown in elevated pCO_2

Alistair Rogers[1], David S. Ellsworth and Steven W. Humphries

Environmental Sciences Department, Building 490D, Brookhaven National Laboratory, Upton, NY 11973-5000, USA

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Abstract

Rubisco activity can be measured using gas exchange (*in vivo*) or using *in vitro* methods. Commonly *in vitro* methods yield activities that are less than those obtained *in vivo*. Rubisco activity was measured both *in vivo* and *in vitro* using a spectrophotometric technique in mature *Pinus taeda* L. (loblolly pine) trees grown using free-air CO_2 enrichment in elevated (56 Pa) and current (36 Pa) pCO_2. In addition, for studies where both *in vivo* and *in vitro* values of Rubisco activity were reported net CO_2 uptake rate (*A*) was modelled based on the *in vivo* and *in vitro* values of Rubisco activity reported in the literature. Both the modelling exercise and the experimental data showed that the *in vitro* values of Rubisco activity were insufficient to account for the observed values of *A*. A trichloroacetic acid (TCA) precipitation of the protein from samples taken in parallel with those used for activity analysis was co-electrophoresed with the extract used for determining *in vitro* Rubisco activity. There was significantly more Rubisco present in the TCA precipitated samples, suggesting that the underestimation of Rubisco activity *in vitro* was attributable to an insufficient extraction of Rubisco protein prior to activity analysis. Correction of *in vitro* values to account for the under-represented Rubisco yielded mechanistically valid values for Rubisco activity. However, despite the low absolute values for Rubisco activity determined *in vitro*, the trends reported with CO_2 treatment concurred with, and were of equal magnitude to, those observed in Rubisco activity measured *in vivo*.

Key words: Rubisco activity, elevated CO_2.

Introduction

It is well documented that with long-term exposure to elevated pCO_2 the initial stimulation of net CO_2 uptake (A) is often not maintained (Gunderson and Wullschleger, 1994; Curtis, 1996; Drake *et al*., 1997). Such a reduction in photosynthetic capacity in elevated pCO_2, termed acclimation (Drake *et al*., 1997), has been largely attributed to a loss of active Rubisco (Rogers and Humphries, 2000). If photosynthetic acclimation is to be incorporated into models seeking to determine the influence of the terrestrial biosphere on the global carbon cycle then an accurate and quantitative assessment of acclimation is required. Key to this assessment is the measurement of Rubisco activity in a manner that allows quantitative comparison among different experiments and the use of absolute values in modelling exercises.

The activity of Rubisco can be determined *in vivo* using gas exchange (von Caemmerer and Farquhar, 1981; Wullschleger, 1993; Long *et al*., 1996) or with *in vitro* methods (Lilley and Walker, 1974; Ward and Keys, 1989; Reid *et al*., 1997). Both *in vitro* and *in vivo* approaches

[1] To whom correspondence should be addressed. Fax: +1 631 344 2060. E-mail: arogers@bnl.gov

Abbreviations: A, net CO_2 uptake ($\mu mol\ m^{-2}\ s^{-1}$); c_i, CO_2 concentration in the sub-stomatal cavity ($\mu mol\ mol^{-1}$); *FACE*, free-air CO_2 enrichment; I, photosynthetic quantum flux density ($\mu mol\ m^{-2}\ s^{-1}$); J_{max}, maximum *in vivo* rate of electron transport ($\mu mol\ m^{-2}\ s^{-1}$); *Lsu*, large subunit of Rubisco; pCO_2, partial pressure of CO_2; T_{leaf}, leaf temperature (°C); TCA, trichloroacetic acid; $V_{c,max}$, maximum *in vivo* rate of ribulose 1,5 bisphosphate-saturated carboxylation ($\mu mol\ m^{-2}\ s^{-1}$); *WIMOVAC*, Windows Intuitive Model of Vegetation response to Atmosphere and Climate change.

seek to measure the same parameter, yet in the few studies where *in vivo* and *in vitro* values for Rubisco activity are presented they rarely concur, commonly the *in vitro* values are lower than those obtained *in vivo* (Myers *et al*., 1999; Tissue *et al*., 1999; Griffin *et al*., 2000).

The authors hypothesized that the NADH-linked spectrophotometric *in vitro* method underestimates the *in situ* activity of Rubisco and that this underestimation is due largely to an insufficient extraction of Rubisco protein prior to the *in vitro* assay. To address this hypothesis two approaches were used. (1) A model (*WIMOVAC*; Humphries and Long, 1995) was used to predict C_3 photosynthesis in order to determine if the *in vitro* values of Rubisco activity reported in the literature are mechanistically capable of supporting the observed A. (2) Rubisco activity in the needles of *Pinus taeda* (loblolly pine) grown in current and elevated pCO_2 was determined using an *in vitro* method (Tissue *et al*., 1993) and *in vivo* by gas exchange and subsequent analyses of the initial slope of the A–c_i response (Wullschleger, 1993). Four questions linked to this hypothesis have been addressed.

(1) Based on the mechanism of C_3 photosynthesis described earlier (Farquhar *et al*., 1980), do *in vitro* methods underestimate Rubisco activity?

(2) Is underestimation of Rubisco activity attributable to an incomplete extraction of Rubisco protein prior to *in vitro* analyses?

(3) Can *in vitro* measurements be corrected to give mechanistically valid, quantitative values useful in modelling photosynthesis and its limitations?

(4) Are *in vitro* estimates of Rubisco activity qualitatively consistent with *in vivo* values?

Materials and methods

Plant material and growth conditions

The study was conducted at the free-air CO_2 enrichment (*FACE*) site in the Blackwood Division of Duke Forest in Orange County, NC, USA. The site and the *FACE* facility are described elsewhere (Ellsworth, 1999). The mid-sections of current year needles from 16-year-old loblolly pines grown for *c*. 2.5 years in elevated (current + 20 Pa) and current (36 Pa) pCO_2 were sampled on 12 May 1999 for the analysis described below (maximum temperature *c*. 30 °C, maximum I 1800 μmol m^{-2} s^{-1}). Needle surface area was determined geometrically as described previously (Johnson, 1984).

Gas exchange measurements

In situ measurements of the responses of A to pCO_2 (A–c_i curves), were measured with a portable photosynthesis system (Li-Cor model 6400, Lincoln, NE, USA). Sunlit pine needles at the top of the crown were sealed inside the chamber while ensuring that chamber conditions maintained growth pCO_2, light saturation and a constant temperature (28 °C). After a short period of equilibration to chamber conditions, the measurements of A, c_i, and stomatal conductance to water vapour were recorded along with environmental parameters. Chamber pCO_2 was then changed and stepped through seven different concentrations starting close to the CO_2 compensation point and ending in elevated pCO_2. Measurements at each successive pCO_2 were made after complete flushing of the chamber with the desired pCO_2 judged by stabilization of the CO_2 signal. Frequent leak tests were made to minimize bias in the low pCO_2 measurements and Teflon tape was used to seal the chamber for measurements. Measurements were made on needles from one tree in each separate experimental plot for the three replicate plots in current and elevated pCO_2, concurrently with the *in vitro* measurements.

In vitro *Rubisco activity*

Five replicate samples were taken from each treatment replicate. These samples included the needles used in the *in situ* gas exchange measurements. The tip and base sections of each fascicle were discarded, the mid-section was immediately ground for 10 s in extraction buffer (Tissue *et al*., 1993) at 4 °C using a high speed homogenizer (Polytron; Kinematica, Switzerland). Homogenized samples were frozen immediately and stored in liquid nitrogen until analysed. The process of removing needles to freezing in liquid nitrogen took less than 2 min. Samples were thawed and centrifuged at 13 000 *g* for 30 s in a microcentrifuge tube. An aliquot of the supernatant was used immediately for determining the initial and total (fully activated) activity of Rubisco using the spectrophotometric, NADH, enzyme-coupled assay described earlier (Tissue *et al*., 1993). Activation state of Rubisco was calculated as the ratio of initial activity to total activity.

Rubisco content

The supernatant resulting from the Rubisco activity analysis was also used for SDS-PAGE. An aliquot was combined with a solution of 62 mM tri(hydroxymethyl)-aminomethane, 2% (w/v) SDS, 65 mM dithiothreitol, and 10% (v/v) glycerol. The trichloroacetic acid (TCA)/acetone method described previously (Damerval *et al*., 1986) with some adaptations (Rogers *et al*., 1998) was used to precipitate total needle protein from needles sampled in parallel with those used for the Rubisco activity analyses and from the pellets resulting from the Rubisco activity analysis. Proteins were resolved on 12–18% SDS-polyacrylamide gels as described earlier (Nie *et al*., 1995). Gels were loaded on an equal needle surface area basis. The large subunit of Rubisco was detected by staining with Coomassie brilliant blue R-250. The identity of the *Lsu* Rubisco was confirmed by co-electrophoresed molecular weight markers (BioRad, Hercules CA, USA). Quantification of individual bands was performed using a two-dimensional laser scanning densitometer (model 300A Molecular Dynamics, Sunnyvale CA, USA) as described previously (Nie *et al*., 1995).

Protein content

The protein of the supernatant and pellet resulting from the centrifugation of the Rubisco extraction buffer was precipitated for 16 h at 4 °C with 10% TCA. Precipated protein was washed twice with acetone and dissolved in 0.1 M sodium hydroxide. Protein content was determined using a commercial kit (BCA Protein Assay, Pierce, Rockford, IL, USA).

Modelling

The data used for modelling were taken from studies where both the *in vivo* gas exchange method, and the *in vitro* spectrophotometric method (Lilley and Walker, 1974) were used to determine Rubisco activity. Only studies which provided enough information to determine values for A, I, T_{leaf}, pCO_2, $V_{c,max}$, and J_{max} were selected. The *WIMOVAC* modelling system (Humphries and Long, 1995) was used to simulate the effects of elevated pCO_2 on A. The equations used to model leaf photosynthesis (based on those described by Farquhar *et al.*, 1980), are listed in Long and Drake (Long and Drake, 1992) with the exception of the term for electron transport rate that can be found in Evans and Farquhar (Evans and Farquhar, 1991). Dark respiration was assumed to be 1 μmol m^{-2} s^{-1} (McMurtrie and Wang, 1993). and c_i to be 0.7 of growth pCO_2 (Long and Drake, 1992; Drake *et al.*, 1997). For each data set values were entered for I, T_{leaf}, pCO_2, $V_{c,max}$, and J_{max} and then *WIMOVAC* was used to predict A in current pCO_2 (Rogers and Humphries, 2000). The procedure was repeated using the Rubisco activity determined *in vitro* at both initial and total activity. While the resultant modelled A is somewhat sensitive to the kinetic constants assumed, the goal of this study was not to model A precisely, but instead to test the degree of closure between calculations based on gas exchange measurements and enzymatic measurements while using the widely-accepted parameterization of the Farquhar biochemical model of photosynthesis.

Statistical analysis

Differences in A (Tables 2, 4) and Rubisco activity (Fig. 1) were examined by analysis of variance using $P = 0.05$ as the level of significance. An *a posteriori* Tukey test was used to test for significant differences between individual means. All other statistical analyses were performed using Student's *t*-test.

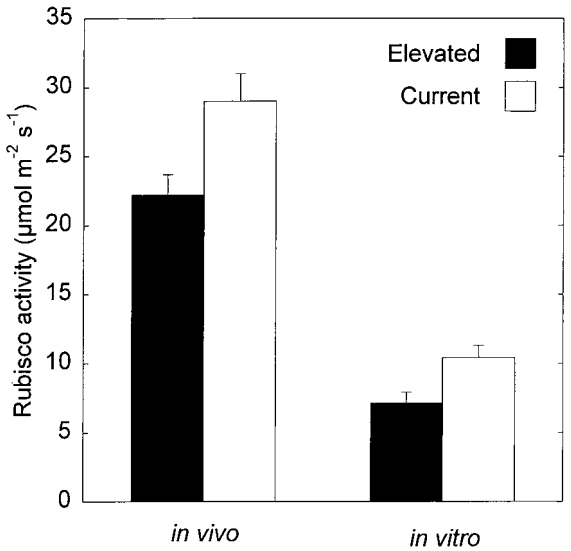


Fig. 1. Mean Rubisco activity (±se, $n = 3$ replicate rings) measured in mature loblolly pines growing in elevated pCO_2 (56 Pa, solid bars) and current pCO_2 (36 Pa, open bars). Estimates of Rubisco activity were made by analysing the response of A to c_i following gas exchange measurements (*in vivo*) and spectrophotometrically following homogenization and freezing of needles (*in vitro*). All means were significantly different from one another ($P < 0.05$).

Results

Literature survey

The mean Rubisco activity reported for *in vitro* values from 10 species was *c.* 50% of that reported from *in vivo* measurements ($t_{(2),44}$, $P < 0.05$; Table 1). The model successfully predicted A when supplied with the Rubisco activity determined *in vivo* (Table 1). For a few studies which reported all the necessary information it was possible to model the A attainable with *in vivo*, *in vitro* and fully activated *in vitro* Rubisco activity (Table 2). Again, for this smaller data set from eight species, *WIMOVAC* successfully predicted the observed A. The modelled A obtained when both initial and fully activated *in vitro* Rubisco activity values were used was significantly lower ($P < 0.05$) than the observed A (Table 2).

Experimental results

The *in vivo* values observed for Rubisco activity were significantly higher than the *in vitro* values ($F_{1,8} = 153$, $P < 0.001$; Fig. 1). Figures 2A and 3A show the significantly reduced content of the *Lsu* Rubisco in the supernatant used for *in vitro* Rubisco activity analysis compared to the amount of the *Lsu* Rubisco recovered using the more thorough TCA method (elevated pCO_2, $P < 0.05$; current pCO_2, $P < 0.05$). Analysis of the pellet and supernatant in samples taken in parallel with those used for the *in vitro* analysis demonstrated that the supernatant contained *c.* 25% of the protein and *c.* 35% of the Rubisco present in the extract prior to centrifugation (Table 3). Rubisco and protein content in the pellet were significantly higher than that in the supernatant ($t_{(2),5}$, $P < 0.1$, and $P < 0.001$, respectively, $n = 6$). There was no effect of pCO_2 on the distribution of Rubisco between the supernatant and the pellet. Growth in elevated pCO_2 resulted in a significant decrease in Rubisco activity ($F_{1,8} = 13.8$, $P < 0.01$). This *c.* 25–30% decrease in Rubisco activity was observed in both the *in vivo* ($V_{c,max}$) and the *in vitro* measurements ($P < 0.05$, Fig. 1). There was no significant effect of elevated pCO_2

Table 1. *The ratio (*R*) of Rubisco activity measured* in vitro *to that measured* in vivo*; and the ratio of the net CO_2 uptake rate modelled using* WIMOVAC *with the* in vivo *value of Rubisco activity ($A_{modelled}$) to the observed CO_2 uptake rate ($A_{observed}$);* Sp = *number of species*, n = *number of data used in comparison*

Comparison	*R*	*Sp,n*	References
in vitro : *in vivo* Rubisco activity	0.47	10,45	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11
$A_{modelled}$: $A_{observed}$	0.98	10,33	1, 2, 3, 5, 6, 7, 8, 9, 10, 11

[1]Habash *et al.* (1995); [2]Li *et al.* (1999); [3]Martindale and Leegood (1997); [4]McKee *et al.* (1995); [5]Myers *et al.* (1999); [6]Paul and Driscoll (1997); [7]Socias *et al.* (1993); [8]Tissue *et al.* (1999); [9]Turnbull *et al.* (1998); [10]van Oosten and Besford (1995); [11]von Caemmerer and Farquhar (1984).

Table 2. *Mean Assimilation (±se, n = 16) either reported directly in the literature or modelled from reported values of Rubisco activity using the* WIMOVAC *system to simulate C_3 photosynthesis*

Data used for this comparison were taken from the studies cited in Table 1 that determined Rubisco activity using the NADH linked spectrophotometric assay. Means with a common letter are not significantly different ($P < 0.05$).

	Assimilation (μmol m^{-2} s^{-1})
Reported	11.20 ± 1.9 a
Modelled using *in vivo* Rubisco activity	10.30 ± 1.6 ac
Modelled using *in vitro* Rubisco activity	4.10 ± 0.8 b
Modelled using fully activated *in vitro* Rubisco activity	5.70 ± 1.1 bc

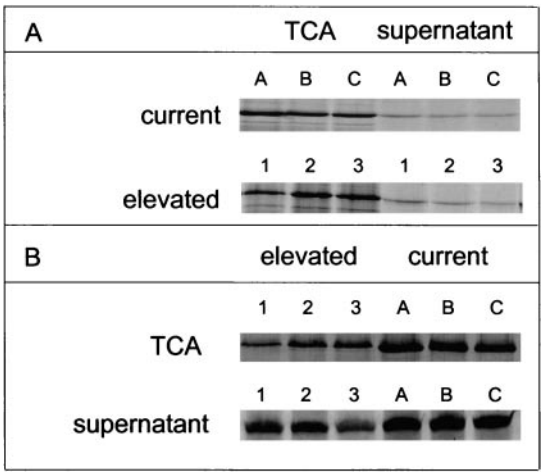


Fig. 2. Sections of Coomassie Blue stained, SDS-PAGE gels showing the levels of the large sub unit (*Lsu*) of Rubisco measured in mature loblolly pines growing in elevated (56 Pa) and current (36 Pa) pCO_2, extracted using two different methods. (A) The comparison between the amount of *Lsu* Rubisco present in the TCA extract and the amount present in the supernatant used to determine the *in vitro* Rubisco activity at both elevated and current pCO_2. (B) The comparison between the levels of *Lsu* Rubisco in elevated and current pCO_2 in protein extracted using a TCA/acetone method (TCA) and in protein taken from the supernatant used to estimate the *in vitro* Rubisco activity (supernatant). Valid comparisons are only possible within a gel. Numbers 1, 2 and 3 indicate the three replicate rings in elevated pCO_2. The letters A, B and C indicate the three replicate rings in current pCO_2.

on the activation state of Rubisco (elevated 46 ± 7%; current 52 ± 9%; $t_{(2),2}$, $P > 0.05$), but the relative levels of the *Lsu* Rubisco in elevated pCO_2 were *c*. 25–30% lower than those in current pCO_2 (Figs 2, 3). This significant reduction in Rubisco content in elevated pCO_2 was observed in the extract used to determine the *in vitro* Rubisco activity ($t_{(1),2}$, $P < 0.05$, Figs 2B, 3B) and in a TCA/acetone total protein extraction ($t_{(2),2}$, $P < 0.05$, Figs 2B, 3B).

Correction of in vitro *Rubisco activity*

The difference between the *Lsu* content of the TCA/acetone extract and the supernatant (Figs 2, 3) was used

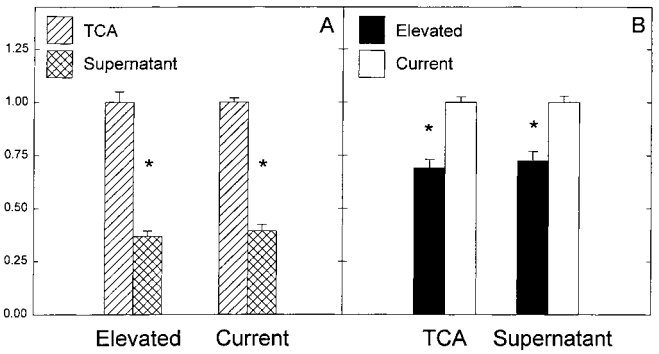
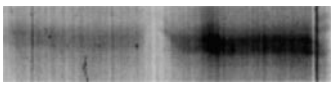


Fig. 3. Bars show the mean levels of the *Lsu* Rubisco (±se, $n = 3$ replicate rings) quantified from the SDS-PAGE gels in Fig. 2. Comparisons are only possible within a pair of bars ($^*t_{(2),2}$, $P < 0.05$). (A) The levels of *Lsu* Rubisco in the TCA/acetone protein isolation (TCA) compared with the levels in the supernatant used for Rubisco activity analysis (supernatant) in elevated and current pCO_2. (B) The levels of the *Lsu* Rubisco in elevated (56 Pa) and current (36 Pa) pCO_2 determined in using a TCA/acetone extraction method and determined from the supernatant used for the *in vitro* Rubisco activity analysis.

Table 3. *Rubisco and protein content of the supernatant and pellet resulting from centrifugation of the Rubisco extraction buffer*

Analysis was performed on needles taken in parallel with those used for activity analysis. The coomassie blue stained *Lsu* Rubisco band from SDS-PAGE is shown for one representative replicate. The results of quantifying all six replicates using laser densitometry are shown below.

	Supernatant	Pellet	Percentage in supernatant
Lsu Rubisco			33 ± 4
Protein (mg)	3.2 ± 0.4	10.6 ± 0.4	23 ± 3

to correct *in vitro* values of Rubisco activity for Rubisco not present in the supernatant using a multiplier (i.e. 2.63). Although slightly smaller, the corrected *in vitro* values for Rubisco activity were not significantly different from the *in vivo* values ($P > 0.05$; data not shown). Table 4 shows the observed A and the result of using *WIMOVAC* to predict A using either the *in vitro, in vivo* or corrected *in vitro* value for Rubisco activity. Regardless of pCO_2 treatment, the A predicted using the *in vitro* value for Rubisco activity was significantly smaller than the observed A ($P < 0.001$). The values of A modelled using the *in vivo* and corrected *in vitro* Rubisco activities were not significantly different from each other or the observed A ($P > 0.05$; Table 4).

Discussion

Does the in vitro *assay underestimate Rubisco activity?*

Following a literature search, the *in vitro* measurements of Rubisco activity were used in *WIMOVAC* to predict A.

Table 4. *Mean (±se, n=3) observed and modelled* A *for the plants described in Fig. 2*

A was modelled using the *WIMOVAC* system to simulate C_3 photosynthesis. Means with a common letter are not significantly different ($P<0.05$).

pCO_2	Observed *A*	Estimate of Rubisco activity used for modelling *A*		
		In vivo	*In vitro*	Corrected *in vitro*
Elevated	6.6±0.6 a	6.4±0.5 ac	1.6±0.3 b	5.3±0.7 ac
Current	5.4±0.4 bc	5.4±0.4 ac	1.5±0.2 b	5.0±0.4 ac

The predicted *A* was *c*. 65% lower than the observed *A*, clearly demonstrating that the observed *A* was mechanistically impossible if the *in vitro* estimate of Rubisco activity was correct. It has been suggested that the low values of Rubisco activity reported using the NADH spectrophotometric assay are due to a loss of activation associated with the extraction of Rubisco (Sage *et al*., 1993; Theobald *et al*., 1998). However, when the fully activated *in vitro* values for Rubisco activity were used in the model instead of the initial values, the predicted *A* was still *c*. 50% lower than the observed *A* (Table 2). Even when maximal activity (the activity of the fully activated enzyme in the absence of inhibitors; Parry *et al*., 1997) was measured in spring wheat, a value was reported that was still *c*. 20% lower than that required to support the *A* observed in the same plants (Theobald *et al*., 1998).

The experimental data support the conclusions drawn from the literature search. Figure 1 shows that the value for Rubisco activity obtained using the *in vitro* method is lower than that obtained using the *in vivo* method and when used for modelling *A* the *in vitro* values were found to be too low to account for the observed *A* (Table 4). Despite the low activation state (*c*. 50%) the observed value for the fully activated Rubisco was still not sufficient to account for the observed *A* (data not shown). Clearly the *in vitro* assay underestimates Rubisco activity.

Is underestimation of Rubisco activity attributable to an incomplete extraction of Rubisco protein prior to in vitro *analyses?*

The protein in needles sampled in parallel with the Rubisco activity assay was precipitated using a TCA/acetone method. This method is a thorough total protein extraction method (Damerval *et al*., 1986; Rogers *et al*., 1998), which, it was assumed, would precipitate all the protein in the needle samples. Figures 2A and 3A clearly show that the amount of *Lsu* Rubisco present in the supernatant used for the spectrophotometric assay of Rubisco activity is lower than the amount in needles sampled in parallel using the TCA/acetone method. As a confirmation the distribution of total protein and Rubisco between the pellet and supernatant was analysed. A large proportion of Rubisco was present in the pellet supporting the hypothesis that Rubisco is underrepresented in the supernatant (Table 3). The difference in the distribution between the supernatant and pellet of total protein and Rubisco is probably due to insoluble proteins that are not represented in the assay buffer. This also suggests that it is unlikely that the low Rubisco activity values obtained with the *in vitro* method are due specifically to insoluble forms of Rubisco which are not present in the supernatant (Crafts-Brandner *et al*., 1991; Crafts-Brandner and Salvucci, 1994). Using a similar protocol for Rubisco activity analysis in soybean, it has been demonstrated that the relative quantity of Rubisco in the supernatant and pellet fractions were similar to the distribution of Rubisco activity (Crafts-Brandner *et al*., 1991). This work further supports this study's hypothesis that the 'missing' activity is associated with Rubisco present in the pellet. It is concluded that the rapid, one-step homogenization procedures used for Rubisco isolation, in an attempt to preserve enzyme activation state, fail to isolate a large proportion of the Rubisco present in the needles and lead to an underestimation of Rubisco activity when expressed on a leaf area basis.

Can in vitro *measurements be corrected to give mechanistically valid, quantitative values?*

Since TCA extracts co-electrophoresed with the supernatant used for the activity analysis allowed the determination of relative Rubisco content in both extracts (Fig. 2A), the activity value could be corrected for the Rubisco not represented in the supernatant. Table 4 shows that the uncorrected values of *in vitro* Rubisco activity are not sufficient to account for the observed *A*. However, if these values are corrected as described above the modelled *A* is comparable to, and not significantly different from, the observed *A*. This suggests that, at least for loblolly pines, TCA/acetone extractions made in parallel with activity measurements can be used to correct for the poor representation of Rubisco in the buffer used for activity analysis and provide mechanistically valid, quantitative estimates of Rubisco activity.

Are in vitro *estimates of Rubisco activity qualitatively consistent with* in vivo *values?*

Growth in elevated pCO_2 resulted in a significant *c*. 25% reduction in the *in vitro* measured Rubisco activity (Fig. 1) with no change in the activation state of the

enzyme due to pCO_2 treatment. This decrease observed with the *in vitro* assay was also measured *in vivo*. This suggests that although the absolute values obtained using the *in vitro* method may not be mechanistically consistent with the observed *A* the relative treatment effects reported are still valid. This qualitative agreement between the values of Rubisco activity reported in studies using *in vitro* and *in vivo* methods is well documented (Li *et al*., 1999; Myers *et al*., 1999; Tissue *et al*., 1999; Griffin *et al*., 2000). Furthermore, Figs 2B and 3B clearly show that the decrease of the *Lsu* Rubisco in elevated pCO_2 is visible in both protein extracts and is of the same magnitude.

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