Bundle Sheath Diffusive Resistance to CO2 and Effectiveness of C4 Photosynthesis and Refixation of Photorespired CO2 in a C4 Cycle Mutant and Wild-Type Amaranthus edulis

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A mutant of the NAD-malic enzyme-type C4 plant, Amaranthus edulis, which lacks phosphoenolpyruvate carboxylase (PEPC) in the mesophyll cells was studied. Analysis of CO2 response curves of photosynthesis of the mutant, which has normal Kranz anatomy but lacks a functional C4 cycle, provided a direct means of determining the liquid phase-diffusive resistance of atmospheric CO2 to sites of ribulose 1,5-bisphosphate carboxylation inside bundle sheath (BS) chloroplasts (rbs) within intact plants. Comparisons were made with excised shoots of wild-type plants fed 3,3-dichloro-2-(dihydroxyphosphinoyl-methyl)-propenoate, an inhibitor of PEPC. Values of rbs in A. edulis were 70 to 200 m2 s−1 mol−1, increasing as the leaf matured. This is about 70-fold higher than the liquid phase resistance for diffusion of CO2 to Rubisco in mesophyll cells of C3 plants. The values of rbs in A. edulis are sufficient for C4 photosynthesis to elevate CO2 in BS cells and to minimize photorespiration. The calculated CO2 concentration in BS cells, which is dependent on input of rbs, was about 2,000 bar under maximum rates of CO2 fixation, which is about six times the ambient level of CO2. High re-assimilation of photorespired CO2 was demonstrated in both mutant and wild-type plants at limiting CO2 concentrations, which can be explained by high rbs. Increasing O2 from near zero up to ambient levels under low CO2 resulted in an increase in the gross rate of O2 evolution measured by chlorophyll fluorescence analysis in the PEPC mutant; this increase was simulated from a Rubisco kinetic model, which indicates effective refixation of photorespired CO2 in BS cells.
place. A possible means of estimating this is to combine measurements of CO\textsubscript{2} fixation with information on the kinetic properties of Rubisco. Ribulose 1,5-bisphosphate (RuBP) carboxylation and oxygenation in BS chloroplasts are competing reactions, and information is available on the Rubisco CO\textsubscript{2} to O\textsubscript{2} affinity ratio.

In this study, we have used the PEPC mutant of *Amaranthus edulis* LaC\textsubscript{4} 2.16 (Dever et al., 1995; Marocco et al., 1998a, 1998b), which has a defective C\textsubscript{4} cycle and requires direct diffusion of atmospheric CO\textsubscript{2} into the BS cells for CO\textsubscript{2} assimilation and growth. In this mutant, the primary carboxylase for fixing atmospheric CO\textsubscript{2} is Rubisco, which is located in the BS chloroplast. The purpose of this work was to use gas exchange measurements on the *A. edulis* mutant for direct estimation of BS cell resistance to CO\textsubscript{2}, and to determine the dependence of \( r_{bs} \) on the developmental stage of the leaf. For comparison, data were also obtained with wild-type plants by feeding the PEPC inhibitor 3,3-dichloro-2-(dihydroxyphosphinoyl-methyl)-propenoate (DCDP) to prevent operation of the C\textsubscript{4} cycle.

**RESULTS AND DISCUSSION**

Figure 1 outlines important aspects of photosynthesis in the PEPC mutant of *A. edulis* relative to the experimental approach for determining the BS-diffusive resistance to CO\textsubscript{2} (\( r_{bs} \)). Because the C\textsubscript{4} cycle is inoperative, the mechanism of CO\textsubscript{2} fixation and energy requirements are considered the same as in C\textsubscript{3} plants. Fixation of atmospheric CO\textsubscript{2} in the mutant by Rubisco in the C\textsubscript{3} cycle, requires diffusion of CO\textsubscript{2} from the mesophyll to BS cells. CO\textsubscript{2} is considered the primary species of inorganic carbon diffusing to BS cells and supplying CO\textsubscript{2} to Rubisco. Although CO\textsubscript{2} will be converted rapidly to bicarbonate in the cytosol of mesophyll cells via carbonic anhydrase, the diffusion pathway for bicarbonate will be limited because it is not used by Rubisco and BS cells lack, or have negligible levels of, carbonic anhydrase (Ku and Edwards, 1975). Also, in wild-type *A. edulis*, where CO\textsubscript{2} is concentrated in the BS cells by the C\textsubscript{4} cycle through C\textsubscript{4} acid decarboxylation, CO\textsubscript{2} is considered the primary form of inorganic carbon leaking from BS to mesophyll cells (Jenkins et al., 1989b). Reaction of O\textsubscript{2} with RuBP in the photosynthetic carbon oxidation cycle in the BS chloroplasts will result in the production of the photorespiratory products glycerate, CO\textsubscript{2}, and ammonia through metabolism in mitochondria and peroxisomes. According to the known compartmentalization of carbon assimilation in C\textsubscript{4} plants (Kanai and Edwards, 1999), the only function of mesophyll chloroplasts in carbon assimilation in the mutant may be the conversion of glycerate, the product of photorespiration in BS cells, to triose phosphate and conversion of some of the 3-phosphoglycerate (PGA), generated by Rubisco in BS cells, to triose phosphate.

**BS-Diffusive Resistance to CO\textsubscript{2} (\( r_{bs} \))**

The resistances involved in uptake of atmospheric CO\textsubscript{2} by the mutant are described in Equation 10 (“Materials and Methods”). If the CO\textsubscript{2} response curve of CO\textsubscript{2} assimilation (measured at light saturation) is plotted against the intercellular CO\textsubscript{2} partial pressure (\( C_{i} \)), which equilibrates with the liquid phase at the cell wall, the initial slope of the curve is determined by the average physical liquid phase conductance and by the carboxylation efficiency of Rubisco (Fig. 2). Gas phase resistance to CO\textsubscript{2}, \( r_{s} \), was calculated based on transpiration measurements. Typical values of \( r_{s} \) for the mutant plants were in the

![Figure 1. Schematic representation of the movement of gases and metabolites in the PEPC mutant of *A. edulis*. CO\textsubscript{2} diffuses into BS cell chloroplasts where it enters the C\textsubscript{3} cycle. Equilibrium between HCO\textsubscript{3}\textsuperscript{-} and CO\textsubscript{2} in mesophyll cell is fast, but it is slow in BS because of lack of carbonic anhydrase activity. Glycerate, ammonia, and CO\textsubscript{2} are generated by the photosynthetic carbon oxidation (PCO) cycle. Glycerate metabolism and partial reduction of PGA in mesophyll cells may account for use of some photochemically generated energy in mesophyll chloroplasts.](image-url)
range of 5 to 10 m² s⁻¹ mol⁻¹ (2–4 s cm⁻¹). Also, the expected Rubisco CO₂ response curve is shown in Figure 2 in the absence of liquid phase resistance; this demonstrates the contribution of the resistance of RuBP carboxylase (chemical resistance), \( r_{ct} \), to the total CO₂ flux resistance. The Rubisco response was generated taking the maximum velocity of RuBP carboxylase (\( V_c \)) as 1.2 times the CO₂-saturated rate of CO₂ fixation (\( A_{\text{max}} \)) based on Rubisco extractable activity measurements. Also, there is a decrease in RuBP pool with increasing CO₂, which could account for \( A_{\text{max}} \) being lower than \( V_c \) of Rubisco if RuBP becomes limiting (as shown later in Fig. 7). In general, \( r_c \) for mutant leaves was a minor component (from 10 to 15 m² s⁻¹ mol⁻¹) compared with \( r_{bs} \) (152 m² s⁻¹ mol⁻¹ in Fig. 2).

Analogous experiments to those in Figure 2 were performed on leaves of different maturity. The value of \( r_{bs} \) increased with plant age and reached its highest value during grain filling when the leaves were pale green, showing early signs of senescence and lower maximum rates of CO₂ fixation (Table I). Although BS resistance was calculated assuming \( V_c \) is 1.2 times \( A_{\text{max}} \), a sensitivity analysis taking \( V_c/A_{\text{max}} = 1.0 \) and 1.4 showed that this results in a change in the calculated \( r_{bs} \) values of only 3% to 5% in young leaves, and approximately 2% in mature leaves. Interestingly, during the grain filling stage, the leaves still maintained a reasonably high-CO₂ assimilation capacity. The liquid phase resistance from the mesophyll cells to Rubisco in the mutant A. edulis (72–181 m² s⁻¹ mol⁻¹; Table I) is about 70-fold higher than that of C₃ plants (approximately 1–3 m² s⁻¹ mol⁻¹; Evans et al., 1994; Laisk and Loreto, 1996). This high-diffusive resistance in C₄ plants may be attributed to the relatively low BS cell surface area per unit leaf area and structural properties of BS cell walls (Evans and von Caemmerer, 1996). Models of C₄ photosynthesis indicate the rate of CO₂ assimilation under low CO₂ drops rapidly below \( r_{bs} \) values of 50 to 100 m² s⁻¹ mol⁻¹.

### Table 1. Leaf age-dependent differences in \( A_{\text{max}} \) and resistance to CO₂ in the A. edulis PEPC mutant plants

<table>
<thead>
<tr>
<th>Leaf Description</th>
<th>( A_{\text{max}} )</th>
<th>Mesophyll to BS Resistance to CO₂ ( (r_{bs} + r_c) )</th>
<th>Liquid Phase Resistance to CO₂ ( r_{bs} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young, 30% expanded</td>
<td>25.1 ± 1.4</td>
<td>89 ± 16</td>
<td>72.4</td>
</tr>
<tr>
<td>Young, 70% expanded</td>
<td>26.6 ± 1.8</td>
<td>103 ± 16</td>
<td>86.3</td>
</tr>
<tr>
<td>Mature, vegetative, 100% expanded</td>
<td>30.6 ± 1.0</td>
<td>127 ± 6</td>
<td>113.4</td>
</tr>
<tr>
<td>Grain filling stage, 100% expanded</td>
<td>20.6 ± 1.2</td>
<td>201 ± 17</td>
<td>180.8</td>
</tr>
</tbody>
</table>

Figure 2. Example of calculating BS cell resistance from \( A \) versus \( C_i \) curves measured at low O₂ (0.3 mbar) and PFD of 1,800 μmol m⁻² s⁻¹ in PEPC mutant. The inverse of the initial slope of \( A/C_i \) curve is the sum of the diffusive resistance from the cell wall to the sites of Rubisco and of the chemical RuBP carboxylation resistance. A simulated Rubisco CO₂ response curve without diffusive resistance is shown for comparison. Rubisco resistance can be calculated as \( K_c/V_c \). \( V_c \) was taken as \( A_{\text{max}} \).
The resistance observed in the mutant might not reflect the true value of the wild type if the mutation alters the structure of the BS cells. To test this, we used the PEPC inhibitor DCDP, feeding it into the petiole to block the C4 cycle in wild-type plants (method of Jenkins et al., 1989a). The strong reduction in carboxylation efficiency caused by 4 mM DCDP, without a biphasic response (Fig. 3), suggests that PEPC is almost completely inhibited. CO2 response curves were measured at 2% (v/v) O2 before applying DCDP and immediately after photosynthesis declined at ambient CO2 as PEPC was inhibited (Fig. 3). The results show that the calculated BS resistances are similar to those obtained with the PEPC mutant. We measured A/Ci response curves (Ci determined from analysis of transpiration, which eliminates stomatal resistance) and calculated the resistance from the initial slope with Vc for Rubisco equal to 1.2 Amax estimated from CO2-saturated rates in the presence of DCDP. In calculating rb, it is important to eliminate stomatal resistance and to account for any partial inhibition of Rubisco, and Vc by DCDP. Jenkins et al. (1989a) calculated a permeability coefficient for CO2 from the atmosphere to BS cells (the reciprocal for the total diffusive resistance, rb) from measurements of photosynthetic O2 evolution in the presence of DCDP at 1.6% (v/v) CO2 by dividing the photosynthetic rate by the difference between atmospheric CO2 (Ca) and estimates of Cbs. The calculated value of rb from the study of Jenkins et al. in A. edulis is 556 s cm⁻¹ (or 1,373 m² s⁻¹ mol⁻¹), which is about 10-fold higher than values of rb in the present study. Because rb includes stomatal and BS-diffusive resistance, high stomatal resistance in excised leaves could contribute to high rb values. Our A/Ci response curves with the PEPC mutant saturate sharply at intercellular CO2 concentrations about 1%, whereas up to 5% (v/v) ambient CO2 was required in experiments by Jenkins et al. (1989). Also, the calculation of rb is dependent on input of Vc of Rubisco to calculate Cbs. Using the value of Vc from analysis of wild-type plants (Jenkins et al., 1989), rather than Vc in the presence of DCDP, will also overestimate Vc and the calculated diffusive resistance values (see also He and Edwards, 1996).

Light microscopy of leaf anatomy indicates that the wild-type plants grown under both 370 μbar and 10 mbar and the PEPC mutant grown under 10 mbar of CO2 all have Kranz-type leaf anatomy (results not shown; Dever et al., 1995). In wild-type plants grown at 10 mbar of CO2, the BS cell walls at the intercellular space are very thick relative to the walls of the

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**Figure 3.** The response of the rates of CO₂ assimilation (A, O₂) and gross rate of O₂ evolution from PSII (Jₐ₂, A, D) on wild type and PEPC mutant with and without feeding DCDP. Measurements were made on leaves of excised plants under 20 mbar O₂. The calculated values of rb in wild-type plants in presence of DCDP and in PEPC mutant with and without DCDP are shown. A and B, Wild-type plants grown at 370 μbar CO₂; C and D, wild-type plants grown at 10 mbar CO₂; E and F, PEPC mutant grown at 10 mbar CO₂.
mesophyll and cross walls (Fig. 4); similar results were obtained with wild type grown under ambient CO₂. In the PEPC mutant, the BS cell walls are also much thicker than those of mesophyll cells, and cross walls include normal plasmodesmata (arrow). This suggests there are no structural differences between mutant and wild type.

Cellular Conductance and the Temperature Dependence of Photosynthesis under Limiting and Saturating CO₂

CO₂ response curves of photosynthesis were measured in wild-type and mutant A. edulis at leaf temperatures from 15°C to 35°C. Figure 5, A and B, describes the temperature response of A max determined from CO₂-saturated rates, and cellular conductance for CO₂ g, determined from the initial slope of the net rate of CO₂ assimilation (A/C) curves. In this case, conductance instead of resistance (g = 1/r) was used, because it is linearly related to the diffusion flux. In wild-type plants, the cellular conductance for CO₂, g wt, was a function of liquid phase diffusion and carboxylation by PEPC in the mesophyll cell. For the mutant, g mut was the total conductance from mesophyll to BS cells, including liquid phase (g bs) and Rubisco (g c), as determined earlier.

A max increased with increasing temperature, with a Q₁₀ value (the factor by which a reaction increases with a 10°C increase in temperature) of 2 for the mutant and 1.9 for the wild type between 20°C and 30°C. These values of Q₁₀ and activation energies are as expected if photosynthesis under saturating CO₂ and light is controlled by enzymatic processes. Also, the activation energy for A max in the mutant (13.5 kcal mol⁻¹) was close to the in vitro Rubisco activation energy of 13 kcal mol⁻¹ (calculated from Jordan and Ogren, 1984). If RuBP is
saturating for photosynthesis under high light, the $Q_{10}$ values obtained would be consistent with Rubisco, rather than a diffusion limitation, being the major limiting factor for light- and $CO_2$-saturated photosynthesis in the mutant.

In the mutant, $g_{m\text{mut}}$ (which includes $g_{bs}$ and Rubisco conductance) and $g_{bs}$ had a linear response to increasing temperature. For $g_{bs}$, the $Q_{10}$ values were 1.3 between 20°C and 30°C (Fig. 5B), which coincides with the temperature sensitivity of diffusion of small molecules in solutions that have a $Q_{10}$ value of 1.3 (Nobel, 1991). The agreement between the measured and expected $Q_{10}$ value for $g_{bs}$ provides confidence that we are correctly measuring diffusive resistance of $CO_2$ to BS cells. Because $CO_2$ must diffuse to BS cells for fixation in the mutant, the cellular conductance values for the mutant are much lower than for the wild type.

In wild-type plants, $CO_2$ is fixed initially in mesophyll cells, and the temperature response of the mesophyll conductance, determined from the initial slope of the $CO_2$ response curve, showed a saturating curve rather than a linear response, indicating that biochemistry is involved (Fig. 5A). The effect of temperature on the initial slope of the $A/C_i$ response in wild-type $A. edulis$ depends on liquid phase diffusion and PEPC in mesophyll cells. The relative insensitivity of the mesophyll conductance in the wild type to temperature indicates control by biochemistry. This could be attributable to regulation of PEPC by temperature-dependent changes in $K_m$ for phosphoenolpyruvate (PEP; the substrate PEP is lower under limiting $CO_2$ [Leegood and von Caemmerer, 1988]), by allosteric effectors, and/or by covalent modification of the enzyme (phosphorylation/dephosphorylation). There also may be a temperature-dependent effect on PEP because the level is reported to increase with increasing temperature under normal atmospheric levels of $CO_2$ (Labate et al., 1990).

**CO$_2$ Response and Partitioning of Photochemical Electron Flow**

The $CO_2$ response curves for $CO_2$ fixation ($A$), gross rates of $O_2$ evolution ($J_{O2}$), and net rates of $O_2$ evolution ($J_{O2-net}$) of the mutant and wild-type leaves were measured at saturating light (Fig. 6). Measurements of $A$ and $J_{O2}$ were made at 210 mbar of $O_2$, representing current atmospheric levels, and measurements of $A$, $J_{O2}$, and $J_{O2-net}$ at near zero levels of $O_2$ (0.3 mbar), with an interest in studying $O_2$-dependent processes. To overcome the high-diffusive resistance from the atmosphere to the BS cells in the mutant, $CO_2$ concentrations as high as 2% (20 mbar) were required (Fig. 6, A and C), whereas near atmospheric levels were saturating for the wild type (Fig. 6, B and D). The mutant plants of $A. edulis$ had about 100 times lower initial slopes in $A/C_i$ curves compared with the wild type (Fig. 6, A versus B and C).

![Figure 6](https://www.plantphysiol.org/cn/6263856){#fn-6}

In the mutant at 0.3 mbar of $O_2$, it is obvious that rates of $A$, $J_{O2}$, and $J_{O2-net}$ were very similar in both the mutant and wild type at 0.3 mbar $O_2$ (Fig. 6, A and B). At 210 mbar of $O_2$, $J_{O2}$ was substantially higher than $A$ in both the mutant and wild type (Fig. 6, C and D). This is clearly shown in Figure 6, E and F, where $J_{O2-A}$ is plotted in response to varying $CO_2$ at 0.3 and 210 mbar of $O_2$. $J_{O2-A}$ can potentially be accounted for by dark-type mitochondrial respiration ($R_d$), photosynthesis (1.5 velocity of RuBP oxygenase [$v_o$]), photosystem (PS) II-dependent $O_2$ evolution associated with the Mehler-peroxidase reaction ($J_{O2NA}$), and $O_2$ evolution associated with nitrogen assimilation ($J_{O2NA}$, see Eqs. 3–6).

In the mutant at 0.3 mbar of $O_2$, it is obvious that most of the PSII activity ($J_{O2}$) can be accounted for by $CO_2$ fixation and that $R_d$, $v_o$, $J_{O2MB}$, and $J_{O2NA}$ must be low (Fig. 6, A and E). Because a partial pressure of 0.3 mbar of $O_2$ in the atmosphere is extremely low, a correspondingly low level is expected in the mesophyll cells within the leaf. However, even under very low external levels of $O_2$, the $O_2$ level in the BS cells will increase when $O_2$ is generated from PSII activity under a high-BS cell-diffusive resistance. At 0.3 mbar of $O_2$ in the atmosphere and based on the average
value for BS cell resistance determined in this study (described later), the calculated level of O₂ in BS cells at CO₂-saturated rates of photosynthesis was about 30 mbar. From Equation 14, if we take \( A = 30 \mu \text{mol m}^{-2} \text{s}^{-1} \), \( r_{bs} = 30 \text{ s cm}^{-1} \), and \( a_w = 0.79 \), \( a_w \) is a constant that takes into account the difference in O₂ and CO₂ diffusivities [at 25°C, \( a_w = 0.79 \); Farquhar, 1983]), and \( b = 0.5 \), then the concentration of O₂ would be 35 \( \mu \text{M} \) (equivalent to about 30 mbar of O₂ in the gas phase; O₂ = 0 + 0.79*0.5*30*A/10 = 35 \( \mu \text{M} \)). \( R_d \) from measurements in the dark under normal atmospheric conditions is 2 to 3 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (data not shown). On average, \( I_{O2} \) for nitrate assimilation to Glu is estimated to be about 5% of \( A \), not considering re-assimilation of ammonia from photorespiration (see Edwards and Baker, 1993). Thus, \( R_d \) in vascular tissue plus nitrate assimilation could easily account for the difference between \( I_{O2} \) and \( A \) at 0.3 mbar of O₂. Some rise in values of \( I_{O2} \) under limiting CO₂ at 0.3 mbar of O₂ would be expected, because \( A \) decreases relative to \( R_d \) (Fig. 6E). These results indicate that \( I_{O2} \) and \( v_{m} \) must be very low in the mutant under 0.3 mbar of O₂ in the atmosphere.

In the mutant at 210 mbar of O₂, the values of \( I_{O2} \) were much greater than at 0.3 mbar of O₂, particularly with decreasing levels of CO₂ (Fig. 6E). The logical explanation for this effect is that the mutant, which lacks a C₄ cycle, has increasing rates of photorespiration under limiting CO₂ just as C₃ plants do (see also Lacuesta et al., 1997; Maroco et al., 1998a), which causes a corresponding increase in \( I_{O2} \). Under high CO₂ and under 210 mbar of O₂, the mutant may have some additional dark-type respiration in mesophyll cells, resulting in larger values of \( I_{O2} \) than at 0.3 mbar of O₂.

In the wild-type plant under 0.3 mbar of O₂ (Fig. 6F), the value of \( I_{O2} \) was about 4 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), which was higher than in the mutant, and was independent of the level of CO₂. As with the mutant, nitrate assimilation and \( R_d \) in BS tissue may partly account for the difference. However, the Mehler reaction or photorespiration may also contribute in the wild-type plant. In a recent study, there was evidence for significant Mehler reaction in wild-type \( A. edulis \) under rather low levels of O₂ (between 0.2 and 20 mbar; Laish and Edwards, 1998). In NAD-malic enzyme (NAD-ME) species like \( A. edulis \), the Mehler reaction is proposed to function in mesophyll chloroplasts and contribute to generation of ATP for the C₄ cycle (Furbank and Badger, 1982; Laish and Edwards, 1998). With increasing CO₂, there may be a rise in the rate of the Mehler reaction as the rate of the C₄ cycle increases, whereas with decreasing CO₂, there may be a rise in photorespiration; together these effects could result in values of \( I_{O2} \) being reasonably constant in NAD-ME-type species with varying CO₂ (see Furbank and Badger, 1982).

In the wild-type plant under 210 mbar of O₂, \( I_{O2} \) was higher than \( A \) across the CO₂ response curve (Fig. 6D); the pattern of change with increasing CO₂ is very similar to that of Canvin et al. (1980) from O₂ isotope analysis of photosynthesis in \( A. edulis \). \( I_{O2} \) at 210 mbar was greater than at 0.3 mbar of O₂, and a sharp increase in \( I_{O2} \) occurred at very low levels of CO₂. Above approximately 0.025 mbar of CO₂, \( I_{O2} \) was constant at about 8 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Fig. 6F). As noted above, this can be explained by O₂-dependent photorespiration at extremely low levels of CO₂ and increasing Mehler reaction at higher CO₂. The larger \( I_{O2} \) values at high CO₂ under 210 mbar versus 0.3 mbar of O₂ may be accounted for by O₂-dependent dark respiration and/or the Mehler reaction.

To evaluate Rubisco kinetics in mutant leaves relative to \( A \) and \( I_{O2} \), we analyzed RuBP content and RuBisco activity (Fig. 7). With increasing CO₂ from zero up to about 0.8 mbar there was an increase in RuBP content, above which it decreased. The initial...
extractable activity of Rubisco in the mutant was higher than $A_{\text{max}}$ at saturating CO$_2$ and decreased slightly at the lower CO$_2$ concentrations (Fig. 7, C and D). The decrease at low CO$_2$ correlated with a decrease in the state of activation of the enzyme. In the wild type (results not shown), leaf RuBP content was similar to the mutant at low CO$_2$ (at CO$_2$ = 34 μbar, RuBP was 56 μmol m$^{-2}$) and decreased at high CO$_2$ (at CO$_2$ = 4.8 mbar, RuBP was 47 μmol m$^{-2}$), which is in close agreement with the values of Leegood and von Caemmerer (1988). Considering a Rubisco active site turnover rate of 2.8 s$^{-1}$ (Woodrow and Berry, 1988), the number of active sites per leaf area in A. edulis would be about 15 to 20 μmol m$^{-2}$. The RuBP concentration across the A/C$_4$ curve at light saturation exceeded the number of active sites by about three times, which suggests the RuBP is at saturating levels. However, the observation that the RuBP concentration decreased at high CO$_2$ (Fig. 7) and with the known competitive interaction of some chloroplast metabolites with RuBP (e.g. PGA; Servaites and Geiger, 1995), it is possible that $A_{\text{max}}$ is partly limited by RuBP regeneration.

Light Response and Partitioning of Photochemical Electron Flow

The response of photosynthesis to light under very high CO$_2$ (4%, 40 mbar) in the mutant gave about the same maximal quantum yield for O$_2$ evolution ($I_{O2\text{-net}} \Delta \text{O}_2$) as in the wild type (0.064 versus 0.066; Fig. 8, A and B). The quantum yield for O$_2$ evolution for wild-type A. edulis was higher than measured by Ehleringer and Bjorkman (1977) for CO$_2$ fixation in NAD-ME-type C$_4$ species (0.054). Higher values for the wild type may be explained by the use of highly saturating CO$_2$ and very low O$_2$, which would restrict O$_2$-dependent use of energy, and by the fact that O$_2$ evolution, rather than CO$_2$ uptake, was measured. In the wild-type plant under very high CO$_2$, the responses of $I_{O2}$ and $I_{O2\text{-net}}$ to increasing light were very similar, indicating there was little photorespiration and Mehler reaction under this condition. It is uncertain why the Mehler reaction would be restricted under such high levels of CO$_2$ in the wild type. However, in the wild-type plant under 4% (v/v) CO$_2$, direct diffusion of CO$_2$ to BS cells would occur, bypassing the C$_4$ cycle, because in mutant plants, which lack a C$_4$ cycle, photosynthesis is saturated by 2% (v/v) CO$_2$.

In the mutant, under saturating levels of CO$_2$, which prevent photorespiration, we would expect maximum quantum yields of O$_2$ evolution similar to those of C$_3$ plants. Instead, the values in the mutant were lower than in C$_3$ plants under saturating CO$_2$ and similar to those of the A. edulis wild type. This suggests that the absorbed energy, which is used in the wild type in mesophyll chloroplasts for the C$_4$ cycle (ATP for conversion of pyruvate to PEP, and NADPH to the degree malate is synthesized), may be dissipated as heat in the mutant if there is no other means for using it in carbon assimilation. In the mutant, there is no requirement for energy in mesophyll cells in carbon assimilation, unless part of the PGA and glyceroate formed via RuBP carboxylase and oxygenase activities in BS chloroplasts is shuttled to mesophyll cells for reduction (Fig. 1).

In the mutant under CO$_2$ which is limiting for photosynthesis (2 mbar), and at 210 mbar of O$_2$, $I_{O2}$ was much higher than $A$, and the maximum quantum yield under limiting light was higher for $I_{O2}$ than for $A$ (Fig. 8C). This can be explained by the mutant having a high level of photorespiration and responding like a C$_3$-type species under limiting CO$_2$.

In the wild type under atmospheric levels of CO$_2$ and 210 mbar O$_2$, the light response curves showed a higher quantum yield (from initial slopes) and a higher light-saturated rate for $I_{O2}$ than for $A$ (Fig. 8D). This may be attributed to the Mehler reaction increasing with increasing light and generating ATP for the C$_4$ cycle under 210 mbar O$_2$, which could largely account for the difference between $I_{O2}$ and $A$. With the wild-type plant having a functional C$_4$ cycle, photorespiration and dark-type respiration are expected to be minor components of the difference between $I_{O2}$ and $A$. A previous study indicated that the Mehler reaction is functioning in A. edulis but is insufficient to supply the ATP needed to support the C$_4$ cycle (Laisk and Edwards, 1998). Thus, both the Mehler reaction and PSI-mediated cyclic electron flow may generate the ATP, with some flexibility in the magnitude of each. In contrast to results under

Figure 8. Light response of PEPC mutant and wild-type A. edulis O$_2$ evolution ($I_{O2\text{-net}}$, □) at highly saturating levels of CO$_2$ (40 mbar CO$_2$ (O$_2$ = 0.3 mbar), and CO$_2$ uptake (A, ◦) at limiting CO$_2$ (2 mbar for PEPC mutant and 0.36 mbar for wild type) and 210 mbar O$_2$ pressure. Leaf temperature was 28°C. Gross rates of O$_2$ evolution ($I_{O2}$, Δ) were calculated from simultaneous fluorescence measurements as described in “Materials and Methods.”
210 mbar of O₂ (Figs. 6D and 8D), there was no evidence for function of the Mehler reaction under low O₂ (Fig. 8B), which suggests the ATP needed to support the C₄ cycle is provided by PSI-dependent cyclic electron flow.

**O₂ Effect on the Partitioning of Photochemical Electron Flow**

It is well known that at low-CO₂ concentrations, the rate of CO₂ assimilation in C₄ plants exhibits low sensitivity to O₂. This is in contrast to C₃ plants, where photorespiration greatly reduces the rate of CO₂ assimilation in response to increasing O₂ (Kanai and Edwards, 1999). Measurements of A and J₀₂ in mutant plants, in which the C₄ cycle is not functional, provide an opportunity to follow more closely the maximum potential for Rubisco oxygenase and the glycolate pathway to function. This is not possible in normal C₄ leaves, where the CO₂ pump operates.

The responses of A and J₀₂ were measured at increasing O₂ concentrations over a range of CO₂-limited concentrations where greater RuBP oxygenase activity is expected for mutant and wild-type plants (Fig. 9). In the wild-type plant at rate-limiting CO₂ levels, high O₂ increased J₀₂, but the CO₂ assimilation rates remained relatively unaffected by O₂ (Fig. 9B and D). The increase in J₀₂ with increasing O₂ under low CO₂ suggests an increase in photorespiration through RuBP oxygenase activity. At a given level of O₂, the value of J₀₂-A (Fig. 9F) remained about the same with increasing levels of CO₂, which, as discussed earlier, may be attributable to the Mehler reaction partially providing ATP to support the C₄ cycle and increasing with increasing rates of CO₂ fixation.

In the mutant, high O₂ increased J₀₂ at the lowest, rate-limiting CO₂ levels, but the CO₂ assimilation rates remained relatively unaffected by O₂ (Fig. 9A and C). The increased electron transport rate with increasing O₂ under limiting CO₂ suggests an O₂-dependent increase in the RuBP oxygenation rate. At the higher levels of O₂, the difference between J₀₂ and A (Fig. 9E) was gradually suppressed by increasing CO₂, which is expected if increasing CO₂ suppresses photorespiration and considering that the mutant does not have a C₄ cycle that could be supported by the Mehler reaction.

For the mutant under limiting CO₂, the activity of J₀₂ is expected to be largely accounted for by the sum of the velocity of RuBP carboxylase (vₚ) and vₒ. We extrapolated J₀₂, measured at different O₂ concentrations, to CO₂ = 0 and plotted the resulting values against BS cell O₂ concentration in an effort to evaluate the effect of Rubisco oxygenase activity on J₀₂ (Fig. 10). Assuming photorespired CO₂ is re-assimilated with the probability determined by the ratio of Rubisco conductance to BS cell conductance, the expected J₀₂ response can be described by the solid line in Figure 10. The experimental points are in good agreement with predicted results based on Rubisco kinetics if re-assimilation is accounted for, except that at the highest O₂ level (480 μM, which is...
about twice the atmospheric level), the oxygenase activity was lower than expected. Analysis of RuBP content in the leaves of the mutant at low CO₂ (0.4 mbar) showed a decrease of RuBP concentration at rate-limiting CO₂ and with increasing O₂ (Fig. 11). Therefore, we suggest that the lower than expected \( \frac{I}{O_2} \) value at O₂ concentration below the \( K_m \) for RuBP oxygenase (\( K_o \) value (640 \( \mu \text{M} \)) in the mutant (Fig. 10) is the result of RuBP becoming partially limiting for CO₂ assimilation. Very similar results were obtained with wild-type plants (Fig. 11). Also, the response of \( \frac{I}{O_2} \) to O₂ under low CO₂ in the wild type is similar to that in \( A. \text{edulis} \) measured at the CO₂ compensation point by O₂ isotope analysis (Canvin et al., 1980).

There was only a small effect of O₂ on the rate of CO₂ assimilation at low-CO₂ concentrations in either the mutant or wild-type \( A. \text{edulis} \) (Fig. 9). The limited effect of O₂ on CO₂ assimilation in the mutant, where there is no C₄ cycle function, can be explained by effective re-assimilation in the BS cells of the CO₂ that is generated from dark-type respiration and photorespiration. It is also evident that in the wild-type plants, CO₂ re-assimilation at low CO₂ affects assimilation kinetics. In this case, refixation of photorespired CO₂ may occur in mesophyll cells via PEPC, if there is leakage of CO₂, as well as in BS cells. However, the effectiveness of the mutant suggests that, under low CO₂, much of the photorespired CO₂ will be directly refixed in BS cells.

**Calculation of CO₂ Concentration and Leakiness of CO₂ in BS Cells of \( A. \text{edulis} \)**

If Rubisco kinetic parameters (\( V_c \), \( K_c \) [the \( K_m \) for RuBP carboxylase], \( K_o \), and \( S \) [the relative specificity factor for carboxylase versus oxygenase]) are known, one can calculate BS CO₂ concentration using a C₄ photosynthesis model (von Caemmerer, 2000). Figure 12 shows calculated BS cell CO₂ concentrations during photosynthesis in wild-type \( A. \text{edulis} \) leaves under varying intercellular CO₂ concentrations at 210 mbar of O₂ calculated with the model of C₄ photosynthesis. Also, shown in Figure 12 are the calculated values of BS cell leakiness during CO₂ fixation in wild-type plants, which range from approximately 0.2 to 0.3 (from Eqs. 16 and 17, using an \( r_{bs} \) value of 113 m² s⁻¹ mol⁻¹). As increasing ambient levels of CO₂ became saturating for photosynthesis, the calculated levels of CO₂ in the BS cells of mature leaves reached levels of approximately 2,000 \( \mu \text{bar} \), which is about six times that normally occurring in the atmosphere.

**CONCLUDING REMARKS**

The PEPC mutant of \( A. \text{edulis} \) has high light- and CO₂-saturated rates of photosynthesis comparable with those of the wild type. The high CO₂ required for growth and the high-diffusive resistance of the PEPC mutant to CO₂ indicate that physical restraints to gases have been conserved in the C₄ anatomy of the mutant. The value of BS cell resistance to CO₂ increased with leaf age in \( A. \text{edulis} \) from 72 to 181 m² s⁻¹ mol⁻¹ (Table I). Similar resistance values for mature leaves were obtained with wild-type plants treated with the PEPC inhibitor DCDP. The results suggest there may be developmental changes in BS cells that increase the diffusive resistance to CO₂ by changes in the liquid phase of the cell or cell wall properties. The concentration of CO₂ in BS cells under high rates of photosynthesis is estimated to be about six times current ambient levels, sufficient to largely repress photorespiration. Re-assimilation of CO₂ from dark-type respiration and photorespiration in BS cells is an important component of both PSII activity and consumption of reductive power at very low-CO₂ concentrations in \( A. \text{edulis} \).
The yield of PSII was measured by chlorophyll fluorescence using a fluorometer (PAM 101, Walz, Effeltrich, Germany). The gross rate of O$_2$ evolution from PSII ($V_{II}$) was found by analysis of several experiments on A/C$_i$ responses.

Gas Exchange (A and $I_{O_2-net}$)

Leaf gas exchange was measured with the FastEst gas system (FastEst, Tartu, Estonia; described in detail in Laik and Oja, 1998). The system was equipped with a CO$_2$ analyzer (6251, LI-COR, Lincoln, NE) and a S-3A O$_2$ ceramic heated zirconium oxide analyzer (Applied Electrochemistry Inc., Sunnyvale, CA). Leaf gas exchange characteristics, net rates of CO$_2$ fixation (A), C$_i$ PFD, and leaf temperature were determined as in Laik and Loreto (1996). For measurements of A under high levels of CO$_2$, there is an increase in noise to signal ratio in measuring CO$_2$ with an infrared gas analyzer. To improve measurements of A, sampling time was 0.1 s for 3 min resulting in 1,800 data points, which were averaged. For example, in one experiment A measured at 3% (v/v) CO$_2$ was 41.9 mmol m$^{-2}$ s$^{-1}$ with a SE with n = 1,800 of ± 0.12. The S-3A O$_2$ analyzer provides an accurate measure of the net rate of O$_2$ evolution ($I_{O_2-net}$) under low levels of atmospheric O$_2$ (less than 10 mbar), independent of CO$_2$ concentration.

Measurement of Chlorophyll Fluorescence and Calculation of PSII Activity ($I_{O2}$)

The yield of PSII was measured by chlorophyll fluorescence using a fluorometer (PAM 101, Walz, Effeltrich, Germany). The gross rate of O$_2$ evolution from PSII ($I_{O2}$) was calculated as:

$$I_{O2} = APFD \times Y_{II} \times (F_m - F_n) / 4$$

where ($F_m - F_n$) is the yield of PSII (e$^-$ quanta absorbed), $F_m$ is fluorescence yield of steady-state photosynthesis, $F_n$ is maximal fluorescence yield by exposure to a 1-s pulse of 15,000 mmol m$^{-2}$ s$^{-1}$ light and APFD is the absorbed photosynthetic quantum flux density at steady state (Genty et al., 1989). For estimating the relative optical cross section of PSII ($Y_{II}$), the method proposed by Laik and Loreto (1996) was used. $Y_{II}$ was found by extrapolating a plot of $F_m / F_n$ versus quantum yield of O$_2$ evolution measured with an O$_2$ electrode ($I_{O2-net}$) at different light intensities, to $F_m / F_n = 0$. The measurements were made under a low-O$_2$ background (0.025%) and high CO$_2$ so that respiratory uptake of O$_2$ would be minimized and the O$_2$ evolution measured would reflect essentially all PSII activity. The calculated values of $Y_{II}$ for wild-type and mutant plants were 0.44 to 0.55 and 0.41 to 0.48, respectively. For calculations of APFD, the light reflected and transmitted by the leaf was measured using an integrating sphere (Labsphere, North Sutton, NH). For the mutant leaf, the average fractional absorption of incident light was 0.82, whereas for the wild type, the value was 0.85.

In mature leaves of mutant plants, the chlorophyll (a+b) content was 156 mg m$^{-2}$ compared with 309 mg m$^{-2}$ in the ambient CO$_2$-grown wild type. The fresh weights of the mutant and wild-type leaves were identical, 22 mg cm$^{-2}$, but the mutant had less dry weight per leaf area, 2.9 mg cm$^{-2}$, compared with 4.4 mg cm$^{-2}$ in the wild type (leaves were sampled at midday).

Equations and Calculation of Leaf Photosynthesis Parameters

O$_2$ Evolution in Mutant and Wild Type

O$_2$ evolution associated with linear electron transport rate can be expressed as

$$I_{O2} = \nu_i + \nu_o + J_1$$

where $\nu_i$ and $\nu_o$ are RuBP carboxylation and oxygenation rates, respectively, and $J_1$ is the use of electrons in other processes (e.g. Mehler reaction and nitrogen reduction). The following equations illustrate the main factors in considering the relationship between $I_{O2}, I_{O2-net}$, and $A$ in C$_3$ and C$_4$ plants, because there is no net consumption of reductive power in the C$_4$ cycle of malic enzyme-type species (see Edwards and Baker, 1993).

$$A = \nu_i - 0.5\nu_o - R_d$$

$$J_{O2} = \nu_i + \nu_o + I_{O2-net} + I_{O2-net}$$

$$I_{O2-net} = A + I_{O2-net}$$
Analysis of Parameters of CO₂ Fixation and rₚᵣ in PEPC Mutant

We assume that light-saturated CO₂ uptake in the mutant A. edulis plants is limited primarily by physical diffusive resistance to CO₂ and Rubisco activity (see Fig. 1). Values for the diffusive resistance to CO₂ carboxylation resistance, and RuBP carboxylation and oxygenation velocities can be calculated from leaf gas exchange measurements using the biochemical model of Rubisco developed by Farquhar et al. (1980). According to their model for C₄ photosynthesis, the CO₂ assimilation rate in the mutant can be described according to equation 3 above, where \( A = v_c - 0.5 v_o - R_w \). Assuming Rubisco saturation by RuBP,

\[
v_c = \frac{V_c \cdot C_c}{C_c + K_c(1 + O_2/K_c)}
\]

(7)

\[
v_o = \frac{V_o \cdot O_2}{O_2 + K_o(1 + C_o/K_o)}
\]

(8)

\[
V_o = \frac{V_c \cdot K_o}{K_o - S}
\]

(9)

where \( v_c \) and \( v_o \) are maximum carboxylation and oxygenation velocities, respectively, \( K_c \) and \( K_o \) are carboxylation and oxygenation Michaelis constants, respectively, \( C_o \) and \( O_2 \) are CO₂ and O₂ concentrations at Rubisco active sites, respectively, and \( S \) is Rubisco specificity for CO₂ relative to O₂. Total resistance to CO₂ flux in the mutant, \( r_c \), can be described as the sum of three resistances

\[
r_c = r_g + r_m + r_o
\]

(10)

where \( r_g \) is gas phase resistance (boundary layer and stomatal), \( r_m \) is liquid phase resistance (effectively BS resistance), and \( r_o \) describes carboxylation resistance of Rubisco. Gas phase resistance, \( r_g \), was calculated from transpiration data. Intercellular CO₂ partial pressure, \( P_c \), equals

\[
C_c = C_o - r_g A
\]

(11)

where \( C_o \) is ambient CO₂ and \( A \) is net CO₂ assimilation rate. Intercellular CO₂ partitions between the gas phase and the cell wall liquid phase, \( C_c \), is

\[
C_c = \beta_c C_i
\]

(12)

giving soluble CO₂ where \( \beta_c \) is the CO₂ solubilization coefficient. The CO₂ concentration at the sites of carboxylation in the mutant, \( C_i \), is

\[
C_i = C_o - r_o A
\]

(13)

O₂ concentration in the BS chloroplasts can be described as

\[
O_2 = O_2 + \alpha_o \cdot A
\]

(14)

where O₂ concentration at the mesophyll cell wall is

\[
O_2 = \beta_o O_2
\]

(15)

\( \alpha_o \) is a constant that takes into account the difference in O₂ and CO₂ diffusivities (at 25°C \( \alpha_o = 0.79 \); Farquhar, 1983), \( h \) is the relative proportion of O₂ evolution in BS cells, \( O_2 \) is the intercellular concentration of O₂, and \( \beta_c \) is the O₂ partitioning factor between gaseous and liquid phase.

DCDP Feeding Experiments

A leaf petiole was cut under water, and the CO₂ response curve was measured at 2% (v/v) oxygen (Fig. 3, A-C). Water was then replaced by a 4 mM solution of DCDP (PEPC inhibitor). After DCDP caused a decrease of photosynthesis to a stable level under atmospheric levels of CO₂ (about 20 min), the CO₂ response was measured (Fig. 3, B, D, and F). Measurement of photosynthesis on excised leaves can be problematic; however, using the FastEst gas exchange system the \( A/C \) response curves could be run in 20 min. The maximum rates of CO₂ fixation and response curves of control plants were similar to intact plants.

C₄ Photosynthesis and Bundle Sheath Diffusive Resistance to CO₂

Calculating BS Cell CO₂ Concentration and Leakiness in Wild-Type Plants

The mechanistic model of C₄ photosynthesis developed by von Caemmerer (2000) was used for estimating concentrations of CO₂ in BS cells. The model requires inputs for Rubisco kinetic parameters. The kinetic constants for A. edulis Rubisco (\( K_c = 16 \mu M, K_o = 640 \mu M, \text{ and } S = 82 \text{ at } 25^\circ C \) were obtained from the work of Jordan and Ogren (1983) and corrected for temperature according to Woodrow and Berry (1988). \( V_o \) was taken equal to the CO₂-saturated assimilation rate at a PFD of 1,800 \( \mu mol \text{ m}^{-2} \text{ s}^{-1} \).

Leakiness of CO₂ from BS cells during photosynthesis in wild-type plants was calculated as a fraction of the rate of the C₄ cycle based on the above estimates of BS cell CO₂ concentration and measured values of \( r_m \) in the A. edulis mutant, where the rate of leakage of CO₂ per unit leaf area (\( L_o \)) equals

\[
L_o = \frac{[CO₂]_{BS} - [CO₂]_{BS}}{r_m}
\]

(16)

with \([CO₂]_{BS}\) and \([CO₂]_{BS}\) representing the level of CO₂ in BS cells versus the external concentration in the intercellular air space, respectively. Leakiness (\( L_o \)), from BS cells as a fraction of the rate of the C₄ cycle is defined as

\[
L = L_o/(L_o + A)
\]

(17)

Measurement of RubBP Content

Leaf metabolism was stopped by quick filling of the leaf chamber with cooled ethanol (approximately ~90°C). The frozen leaf (8 cm²) was then removed from the chamber and ground into a fine powder in a small mortar under liquid nitrogen. The powder was then transferred into 3 mL of 1 M HClO₄ and extracted for 15 min. The extract was neutralized with 5 M KOH and centrifuged 3 min at 5,000g. The supernatant was stored under liquid N₂ until analyzed. RubBP content was determined using the method of ¹⁴C incorporation into acid stable product as in Prinsley et al. (1986) using purified Rubisco from tobacco (Nicotiana tabacum).

Rubisco and Chlorophyll Content

For Rubisco activity measurements, the leaves were sampled the same way as for RubBP determination. The frozen leaf powder was then transferred into CO₂-free 100 mM HEPES/KOH buffer (pH = 7.9). The Rubisco extraction buffer (1 mL per cm² of leaf) contained 20 mM MgCl₂, 2 mM EDTA, and 5 mM dithiothreitol. It was then ground and homogenized in a Broek Tissue Grinder (Corning, Palo Alto, CA) for about 20 s and divided into two parts, the first of which was immediately assayed by injecting 0.1 mL into a 0.9-mL assay medium and running the reaction 30 s at 28°C. This was taken as the in vivo activity of Rubisco. The assay media had the following final composition: 100 mM HEPES/KOH (pH = 7.9), 10 mM MgCl₂, 2 mM EDTA, 5 mM dithiothreitol, 1 mM RuBP, and 10 mM NaHCO₃ + NaH¹⁴CO₃ with specific activity of 0.5 Ci mol⁻¹. The second assay, which was run after incubating the enzyme preparation 15 min in the presence of 10 mM NaHCO₃, was taken as the fully carbamylated activity. It was shown, by adding purified Rubisco with known activities to leaf material before grinding, that there was no loss of activity attributable to extraction procedures. However, the possibility that some loss of activity may have occurred because of incomplete solubilization of Rubisco from the leaf material cannot be ruled out. The chlorophyll content of the leaves was determined according to Porra et al. (1989) using 80% (v/v) acetone extract.

ACKNOWLEDGMENTS

We thank Dr. Colin Jenkins (Commonwealth Scientific and Industrial Research Organization, Canberra, Australia) for providing PEPC inhibitor DCDP. We appreciate discussion and some preliminary work with Dr. João Maroco (Instituto Superior de Agronomia, Lisbon, Portugal) on this project.

Received May 8, 2002; returned for revision June 16, 2002; accepted June 30, 2002.

LITERATURE CITED

CORRECTIONS

Vol. 130: 964–976, 2002

Kiirats O., Lea P.J., Franceschi V.R., and Edwards G.E. Bundle sheath diffusive resistance to CO₂ and effectiveness of C₄ photosyntheses and refixation of photrespired CO₂ in a C₄ 0 cycle mutant and wild-type *Amaranthus edulis*.

The correct Figure 12 for the above article appears below.

![Figure 12](image_url)

Figure 12. CO₂ response for CO₂ assimilation rate at leaf temperature of 28°C; PFD = 1,800 μmol m⁻² s⁻¹ (●), calculated CO₂ partial pressure in BS cells (←), and leakiness of CO₂ from BS cells (△). The CO₂ level in BS cells was calculated according to von Caemmerer (2000), and the leakiness was calculated according to equations 16 and 17 using ᵣₙₐ value of 113 m² s⁻¹ mol⁻¹. Similar results were obtained from analysis of several experiments on A/CO₂ responses.


The labeling of two bar graphs in Figure 5 of the above article is incorrect. The bar graph labeled 50 E’ M SeCys should be labeled 50 E’ M SeO₄²⁻. The graph labeled 50 E’ M SeO₄²⁻ should be labeled 50 E’ M SeCys. The authors apologize for this error.


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