Measurements of the Engagement of Cyanide-Resistant Respiration in the Crassulacean Acid Metabolism Plant *Kalanchoë daigremontiana* with the Use of On-Line Oxygen Isotope Discrimination

Sharon A. Robinson, Dan Yakir, Miquel Ribas-Carbo, Larry Giles, C. Barry Osmond, James N. Siedow, and Joseph A. Berry

Department of Botany, Duke University, Durham, North Carolina 27706 (S.A.R., D.Y., M.R.-C., L.G., C.B.O., J.N.S.); and Carnegie Institution of Washington, Department of Plant Biology, 290 Panama Street, Stanford, California 94305–1297 (J.A.B.)

**ABSTRACT**

Discrimination against $^{18}$O during dark respiration in tissues of *Kalanchoë daigremontiana*, *Medicago sativa*, and *Glycine max* was measured using an on-line system that enabled direct measurements of the oxygen fractionation of samples in a gas-phase leaf disk electrode unit. Discrimination factors for cytochrome pathway respiration were 18.6 to 19.8% for all tissues. However, discrimination in cyanide-resistant respiration was significantly higher in green tissues (30.4–31.2%) compared with nongreen tissues (25.3–25.9%). Using these discrimination factors, the partitioning of electron transport to these pathways was calculated from measurements of discrimination in the absence of inhibitors. Changes in flux through the alternative pathway were measured during the light and dark phases of Crassulacean acid metabolism in leaf disks of *K. daigremontiana*. The flux of electrons through the alternative pathway was higher during decarboxylation than during the other phases of Crassulacean acid metabolism. The increase in alternative pathway electron flux accounted for all of the increased respiration in the light phase. Despite this increase, simultaneous measurements of malate concentration and respiratory flux confirm that only a small proportion of the total malate decarboxylation occurs in the mitochondria.

Mitochondrial electron transport in higher plants can proceed by the phosphorylating cytochrome pathway or by the largely nonphosphorylating alternative pathway (4). The branch point for these two pathways is known to be at the level of ubiquinone, and electron transport through the alternative pathway is nonphosphorylating from this point on (17). The physiological basis for this apparently wasteful process is still unclear, but it has been suggested that it operates as an energy overflow mechanism, oxidizing cellular substrate in excess of that needed for growth, storage, or ATP synthesis (2, 13, 14, 21). Previous studies have established a decrease in the cyanide sensitivity of leaf respiration in plants showing CAM (12), especially during the first daylight hours (20). It has been suggested that engagement of the alternative pathway in CAM mitochondria during the light period would allow malate oxidation to proceed simultaneously with photophosphorylation.

Studies of the engagement of the alternative pathway in intact tissues have been based on titration of $O_2$ uptake with inhibitors that block either the Cyt or alternative pathway. However, this approach is based on the problematic assumption that the inhibitors are specific (16). In 1989, Guy et al. reported a new approach based on the observation that the alternative pathway discriminates against $^{18}$O to a greater extent than the Cyt pathway (7). Measurements using inhibitors during respiration by various plant materials, ranging from single-celled organisms to higher plant mitochondria and mutants lacking either the Cyt or alternative pathways (8, 23), established discrimination values of 17.1 to 20.0% for the Cyt pathway and 23.5 to 25.5% for the alternative pathway. Although inhibitors were needed to establish these initial reference points for discrimination, the technique subsequently allows estimation of the partitioning of electron flow between the two mitochondrial pathways during steady-state respiration in the absence of inhibitors (8).

This method has now been developed further to allow on-line measurements of respiratory $^{18}$O discrimination in the gas phase surrounding intact tissue (D. Yakir and L. Giles, unpublished). Because this system allows relatively fast measurements, the different isotopic signatures can also be used to study the effect of physiological and developmental factors on the flux of electrons through the alternative pathway. In this report, we have used measurements of isotopic discrimi-
Figure 1. Schematic diagram of the gas-phase system for on-line measurements of $^{18}$O discrimination during respiration. For explanation, see text.

Materials and Methods

Plant Materials and Methods

Kalanchoe daigremontiana plants were vegetatively propagated from stocks that had been maintained at Duke University for several years. Experimental plants were grown in the Duke University Phytotron with a 10-h/27°C day with 350 μmol photons m$^{-2}$ s$^{-1}$ of PAR, and a 14-h/17°C night. Soybean plants (Glycine max L. Centennial) were grown in temperature-regulated glasshouses (26°C day, 20°C night) at Duke University with a 16-h light period (natural light supplemented with incandescent). Cotyledons were taken from 10- to 14-d-old plants. Plants were grown in a mixture of gravel and vermiculite (2:1, v/v) and were irrigated daily with nutrient solution (half-strength Hoagland solution). Medicago sativa L. seedlings (alfalfa sprouts) were purchased from a local supermarket and kept in the dark for the duration of the experiment.

Leaf discs (10 cm$^2$), whole cotyledons, and alfalfa sprouts (1 g) were weighed and placed directly in the oxygen electrode chamber between pieces of gauze and dampened medical wipes. Soybean roots were washed to remove soil and weighed before being placed in the chamber. During inhibitor treatments, KCN was applied by sandwiching tissues between medical wipes saturated with 1 mM KCN, and tissues were soaked in 2 to 10 mM SHAM solution. Respiratory rates were determined at 25°C except in the case of dark phase measurements of K. daigremontiana, which were carried out at 17°C.

The concentration of malic acid in K. daigremontiana was estimated from titratable acidity of discs extracted after determination of discrimination values. Discs were weighed and killed by boiling in 10 mL of 80% ethanol for 5 min, 50 mL of 20% ethanol was then added, and the samples were boiled for a further 20 min to reduce the volume. Cooled extracts were titrated to pH 7.0 with 10 mM KOH (CO$_2$ free).

On-Line Oxygen Uptake and Fractionation

Oxygen consumption and fractionation were determined by on-line measurements of gaseous samples withdrawn from a leaf disc electrode unit (LDI Hansatech Instruments Ltd., Kings Lynn, Norfolk, UK), shown in Figure 1. Tissue samples (leaf discs, cotyledons, roots, and whole seedlings) were placed on a moist pad in the chamber in the dark and allowed to equilibrate with the inlet vent open. The inlet vent was then closed and the tissue was allowed to respire. The respiratory rate (oxygen uptake) was monitored on a chart recorder, which allowed us to take samples at appropriate times and ensure that sufficient oxygen remained in the chamber.

Samples were withdrawn at regular time intervals (10–20 min depending on respiratory rate) into a pre-evacuated volume (100 μL) through a capillary. After a 30-s delay to ensure that the sample volume had been filled, the capillary was closed and the helium flow was switched to the sample loop using a four-way valve. Carbon dioxide and water vapor were removed from the He stream and the O$_2$, Ar, and N$_2$ gases were separated by GC (NA 1500 Carlo Erba Instrumentazione, Milan, Italy) using a 915 × 6 mm diameter molecular sieve MS 5-Å (80–100 mesh) column heated to 50°C at a flow rate of 30 mL min$^{-1}$ of He carrier gas. The components were detected using a thermal conductivity detector and integrated using a Hewlett Packard (Model 3392A) integrator; retention times were 3.84 min for Ar and O$_2$ and 6.10 min for N$_2$. The fraction (f) of unreacted oxygen in the sample was determined from the integrated areas of the peaks, according to the formula:

$$f = \frac{[O_2 + Ar]}{[N_2]} \times 0.01196$$

where $[O_2 + Ar]$ is the integrated area of the combined O$_2$ and Ar peak, [N$_2$] is the integrated area of the N$_2$ peak, and 0.01196 is the proportion of Ar/N$_2$ in air. The estimate of $f$ is independent of the sample volume.
The isotope ratio $^{18}$O/$^{16}$O was determined directly from the ratio of masses 32 and 34 using a SIRA Series II isotope ratio mass spectrometer (VG ISOGAS, Middlewich, UK) operated in continuous flow mode. The reproducibility of measuring the fraction and isotope ratio of oxygen in air introduced through the empty chamber was better than ±0.2% and ±0.3%, respectively.

Calculations of Oxygen Isotope Discrimination

Oxygen discrimination was calculated as described by Guy et al. (7). Discrimination was calculated as the slope of a linear regression through the origin of $(\ln R/\ln R_o) \times 1000$ versus $-\ln f$, where $R_o$ and $R$ are the isotope ratios of the reference sample and a subsequent sample, respectively, and $f$ is the fraction of $O_2$ remaining in the chamber (see ref. 8 for details). Discrimination for each tissue was calculated from at least six samples.

The partitioning of electrons to the alternative pathway in the absence of inhibitors ($A$) was calculated as outlined by Guy et al. (7):

$$A = \frac{(D_a - D_c)}{(D_a - D_o)} \times 100$$

where $D_a$ is the net uninhibited discrimination on a linear scale between $D_o$ (discrimination by the alternative oxidase) and $D_c$ (discrimination by the Cyt oxidase). The flux of electrons through the alternative pathway was calculated by multiplying the total respiratory rate in the absence of inhibitors by the partitioning factor, $A$.

RESULTS AND DISCUSSION

Oxygen Isotope Discrimination during Respiration of Green and Nongreen Tissues

The on-line gas phase method has advantages over the previous method (7, 8), which involved conversion of $O_2$ to $CO_2$ before measurement. It is considerably faster, allowing an experimental determination of discrimination values in approximately 1 h. It has fewer preparative steps and, therefore, less chance of contamination or error. It has a much smaller volume requirement (5 mL rather than 150–400 mL), and using the gas phase overcomes some of the diffusional limitations encountered in previous studies. The method is also useful with bulky tissues, such as leaves of CAM plants, because it minimizes wounding during tissue slice preparation. Wounding stimulates alternative pathway oxidation in CAM plants (12).

To compare previous measurements in the aqueous phase (7, 8) with the gas phase measurements described here, a similar experiment was performed using whole seedlings of M. sativa. Discrimination values in the presence of KCN and SHAM and in the absence of inhibitors are shown in Figure 2. These results are in the range described previously (7). The control values are almost identical to those obtained for SHAM-treated tissues and indicate that electron flow is almost exclusively through the Cyt pathway.

Discrimination values were also obtained for leaf discs of soybean and Kalanchoë and for soybean cotyledons and roots using the gas-phase method. Table I shows the discrimination values obtained for these tissues. If electron transport through the alternative pathway was blocked by SHAM, discrimination values for all tissues were in the range of 18.6 to 19.8%, very similar values to those reported by Guy et al. (7). However, when electron transport through the Cyt pathway was blocked by KCN, the discrimination values did not always conform to those previously described. The values were similar (25.3–25.9%) for nongreen tissues, but discrimination values obtained for green tissues were significantly higher (30.4–31.2%). Figure 3 shows the values obtained for leaf discs of K. daigremontiana in the presence and absence of KCN and SHAM. Discrimination by the alternative pathway is 5.3% higher than that for the nongreen alfalfa sprouts. Indeed, in Kalanchoë, the $D$ values in the absence of inhibitors were frequently higher than those obtained with KCN treatments in nongreen tissues. All incubations were conducted in total darkness to avoid complications from photosynthetic $O_2$ production. This result does not, therefore, appear to be an artifact arising from inhibitor treatments of green tissues, and it would appear that the discrimination of the alternative oxidase in green tissues is greater than that in nongreen tissues of higher plants.

The possibility that chlororespiration was responsible for the higher $D$ value in green tissues has been considered; however, this could not be the case if chlororespiration is cyanide sensitive. Reports in the literature (3, 6) suggest that there may be both cyanide- and SHAM-sensitive chlororespiration. Measurements of respiration in mitochondria from leaves and nongreen tissues are needed to resolve this question.
Table I. Oxygen Isotope Discrimination during Respiration of Plant Tissues in the Gas Phase

<table>
<thead>
<tr>
<th>Plant Material</th>
<th>+SHAM</th>
<th>Control</th>
<th>+CN*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kalanchoe leaf discs</td>
<td>19.8</td>
<td>21.1–27.1</td>
<td>31.2</td>
</tr>
<tr>
<td>Soybean cotyledons</td>
<td>-</td>
<td>25.2</td>
<td>31.2</td>
</tr>
<tr>
<td>Soybean leaf discs</td>
<td>-</td>
<td>24.7</td>
<td>30.4</td>
</tr>
<tr>
<td>Nongreen tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medicago sativa seedlings</td>
<td>19.7</td>
<td>19.6</td>
<td>25.9</td>
</tr>
<tr>
<td>Soybean roots</td>
<td>18.6</td>
<td>21.7</td>
<td>25.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acidification</th>
<th>Deacidification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration (µmol O₂ uptake m⁻² s⁻¹)</td>
<td>1.81 ± 0.69</td>
<td>2.56 ± 0.40</td>
</tr>
<tr>
<td>Discrimination (%)</td>
<td>22.38 ± 1.24</td>
<td>25.00 ± 0.99</td>
</tr>
<tr>
<td>Cytochrome pathway flux (µmol O₂ uptake m⁻² s⁻¹)</td>
<td>1.33 ± 0.63</td>
<td>1.40 ± 0.37</td>
</tr>
<tr>
<td>Alternative pathway flux (µmol O₂ uptake m⁻² s⁻¹)</td>
<td>0.52 ± 0.11</td>
<td>1.16 ± 0.24</td>
</tr>
</tbody>
</table>

These results suggest that the alternative oxidase of green tissues differs from that found in nongreen tissues. Differences in the apparent mol wt of isozymes of the alternative oxidase of these tissues have been noted (11, 18). Further investigation of the link between higher discrimination values for the alternative oxidase and the development and greening of tissues is obviously required.

Measurement of the Changes in Discrimination during the Light and Dark Phases of CAM Metabolism

Having ascertained the oxygen isotope discrimination of the alternative and Cyt pathways in leaf discs of *K. daigremontiana* with the use of inhibitors, we were able to investigate changes in the flux through the alternative pathway at various phases in the CAM cycle.

With the use of on-line measurements of oxygen discrimination, the flux through the alternative pathway during dark respiration was measured at regular intervals throughout the light and dark phases. Table II shows the total respiratory rate, D factors, and partitioning of the respiratory pathways in *Kalanchoe* leaf discs during acidification and deacidification. Discrimination values were higher during deacidification and there was a small increase in total respiratory flux compared with that seen in the dark. The variation in the flux of electrons through the alternative pathway over a 24-h period is shown in Figure 4A. The flux was determined from the respiration rate and the discrimination value and, therefore, gives more information about the actual contribution of the alternative pathway at any particular time. Throughout the 14-h dark period, the flux remained relatively constant at around 0.5 µmol O₂ m⁻² s⁻¹, but the flux increased during the light period, reaching a peak of almost 1.6 µmol O₂ m⁻² s⁻¹ after 4 h in the light. Almost all of the increased respiration during deacidification (Table II) seems to have involved the alternative pathway.

![Figure 3](image-url)  
Figure 3. Discrimination against ¹⁸O during dark respiration by *K. daigremontiana* leaf discs in the absence of inhibitors, with 10 mM SHAM, or with 1 mM KCN. Discrimination is calculated as described in Figure 2. Discrimination factors (D) are shown; correlation coefficients for inhibitor treatments were 0.982 for SHAM, 0.996 for KCN, and 0.997 for the control.

![Figure 4](image-url)  
Figure 4. A, Changes in the flux through the alternative pathway in *K. daigremontiana* leaf discs during light and dark. Data are pooled from two separate experiments. B, Changes in malate concentration of *K. daigremontiana* leaf discs. The solid bar at the bottom of the figure represents the dark period.
Measurements of malate concentration (Fig. 4B) show that the peak in alternative oxidase activity corresponded to the period of rapid malate oxidation. By the end of the light period, most of the malate had been decarboxylated, and engagement of the alternative pathway fell to the levels seen in the dark period. Our results confirm the observations of Rustin and Queiroz-Claret (20), who showed that there was an increase in cyanide-resistant leaf respiration in the first hours of the light phase in K. blossfeldiana. The results on variation in the engagement of the alternative pathway presented here can be used to support suggestions that one of the functions of the alternative pathway is to permit high fluxes in tricarboxylic acid cycle metabolism without the restraints imposed by adenylate charge (12). This is likely to be especially important during deacidification in the light in malic enzyme-CAM plants, when an unspecified proportion of the vacuolar malate may be metabolized to CO₂ via the tricarboxylic acid cycle (5, 15). The CO₂ derived in this way, when mitochondrial oxidative phosphorylation is bypassed, would not alter the energy budget of the cell, and may account for the observation that quantum yields during deacidification are similar to those during C₃ photosynthesis in these CAM plants following deacidification (1).

It is worth considering whether the enhancement of alternative oxidase activity is a significant or crucial factor in facilitating deacidification. Rates of deacidification during the period of maximum alternative oxidase activity were 20 μeq g⁻¹ fresh weight h⁻¹ or 5.5 μmol m⁻² s⁻¹. These were presumably achieved by the concerted action of cytosolic NADP-malic enzyme and mitochondrial NAD-malic enzyme. The maximum rate of respiration, measured in the dark at intervals throughout deacidification, was 3.2 μmol m⁻² s⁻¹. Assuming that the rate of respiration was not stimulated by light, that malate was the principal respiratory substrate, and that it was completely oxidized to CO₂ in the mitochondria with an O₂/CO₂ stoichiometry of 1.0, at most only 0.8 μmol active malate m⁻² s⁻¹, or 14.5% of the total malate oxidation during deacidification, occurred in mitochondria. However, if malate oxidation only proceeded as far as pyruvate and CO₂ (8), then mitochondrial respiration could be responsible for up to 58% of total malate deacidification, of which half could be attributed to alternative oxidase respiration.

Earlier evidence that fumarase randomization and futile cycling of malate do not occur during deacidification (10, 19) favors the view that only a small proportion of malate is oxidized in the mitochondria. Although previous (³¹⁸O/¹⁸O) exchange measurements (22) established high rates of O₂ uptake in the light in CAM plants (about 50% of O₂ evolution), these appear to be mediated by the Mehler reaction rather than by mitochondrial metabolism. The feasibility of evaluating (¹⁸O) fractionation in the light during deacidification is being explored to resolve these questions.

LITERATURE CITED