Temperature Response of Mesophyll Conductance. Implications for the Determination of Rubisco Enzyme Kinetics and for Limitations to Photosynthesis in Vivo

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CO_2 transfer conductance from the intercellular airspaces of the leaf into the chloroplast, defined as mesophyll conductance (g_m), is finite. Therefore, it will limit photosynthesis when CO_2 is not saturating, as in C3 leaves in the present atmosphere. Little is known about the processes that determine the magnitude of g_m. The process dominating g_m is uncertain, though carbonic anhydrase, aquaporins, and the diffusivity of CO_2 in water have all been suggested. The response of g_m to temperature (10°C–40°C) in mature leaves of tobacco (*Nicotiana tabacum* L. cv W38) was determined using measurements of leaf carbon dioxide and water vapor exchange, coupled with modulated chlorophyll fluorescence. These measurements revealed a temperature coefficient (Q_{10}) of approximately 2.2 for g_m, suggesting control by a protein-facilitated process because the Q_{10} for diffusion of CO_2 in water is about 1.25. Further, g_m values are maximal at 35°C to 37.5°C, again suggesting a protein-facilitated process, but with a lower energy of deactivation than Rubisco. Using the temperature response of g_m to calculate CO_2 at Rubisco, the kinetic parameters of Rubisco were calculated in vivo from 10°C to 40°C. Using these parameters, we determined the limitation imposed on photosynthesis by g_m. Despite an exponential rise with temperature, g_m does not keep pace with increased capacity for CO_2 uptake at the site of Rubisco. The fraction of the total limitations to CO_2 uptake within the leaf attributable to g_m rose from 0.10 at 10°C to 0.22 at 40°C. This shows that transfer of CO_2 from the intercellular air space to Rubisco is a very substantial limitation on photosynthesis, especially at high temperature.

In C3 plants, the diffusion of CO_2 from the atmosphere to the active site of Rubisco follows a complex pathway involving as many as eight discrete conductance components (Nobel, 1999). Most commonly, this pathway is simplified into three main components: boundary layer, stomatal conductance, and mesophyll conductance (g_m; Farquhar and Sharkey, 1982). Boundary layer conductance depends on several leaf physical and environmental properties, in particular, size, surface structures, stomatal location, and air movement around the leaf, whereas stomatal conductance is primarily influenced by stomatal pore numbers and dimensions. The flexible and dynamic qualities of the stomatal pores provide the leaf with physiological control of CO_2 influx and water efflux (Farquhar and Sharkey, 1982). Estimates of boundary layer and stomatal conductances to CO_2 are based on water vapor released from the leaf because water and CO_2 share the same gaseous diffusion pathway (e.g. von Caemmerer and Farquhar, 1981). As a result, it has long been known that limitations of diffusion through the stomata and boundary layer are purely physical (Penman and Schofield, 1951).

g_m, defined as the conductance of CO_2 transfer from the intercellular leaf airspaces to the site of carboxylation, was initially assumed large enough to have a negligible impact on photosynthesis (Farquhar et al., 1980). More recent research suggests that g_m may be sufficiently small to significantly decrease the concentration of CO_2 at the site of carboxylation (C_c) relative to that in the intercellular space (C_i), thereby limiting photosynthesis (Harley et al., 1992; Loreto et al., 1992; Evans et al., 1994; von Caemmerer et al., 1994; Eichelmann and Laisk, 1999; von Caemmerer, 2000). Many physiological and anatomical leaf characteristics have been correlated with g_m, including, but not limited to, photosynthetic potential (von Caemmerer and Evans, 1991; Loreto et al., 1992), stomatal conductance (Loreto et al., 1992), and chloroplast surface area exposed to intercellular air spaces (von Caemmerer and Evans, 1991; Evans et al.,

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1994). In addition to these correlations, previous studies suggest that g_m is closely associated with carbonic anhydrase (CA) activity (Markus et al., 1981; Volokita et al., 1981, 1983; Tsuzuki et al., 1985; Makino et al., 1992; Price et al., 1994; Sasaki et al., 1996). The processes determining g_m may be indicated by ascertaining the temperature response of g_m. If it is driven purely by diffusion, then g_m should have a temperature coefficient (Q_{10}) close to that of the diffusivity of CO_2 in pure water. The Wilke-Chang equation predicts a Q_{10} of 1.25 at 25°C, varying little across the biologically relevant temperature range. This is in close agreement with a range of measurements (Tamimi et al., 1994). If an enzyme, such as CA, is required for the effective transfer of CO_2 to the site of carboxylation, then conductance should be more sensitive to temperature, with a Q_{10} value close to or above 2 (Nobel, 1999). Although the temperature dependence of CO_2 diffusion through aquaporin membrane channels has not been reported, diffusion of ammonia through aquaporins shows a Q_{10} of 2.07 (calculated from Niemietz and Tyerman, 2000). Assuming that the much larger molecules of CO_2 could not move through the pore more readily, then if transfer through aquaporins were the major determinant of CO_2 transfer to the site of carboxylation, a Q_{10} for g_m of 2 or above would again be expected.

Previously, we have used transgenically modified tobacco (*Nicotiana tabacum* L. cv W38) with low Rubisco content to determine the in vivo temperature responses of Rubisco kinetic parameters (Bernacchi et al., 2001). These responses, integrated into the model describing Rubisco-limited photosynthesis (Farquhar et al., 1980), improved predicted rates of photosynthesis over a wide range of temperature relative to predictions using earlier temperature responses developed from in vitro studies. Our earlier study reported apparent kinetic parameters based on intercellular CO_2 concentrations. With g_m known, CO_2 concentration at the site of carboxylation may be calculated and the actual kinetic constants determined for each temperature in vivo (von Caemmerer et al., 1994). With the actual Rubisco kinetic constants known, it is in turn possible to quantify the limitation that g_m imposes on photosynthesis at each temperature.

The objectives of this study were to: (a) provide insight into the mechanisms controlling g_m by discovering how it varies with leaf temperature, (b) determine in vivo temperature-dependent changes in Rubisco enzyme kinetics by determining C_c from g_m, and (c) quantify the limitation that g_m imposes upon photosynthesis from 10°C to 40°C. The latter will be addressed specifically for Rubisco-limited photosynthesis, which is the most common limitation of light-saturated C3 photosynthesis (Rogers and Humphries, 2000) and the most responsive to CO_2 concentration at the site of carboxylation (von Caemmerer, 2000).

RESULTS

Temperature Response of g_m

Two methods were used to determine g_m, depending on whether J varies with C_i or not. The constant and variable methods yielded very similar estimates of g_m: 0.1075 and 0.095 mol m^{-2} s^{-1} bar^{-1}, respectively, at 25°C. Both methods showed a similar high dependence of g_m on temperature ($F_{2,28} = 25.45$, $P < 0.001$) and a Q_{10} of 2.2 between 10°C and 35°C (Fig. 1). g_m increased exponentially with temperature until 35°C to 37.5°C where it peaked, declining at higher temperature (Fig. 1).

Rubisco Kinetics

The temperature responses of the photosynthetic CO_2 compensation point (Γ^*) determined in this study are shown in Figure 2A and Table I. Michaelis constants for carboxylation (K_c) and oxygenation (K_o), calculated from a C_c increase exponentially with temperature; these values are 25% to 35% lower than K_c and 20% to 50% lower than K_o calculated previously from the intercellular CO_2 concentrations (C_i; Bernacchi et al., 2001; Fig. 2, b and c; Table I).

Limitation of Photosynthesis by g_m

The limitation imposed on photosynthesis by g_m (l_{gm}) is expressed as the proportionate decrease in A

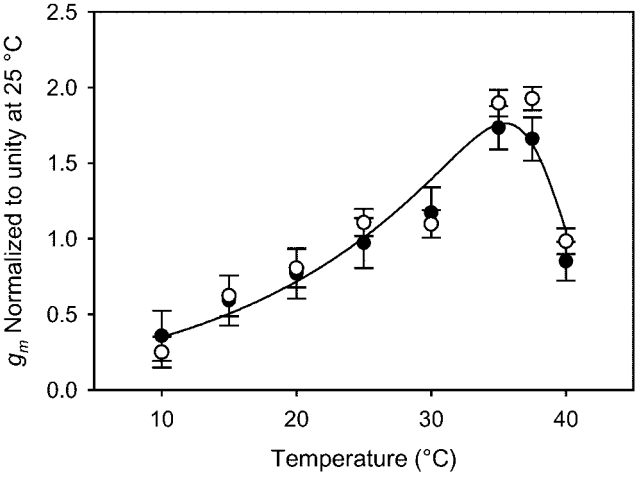

Figure 1. Temperature response of g_m normalized to unity for measurements made by the variable J method at 25°C, determined from simultaneous measurements of gas exchange and chlorophyll fluorescence. g_m was estimated using both the constant J (g_m at 25°C = 0.1075 mol m^{-2} s^{-1} bar^{-1}; white symbols) and variable J methods (g_m at 25°C = 0.095 mol m^{-2} s^{-1} bar^{-1}; black symbols). The continuous line represents the function:

$$g_m = \frac{e^{(c-\Delta H_a/RT_k)}}{1 + e^{[(\Delta S \cdot T_k - \Delta H_d)/RT_k]}}$$

fitted to all the illustrated points. Each point is the mean of at least three replicate plants (±1 SE).

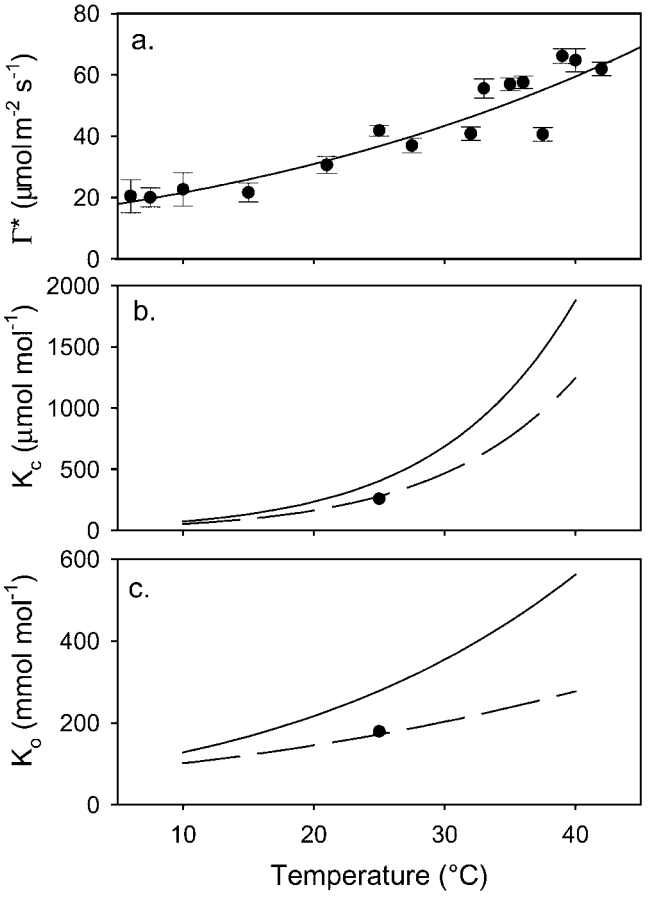

Figure 2. a, Temperature response of Γ* measured using mass spectrophotometry at the CO_2 compensation point when chloroplast CO_2 concentration (C_c) is equal to intercellular CO_2 concentration (C_i). Values represent the mean of two to nine individual leaves (±1 SE of the population mean). b and c, K_c and K_o as a function of temperature and calculated as apparent values based on C_i (solid lines) and actual values based on C_c (broken lines). Points represent K_c and K_o determined previously and independently using similar methods but for a single temperature, 25°C, from von Caemmerer et al. (1994).

caused by the measured, compared with infinite, g_m (Equation 13). This limitation rises as a proportion from 0.08 at 10°C to 0.22 at 40°C (Fig. 3).

DISCUSSION

Temperature Response of g_m

This study showed that g_m determined in vivo is more dependent on temperature than could be explained by simple diffusion in water. Both methods used in this study to estimate the temperature response of g_m require that the response of A to C_i is well described by the model presented by Farquhar et al. (1980). The presence of other processes that are not incorporated into the leaf model of photosynthesis, such as photoinhibition or triose phosphate limitation, may alter this response. However, chlorophyll fluorescence measurements suggested that neither process influenced A under the measurement conditions.

The observed Q_{10} of approximately 2.2 (Fig. 1) shows that g_m does not conform to transfer dominated by simple diffusion, but suggests that an enzyme or other protein-facilitated process is involved. One possible explanation is that CA is facilitating the transfer of CO_2 into the chloroplast (Tsuzuki et al., 1985). Numerous studies demonstrate that CA is present and active in the mesophyll (Markus et al., 1981; Volokita et al., 1981, 1983; Tsuzuki et al., 1985; Sasaki et al., 1996). Studies also correlate Rubisco content with CA activity (Sasaki et al., 1996) and g_m (von Caemmerer et al., 1991; Loreto et al., 1992), suggesting that CA and Rubisco are mutually regulated (Sasaki et al., 1996). However, limitation of CO_2 transfer by CA was brought into question by the observation that antisense reduction of CA activity to 2% of wild-type levels failed to produce any reduction in light-saturated photosynthesis in the current ambient CO_2 concentration (Price et al., 1994). Therefore, a controlling role for CA in transfer of CO_2 could be possible if a different isoform of CA, not addressed by Price et al. (1994), exists, which is specifically involved in the transfer of CO_2 in the leaf. Another possible explanation for the high Q_{10} is that aquaporins increase the CO_2 permeability of the cell membranes (Cooper and Boron, 1998; Terashima and Ono, 2002). In a recent study, CO_2 diffusion into the chloroplast was inhibited by $HgCl_2$ characteristic of aquaporin involvement (Terashima and Ono, 2002). The deactivation of g_m at higher temperatures would, therefore, involve either direct denaturation of the aquaporin proteins or altered membrane physical properties resulting in a loss in aquaporin function.

Rubisco Kinetics

The kinetic parameters of Rubisco are commonly calculated from the response of A to C_i (e.g. McMurtrie and Wang, 1993; Harley and Baldocchi, 1995; Bernacchi et al., 2001). Although this is pragmatic for modeling leaf and canopy photosynthesis, it will not reveal the actual in vivo kinetic parameters of Rubisco if C_c is significantly lower than C_i. Here, we show that over the temperature range of 10°C to 40°C, g_m is both significant and variable with temperature. As a result, C_c is always lower than C_i. We have used the temperature response of g_m to calculate C_c and, in turn, recalculate the kinetic parameters of Rubisco. This recalculation based on the actual CO_2 concentration at Rubisco shows that K_c and K_o are overestimated by the use of C_i and that part of their apparent dependence on temperature is an artifact of the dependence of g_m on temperature (Fig. 2, b and c). von Caemmerer et al. (1994) made similar calculations with tobacco plants, but at just one temperature. These estimates of K_c and K_o at 25°C are within 8% and 5%, respectively, of those measured independently here (Fig. 2, b and c).

Table I. *The scaling constant (c) and energies of activation (Δ H_a), deactivation (Δ H_d), and entropy (Δ S) describing the temperature responses for mesophyll conductance and Rubisco enzyme kinetic parameters* [*parameter* = $e^{(c - \Delta Ha/RTk)}$ *or parameter* = $e^{(c - \Delta Ha/RTk)/(1 + \exp((\Delta STk - \Delta Hd)/RTk))}$]

nr, No statistically significant deactivation was detected at 40°C.

Parameter	Value at 25°C	*c*	ΔH_a	ΔHd	ΔS
g_m	1	20.0	49.6	437.4	1.4
Γ*	37.43	13.49	24.46	nr	nr
K_c	272.38	38.28	80.99	nr	nr
K_o	165.82	14.68	23.72	nr	nr

Limitation of Photosynthesis by g_m

Photosynthesis is limited increasingly by g_m as temperature rises, despite the exponential increase in g_m (Fig. 3). Previously, we have shown an exponential increase in maximum in vivo Rubisco activity ($V_{c,max}$) up to 40°C in tobacco (Bernacchi et al., 2001). The peak and subsequent decrease in g_m above 35°C suggests a lower energy of deactivation for g_m than Rubisco. Studies of CA levels in intact leaves have suggested Rubisco and CA activity are coordinated under various growth conditions (Porter and Grodzinski, 1984; Peet et al., 1986; Makino et al., 1992). However, this would not explain the different responses observed here at high temperature.

The exponential increase in $V_{c,max}$ demonstrated by Bernacchi et al. (2001) is inconsistent with studies that show a decrease in $V_{c,max}$ above 35°C (Harley and Tenhunen, 1991; Crafts-Brandner and Salvucci, 2000). These inconsistencies in $V_{c,max}$ at higher temperatures may result from the use of antisense Rubisco. In wild-type plants, a decrease in g_m at high temperature restricting supply of CO_2 to Rubisco could produce an apparent decrease in $V_{c,max}$ estimated from leaf gas exchange. In plants containing only 10% of the wild-type Rubisco, however, a much larger decrease in g_m would be needed to affect the apparent $V_{c,max}$ estimated from the A/C_i response. Further, it is well documented that Rubisco activase becomes more limiting at higher measurement temperatures for wild-type plants (Crafts-Brandner and Salvucci, 2000); however, this is not likely in tobacco plants that contain only 10% wild-type levels of Rubisco but normal levels of activase.

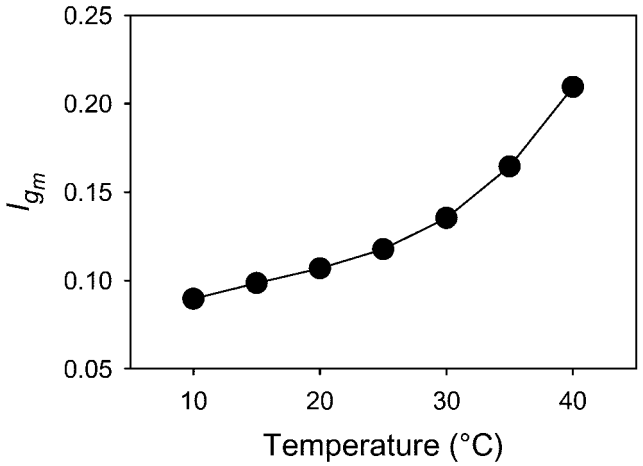

Figure 3. Temperature response of the limitation imposed upon photosynthesis by g_m:

$$l_{gm} = (A_{cc} - A_{ci})/A_{cc}$$

where A_{cc} and A_{ci} are values of A estimated graphically using the actual g_m and infinite g_m, respectively.

The temperature responses for Rubisco kinetic parameters provided in this study, when implemented into the biochemical model of photosynthesis of Farquhar et al. (1980), allow estimation of photosynthesis at the chloroplast level based on in vivo measurements over a wide range of temperatures. Using these parameters to scale photosynthesis to the leaf, canopy, or ecosystem levels requires the temperature response of g_m to be included in the models. We contend that using apparent values for Rubisco kinetic parameters, as derived from plots of photosynthesis versus C_i (Bernacchi et al., 2001), are sufficient for modeling photosynthesis for most systems. The in vivo estimates of these parameters based on the chloroplastic CO_2 concentrations, as derived in this study, provide improved parameters for modeling systems where g_m is sufficiently low that photosynthesis strongly deviates from model predictions when parameterized according to Bernacchi et al. (2001).

In conclusion, the temperature response of g_m provides evidence that the transfer of CO_2 from the leaf intercellular airspace into the chloroplast is controlled by a protein-facilitated step. CA and aquaporins are candidates because many reports show correlations between these proteins and CO_2 uptake. The limitation to photosynthesis imposed by g_m is also shown to increase from 10% to 22% as temperature increases from 10°C to 40°C. These results show that at all temperatures, and more so at higher temperatures, photosynthesis is significantly limited by the rate of CO_2 movement from the intercellular space into the chloroplast.

MATERIALS AND METHODS

Plant Material

Tobacco (*Nicotiana tabacum* L. cv W38) plants were germinated and grown in environmentally controlled greenhouses located at the University of Illinois (Urbana). Seeds were sown in 1-L plastic containers and were

individually transplanted into 1.5-L round pots approximately 2 weeks after emergence. The growth medium consisted of a soilless mix (Sunshine Mix No. 1, SunGro Horticulture, Inc., Bellevue, WA). The plants were watered regularly and were fertilized weekly with approximately 300 μL L^{-1} NPK 15:5:15 (Peters Excel, The Scotts Co., Marysville, OH) to pot saturation. Greenhouse air temperatures were set to 25°C for the 16-h photoperiod and 18°C for night. Sunlight was supplemented with high-pressure sodium lamps to maintain a minimum photon flux of 500 μmol m^{-2} s^{-1} at plant height.

Gas Exchange and Fluorescence

Leaf gas exchange measurements were coupled with measurements of chlorophyll fluorescence using an open gas exchange system (LI-6400; LI-COR, Inc., Lincoln, NE) with an integrated fluorescence chamber head (LI-6400–40 leaf chamber fluorometer; LI-COR, Inc.). The gas exchange system allowed for independent control of [CO_2], light, and humidity. The leaf chamber was modified by replacing the heat sinks on both Peltier thermoelectric cooling elements with metal blocks containing water channels. These in turn were connected to a heating/cooling circulating water bath (Endocal RTE-100, Neslab Instruments, Inc., Newington, NH). This modification allowed maintenance of leaf temperature at any preset value between 10°C and 40°C.

Photochemical efficiency of photosynthesis (Φ_{PSII}) was determined by measuring steady-state fluorescence (F_s) and maximum fluorescence during a light saturating pulse of >7 mmol m^{-2} s^{-1} (F'_m) on light-adapted leaves following the procedures of Genty and Briantais (1989):

$$\Phi_{PSII} = 1 - F_s/F'_m \quad (1)$$

The rate of electron transport (J) through the leaf was then calculated as:

$$J = \Phi_{PSII} \cdot Q \cdot \alpha_l \cdot \beta \quad (2)$$

where α_l is the leaf absorptance and β is the fraction of absorbed quanta that reaches photosystem II (assumed 0.5 for C3 plants; Ögren and Evans, 1993), and Q is photosynthetically active photon flux density. Leaf absorptance (α_l) was calculated as:

$$\alpha_l = \alpha_b B + \alpha_r(1 - B) \quad (3)$$

Terms α_b and α_r, which represent the measured leaf absorptance at the blue and red light wavelengths emitted from the gas exchange system light source, were measured with an integrating sphere and spectroradiometer (LI 1800; LI-COR, Inc.). B is the proportion of light in the blue wavelengths. Because the ratio of red to blue light varied based on levels of Q, values for α_l were calculated for each level.

Measurements were made on the youngest fully expanded leaf before stem elongation so that measurements were limited to one developmental stage. Photosynthesis was found to be saturating between 500 and 750 μmol m^{-2} s^{-1}, depending on measurement temperature; therefore, all measurements were made at between 900 and 1,200 μmol m^{-2} s^{-1} to ensure light saturation. Q was controlled using a red-blue light source built into the leaf fluorescence cuvette (LI-6400–40, LI-COR, Inc.). The amount of blue light was maximized to prevent stomatal closure, particularly at higher leaf temperature. The vapor pressure deficit was maintained between 0.5 and 2.0 kPa; this range had little effect on stomatal conductance. Leakage of CO_2 into and out of the empty chamber was determined for the range of CO_2 concentrations used in this study and used to correct measured leaf fluxes. Values for A and C_i were calculated using the equations of von Caemmerer and Farquhar (1981).

Measurements of gas exchange and chlorophyll fluorescence were made in 5°C increments from 10°C to 40°C. Responses of A versus C_i coupled with fluorescence were made on at least three plants per temperature increment. Photosynthesis was induced in saturating light and at 400 μmol mol^{-1} CO_2 surrounding the leaf (C_a). The C_a was lowered stepwise from 400 to 50 μmol mol^{-1} and then increased again from 400 to 1,600 μmol mol^{-1}. Measurements consisted of no less than 10 different C_a for each curve. In total, over 30 curves were used to obtain the relationship of g_m with temperature. These responses of A and J to C_i were then used to estimate g_m.

Estimation of g_m

Two methods using simultaneous gas exchange and fluorescence measurements were employed to estimate g_m. The first, the constant J method, was used when J was constant over a range of [CO_2], i.e. when photosynthesis was limited by the regeneration of ribulose-1,5-bisphosphate (Harley et al., 1992). Electron transport (J) estimated from chlorophyll fluorescence is a function of A, C_i, Γ^*, and g_m (Di Marco et al., 1990; Harley et al., 1992). Using Γ^* for a given temperature from Bernacchi et al. (2001) and the response of A to C_i measured here under conditions where J is constant, the equation:

$$J = (A + Rd) \cdot \frac{4 \cdot ((C_i - A/g_m) + 2\Gamma^*)}{(C_i - A/g_m) - \Gamma^*} \quad (4)$$

was solved for g_m at a range of C_i using the method of Loreto et al. (1992).

The second method for estimating g_m, termed the variable J method (Bongi and Loreto, 1989; Harley et al., 1992), uses A and R_d measured from gas exchange and J estimated from fluorescence via Equation 2 and used to solve for g_m after Harley et al. (1992):

$$g_m = \frac{A}{C_i - \dfrac{\Gamma^* \cdot (J + 8 \cdot (A + R_d))}{J - 4 \cdot (A + R_d)}} \quad (5)$$

Each method was used to calculate g_m for each leaf and all temperatures. The presence of alternative electron sinks may underestimate g_m; however, a previous study on tobacco plants demonstrated a lack of alternative electron sinks over a wide range of temperatures (Badger et al., 2000). Both methods for estimating g_m require that the specificity factor of Rubisco for CO_2 and O_2, represented by Γ^*, is known. The response of Γ^* to temperature described previously by Bernacchi et al. (2001) was used.

Temperature Response of g_m

The response of g_m to temperature was fit using the equation:

$$g_m = \frac{e^{(c - \Delta H_a/RT_k)}}{1 + e^{[(\Delta S \cdot T_k - \Delta H_d)/RT_k]}} \quad (6)$$

where c is a scaling constant, ΔH_a is the energy of activation, ΔS is an entropy term, and ΔH_d is a term for deactivation (Harley and Tenhunen, 1991). R is the molar gas constant (.008314 kJ J^{-1} mol^{-1}) and T_k is the leaf absolute temperature (Harley and Tenhunen, 1991). The exponential increase in Equation 6 is related to the temperature coefficient Q_{10} (Nobel, 1999) as follows:

$$Q_{10} = \sqrt{\frac{T_k + 10}{T_k}} e^{(10 \cdot \Delta H_a/[RT_k(T_k + 10)])} \quad (7)$$

All regressions of g_m with temperature were statistically analyzed using ANOVA (regression analysis module, SigmaPlot 6.1, SPSS, Inc., Chicago).

Estimation of K_c and K_o from C_c

By combining the relationship of A to C_i (Equation 8) parameterized by the measurements of Bernacchi et al. (2001) with the measurements of g_m made here, it was possible to recalculate the kinetic parameters of Rubisco by substituting C_c calculated from Equation 9 for C_i in Equation 8.

$$A = (1 - \Gamma^*/C_i) \frac{V_{c\,max} \cdot C_i}{C_i + K_c(1 + O/K_o)} - R_d \quad (8)$$

$$C_c = C_i - A/g_m \quad (9)$$

To link Equations 8 and 9, it is necessary to determine the relationship between g_m and $V_{c,max}$ at 25°C. This was determined from carbon isotope discrimination as $g_m = 0.0045\ V_{c,max}$ (Evans et al., 1986; von Caemmerer et al., 1994). K_c and K_o were then recalculated by fitting the relationships of A

to C_c using Equation 8 with C_i replaced by C_c, and Γ* determined from oxygen isotope exchange, as described below.

Γ* Estimated from C_c

Tobacco plants were grown in a greenhouse as described by Ruuska et al. (2000). O_2 exchange was measured on wild-type tobacco leaf discs using a temperature-controlled leaf chamber in a closed system incorporating a mass spectrometer (ISOPRIME, Micromass Ltd., Manchester, UK) as described by Maxwell et al. (1998). Discs were cut from illuminated leaves. The chamber, containing the leaf disc, was first darkened and then flushed with nitrogen. Known volumes of $^{18}O_2$ and CO_2 were added to give an atmosphere of 20% (v/v) $^{18}O_2$ and 0.3% (v/v) CO_2. The leaf disc was illuminated (1,800 μmol m^{-2} s^{-1} at the leaf surface) and photosynthesis was allowed to proceed until CO_2 was depleted to the compensation point. Then the light was turned off and respiratory O_2 and CO_2 exchange recorded. Gas exchange was measured with the mass spectrometer by continuously monitoring $^{16}O_2$ (mass 34), $^{18}O_2$ (mass 36), and CO_2 (mass 44). Gross O_2 evolution, gross O_2 uptake, and net O_2 exchange were calculated from the changes in $^{16}O_2$ and $^{18}O_2$ concentration (Canvin et al., 1980). Γ* was calculated from the $^{16}O_2$ and $^{18}O_2$ exchange at the compensation point, Γ, with the following equations:

$$\Gamma* = \frac{\Gamma}{2}\frac{V_o}{V_c} \tag{10}$$

where V_o and V_c are the rates of Rubisco oxygenation and carboxylation,

$$V_o = (^{18}O_2\ uptake - R_d)/1.5 \tag{11}$$

and

$$V_c = {}^{16}O_2\ evolution - V_o \tag{12}$$

R_d is the $^{18}O_2$ uptake in the dark. The factor 1.5 assumes that for every two O_2 consumed by Rubisco oxygenation, one is consumed by glycolate oxidation (Badger, 1985). These calculations of Γ* assume that consumption of O_2 by all other processes, including the Mehler reaction, is negligible (Ruuska et al., 2000).

Limitation of Photosynthesis by g_m

Bernacchi et al. (2001) determined the responses of A to C_i from three leaves per temperature from 10°C to 40°C in 5°C increments. Using g_m determined here across the same temperature range for tobacco grown in the same environments, C_c is calculated for each of these measurements of A. Using the A versus C_c relationships derived, $V_{c,max}$, K_c, K_o, and Γ* were recalculated for each temperature. From the response of A to C_c, the limitation (l_{gm}) imposed on photosynthesis by diffusion of CO_2 from the substomatal cavity to Rubisco was calculated as:

$$l_{gm} = \frac{(A_{cc} - A_{ci})}{A_{cc}} \tag{13}$$

where A_{cc} and A_{ci} are values of A estimated graphically using the actual g_m and assuming infinite g_m, respectively. This approach is derived by analogy to that of Farquhar and Sharkey (1982) for determining stomatal limitation from A/C_i responses. Equation 13 calculates g_m limitation in the same way from the A/C_c response. l_{gm} was calculated at each temperature from 10°C to 40°C in 5°C increments.

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