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

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CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ uptake at the site of Rubisco. The fraction of the total limitations to CO₂ 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 CO₂ from the intercellular air space to Rubisco is a very substantial limitation on photosynthesis, especially at high temperature.

In C3 plants, the diffusion of CO₂ 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 CO₂ influx and water efflux (Farquhar and Sharkey, 1982). Estimates of boundary layer and stomatal conductances to CO₂ are based on water vapor released from the leaf because water and CO₂ 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 CO₂ 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 CO₂ at the site of carboxylation ($C_i$) relative to that in the intercellular space ($C_c$), 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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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 (\( I^* \)) determined in this study are shown in Figure 2A and Table I. Michaelis constants for carboxylation (\( K_c \)) and oxygenation (\( K_o \)), calculated from \( C_i \) 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 \( g_m \) (\( \Delta g_m \)) is expressed as the proportionate decrease in \( A \)

\[
\Delta g_m = \frac{e^{-\Delta g_m/(RT)}}{1 + e^{-(\Delta g_m)/(RT)}}
\]

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

Figure 1. Temperature response of \( g_m \) normalized to unity at 25°C.
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).
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 Goddard, 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.

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

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**Table 1.** The scaling constant (c) and energies of activation ($\Delta H_a$), deactivation ($\Delta H_d$), and entropy ($\Delta S$) describing the temperature responses for mesophyll conductance and Rubisco enzyme kinetic parameters [parameter = $e^{(c - \Delta H_a/kT)}/H_20849 + e^{(c - \Delta H_d/kT)/(1 + e^{(c - \Delta H_d/kT)})}$]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value at 25°C</th>
<th>c</th>
<th>$\Delta H_a$</th>
<th>$\Delta H_d$</th>
<th>$\Delta S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_m$</td>
<td>1</td>
<td>20.0</td>
<td>49.6</td>
<td>437.4</td>
<td>1.4</td>
</tr>
<tr>
<td>$P^*$</td>
<td>37.43</td>
<td>13.49</td>
<td>24.46</td>
<td>nr</td>
<td>nr</td>
</tr>
<tr>
<td>$K_c$</td>
<td>272.38</td>
<td>38.28</td>
<td>80.99</td>
<td>nr</td>
<td>nr</td>
</tr>
<tr>
<td>$K_n$</td>
<td>165.82</td>
<td>14.68</td>
<td>23.72</td>
<td>nr</td>
<td>nr</td>
</tr>
</tbody>
</table>
 individuall y transplanted into 1.5-L round pots approximately 2 weeks after emergence. The growth medium consisted of a soill ess mix (Sunshine Mix No. 1, SunGro Horticulture, Inc., Bellevue, WA). The plants were watered regularly and were fertilized weekly with approximately 300 μL m⁻² 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 [CO₂], 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 (Φₚₑₚₚ) was determined by measuring steady-state fluorescence (Fₚ) and maximum fluorescence during a light saturating pulse of >7 mmol m⁻² s⁻¹ (Fₚₚₚ) on light-adapted leaves following the procedures of Genty and Briantais (1989):

\[
Φₚₑₚₚ = 1 - Fₚ/Fₚₚₚ
\] (1)

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

\[
j = Φₚₑₚₚ \cdot Q \cdot αₖ \cdot β
\] (2)

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

\[
αₖ = α_{λ}β + α_{λ}(1 - β)
\] (3)

Terms \(α_{λ}\) and \(α_{λ}β\), which represent the measured leaf absorbance 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 \(α_{λ}\) 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 900 to 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.

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 [CO₂], 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 \(A\), \(C\), \(P^*\), and \(g_m\) (De Marco et al., 1990; Harley et al., 1992). Using \(P^*\) for a given temperature from Bernacchi et al. (2001) and the response of \(A\) to \(C\), measured here under conditions where \(J\) is constant, the equation:

\[
J = (A + Rd) \cdot \frac{A^2}{C} \cdot (A + Rd) = \frac{4}{(C - A/g_m) + 2P^*} - P^* - \frac{4}{(A - P^*)} - 1
\] (4)

was solved for \(g_m\) at a range of \(C\) 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):

\[
g_m = \begin{align*}
&\frac{A}{C_i - \frac{1}{2} \cdot (1 + \frac{8}{(A + Rd) - 4} + \frac{A + Rd}{A})} \quad (5)
\end{align*}
\]

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 CO₂ and O₂, represented by \(P^*\), is known. The response of \(P^*\) 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:

\[
g_m = \frac{e^{-\Delta S/(RT)}}{1 + e^{4(\Delta S)/(RT)}}
\] (6)

where \(c\) is a scaling constant, \(\Delta H_S\) is the energy of activation, \(\Delta S\) is an entropy term, and \(\Delta H_D\) is a term for deactivation (Harley and Tenhunen, 1991). \(R\) is the molar gas constant (8.314 kJ mol⁻¹) and \(T_2\) 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:

\[
Q_{10} = \frac{T_1 + 10}{T_1}
\] (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 \(C_c\) (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 \(C_c\) in Equation 8.

\[
A = (1 - \Gamma^* / C_c) \cdot \frac{V_{max}}{C_c + K_c} \cdot \frac{C_c}{C_c + K_o} - R_d
\] (8)

\[
C_c = C_c - A / g_m
\] (9)

To link Equations 8 and 9, it is necessary to determine the relationship between \(g_m\) and \(V_{max}\) at 25°C. This was determined from carbon isotope discrimination as \(g_m = 0.0045 V_{max}\) (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, using Equation 8 with C replaced by C, and Γ* determined from oxygen isotope exchange, as described below.

Γ* Estimated from C

Tobacco plants were grown in a greenhouse as described by Ruuska et al. (2000). O2 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 18O2 and CO2 were added to give an atmosphere of 20% (v/v) 18O2 and 0.3% (v/v) CO2. The leaf disc was illuminated (1,800 μmol m−2 s−1 at the leaf surface) and photosynthesis was allowed to proceed until CO2 was depleted to the compensation point. Then the light was turned off and respiratory O2 and CO2 exchange recorded. Gas exchange was measured with the mass spectrometer by continuously monitoring 18O2 (mass 34), 16O2 (mass 36), and CO2 (mass 44). Gross O2 evolution, gross O2 uptake, and net O2 exchange were calculated from the changes in 16O2 and 18O2 concentration (Canvin et al., 1980). Γ* was calculated from the 16O2 and 18O2 exchange at the compensation point, Γ, with the following equations:

where V and V are the rates of Rubisco oxygenation and carbonization,

V = (18O2 uptake – R)/1.5

and

V = (16O2 evolution – V)

R is the 18O2 uptake in the dark. The factor 1.5 assumes that for every two O2 consumed by Rubisco oxygenation, one is consumed by glycolate oxidation (Badger, 1985). These calculations of Γ* assume that consumption of O2 by all other processes, including the Mehler reaction, is negligible (Ruuska et al., 2000).

Limitation of Photosynthesis by 18g

Bernacchi et al. (2001) determined the responses of A to C from three leaves per temperature from 10°C to 40°C in 5°C increments. Using 18g determined here across the same temperature range for tobacco grown in the same environments, C is calculated for each of these measurements of A. Using the A versus C relationships derived, Ve,max, Ke, Km, and Γ* were recalculated for each temperature. From the response of A to C, the limitation (Ip) imposed on photosynthesis by diffusion of CO2 from the substomat cell to Rubisco was calculated as:

Ip = (AS – A)/(AS)

where AS and A are values of A estimated graphically using the actual 18g and assuming infinite 18g, respectively. This approach is derived by analogy to that of Farquhar and Sharkey (1982) for determining stomatal limitation from A/Cg responses. Equation 10 calculates 18g limitation in the same way as the A/Cg response. Ip was calculated at each temperature from 10°C to 40°C in 5°C increments.

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LITERATURE CITED


