Possible explanation of the disparity between the *in vitro* and *in vivo* measurements of Rubisco activity: a study in loblolly pine grown in elevated pCO₂

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Received 19 February 2001; Accepted 21 March 2001

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 spectro-photometric technique in mature *Pinus taeda* L. (loblolly pine) trees grown using free-air CO₂ enrichment in elevated (56 Pa) and current (36 Pa) pCO₂. In addition, for studies where both *in vivo* and *in vitro* values of Rubisco activity were reported net CO₂ 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 CO₂ treatment concurred with, and were of equal magnitude to, those observed in Rubisco activity measured *in vivo*.

Key words: Rubisco activity, elevated CO₂.

Introduction
It is well documented that with long-term exposure to elevated pCO₂ the initial stimulation of net CO₂ uptake (A) is often not maintained (Gunderson and Wullschleger, 1994; Curtis, 1996; Drake et al., 1997). Such a reduction in photosynthetic capacity in elevated pCO₂, 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 vitro* 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

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Abbreviations: A, net CO₂ uptake (µmol m⁻² s⁻¹); cᵢ, CO₂ concentration in the sub-stomatal cavity (µmol mol⁻¹); FACE, free-air CO₂ enrichment; I, photosynthetic quantum flux density (µmol m⁻² s⁻¹); Jₘₘₙₓ, maximum *in vivo* rate of electron transport (µmol m⁻² s⁻¹); Lsu, large subunit of Rubisco; pCO₂, partial pressure of CO₂; Tₑₕₑₚ, leaf temperature (ºC); TCA, trichloroacetic acid; Vₑₜₚₐₓ, maximum *in vivo* rate of ribulose 1,5 bisphosphate-saturated carboxylation (µmol m⁻² s⁻¹); WIMOVAC, Windows Intuitive Model of Vegetation response to Atmospheric 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 C₃ 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* (lobolly pine) grown in current and elevated pCO₂ 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–cᵣ response (Wullschleger, 1993). Four questions linked to this hypothesis have been addressed.

1. Based on the mechanism of C₃ 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 CO₂ 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 lobolly pines grown for c. 2.5 years in elevated (current+20 Pa) and current (36 Pa) pCO₂ were sampled on 12 May 1999 for the analysis described below (maximum temperature c. 30 °C, maximum J 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 pCO₂ (A–cᵣ 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 pCO₂, light saturation and a constant temperature (28 °C). After a short period of equilibration to chamber conditions, the measurements of A, cᵣ, and stomatal conductance to water vapour were recorded along with environmental parameters. Chamber pCO₂ was then changed and stepped through seven different concentrations starting close to the CO₂ compensation point and ending in elevated pCO₂. Measurements at each successive pCO₂ were made after complete flushing of the chamber with the desired pCO₂ judged by stabilization of the CO₂ signal. Frequent leak tests were made to minimize bias in the low pCO₂ 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 pCO₂, 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 (Dameval 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. Precipitated 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_kat, 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_kat, 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

<table>
<thead>
<tr>
<th>Comparison</th>
<th>R</th>
<th>Sp.n</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>in vitro : in vivo Rubisco activity</td>
<td>0.47</td>
<td>10.45</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11</td>
</tr>
<tr>
<td>A_{modelled} : A_{observed}</td>
<td>0.98</td>
<td>10.33</td>
<td>1, 2, 3, 5, 6, 7, 8, 9, 10, 11</td>
</tr>
</tbody>
</table>

1Habash et al. (1995); 2Li et al. (1999); 3Martindale and Leegood (1997); 4McKee et al. (1995); 5Myers et al. (1999); 6Paul and Driscoll (1997); 7Socias et al. (1993); 8Tissue et al. (1999); 9Turnbull et al. (1998); 10van Oosten and Besford (1995); 11von 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 C3 photosynthesis

<table>
<thead>
<tr>
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<th>Assimilation (µmol m⁻² s⁻¹)</th>
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<tbody>
<tr>
<td>Reported</td>
<td>11.20 ± 1.9 a</td>
</tr>
<tr>
<td>Modelled using in vivo Rubisco activity</td>
<td>10.30 ± 1.6 ac</td>
</tr>
<tr>
<td>Modelled using in vitro Rubisco activity</td>
<td>4.10 ± 0.8 b</td>
</tr>
<tr>
<td>Modelled using fully activated in vitro Rubisco activity</td>
<td>5.70 ± 1.1 bc</td>
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</table>

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) pCO₂ 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 pCO₂. (B) The comparison between the levels of Lsu Rubisco in the supernatant and pelleted pCO₂ 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 current pCO₂.

A TCA supernatant

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>A</th>
<th>B</th>
<th>C</th>
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<tr>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
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B elevated current

<table>
<thead>
<tr>
<th></th>
<th>elevated</th>
<th>current</th>
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<tr>
<td>1</td>
<td>2</td>
<td>3</td>
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</table>

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 (tₚ,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 pCO₂. (B) The levels of the Lsu Rubisco in elevated (56 Pa) and current (36 Pa) pCO₂ 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

<table>
<thead>
<tr>
<th></th>
<th>Supernatant</th>
<th>Pellet</th>
<th>Percentage in supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lsu Rubisco</td>
<td>3.2 ± 0.4</td>
<td>10.6 ± 0.4</td>
<td>33 ± 4</td>
</tr>
</tbody>
</table>

Protein (mg) 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 vivo, in vitro or corrected in vitro value for Rubisco activity. Regardless of pCO₂ 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.
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.

**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 C3 photosynthesis. Means with a common letter are not significantly different ($P<0.05$).

<table>
<thead>
<tr>
<th>pCO₂</th>
<th>Observed $A$</th>
<th>Estimate of Rubisco activity used for modelling $A$</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>In vivo</td>
</tr>
<tr>
<td>Elevated</td>
<td>$6.6\pm0.6\ a$</td>
<td>$6.4\pm0.5\ ac$</td>
</tr>
<tr>
<td>Current</td>
<td>$5.4\pm0.4\ bc$</td>
<td>$5.4\pm0.4\ ac$</td>
</tr>
</tbody>
</table>

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 $pCO₂$ 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.

Acknowledgements

We thank David Tissue for instruction in Rubisco activity analysis, Divine Adika and Elke Naumburg for technical assistance and George Hendrey, Keith Lewin, John Nagy and Andrew Palmiotti for dedication in constructing and operating the FACE system. This research was performed under the auspices of the US Department of Energy under contract no. DE-AC02-98CH10886.

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