Estimating the Excess Investment in Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase in Leaves of Spring Wheat Grown under Elevated CO₂[1]

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Wheat (*Triticum aestivum* L.) was grown under CO₂ partial pressures of 36 and 70 Pa with two N-application regimes. Responses of photosynthesis to varying CO₂ partial pressure were fitted to estimate the maximal carboxylation rate and the nonphotorespiratory respiration rate in flag and preceding leaves. The maximal carboxylation rate was proportional to ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content, and the light-saturated photosynthetic rate at 70 Pa CO₂ was proportional to the thylakoid ATP-synthase content. Potential photosynthetic rates at 70 Pa CO₂ were calculated and compared with the observed values to estimate excess investment in Rubisco. The excess was greater in leaves grown with high N application than in those grown with low N application and declined as the leaves senesced. The fraction of Rubisco that was estimated to be in excess was strongly dependent on leaf N content, increasing from approximately 5% in leaves with 1 g N m^{-2} to approximately 40% in leaves with 2 g N m^{-2}. Growth at elevated CO₂ usually decreased the excess somewhat but only as a consequence of a general reduction in leaf N, since relationships between the amount of components and N content were unaffected by CO₂. We conclude that there is scope for improving the N-use efficiency of C₃ crop species under elevated CO₂ conditions.

The effects of the increasing atmospheric CO₂ concentration on photosynthesis has stimulated an enormous volume of research, which has been extensively reviewed (Lawlor and Mitchell, 1991; Bowes, 1993; Drake et al., 1997). One aspect of particular interest for the possibility of crop improvement is the way in which a change in CO₂ partial pressure alters the balance of limitation between processes that determine the instantaneous A in C₃ plants. At high light, photosynthesis can be limited by the carboxylation capacity or by the light-saturated capacity for regeneration of RuBP, and the optimal use of resources invested in photosynthetic components (i.e. that which gives the maximal rate of photosynthesis) for a given environment occurs when these capacities are balanced (Farquhar and Sharkey, 1994). When CO₂ partial pressure increases,

the carboxylation capacity increases more than the light-saturated capacity for RuBP regeneration; therefore, the theoretical optimal balance of investment in photosynthetic components is altered (Sage, 1994; Webber et al., 1994; Medlyn, 1996).

Optimization of these components has considerable implications for N-use efficiency of leaf photosynthesis (Makino et al., 1997), since photosynthetic components involved in determining the carboxylation capacity (Rubisco) and light-saturated RuBP-regeneration capacity (e.g. thylakoid ATP-synthase and the Cyt b/f complex) account for a substantial fraction of leaf N (Evans and Seemann, 1989). For a doubling of CO₂ partial pressure from the current ambient conditions (36 Pa), a 30% to 40% increase in the ratio of light-saturated RuBP-regeneration capacity to carboxylation capacity has been predicted for optimal N-use efficiency (Webber et al., 1994; Medlyn, 1996). Since N is likely to be more limiting to growth at elevated CO₂, this increase in ratio would normally be expected to be achieved by a specific decrease in the amount of Rubisco.

Experimental evidence from gas-exchange studies suggests that such optimization occurs only infrequently in C₃ plants grown at elevated CO₂ (Makino, 1994; Sage, 1994; Medlyn, 1996). However, many studies have found evidence of acclimatory decreases in A and in amount of components on a leaf-area basis in response to growth at elevated CO₂ (for review, see Webber et al., 1994; Drake et al., 1997). Similarly, at subambient CO₂, Rubisco content per unit leaf area may increase (Rowland-Bamford et al., 1991), but there is little evidence for an increase in carboxylation capacity relative to other capacities (Sage and Reid, 1992). It therefore seems that many of the observations reflect a general reduction in all photosynthetic components due to a source-sink imbalance at higher CO₂ rather than to an optimization between photosynthetic components. This is consistent with the hypothesis that the mechanism for the effect of elevated CO₂ on amounts of photosynthetic components is a response to carbohydrate buildup in the leaves (van Oosten and Besford, 1996).

Abbreviations: A, photosynthetic rate; A_g, gross photosynthetic rate; CABP, 2-carboxyarabinitol-1,5-bisphosphate; CF₁, thylakoid coupling factor 1; g_w, conductance for diffusion of CO₂ from the intercellular space to the carboxylation site; p_a, external CO₂ partial pressure; p_i, internal CO₂ partial pressure; RuBP, ribulose-1,5-bisphosphate.

Transcription for both a subunit of thylakoid ATP-synthase and the Rubisco small subunit was decreased by cold-girdling, which decreased carbohydrate export from the leaf (Krapp and Stitt, 1995).

In one study (Nie et al., 1995) there was apparent support for an optimization response in wheat crops grown in a free-air CO_2-enrichment facility. While there was no effect of elevated CO_2 on the amount of Rubisco when the leaf emerged, there was a subsequent effect, because Rubisco was decreased relative to other photosynthetic components. However, important recent work (Nakano et al., 1997) has shown that in young rice leaves the effect of elevated CO_2 partial pressure appears to operate entirely via a reduction in leaf N. Since leaves with less N contain a smaller fraction of Rubisco, there is a de facto readjustment in favor of RuBP regeneration compared with carboxylation capacity, which is dependent on N-supply conditions. This result could explain the variability in experimental findings on the occurrence of the theoretical optimization (Sage, 1994), since any rebalancing will depend on whether a reduction in leaf N occurs at elevated CO_2 under the experimental conditions.

The issue is of particular importance for C_3 crop species, because if they do not optimize their investment in photosynthetic components in response to growth CO_2 partial pressure, there may be opportunities to increase the N-use efficiency of photosynthesis by genetic manipulation for the higher CO_2 environments of the future. Specifically, if there is no optimization under agronomic conditions, there will be excess investment in Rubisco relative to RuBP-regeneration capacity at high CO_2 concentrations. Makino et al. (1997) recently demonstrated that N-use efficiency of leaf photosynthesis in rice, measured during short-term exposure to elevated CO_2 concentrations, was greater in lines that had been transformed to specifically reduce the amount of Rubisco compared with the wild type. The extent to which this would be advantageous in plants grown at elevated CO_2 is dependent on how much Rubisco remains in excess when given the opportunity to acclimate. The aim of this study was to estimate the amount of Rubisco that was in excess of the requirement for leaf photosynthesis in wheat plants grown at elevated CO_2 partial pressures and various N-supply treatments under conditions representative of the field. To this end, a large number of gas-exchange measurements were combined with estimations of Rubisco and thylakoid ATP-synthase contents, the latter being chosen as the largest single component of RuBP regeneration (Evans and Seemann, 1989).

MATERIALS AND METHODS

Plant Culture

Data are presented from three experiments on spring wheat (*Triticum aestivum* L. cv Minaret) grown in controlled environments. Experiments 1 and 2 were designed to estimate the amount of excess Rubisco at elevated CO_2 under different N-supply regimes, and experiment 3 was designed to relate the estimated excess Rubisco to its activation state. For all experiments, nine pregerminated seeds were sown in each 10-L plastic bag containing sintered agrillite rooting medium and Perlite. Groups of six bags were placed together in boxes on wheels, arranged to form four replicate arrays of three by four boxes, each assigned to a separate growth chamber (described by Lawlor et al., 1993). In experiments 1 and 2, plants were grown at two N supplies and at CO_2 partial pressures of 36 Pa (ambient) or 70 Pa (elevated). In experiment 3, all plants were grown with a total N supply of 10 g m^{-2} and a CO_2 partial pressure of 36 Pa (ambient) or 100 Pa (elevated); the more extreme elevated CO_2 was chosen to ensure significant effects on activation.

Nutrient solution (described by Delgado et al., 1994) was applied twice weekly from approximately 10 to 70 d after sowing; the nitrate concentrations were 12.5 and 4.6 mM for high- and low-N regimes, respectively, for experiments 1 and 2, and 6.25 mM for experiment 3. Nitrate concentrations were varied by substituting chloride. Following eight nutrient applications, the concentrations of solutions were quadrupled in all experiments for the remaining applications. The total N application was equivalent to 8 and 18 g m^{-2} in experiment 1, 8 and 21 g m^{-2} in experiment 2 for the low- and high-N treatments, and 9.8 g m^{-2} in experiment 3. Although the total N applied was similar in experiments 1 and 2, the nature of the application regimes were different, since the last application was 30 d before anthesis in experiment 1 and 8 d before anthesis in experiment 2. The exact nature of the N-application regimes was not critical for the hypotheses being tested, but this change between experiments was made to induce a wider range of leaf N contents in experiment 2. Plants were watered daily with demineralized water and grown with day/night temperature regimes of 20°C/7°C from the start of tillering until maturity (before this time the temperatures were 12°C/6°C), a 16-h photoperiod, and constant artificial PPFD of 580 µmol quanta m^{-2} s^{-1} at plant height. To minimize positional effects, boxes were moved within and between chambers every 10 to 11 d. To mimic a continuous crop with an even canopy structure, reflective screens were placed from the soil surface to the top of the canopy around the arrays. Gaps caused by destructive sampling were filled with bags containing plants of the same treatment.

Gas-Exchange Measurement and Analyses

The response of A to varying p_i in the leaf was determined in a six-chamber, open-circuit gas-exchange system as previously described (Lawlor et al., 1989), with a leaf temperature of 20°C, a leaf-to-air vapor-pressure deficit of 1.5 kPa, and a PPFD of 1400 to 1500 µmol quanta m^{-2} s^{-1}. Saturating PPFD was used because the study concerned the effect of growth CO_2 on the balance of carboxylation and RuBP-regeneration capacities, which is dictated by the amount of the components. Responses were determined in six replicate leaves for each treatment and occasion. In experiment 1, responses were determined in flag−1 (leaf preceding flag) and flag leaves on three and four occasions, respectively; in experiment 2 responses were determined in flag−2 (leaf preceding flag−1), flag−1, and flag leaves on three, three, and six occasions, respectively; and in exper-

iment 3 responses were determined in flag leaves on seven occasions.

The first measurement of each leaf was when the ligule had just become visible. Light-saturated photosynthesis was estimated at a p_i of approximately 2, 4, 7, 10, 14, 25, 50, 60, and 68 Pa for each leaf. In addition, the sensitivity of leaf photosynthesis to changing O_2 partial pressure from ambient (20 kPa) to 2 kPa was determined at a p_a of 70 Pa. Light-saturated photosynthesis was assumed to be determined by the RuBP-saturated kinetics of Rubisco for p_i <35 Pa (Makino et al., 1988), and points in this region were fitted to Equation 1 below. In about 10% of cases this procedure gave a poor fit ($r^2 < 0.90$), in which case the fit was restricted to points with a $p_i < 25$ Pa, which invariably increased r^2 to >0.90. The responses were fitted assuming a finite g_w (Loreto et al., 1992) so that A_g is given by:

$$A_g = \frac{1}{2}(K \cdot g_w + p_i \cdot g_w + R_d + V_{cmax} - \Delta),$$

$$\Delta = \sqrt{\begin{array}{l} K^2 \cdot g_w^2 + 2 \cdot K \cdot g_w^2 \cdot p_i + 2 \cdot K \cdot g_w \cdot R_d \\ + 2 \cdot K \cdot g_w \cdot V_{cmax} + p_i^2 \cdot g_w^2 + 2 \cdot p_i \cdot g_w \cdot R_d \\ - 2 \cdot V_{cmax} \cdot p_i \cdot g_w + R_d^2 - 2 \cdot V_{cmax} \cdot R_d + V_{cmax}^2 \\ + 4 \cdot V_{cmax} \cdot \Gamma^* \cdot g_w \end{array}} \quad (1)$$

where K is the effective Michaelis-Menten constant for CO_2 at 20 kPa O_2, Γ^* is the photorespiratory compensation point, V_{cmax} is the maximal carboxylation rate, and R_d is the rate of nonphotorespiratory respiration. A similar equation was derived by von Caemmerer and Evans (1991). The values of K and Γ^* at 20°C were assumed to be constant for all leaves and were taken from the literature: K of 47.0 Pa is the value for wheat at 25°C taken from Makino et al. (1988); temperature dependence is from A.J. Keys, (unpublished data) and Machler et al. (1980); and Γ^* of 3.4 Pa is from Brooks and Farquhar (1985). The value of g_w cannot practically be estimated by fitting $A - p_i$ responses (von Caemmerer et al., 1994); therefore, three different approaches were tried for this parameter for all response curves. In the first, g_w was assumed to have a constant value for all leaves; in the second, it varied between leaves such that the CO_2 partial pressure at the site of carboxylation was 0.55 of atmospheric ambient (i.e. 20 Pa when $p_a =$ 36 Pa; Farquhar and Sharkey, 1994); in the third, an initial value of 5.0 μmol m^{-2} s^{-1} Pa^{-1} at the time of leaf emergence, which declined as the leaf senesced to a value of 2.0 μmol m^{-2} s^{-1} Pa^{-1}, was used based on the dependence on leaf age found for wheat flag leaves (Loreto et al., 1994). Since the last approach gave the best r^2 values (>0.95 for 96% and >0.99 for 74% of curves), all of the data presented from the 508 response curves in the three experiments were obtained using this approach. (However, because g_w was not measured, the sensitivity of our conclusions to the assumed value of g_w is addressed in "Discussion.") For each $A - p_i$ response curve, nonlinear regression (Genstat5, Rothamsted Experimental Station, Hertfordshire, UK) was used to estimate the values of only two parameters in Equation 1, V_{cmax} and R_d.

For any observed value of A at a given p_i, the minimum value of V_{cmax} required to achieve this ($V_{cmax,req}$) from Equation 1 is:

$$V_{cmax,req} = A_g \cdot \frac{p_i \cdot g_w + K \cdot g_w - A}{p_i \cdot g_w - \Gamma^* \cdot g_w - A} \quad (2)$$

The amount of apparent excess investment in Rubisco (r_{xs}) compared with that needed to maintain the A is given by:

$$r_{xs} = \frac{V_{cmax} - V_{cmax,req}}{8 \cdot k_{cat}} \cdot 0.55 \quad (3)$$

where k_{cat} is the maximal rate of carboxylation per active site on fully activated Rubisco, 8 is the number of active sites per molecule, and 0.55 is the molecular mass (g μmol^{-1}) for Rubisco. Equations 2 and 3 were used to estimate the theoretical excess in Rubisco content compared with that required to maintain the observed assimilation rate with the p_a set at the elevated CO_2 growth value. Thus, Equation 2 was used with the observed p_i and A values corresponding to $p_a = 70$ Pa for experiments 1 and 2 and $p_a = 100$ Pa for experiment 3. In only 4% of the $A -$ p_i responses were these estimated values of r_{xs} less than 0, and these were within the error of the fitting procedure, suggesting that the assumed values of K, Γ^*, and g_w were reasonable.

The k_{cat} for Rubisco was estimated by the coefficient relating V_{cmax} to the Rubisco content, estimated by linear regression forced through the origin, and converted to moles of CO_2 per mole active site per second. The k_{cat} for thylakoid ATP-synthase can be estimated from the relationship between $A_{g,70}$ and ATP-synthase content. The rate of ATP synthesis (V_{ATP}) required to maintain a A at $p_a = 70$ Pa was reported previously (eq. 16.27, Farquhar and von Caemmerer, 1982):

$$V_{ATP} = \frac{3 \cdot p_c + 7 \cdot \Gamma^*}{p_c - \Gamma^*} \cdot A_{g,70} \quad (4)$$

where p_c is the CO_2 partial pressure at the site of carboxylation, given by $p_i - A/g_w$. Since estimates of p_c did not vary much, V_{ATP} was well approximated by $3.68(A_{g,70})$. Linear regression forced through the origin of $A_{g,70}$ on the amount of thylakoid ATP-synthase gives a coefficient that is therefore approximately proportional to k_{cat}. This is multiplied by the 3.68 factor and by 0.555 g μmol^{-1} to convert to moles of ATP per mole of ATP-synthase per second.

Biochemical Assays

The activity and amount of Rubisco and the amounts of thylakoid ATP-synthase (specifically the CF$_1$ α- and β-subunits), soluble protein, chlorophyll, and total leaf N were determined for the same leaf samples that had been used for gas-exchange measurement. Unless otherwise stated, all operations were done between 0°C and 4°C. The leaves were cut into strips and divided for analysis of total N, Rubisco, and ATP-synthase. Requirement for material meant that only two of these were possible on a given sample. Total N, chlorophyll, and soluble protein contents were determined by the Dumas combustion method and the Arnon and Bradford methods, as previously described (Lawlor et al., 1989).

Figure 1. The estimated rate (see text) of $A_{g,70}$ versus time as days relative to anthesis in wheat leaves in two experiments. Data for flag leaves are presented in separate graphs from preceding leaves for clarity. Plants were grown in different N-application and CO_2 environments, as denoted in the key. For all plants, photosynthesis was measured at a leaf temperature of 20°C, a PPFD of 1500 μmol quanta m^{-2} s^{-1}, and a leaf-to-air vapor-pressure deficit of 1.5 kPa. Error bars represent SE of differences between mean values shown from the $CO_2 \times N$ analysis of variance for each occasion ($n = 6$).
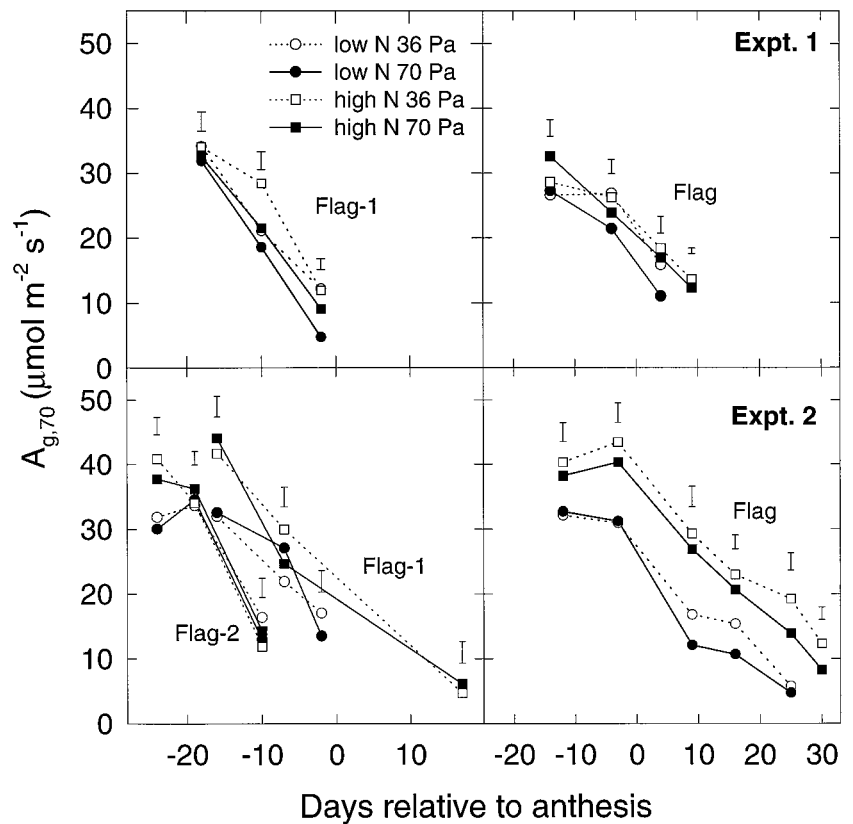
The amount of Rubisco was determined from the binding of [^{14}C]CABP using a modified method of Yokota and Canvin (1985). One-half of the blade from each leaf sample (approximately 50 mm^2) was homogenized in 2 mL of a 50 mM Bicine buffer, pH 7.5, containing 20 mM $MgCl_2$, 1 mM EDTA, and 50 mM β-mercaptoethanol. Part of this homogenate was removed for the determination of chlorophyll and soluble protein content. The remainder was clarified at 10,000g for 3 min, and 200 μL of the supernatant was incubated for 15 min in 100 μL of a 4× activating buffer containing 400 mM Bicine, pH 8.0, 80 mM $MgCl_2$, 40 mM $NaHCO_3$, and 200 mM β-mercaptoethanol. To this 40 μL of 1 M Na_2SO_4, 35 μL of water, and 25 μL of 2.3 mM [^{14}C]CABP (37 GBq mol^{-1}) were added. Protein was precipitated by adding 288 μL of 60% (w/v) PEG, which was left to stand on ice for 30 min, followed by clarification at 10,000g for 10 min. The pellet was washed twice in 20% (w/v) PEG and then resuspended in 1 mL of 1% (v/v) Triton X-100 and left overnight at room temperature prior to the addition of scintillant and counting (model 2500 TR liquid-scintillation analyzer, Packard Instruments, Meriden, CT).

Initial, total, and maximal activities of Rubisco were determined using the method of Parry et al. (1997). Rubisco from leaves (40–50 mm^{-2}) was rapidly extracted in 1 mL of CO_2-free buffer containing 50 mM Bicine, pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA, and 50 mM β-mercaptoethanol. Extracts were clarified at 10,000g for 2 min and the supernatant was immediately assayed for Rubisco activity. Initial activity was determined at 25°C by adding 20 μL of extract

to 980 μL of a CO_2-free assay buffer containing 100 mM Bicine, pH 8.2, and 20 mM $MgCl_2$ to which [^{14}C]$NaHCO_3$ (4.6 kBq μmol^{-1}) and RuBP had been added to concentrations of 10 and 0.4 mM, respectively, immediately prior to adding the extract. Total activity was determined at 25°C by incubating 20 μL of extract for 3 min in 980 μL of the same assay buffer without RuBP and with free CO_2 to allow for the carbamylation of all available active sites. The assay was started by adding RuBP to 0.4 mM as above.

For determination of maximal activity, 100 μL of extract was incubated for 30 min in an equal volume of 400 mM Na_2SO_4 at 4°C to remove any tight-binding inhibitors that may have been present. This was followed by centrifugation at 400g for 2 min at 4°C using Sephadex G25 medium (Pharmacia) equilibrated with extraction buffer in a small polystyrene column (Pierce). Maximal activity was determined using the protocol for total activity, beginning with the 3-min incubation in the absence of RuBP. All assays were stopped after 1 min by adding 100 μL of 10 M formic acid to liberate any unfixed $^{14}CO_2$ and thereafter evaporated to dryness. Determination of ^{14}C was by liquid-scintillation spectrometry.

The content of CF_1 α- and β-subunits was quantified in a selection of the remaining leaf halves by extraction in a 50 mM Bicine buffer, pH 7.6, which contained 20 mM EDTA, 1 mM $MgCl_2$, and 50 mM β-mercaptoethanol. Again, part of the homogenate was removed for the determination of chlorophyll and soluble protein, and each sample was clarified at 10,000g for 3 min. Samples were solubilized at a SDS to protein ratio of 4:1 in 250 mM Tris buffer, pH 7.6,
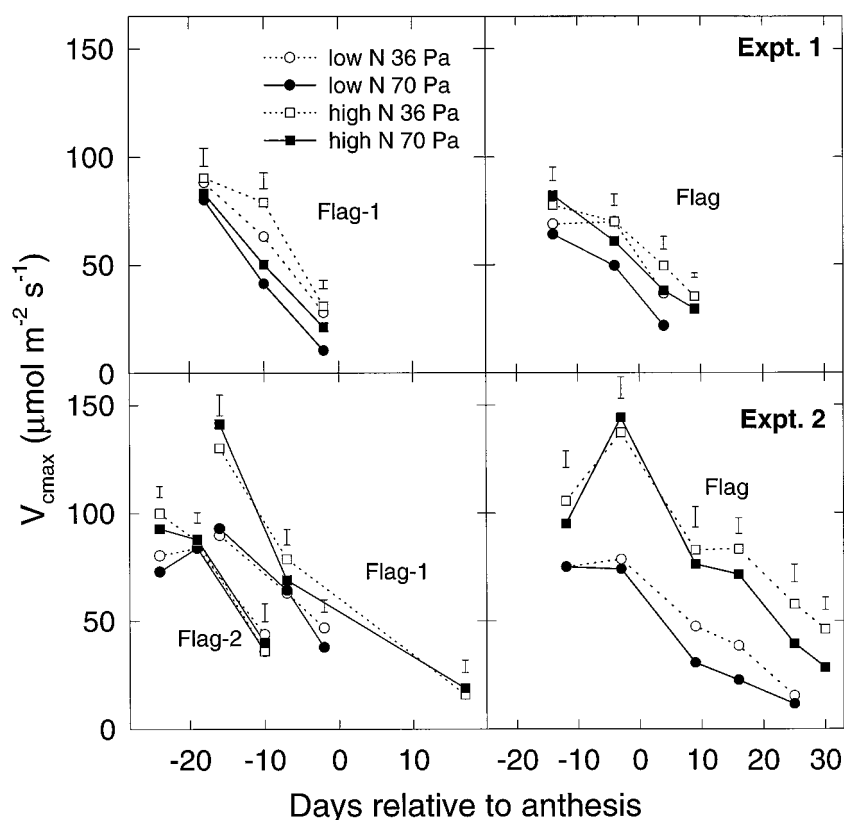
Figure 2. The estimated rate (see text) of V_{cmax} versus time as days relative to anthesis for wheat leaves in two experiments. Treatments were as described in Figure 1. Error bars are defined as in Figure 1.

containing 250 mM DTT, 10% (w/v) SDS, 0.2% (w/v) bromphenol blue, and 10% (w/v) glycerol. Denatured CF_1 was electrophoresed on a 12% discontinuous gradient of SDS-polyacrylamide minigel (Bio-Rad), and separated proteins were electroblotted for 90 min at 1.8 mA cm^{-2} onto a PVDF membrane (Millipore) in a cell (TransBlot, Bio-Rad) containing 25 mM Tris, pH 8.3, 192 mM Gly, and 20% methanol. The membrane was blocked and developed with a chemiluminescent detection system (Aurora, ICN) using primary polyclonal antibody against the α- and β-subunits of CF_1 and the manufacturer's protocol.

Bands visualized onto radiographic film were scanned and quantified using a software package (SigmaGel, Jandel Scientific, Sausalito, CA). CF_1 standard run alongside samples was prepared to approximately 90% purity from 10-d-old wheat seedlings using the method of Moase and Green (1981). The standard was further purified on a Sephacryl S-300 column (Pharmacia) that had been pre-equilibrated with several washes of buffer containing 40 mM Tricine-NaOH, pH 8.0, 1 mM ATP, and 1 mM EDTA. Crude CF_1 was applied to the column and eluted using a 0.1 to 0.5 M NaCl gradient. Total CF_1 obtained was assessed at 98% purity, of which 63% represented the α- and β-subunits. Assuming that all CF_1 α- and β-subunits are present in the thylakoid ATP-synthase complex, the amount of this complex was estimated by multiplying CF_1 α and β content by 1.61 g ATP-synthase per g CF_1 α and β (Moase and Green, 1981).

RESULTS

The As measured at 70 Pa p_a and high light corrected for nonphotorespiratory respiration ($A_{g,70}$) are shown in Figure 1 for various leaves in experiments 1 and 2. In each case, the first point for each leaf corresponds approximately to full leaf expansion (ligule just visible), and there was no significant effect of growth-CO_2 conditions on A_g at this time. When there were significant effects (e.g. at 15 d after anthesis in experiment 2), growth in elevated CO_2 decreased the A. The effect of the N treatment was more pronounced in flag leaves in experiment 2 than for the other leaves in experiments 1 and 2. The pattern of effects was very similar for the carboxylation capacity, V_{cmax}, as estimated from $A - p_i$ responses (Fig. 2). Again, elevated CO_2 treatment did not affect leaves at full expansion or shortly afterward but did induce a more rapid subsequent decline.

The relationship between A_g measured at 70 Pa CO_2 partial pressure and the ATP-synthase content of the same leaf samples is shown in Figure 3. The line shown is a linear regression through the origin ($r^2 = 0.65$), and from the slope, k_{cat} was estimated using Equation 4 at 200 mol ATP mol^{-1} ATP-synthase s^{-1} (SE = 6). The relationship between V_{cmax} estimated from $A - p_i$ responses and Rubisco content of the same leaf samples was close to the theoretical proportionality (Fig. 4). The linear regression through the origin ($r^2 = 0.83$) gave an overall k_{cat} estimate of 2.79 s^{-1} (SE = 0.06).
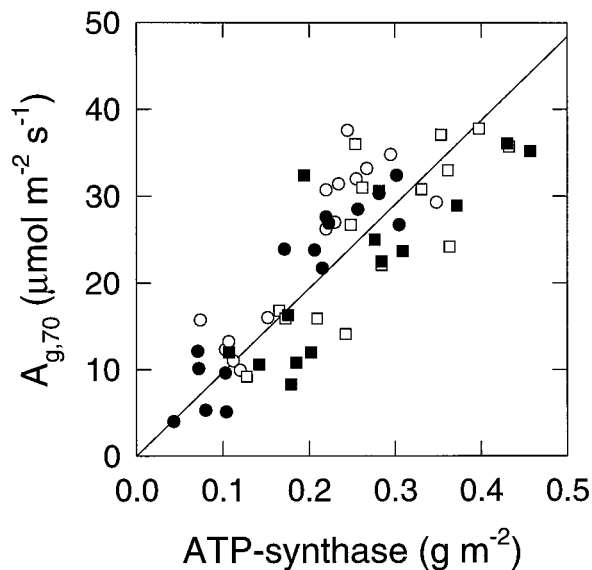
Figure 3. Rate of $A_{g,70}$ versus ATP-synthase content for flag 1 and flag leaves in experiment 1. Photosynthesis was measured at a leaf temperature of 20°C, a PPFD of 1500 μmol PPFD m^{-2} s^{-1}, and a leaf-to-air vapor-pressure deficit of 1.5 kPa. Plants were grown with a total application of eight (circles) and 18 (squares) g N m^{-2} under 36 (open symbols) and 70 (closed symbols) Pa CO_2. The line is a linear regression forced through the origin: $y = 158x$ ($r^2 = 0.65$).

Chlorophyll, Rubisco, and ATP-synthase content were related to leaf N content (Fig. 5). Chlorophyll was approximately proportional to the leaf N content (Fig. 5b), whereas Rubisco had a positive x intercept (Fig. 5a), suggesting that the fraction of leaf N represented by Rubisco increases as leaf N content increases. ATP-synthase against leaf N (Fig. 5d) had a smaller x intercept than for Rubisco. Figure 6 shows the relationship between ATP-synthase and
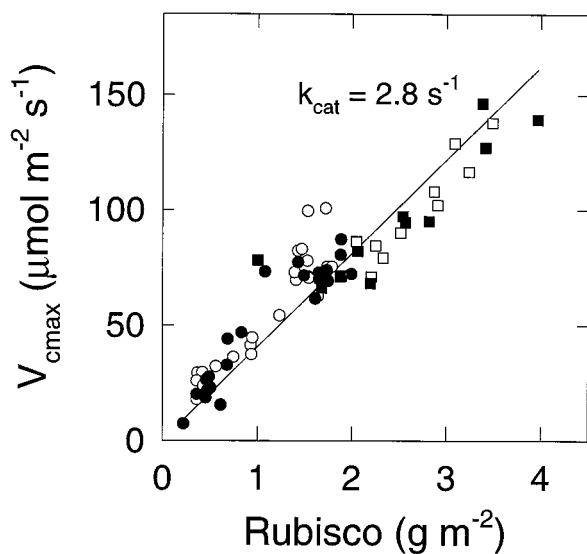

Figure 4. Rate of estimated (see text) V_{cmax} versus Rubisco content for the first four occasions of flag leaves from experiment 2. Conditions and treatments were as described in Figure 3. The line is a linear regression forced through the origin: $y = 40.6x$ ($r^2 = 0.83$).
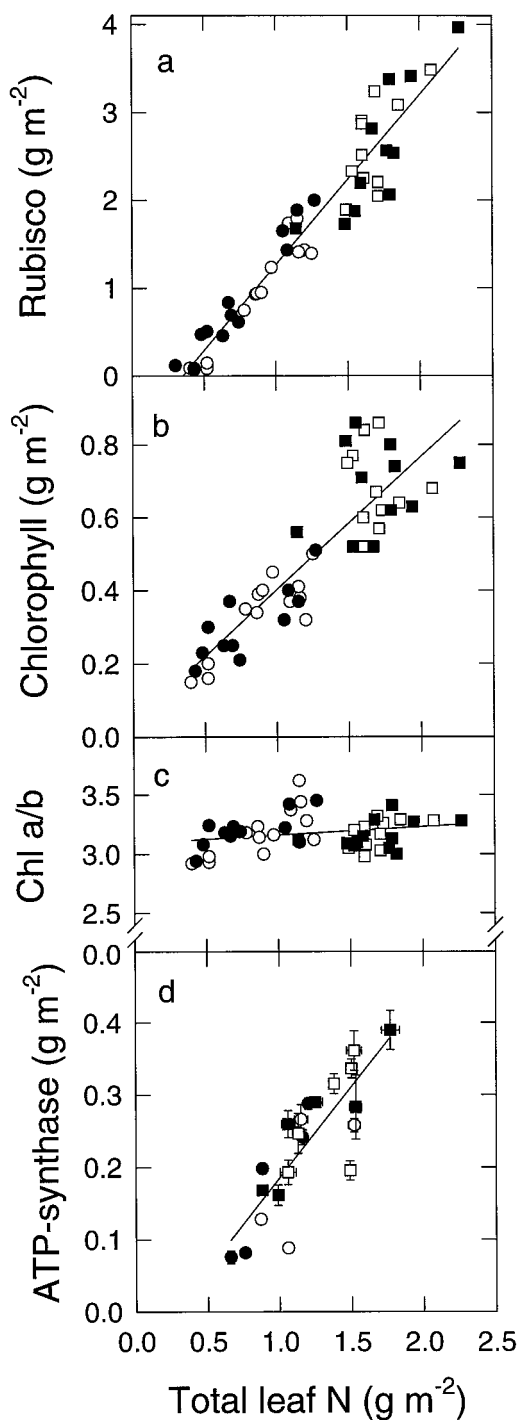

Figure 5. Amounts of Rubisco and chlorophyll, chlorophyll a/b ratio, and ATP-synthase content versus total leaf N content. For ATP-synthase (d), points are the means of three N values versus the means of three ATP-synthase values in leaves of the same occasion and treatment, with SE shown for both axes of each. Plants were grown at either low (circles) or high (squares) N applications and at 36 (open symbols) or 70 Pa CO_2 (closed symbols). For Rubisco (a), $y = 1.944x - 0.684$ ($r^2 = 0.92$). For chlorophyll (b), $y = 0.364x$ ($r^2 = 0.75$). For ATP-synthase (d), $y = 0.15x - 0.04$ ($r^2 = 0.69$).
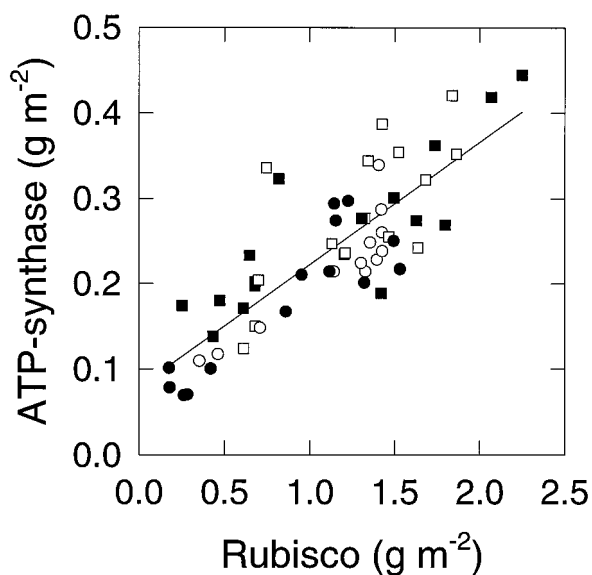
Figure 6. The relationship between ATP-synthase and Rubisco contents measured in the same leaf (flag−1 or flag) of experiment 1. The line is a linear regression: $y = 0.14x + 0.08$ ($r^2 = 0.68$). Symbols are as described in Figure 5.

Rubisco content determined in the same flag−1 and flag leaves of various ages in the different treatments. It is clear that Rubisco and ATP-synthase are not simply proportional and that the mass ratio of Rubisco to ATP-synthase increases from about 3.8 at low Rubisco content to 5.75 at high Rubisco content. There were no significant effects of N or CO_2 treatment on this relationship.

Given the relationship between V_{cmax} and Rubisco content (Fig. 4), Equation 3 was used to estimate the apparent excess Rubisco content compared with that required to maintain the observed rate of photosynthesis at 70 Pa atmospheric CO_2 (Fig. 7). For all leaves and for both experiments, the excess was consistently less at low N. Where elevated CO_2 treatment had a significant effect (flag−1 leaves in experiment 1 and flag leaves in all experiments), it decreased the excess. In some cases (e.g. experiment 2: low N, 70 Pa, flag leaves 15 d after anthesis), the estimated excess was not significantly greater than 0. It is also possible to use Equation 3 to estimate excess Rubisco at ambient CO_2. This was only significantly different from 0 for the last three points for high-N flag leaves in experiment 2, decreasing from approximately 0.4 to approximately 0.2 g m^{-2} (data not shown).

In experiment 3 leaves used for gas exchange were freeze-clamped at high light and at their growth p_a and the activity of isolated Rubisco was determined in vitro, which was related to the Rubisco content. In Figure 8 the in vitro activities are compared with the V_{cmax} required to achieve the observed A measured at the growth CO_2 partial pres-
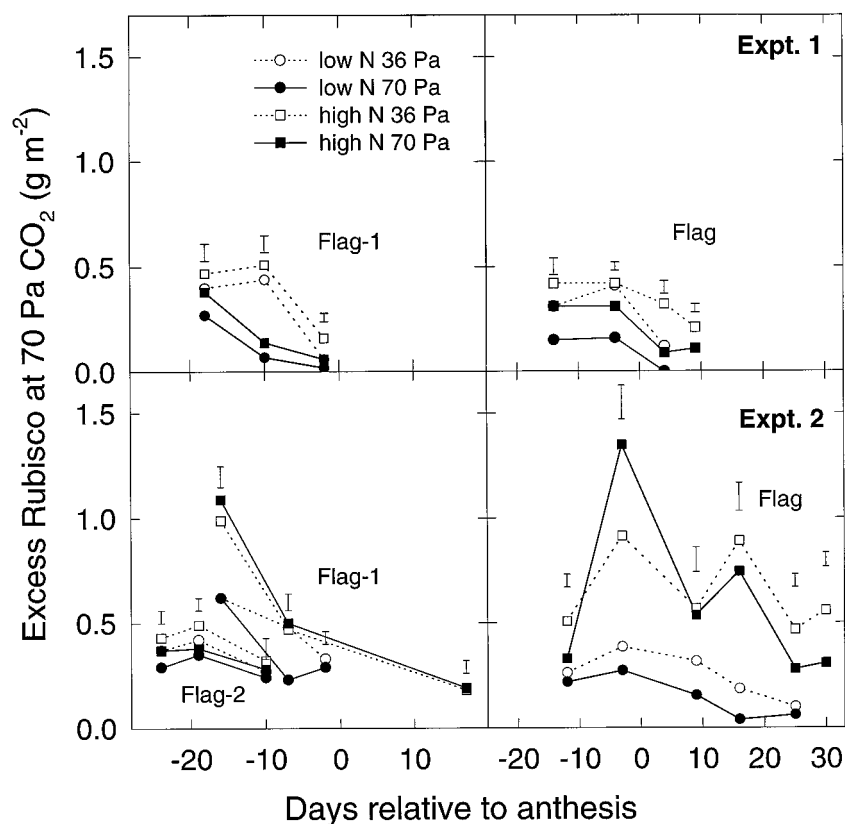

Figure 7. The estimated excess (see text) of Rubisco at 70 Pa CO_2 atmospheric partial pressure versus time as days relative to anthesis for various wheat leaves from two experiments. Treatments were as described in Figure 1. Error bars are defined as in Figure 1.
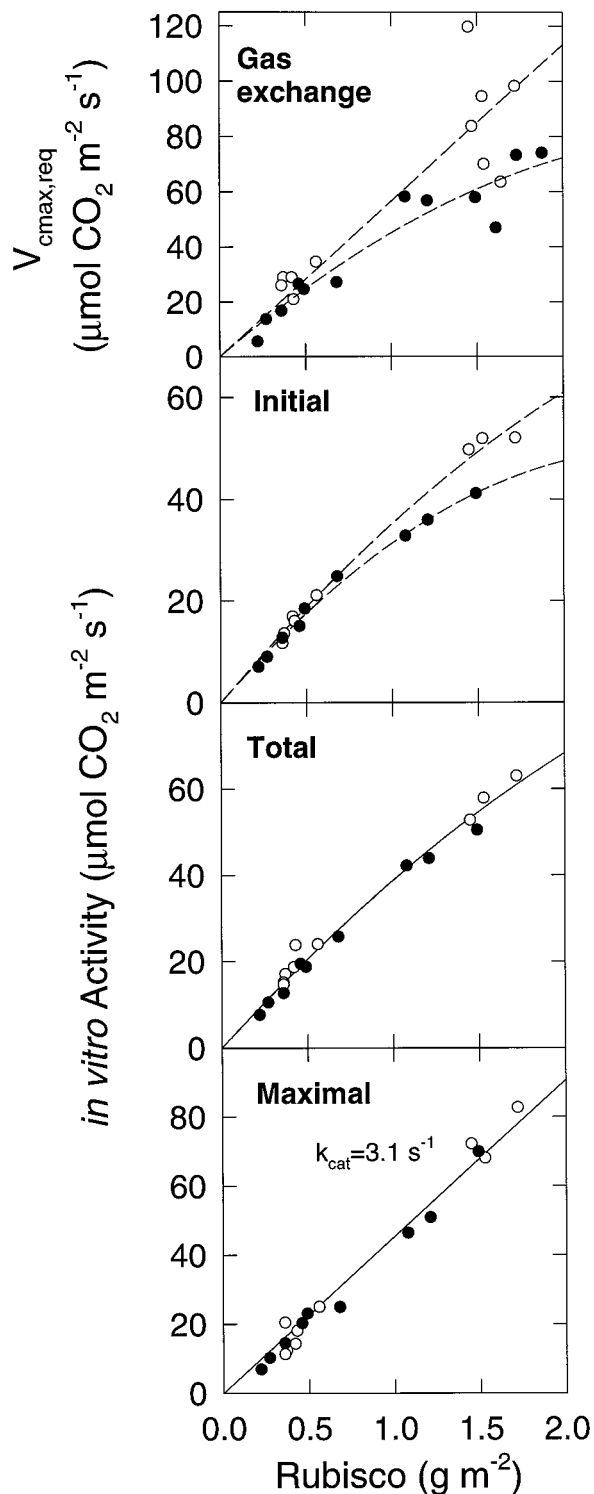
sure, estimated from the gas-exchange parameters using Equation 2. Total activity refers to that determined after complete activation of the enzyme and maximal activity after treatment to remove any inhibitors bound to the active site (Parry et al., 1997). The initial and total activities show a distinct curvilinear relationship, with the amount determined by [^{14}C]CABP binding, whereas maximal activity is proportional, giving an estimated k_{cat} of 3.1 s^{-1} at 25°C. Although the relationships between total and maximal activity and Rubisco content are independent of growth-CO_2 treatment, the lines for initial activity for the two treatments diverge at high Rubisco content. A similar pattern is seen for the estimates of required V_{cmax}, which diverge at higher Rubisco contents.

DISCUSSION

A − p_i Responses

The effect of elevated-CO_2 growth treatment on the A measured at p_a = 70 Pa was small, and where there was an effect, it was almost invariably a decrease (Fig. 1). Carboxylation capacity, as estimated by the V_{cmax} parameter, was also decreased (Fig. 2). Many studies of the effects of elevated CO_2 have observed decreases in photosynthetic capacity (Sage, 1994; Webber et al., 1994; Sicher and Bunce, 1997), except when N supply was not limited (Habash et al., 1995). We found no consistent differences in the effect of CO_2 on these parameters between N treatments. The application of N ended 30 d before anthesis in experiment 1 compared with 8 d before anthesis in experiment 2, and this resulted in the N treatment exerting more of an effect on leaf area in experiment 1 and more on leaf N content in experiment 2. Consequently, effects of N treatment on leaf-photosynthetic parameters are greater in experiment 2. The effect of CO_2 treatment also differed somewhat between experiments; in experiment 2 there was no effect on $A_{g,70}$ or V_{cmax} in leaves prior to the flag leaf or in flag leaves up to 10 d after anthesis (similar to Delgado et al., 1994), whereas effects appeared earlier in experiment 1. There was no significant effect of CO_2 in any leaf at full emergence (the first point measured).

Amounts of Photosynthetic Components

A_g at 70 Pa CO_2 and ATP-synthase content were well correlated (Fig. 3) and resulted in a k_{cat} estimate for ATP-synthase (200 s^{-1}) comparable to that obtained in vitro (160 s^{-1}; Fromme and Graber, 1989). A strong relationship was expected because effectively all products of photosynthetic electron transport are used in RuBP regeneration for pho-

Figure 8. Estimates of in vivo and in vitro Rubisco activity versus Rubisco content in flag leaves of different ages measured between 22 d before anthesis and 18 d after anthesis in experiment 3. Plants were grown at 36 (open symbols) and 100 (closed symbols) Pa CO_2 partial pressure. Top, V_{cmax} required to maintain observed A at a PPFD of 1500 μmol quanta m^{-2} s^{-1} and the growth CO_2 partial pressure estimated from the $A − p_i$ response using Equation 2. Bottom, Initial, total, and maximal in vitro activities determined at 25°C of Rubisco isolated from leaves freeze-clamped under a PPFD

of 1500 μmol quanta m^{-2} s^{-1} and the growth atmospheric CO_2 partial pressure. Lines are regressions forced through the origin. Separate regressions were used for the two treatments where this improved the r^2 value (dashed lines); otherwise a single regression was used (solid line). Second-order linear regressions were used where this improved the r^2 value (gas-exchange, elevated-CO_2 treatment, and initial and total, both treatments); otherwise, first-order regression was used.

tosynthesis and photorespiration (Habash et al., 1995), and the A is usually limited by RuBP regeneration at the p_i used here (45–55 Pa) (Makino et al., 1988). Although under these conditions the rate can also be limited by triose-phosphate-utilization capacity (Farquhar and Sharkey, 1994), in our experiments the stimulation of $A_{g,70}$ on decreasing O_2 partial pressure to 2 kPa was mostly close to the theoretical expectation for RuBP-regeneration-limited photosynthesis (mean stimulation 18%; data not shown).

The relationship between the estimated in vivo V_{cmax} and the Rubisco content used to derive the estimate of Rubisco k_{cat} of 2.8 s^{-1} at 20°C (Fig. 4) was strong ($r^2 = 0.83$). The value is in line with in vivo estimates for tobacco of 3.5 s^{-1} (25°C; von Caemmerer et al., 1994) and for wheat of 3.3 s^{-1} (23°C; Evans and Austin, 1986) and an in vitro estimate for wheat of 3.0 s^{-1} (25°C; Makino et al., 1988), given the temperature dependence that would be expected to increase the value by 40% going from 20°C to 25°C (Machler et al., 1980; Makino et al., 1988). The fraction of leaf N invested in Rubisco increases with leaf N content from 21% to 26% in the range 1 to 2 g N m^{-2} (Fig. 5d) in a manner similar to previous findings for wheat flag leaves of varying age (Makino et al., 1988; Lawlor et al., 1989).

The relationship between ATP-synthase and Rubisco content determined in experiment 1 (Fig. 6) was such that the ratio of Rubisco to ATP-synthase increased with increasing Rubisco content independently of treatment. Since the amounts of these components are expected to reflect carboxylation and RuBP-regeneration capacities (supported by the data in Figs. 3 and 4), this suggests that carboxylation capacity increases relative to RuBP-regeneration capacity in leaves with high N content and that this is not affected by elevated CO_2. This is in close agreement with the results of Nakano et al. (1997) using rice. Also, a field experiment on wheat revealed that elevated CO_2 did not affect the ratio of Rubisco to other components, including CF_1, at flag leaf emergence but did induce a decrease in this ratio during grain fill (Nie et al., 1995), which could be interpreted as a more rapid decline in leaf N in the elevated-CO_2-grown leaves.

Excess Investment in Rubisco

The amount of Rubisco in excess of the requirement to maintain photosynthesis at high light and $p_a = 70$ Pa estimated from the gas-exchange data (Fig. 7) showed a clear pattern in all leaves: the excess was greater in high-N compared with low-N leaves and declined as leaves senesced. The effect of elevated CO_2 treatment was more variable but generally decreased the excess. It is therefore clear that some rebalancing of capacities does occur in the elevated-CO_2 treatment, since the excess is lower, but there is still some excess until well into senescence in the low-N treatments and throughout in the high-N treatments. From the results presented above and from the conclusions of Nakano et al. (1997), such rebalancing can be explained purely in terms of elevated-CO_2 treatment inducing a reduction in leaf N. This is associated with a greater than proportional reduction in Rubisco (Fig. 5a) compared with all of the other photosynthetic components, particularly

ATP-synthase (Fig. 6); therefore, there is a de facto rebalancing away from carboxylation to RuBP-regeneration capacities. This hypothesis is supported by the relationship between the data in Figure 7, expressed as the fraction of Rubisco in excess at 70 Pa CO_2 versus leaf N (Fig. 9). The fraction increases from approximately 5% in leaves with 1 g N m^{-2} to approximately 40% in leaves with 2 g N m^{-2} regardless of CO_2 treatment. Thus, any decrease in excess Rubisco due to elevated growth-CO_2 partial pressure appears to be an indirect consequence of decreased leaf N.

The estimates of excess Rubisco refer to the conditions used for the determination of steady-state photosynthesis. The conditions of high light intensity and moderate temperature used are those under which Rubisco would be expected to be the least in excess; therefore, the excess would presumably be greater under variable field conditions at elevated CO_2. (However, this conclusion would not hold for conditions that decrease stomatal conductance, such as water stress.)

Sensitivity to g_w

The above analysis was based on assuming a variable g_w, as described in "Materials and Methods." To investigate the sensitivity of the conclusions based on this assumption, the analysis was repeated with constant g_w values of 2.0 and 6.0 μmol m^{-2} s^{-1} Pa^{-1}, spanning the range estimated in measurements (von Caemmerer and Evans, 1991; Loreto et al., 1994) or infinity. For the regression between V_{cmax} and Rubisco content, k_{cat} estimates of 3.71, 2.53, 2.17 s^{-1} with $r^2 = 0.75$, 0.67, 0.56, respectively, were derived, compared with 2.79 s^{-1} with $r^2 = 0.83$ for the data shown in Figure 4. The assumption of a finite g_w, therefore, im-
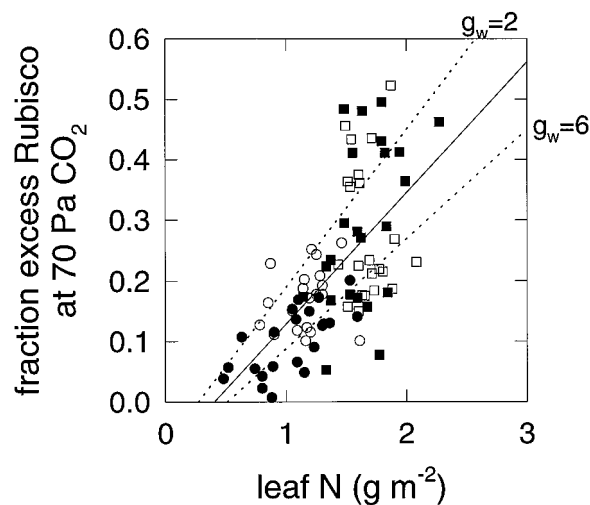

Figure 9. The fraction of Rubisco estimated to be in excess of that required to maintain A at 70 Pa atmospheric CO_2 partial pressure versus total leaf N content, for all leaves in experiments 1 and 2. Plants were grown at low (circles) and high (squares) N applications and at 36 (open symbols) or 70 (closed symbols) Pa CO_2. The solid line is linear regression of all points ($r^2 = 0.41$; $n = 87$). Dotted lines are equivalent regressions for same data when recalculated with g_w = 2 and 6 μmol m^{-2} s^{-1} Pa^{-1} ($r^2 = 0.56$ and 0.45, respectively).

proved the fit between Rubisco content and photosynthetic parameters, as was found previously for wheat (Evans and Austin, 1986; Makino et al., 1988). Whereas the effect on k_{cat} was quite marked, the value of g_w had much less effect on the main aim of estimating the fraction of Rubisco that is in excess at elevated CO_2. The dotted lines in Figure 9 show the corresponding linear regressions with $g_w = 2$ and 6 μmol m^{-2} s^{-1} Pa^{-1}. These values would alter the estimate of 40% excess Rubisco at 2 g N m^{-2} to 50% and 30%, respectively. Even making the unlikely assumption of an infinite g_w gave the same highly significant trend and a corresponding fraction excess of 20% (data not shown).

Rubisco Activation

It is usually assumed that Rubisco in excess of that required to maintain carboxylation will be deactivated, because RuBP concentrations are stable (Sage et al., 1990) and a reduction in the Rubisco activation state at elevated CO_2 has been found in some (Sage et al., 1990; Nakano et al., 1997) but not all (Sicher et al., 1995) studies. The estimated value of the V_{cmax} required to maintain observed A ($V_{cmax,req}$ from Eq. 2) should therefore be directly comparable to the initial activity of Rubisco. Activity parameters determined for Rubisco isolated from leaf sections freeze-clamped under high light and growth-CO_2 partial pressure were compared with $V_{cmax,req}$ values estimated for the same leaf sections under these conditions (Fig. 8).

The pattern of $V_{cmax,req}$ was similar to initial activity, with elevated CO_2 points lying increasingly below ambient CO_2 points as Rubisco content increased, although the absolute initial in vitro activities were much lower. The apparent decrease in activation state (initial activity/maximal activity) with increasing Rubisco content in ambient CO_2 leaves to 60% to 70% was unexpected, since it has been assumed that all Rubisco is utilized at high light and $p_a = 36$ Pa, for which there is good evidence (Makino et al., 1988; Sage et al., 1990) and which resulted in a close relationship between estimated V_{cmax} and Rubisco content (Fig. 4). Part of the decrease in activation state is associated with an inhibitor, as demonstrated by the difference between total and maximal activity (Parry et al., 1997), but initial activity was also somewhat lower than total. Although the conditions used were similar to those used in many other studies, initial activity is known to be affected by pretreatment of the extraction buffer and by the time taken to determine activity (Sage et al., 1993), which may have lowered absolute initial activities in our material, but the difference between treatments probably reflects the in vivo state.

The in vitro maximal activity of Rubisco was closely correlated ($r^2 = 0.98$) with its amount, and the estimated k_{cat} of 3.1 s^{-1} was the same as that estimated previously for wheat at 25°C (Makino et al., 1988). However, even the maximal activity was somewhat less (20% lower at 1.5 g m^{-2} Rubisco) than the $V_{cmax,req}$ for ambient leaves (Fig. 8); estimates of Rubisco activity are often higher in vivo than in vitro (von Caemmerer et al., 1994).

CONCLUSIONS

We have found evidence that there is excess investment in Rubisco over that required for maintaining A at elevated CO_2 and that this excess is greater at high N. Long-term growth at elevated CO_2 does reduce this excess somewhat but apparently only as an indirect consequence of elevated CO_2 causing a decreased leaf N content, which agrees with the findings of Nakano et al. (1997). There is therefore no evidence of a direct mechanism optimizing the balance between carboxylation and RuBP-regeneration capacities in response to long-term growth at elevated CO_2. Makino et al. (1997) observed an increase in A per unit leaf N in rice plants genetically manipulated to reduce Rubisco expression, which was measured under conditions of short-term exposure to elevated CO_2. In agreement with the relationship shown in Figure 9, they found the greatest advantage in leaves with high N content. The work presented here suggests that most of this advantage would be preserved if the plants were grown under conditions of elevated CO_2, since there is still excess investment in Rubisco under these conditions. There is therefore potential for improving the adaptation of crop plants to growth at elevated CO_2.

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