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Electron Partitioning between the Cytochrome and Alternative Pathways in Plant Mitochondria[1]

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The contribution of the cyanide-resistant, alternative pathway to plant mitochondrial electron transport has been studied using a modified aqueous phase on-line mass spectrometry-gas chromatography system. This technique permits direct measurement of the partitioning of electrons between the cytochrome and alternative pathways in the absence of added inhibitors. We demonstrate that in mitochondria isolated from soybean (*Glycine max* L. cv Ransom) cotyledons, the alternative pathway contributes significantly to oxygen uptake under state 4 conditions, when succinate is used as a substrate. However, when NADH is the substrate, addition of pyruvate, an allosteric activator of the alternative pathway, is required to achieve the same level of alternative pathway activity. Under state 3 conditions, when the reduction state of the ubiquinone pool is low, the addition of pyruvate allows the alternative pathway to compete with the cytochrome pathway for electrons from the ubiquinone pool when the cytochrome pathway is not saturated. These results provide direct experimental verification of the kinetics consequences of pyruvate addition on the partitioning of electron flow between the two respiratory pathways. This distribution of electrons between the two unsaturated pathways could not be measured using conventional oxygen electrode methods and illustrates a clear advantage of the mass spectrometry technique. These results have significant ramifications for studies of plant respiration using the oxygen electrode, particularly those studies involving intact tissues.

One of the distinguishing characteristics of respiration in plants is the presence of a cyanide-resistant pathway, alternative to the standard Cyt pathway (Moore and Siedow, 1991). Both pathways use electrons from the ubiquinone pool to reduce O_2 to water. Unlike the Cyt pathway, the alternative pathway does not couple electron transport to the generation of a proton motive force for the synthesis of ATP (Moore et al., 1978). In early studies of alternative pathway regulation, it was proposed that, although both pathways derived electrons from the same pool of reduced ubiquinone, electron flow to the Cyt pathway would be kinetically favored. This was because the redox potential of the primary oxidant on the Cyt pathway was higher than that of the ubiquinone redox couple, whereas the primary oxidant on the alternative pathway had a redox potential lower than that of the ubiquinone (Bahr and Bonner, 1973). This led to the paradigm that showed that the alternative pathway acts as an "energy overflow" capacity and it only becomes engaged when electron flow through the Cyt pathway is essentially saturated (Lambers, 1980). The paradigm also led to the development of methods for assessing engagement of the alternative pathway based on the effect of specific electron transport inhibitors on the observed rate of respiration (Møller et al., 1988).

In recent years, progress has been made in understanding the alternative pathway in several different areas. Characterization of the structure of the alternative oxidase has advanced because of the appearance of an antibody against the oxidase protein (Elthon and McIntosh, 1987; Elthon et al., 1989) and the subsequent isolation and sequencing of cDNAs associated with this protein (Rhoads and McIntosh, 1991; Sakajo et al., 1993; Vanlerberghe and McIntosh, 1994). In addition, Moore et al. (1988) introduced a voltametric technique that allows simultaneous measurements of the reduction state of the ubiquinone pool and the rate of O_2 uptake by isolated mitochondria. This method has facilitated kinetics studies of electron flow through the alternative pathway, providing a more mechanism-based understanding of the relationship between the reduction

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Abbreviations: Q_r/Q_t, fractional reduction of ubiquinone pool; SHAM, salicylhydroxamic acid; τ_a, the fraction of total electron flow partitioning to the alternative pathway; τ_c, the fraction of total electron flow partitioning to the Cyt pathway.

state of the ubiquinone pool and alternative pathway activity (Dry et al., 1989; Siedow and Moore, 1993; James et al., 1994; Ribas-Carbo et al., 1994). Initial studies with the voltametric technique demonstrated that the alternative pathway in isolated mitochondria was not significantly engaged until the ubiquinone pool became at least 40% reduced (Dry et al., 1989; Day et al., 1991).

An important consequence of the energy overflow concept of the operation of the alternative pathway was the use of inhibitors of the alternative pathway such as SHAM to ascertain the activity of the pathway in the absence of inhibitors (Møller et al., 1988), the rationale being that electron flow could not be diverted from the alternative pathway to the already saturated Cyt pathway following addition of the inhibitor. More recently, however, Millar et al. (1993) found that pyruvate can activate the alternative pathway, and this activation by pyruvate was subsequently demonstrated to be the result of an apparent decrease in the K_m of the alternative pathway for reduced ubiquinone (Umbach et al., 1994). This raised the possibility that the alternative pathway could compete directly with the Cyt pathway for electrons from the ubiquinone pool under some metabolic circumstances. Recently, Hoefnagel et al. (1995) compared O_2 and ferricyanide reduction rates in conjunction with inhibitors to demonstrate that such partitioning can take place. In this paper, we report studies using MS to examine this possibility without having to resort to the use of inhibitors.

Guy et al. (1987, 1989) first used MS to show that the Cyt and alternative oxidases discriminate differently against the isotope ^{18}O, with the alternative oxidase showing a fractionation of 25 to 27‰ and the Cyt pathway showing a fractionation of 19 to 20‰ (Guy et al., 1989). The observation that electron flow through the two pathways could be distinguished on the basis of ^{18}O fractionation was significant because it held the promise of allowing direct measurements of electron partitioning between the two pathways without having to add inhibitors of either pathway (Guy et al., 1989). The first measurements were performed using an aqueous phase system that allowed experiments using isolated mitochondria, intact cells, submitochondrial particles, whole seedlings, and unicellular algae (Guy et al., 1989; Weger et al., 1990a, 1990b). However, this system had two major limitations for routine studies: (a) it required the conversion of O_2 to CO_2, which made the technique very cumbersome and time consuming, and (b) any problems with O_2 diffusion between the experimental tissue and the aqueous phase could result in reduced respiratory-linked fractionation (Guy et al., 1989, 1992). In previous work, we reported the development of an on-line gas-phase system that alleviated both of these limitations (Robinson et al., 1992). This system used a closed chamber from which O_2 samples were fed into a gas chromatograph to separate O_2 and N_2 before introducing the sample to a mass spectrometer that measured the $^{34}O_2/^{32}O_2$ ratio directly (Robinson et al., 1992). This modified system facilitated experiments using intact tissues but could not be used with aqueous phase samples, such as isolated mitochondria, cultured cells, or algae.

In this paper we report the development of an on-line aqueous phase, tandem gas chromatograph-mass spectrometer system that allows measurements of O_2 fractionation with samples such as isolated mitochondria or unicellular algae without the drawbacks inherent in the earlier design, and we present studies using this new system that examine the regulation of the partitioning of electron flow between the two pathways in isolated soybean (*Glycine max* L.) cotyledon mitochondria. These studies demonstrate that electrons from the ubiquinone pool may be shared between the alternative and Cyt pathways when the latter is not saturated. We discuss the consequences for measurements of electron partitioning using O_2 electrodes in conjunction with electron transfer inhibitors (Møller et al., 1988) and the role of the alternative pathway in plant mitochondria.

MATERIALS AND METHODS

Plant Material

Soybean (*Glycine max* L. cv Ransom) seeds were treated with 0.5% NaOHCl for 10 min and swollen in distilled water for 2 h with continuous bubbling of air.

Seeds were planted in a 1:1 mixture of sand and perlite and grown in a growth chamber in the Duke University Phytotron at 27/23°C on a 14-h/10-h (light/dark) regime at 1000 μmol photons m^{-2} s^{-1}. Plants were watered twice a day and cotyledons were harvested between 6 and 7 d after planting.

Mitochondrial Isolation

Mitochondria were isolated from soybean cotyledons and purified on Percoll gradients as described by Day et al. (1985) with modifications (Umbach and Siedow, 1993). Protein was estimated by the method of Lowry et al. (1951).

O_2 Uptake Measurements

O_2 uptake by isolated mitochondria (0.1–0.5 mg of protein) was measured polarographically in a reaction medium containing 0.3 M Suc, 5 mM KH_2PO_4, 10 mM Tes, 10 mM KCl, and 2 mM $MgSO_4$ (pH 7.2) in a 1.7-mL Gilson (Middleton, WI) O_2 electrode chamber thermostated at 25°C. Respiration was initiated with either 5 mM succinate or 2 mM NADH. When succinate was used as a substrate, mitochondria were preincubated with 0.15 mM ATP, and succinate dehydrogenase was further activated by a single state 3/state 4 transition initiated by the addition of 150 μM ADP. When NADH was used as a substrate, O_2 uptake was measured in the absence of ADP without going through a state 3/state 4 transition. Pyruvate (5 mM) was used to activate the alternative oxidase when required (Millar et al., 1993). SHAM (2 mM) was used to inhibit the alternative pathway, and either 2 mM KCN or 2 μM myxothiazol was used to inhibit the Cyt pathway. In the inhibitor titration experiments, sequential additions of stocks of 10 mM SHAM and 10 mM KCN were used.

O_2 Extraction and Isotope Analysis

The modified aqueous phase O_2 fractionation system (Fig. 1) consists of a closed cuvette within which respiration of the sample takes place. Aqueous samples are withdrawn sequentially and purged with He. The resulting gases are then separated in a gas chromatograph, and the $^{18}O/^{16}O$ isotope ratio is obtained using MS. All experiments were carried out at room temperature using the air-saturated reaction buffer utilized for measuring O_2 uptake described above. For measurements in state 3, 0.5 mM ADP was added just before the reaction cuvette was sealed.

The reaction cuvette was made of acrylic plastic. The cuvette (volume 25 mL) is equipped with a top plug that descends as samples are withdrawn during the course of the experiment. Valves (Whitey Co., Highland Heights, OH) were used to transfer a 3-mL sample from the reaction cuvette (Fig. 1, No. 1) into an evacuated sample chamber (Fig. 1, No. 2). The valves were then positioned to flush the sample into the degassing chamber with a stream of He (Fig. 1, No. 3) and bubbled for 2 min to purge all gases from the aqueous sample. The resulting He-carried gas sample was passed through magnesium perchlorate (Fig. 1, No. 6) and ascarite (Fig. 1, No. 7) traps, where water and CO_2, respectively, were removed. The remaining gases (N_2, O_2, and Ar) were adsorbed onto a coarse molecular sieve-5A (20 mesh) trap at liquid N_2 temperature (Fig. 1, No. 8). This trap was then switched into the flow path of carrier gas for the gas chromatograph (NA 1500; Carlo Erba Instrumentazione, Milan, Italy) and gases were released by warming the molecular sieve to 90°C for 1 min and separated using a 915- × 6-mm diameter molecular sieve-5A (80–100 mesh) column heated to 50°C at a flow rate of 30 mL min^{-1} of He

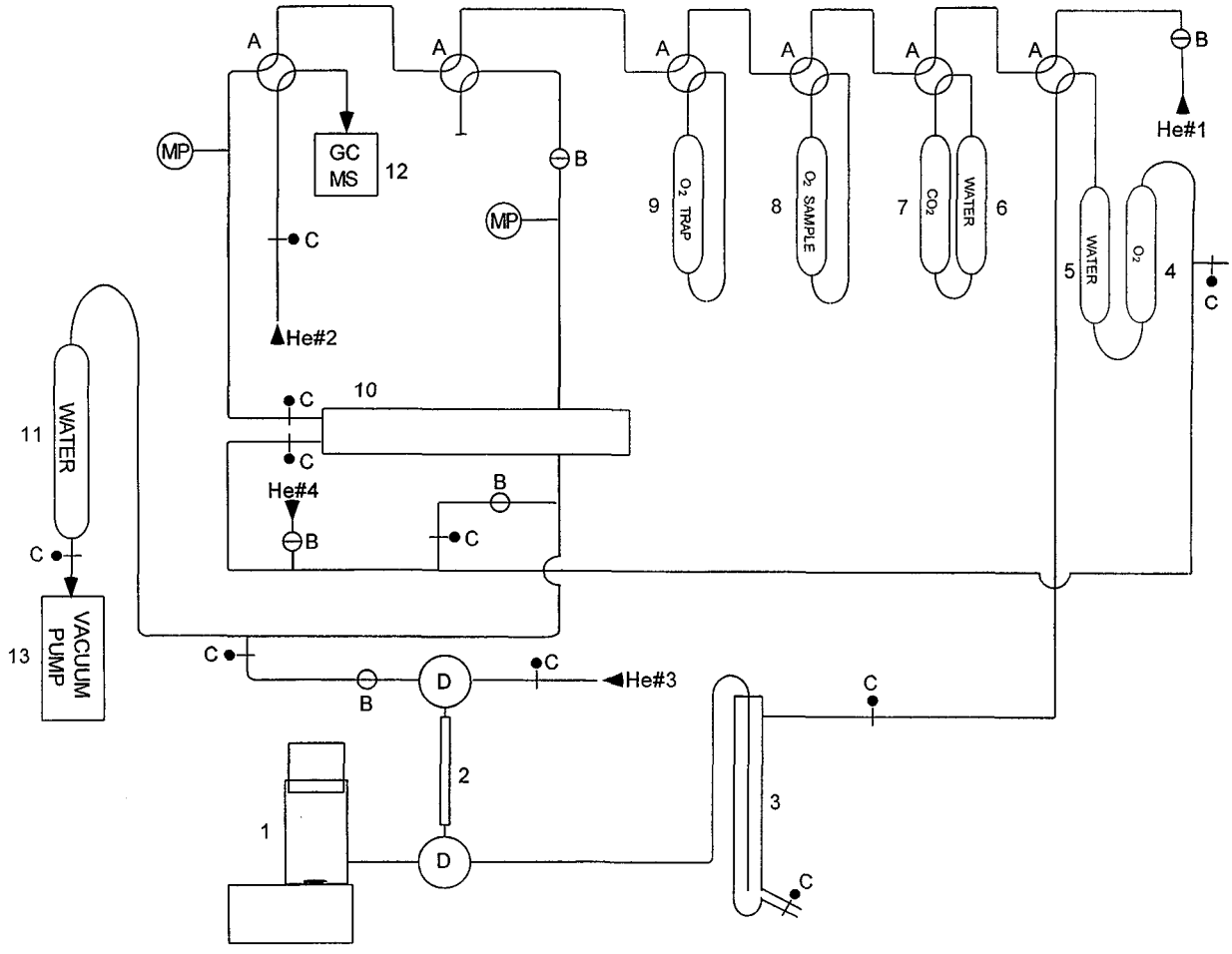

Figure 1. Schematic diagram of the aqueous phase O_2 fractionation system. Labels and symbols represent the following: 1, reaction cuvette with an adjustable plunger and a magnetic stirrer on the bottom; 2, 3-mL sample chamber; 3, degassing chamber where the sample is purged with He to remove gases; 4, O_2 trap held under liquid N_2; 5, liquid N_2 trap for water; 6, water vapor trap (magnesium perchlorate) for the He-carried gas sample; 7, CO_2 trap (ascarite) for the He-carried gas sample; 8, molecular sieve-5A (Fisher Scientific) for trapping of O_2; 9, molecular sieve-5A to trap back-diffused O_2 from either the vacuum line or the system; 10, buffer volume (approximately 1 L) to absorb pressure changes within the whole system; 11, water trap under liquid N_2; 12, gas chromatograph (NA1500; Carlo Erba Instrumentazione) connected in series with a SIRA Series II isotope ratio mass spectrometer (VG ISOGAS); 13, rotary vacuum pump; A, four-way valves; B, on-off valves; C, variable flow valves; D, three-way valves; He#, He sources at regulated flow; MP, manometric pressure.

carrier gas. After the column, the gases passed through a thermal conductivity detector and into a splitter, where approximately 1 mL min^{-1} was introduced directly into a VG-ISOTECH SIRA Series II isotope ratio mass spectrometer (VG ISOGAS, Middlewich, UK) operated in a continuous flow mode. The entire line was kept under positive pressure between 1.0 and 2.3 atm, with several He sources and vacuum exits (Fig. 1). The isotope ratio $^{34}O_2/^{32}O_2$ was determined by integration of the two O_2 peaks, and the O_2/N_2 ratio was determined by integration of the signal from the thermal conductivity detector with a correction for argon contamination.

Glycolate Oxidase

O_2 fractionation during the glycolate oxidase reaction was carried out as described previously by Guy et al. (1993).

Fractionation Calculations

For calculation purposes, O_2 isotope fractionation was first calculated using the fractionation factor (D), which is derived from the slope of a linear regression through the origin of a plot of $(\ln R/R_o) \times 1000$ versus $-\ln f$ (Guy et al., 1989):

$$D(‰) = \frac{\ln R/R_o}{-\ln f} \times 1000$$

where R is the $^{34}O_2/^{32}O_2$ ratio of the sample, R_o is the O_2 isotope ratio of the reference sample, and f is the fraction of O_2 not consumed. The amount of O_2 in the sample was determined from the integrated areas of the peaks off the gas chromatogram using the following equation:

$$[O_2] = \frac{[O_2+Ar]}{[N_2]} - 0.0388$$

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 0.0388 is the ratio of the detector output to the Ar/N_2 in the reaction medium. This assumes that the Ar/N_2 ratio is constant. The concentration of O_2 is normalized to the concentration of N_2 because the latter is not being consumed during respiration. The value of f will be independent of the sample volume (Robinson et al., 1992). For standardization purposes (Guy et al., 1993), we show all fractionation values as D (Farquhar and Richards, 1984). The fractionation factor (D) is converted to D using the following equation (Guy et al., 1989):

$$\Delta(‰) = \frac{D}{1 - (D/1000)} \times 1000$$

The normalization of the data and the calculation of the SE values of the regression were carried out as described by Weger et al. (1990b).

Partitioning Calculation

The calculation of the electron partitioning through the alternative pathway in the absence of inhibitors is obtained as described by Guy et al. (1989).

$$\tau_a = \frac{\Delta_n - \Delta_c}{\Delta_a - \Delta_c}$$

where τ_a is the fraction of the total electron flow going through the alternative pathway, Δ_n is the O_2 fractionation in the absence of inhibitors, Δ_a is the fractionation associated with the alternative pathway, and Δ_c is the fractionation value associated with the Cyt pathway. The latter two values are obtained in separate measurements where electron flow through the Cyt or alternative pathway is completely inhibited by the addition of either 2 mM KCN or 2 mM SHAM, respectively.

The rate of O_2 uptake through each pathway was derived from the values for electron partitioning, as measured by isotope fractionation, multiplied by the absolute rate of O_2 consumption (by GC) over time.

RESULTS

Technical Improvements

The use of an on-line gas chromatograph-mass spectrometer (Robinson et al., 1992) allowed the development of an aqueous phase O_2 fractionation system that is more suit-

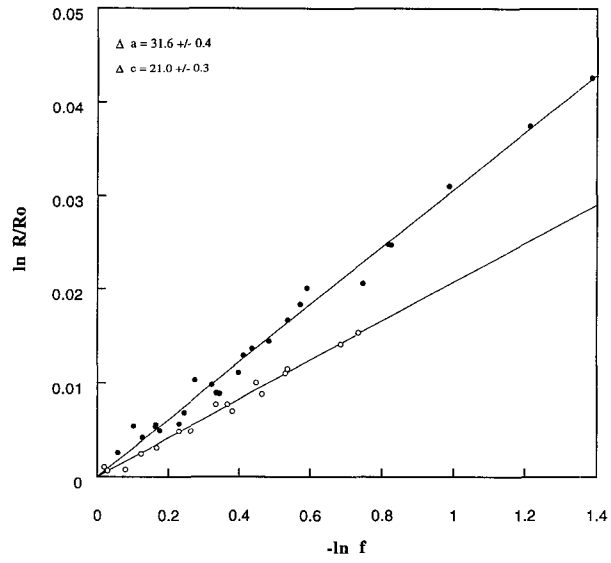

Figure 2. $^{18}O_2$ fractionation during Cyt and alternative pathway electron transport in isolated soybean cotyledon mitochondria. O_2 fractionation by Cyt oxidase (○) was measured in the presence of SHAM (2 mM) under state 3 and state 4 conditions. O_2 fractionation by the alternative oxidase (●) was measured after the addition of either cyanide (2 mM) or myxothiazol (2 μM). In both studies succinate (5 mM) and NADH (2 mM) were used individually as the reducing substrates in the presence or absence of either malonate (3 mM), when succinate was the electron donor, or pyruvate (5 mM). Δ_a and Δ_c are the resulting fractionation values associated with the alternative pathway (n = 25) and the Cyt pathway (n = 16), respectively.

able for biochemical experimentation than the previously described system (Guy et al., 1989, 1992). The key feature of this new system is the ability to inject directly an O_2-containing sample into the GC-MS (Fig. 1), avoiding the rigorous requirement of converting O_2 to CO_2 (Guy et al., 1989, 1992). However, the greatest advantage of the new method is its speed. Samples can be taken and fully processed as frequently as every 6 min, permitting experiments with labile preparations (such as isolated mitochondria) to be conducted under controlled conditions.

To compare the results of the new design with the previous one, we measured O_2 fractionation during the glycolate oxidase reaction. The fractionation (Δ) for glycolate oxidase was found to be 22.9‰ using our system compared with Δ = 22.7‰ reported previously (Guy et al., 1993). Values similar to those reported previously for O_2 fractionation by the Cyt and alternative pathways in *Chlamydomonas reinhardtii* Dangeard (Weger et al., 1990a) were also obtained using our system (P. B. Heifetz and M. Ribas-Carbo, unpublished results), further demonstrating no significant difference between data obtained with the two systems.

O_2 Isotope Fractionation by the Cyt and Alternative Pathways

Measurements of O_2 isotope fractionation by the Cyt and the alternative pathways were determined independently under a wide range of different conditions using mitochondria isolated from soybean cotyledons. Measurements were made using either 5 mM succinate, under both state 3 and state 4 conditions, or 2 mM NADH, in the presence or absence of 0.5 mM ADP, with or without 5 mM pyruvate, and in the presence or absence of 3 mM malonate, an inhibitor of succinate dehydrogenase, when succinate was the substrate. The fractionation due to the Cyt pathway was measured in the presence of 2 mM SHAM to inhibit the alternative pathway, and the fractionation by the alternative pathway was obtained by inhibiting the Cyt pathway with either 2 mM KCN or 2 μM myxothiazol. Figure 2 shows the data used to calculate the fractionation value for each pathway. In the plot, data from many experiments were pooled. The fractionation values were 21.0 and 31.6 for the Cyt and alternative pathways, respectively, and there was no effect of the different experimental conditions on the O_2 fractionation by either pathway. The uniformity of the O_2 isotope fractionation values obtained for each pathway under a wide range of conditions using different mitochondrial preparations confirms the assumption of Guy et al. (1989) that these can be used as standard values for each pathway in subsequent experiments in which no inhibitors are added.

The O_2 isotope fractionation values obtained using soybean cotyledon mitochondria were very similar to those obtained in gas-phase experiments with intact soybean cotyledons, in which values of 19.6‰ (Robinson et al., 1992, 1995) and 32.2‰ (Robinson et al., 1992) were reported for the Cyt and alternative pathways, respectively. However, the fractionation value for the alternative pathway in isolated soybean cotyledon mitochondria did differ from that obtained using mitochondria isolated from *Symplocarpus foetidus* spadices, for which the fractionation for the alternative pathway was only 24.1‰ (Guy et al., 1989). No difference in the fractionation value associated with the Cyt pathway was observed when comparing soybean cotyledon and *S. foetidus* mitochondria (Guy et al., 1989). With intact tissues, we consistently observed that in nongreen tissues the alternative pathway showed fractionation values in the range 24 to 26‰, whereas in green tissues, except *Chlamydomonas* (P. B. Heifetz and M. Ribas-Carbo, unpublished results), the value was in the range 30 to 32‰ (Robinson et al., 1992, 1995; M. Ribas-Carbo, L. Giles, S.A. Robinson, A.M. Lennon, J.N. Siedow and J.A. Berry, unpublished observations). Our studies indicate that the source of this differential fractionation lies at the level of the mitochondrion. The mechanistic origin of the difference in the fractionation values still needs to be established.

Electron Partitioning in the Absence of ADP

The redox state of the ubiquinone pool plays a central role in the regulation of electron partitioning between the Cyt and alternative pathways (Moore and Siedow, 1991; Siedow and Moore, 1993; Ribas-Carbo et al., 1994). It is well established that in the absence of ADP the reduction state of the ubiquinone pool increases (Dry et al., 1989). Until now, it has not been possible to study the dependence of electron flow through the alternative pathway on the redox state of the ubiquinone pool in isolated mitochondria without first inhibiting the Cyt pathway (Dry et al., 1989; Siedow and Moore, 1993; James et al., 1994; Ribas-Carbo et al., 1994; Umbach et al., 1994). It has been established using the aqueous phase system that, in the absence of added pyruvate, electron flow through the alternative pathway does not become significant until the ubiquinone pool reaches 35 to 40% reduction (Dry et al., 1989; Ribas-Carbo et al., 1994). We have been able to ascertain the relative partitioning of electron flow between the Cyt and alternative pathways in the absence of inhibitors of electron transfer.

In isolated soybean cotyledon mitochondria, the partitioning of electron flow in state 4 (−ADP) when succinate was used as a substrate was 0.28 to the alternative pathway and 0.72 to the Cyt pathway (Table I). The fraction of electron flow partitioning to the alternative pathway decreased significantly to 0.15 when the reduction state of the ubiquinone pool decreased following the addition of 3 mM malonate to inhibit succinate dehydrogenase (Table I). Reduction of the ubiquinone pool (Q_r/Q_t) in state 4 approaches values of 0.9 in the absence of malonate and decreases to approximately 0.5 in the presence of 3 mM malonate (Dry et al., 1989; Ribas-Carbo et al., 1994). The observed isotope fractionation supports the hypothesis that the redox state of the ubiquinone pool plays a role in regulating electron partitioning between the two pathways.

When external NADH was used as a substrate the partitioning of electrons between the two pathways differed markedly. The fractionation value in the absence of ADP and pyruvate was 21.9‰, and the partitioning of electrons

Table I. *The partitioning of electrons between the Cyt and alternative pathways in isolated soybean cotyledon mitochondria, in the absence of ADP, measured by O_2 isotope fractionation*

Succinate (5 mM, state 4) and NADH (2 mM, state 2) were added to initiate electron transfer as described in "Materials and Methods." The partitioning was calculated as described in "Materials and Methods" based on the fractionation values for each pathway derived from Figure 2. *n* is the number of O_2 samples taken in every measurement. The rate of O_2 uptake (V_t) and the rates of the Cyt pathway (v_{cyt}) and the alternative pathway (v_{alt}) are expressed as nmol O_2 mg^{-1} protein min^{-1}.

Substrate	Addition	Δ (‰)	*n*	τ_a	V_t	v_{cyt}	v_{alt}
Succinate	None	24.0 ± 0.2	4	0.28	73	53	20
	Malonate (3 mM)	22.6 ± 0.2	3	0.15	13	11	2
	Pyruvate (5 mM)	25.1 ± 0.3	4	0.39	95	58	37
NADH	None	21.9 ± 0.4	3	0.08	88	81	7
	Pyruvate (5 mM)	26.0 ± 0.3	3	0.47	125	66	59

to the alternative pathway was very low (0.08; Table I). These results confirm previous data obtained using the O_2 electrode that indicate that succinate is a more efficient donor to the alternative pathway than is external NADH in mitochondria isolated from tissue other than thermogenic spadices (Hemrika-Wagner et al., 1986).

Effect of Pyruvate on Electron Partitioning to the Alternative Pathway

Pyruvate and other α-keto acids were first described as activators of the alternative pathway (Millar et al., 1993) and were later shown to act by lowering the threshold of Q_r/Q_t needed for engagement of the alternative pathway (Umbach et al., 1994). This effect of α-keto acids in decreasing the apparent K_m of the alternative oxidase for reduced ubiquinone could provide a mechanism, in addition to Q_r/Q_t, for metabolic regulation of respiratory electron transport to the alternative pathway. In an effort to characterize this possibility more fully, we measured the effect of pyruvate on electron partitioning between the Cyt and alternative pathways under state 3 and state 4 conditions. In state 4, using succinate as a substrate, τ_a increased from 0.28 to 0.39 following addition of 5 mM pyruvate (Table I). It is important to notice that, whereas the activity of the alternative pathway increased from 20 to 37 nmol mg^{-1} protein min^{-1}, the activity of the Cyt pathway did not show any significant variation. The effect of pyruvate was more pronounced when NADH was the reducing substrate; the partitioning of electrons to the alternative pathway in the absence of added pyruvate was only 0.08 but increased to 0.47 in its presence (Table I). The actual activity of the alternative pathway increased from 7 to 59 nmol O_2 mg^{-1} protein min^{-1}. Under state 3 conditions with either succinate or NADH as electron donors, where the ubiquinone pool is much more oxidized (Dry et al., 1989), the alternative pathway is almost unable to accept electrons from the ubiquinone pool in the absence of added pyruvate (Table II), consistent with the reported kinetics properties of the alternative pathway in the absence of pyruvate (Dry et al., 1989; Siedow and Moore, 1993; Ribas-Carbo et al., 1994). However, in the presence of pyruvate, 10 to 12% of the total electron flux went through the alternative pathway with the alternative pathway activity ranging between 15 and 21 nmol O_2 mg^{-1} protein min^{-1} (Table II).

The results shown in Table II are significant because they indicate that under state 3 conditions in the presence of pyruvate electrons can partition onto the alternative pathway when the reduction state of the ubiquinone pool is quite low and electron flow through the Cyt pathway is not saturated. These results provide a direct measurement that supports the indirect assays recently used by Hoefnagel et al. (1995) to demonstrate this same phenomenon. The observations in Table II and of Hoefnagel et al. (1995) run counter to the long-standing paradigm that the alternative pathway only becomes active when electron flow to the Cyt pathway is essentially saturated (Bahr and Bonner, 1973; Lambers, 1980).

O_2 Isotope Fractionation versus O_2 Electrode Measurements

A comparison of the rate of O_2 uptake measured in parallel on the same set of isolated soybean cotyledon mitochondria using the standard O_2 electrode assay (Table III) and the mass spectrometer system (Table I) was con-

Table II. *The partitioning of electrons between the Cyt and alternative pathways in isolated soybean cotyledon mitochondria, under state 3 conditions, measured by O_2 isotope fractionation*

Following the addition of succinate (5 mM), 0.5 mM ADP was added to the mitochondrial reaction to ensure that it remained in state 3 throughout the duration of the experiment. Partitioning was calculated as described in "Materials and Methods" based on the fractionation values for each pathway derived from Figure 2, and *n* is the number of O_2 samples taken in every measurement. The rate of oxygen uptake (V_t) and the rates of the Cyt pathway (v_{cyt}) and the alternative pathway (v_{alt}) are expressed as nmol O_2 mg^{-1} protein min^{-1}.

Substrate	Addition	Δ (‰)	*n*	τ_a	V_t	v_{cyt}	v_{alt}
Succinate	None	20.2 ± 0.9	6	0.00	127	127	0
	Pyruvate (5 mM)	22.2 ± 0.4	6	0.12	122	107	15
NADH	None	21.6 ± 0.3	5	0.06	157	148	9
	Pyruvate (5 mM)	22.9 ± 0.2	10	0.10	209	188	21

Table III. *Comparison of the rate of O_2 uptake by isolated soybean cotyledon mitochondria as measured using the O_2 electrode and the O_2 isotope fractionation system*

NADH (2 mM) was used as a substrate in the absence of ADP. Measurements were made simultaneously on the same set of isolated mitochondria using either an O_2 electrode or the O_2 isotope fractionation system (see Table I). *n* is the number of O_2 uptake measurements for each experiment.

Sequential Additions	O_2 Uptake	*n*
	nmol O_2 mg^{-1} protein min^{-1}	
NADH (2 mM)	97 ± 2	5
Pyruvate (5 mM)	120 ± 3	3
SHAM (2 mM)	120 ± 3	3

ducted. Using NADH as a substrate, in the absence of ADP and pyruvate, we measured the O_2 uptake rate with the O_2 electrode (97 nmol O_2 mg^{-1} protein min^{-1}; Table III). In the presence of pyruvate the rate obtained with the O_2 electrode was 120 nmol O_2 mg^{-1} protein min^{-1}. No inhibition was observed following the addition of 2 mM SHAM using the O_2 electrode technique. Taken by itself, this observation would indicate that there should be no electron flow through the alternative pathway. However, measurements with the mass spectrometer system indicate that electron transfer rates were 7 and 81 nmol O_2 mg^{-1} protein min^{-1} in the absence of pyruvate and 59 and 66 nmol O_2 mg^{-1} protein min^{-1} in the presence of pyruvate, to the alternative and Cyt pathway, respectively. The latter observation indicates that the alternative pathway was active in the presence of pyruvate. Electron flow through the Cyt pathway was apparently not saturated, because there was no inhibition of O_2 uptake after addition of SHAM. Electrons going through the alternative pathway must have been redirected to the Cyt pathway following inhibition of the alternative pathway by SHAM. This is an important observation that will have to be taken into account in all future measurements of plant respiration carried out using the response of the O_2 uptake rate to added inhibitors.

Table IV. *Effect of SHAM and KCN on electron partitioning between the Cyt and alternative pathway using the O_2 isotope fractionation system*

These titrations were carried out with the same set of mitochondria used for the O_2 electrode titrations shown in Figure 3. Electron transfer was initiated with 2 mM NADH in the absence of ADP followed by either the addition of 5 mM pyruvate (n = 3) for the SHAM titration or not for the KCN titration. The partitioning was calculated as described in "Materials and Methods" based on the fractionation values derived for each pathway in Figure 2. The rate of O_2 uptake (V_t), the rate of the Cyt pathway (v_{cyt}), and the rate of the alternative pathway (v_{alt}) are expressed as nmol O_2 mg^{-1} protein min^{-1}. SHAM and KCN are measured in μM.

Inhibitor	Δ(‰)	τ_a	V_t	v_{cyt}	v_{alt}
SHAM					
0	24.3 ± 0.6	0.31	120	83	37
100	23.4 ± 0.2	0.23	120	92	28
200	20.9 ± 0.9	0.00	120	120	0
KCN					
0	20.1 ± 0.3	0.00	106	106	0
60	22.4 ± 0.3	0.13	63	55	8
120	30.2 ± 0.8	0.87	55	7	48

KCN and SHAM Titrations

In an effort to test further the ability of the O_2 isotope fractionation technique to accurately measure the partitioning of electrons between the Cyt and alternative pathways, titrations with SHAM and KCN were carried out to observe the switching of electrons from one pathway to the other during the course of the titration (Table IV). Titrations with SHAM in the presence or absence of 2 mM KCN (Fig. 3) and with KCN in the presence or absence of 2 mM SHAM were carried out simultaneously using the O_2 electrode and the O_2 isotope fractionation system with the same set of isolated mitochondria. The apparent K_i for SHAM for inhibition of the alternative pathway was 75 μM (Fig. 3).

When 2 mM NADH was used as a substrate in the presence of 5 mM pyruvate, τ_a decreased from 0.31 in the absence of SHAM to 0.23 with 100 μM SHAM and to an undetectable level in the presence of 200 μM SHAM (Table IV); yet, there was no inhibition of O_2 uptake with addition of SHAM (Fig. 3). With KCN titrations of the Cyt pathway in the absence of pyruvate, τ_a increased from an undetectable level in the absence of KCN to 0.13 in the presence of 60 μM KCN and 0.87 in the presence of 120 μM KCN (Table IV).

DISCUSSION

The O_2 isotope fractionation technique has allowed us to address a series of questions regarding electron partitioning between the Cyt and alternative pathways that could

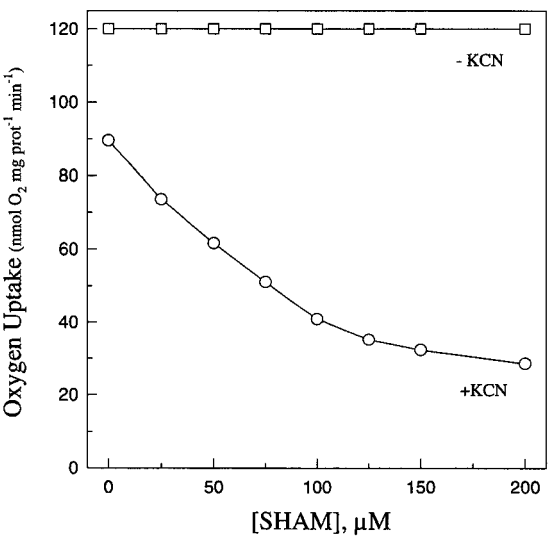

Figure 3. Effect of SHAM on the rate of O_2 uptake in the presence (○) or absence (□) of 1 mM KCN in isolated soybean cotyledon mitochondria. Electron transfer was measured using an O_2 electrode with NADH (2 mM) as a substrate, in the absence of ADP, plus 5 mM pyruvate to obtain maximal engagement of the alternative pathway. These experiments were carried out with the same set of mitochondria used for the SHAM-titration experiments with the mass spectrometer (Table IV).

not be addressed using the O_2 electrode coupled with the application of inhibitors (Møller et al., 1988). Previous studies (Guy et al., 1989, 1992, 1993; Robinson et al., 1992, 1995) indicated that a substantial portion of electron flow took place through the alternative pathway in plant tissues under normal steady-state conditions. This has been difficult to reconcile with the "overflow hypothesis." However, the theoretical underpinnings of the regulation of electron partitioning between the Cyt and alternative pathways have undergone a remarkable reevaluation following the report of the activation of the alternative pathway by pyruvate (Millar et al., 1993). Prior to this work, it was generally assumed that partitioning of electrons onto the alternative pathway was thermodynamically unfavorable relative to the Cyt pathway (Bahr and Bonner, 1973; Siedow and Moore, 1993; Ribas-Carbo et al., 1994). This was supported by the observation that the alternative pathway did not engage significantly until the reduction state of the ubiquinone pool reached a level at which the Cyt pathway was close to saturation (Dry et al., 1989; Moore and Siedow, 1991). The question of whether the alternative pathway could compete for electrons with an unsaturated Cyt pathway was reopened by Umbach et al. (1994), who demonstrated that pyruvate acts to lower the apparent K_m of the alternative pathway for reduced ubiquinone.

In this study, we have addressed several questions relating to the regulation of electron partitioning between the Cyt and alternative pathways. First, we characterized the effect of pyruvate on the contribution of the alternative pathway to total respiration in the absence of ADP. It has long been puzzling that the apparent capacity of the alternative pathway in the presence of inhibitors of the Cyt pathway differs depending on the electron donor used. For example, succinate supports a higher rate of electron transport through the alternative pathway when the Cyt pathway is inhibited than does NADH (Rustin et al., 1984; Hemrika-Wagner et al., 1986; Day et al., 1991; Millar et al., 1993). Our studies of the partitioning of electron flow between the Cyt and alternative pathways in the absence of added inhibitors using the O_2 isotope fractionation technique confirm that the actual electron flow through the alternative pathway differs markedly when using these electron donors. When succinate was used as a substrate, the alternative pathway contributes about 28% to total O_2 uptake with a rate of 20 nmol O_2 mg^{-1} protein min^{-1}, whereas with NADH as a substrate, it contributes only 8% with a rate of 7 nmol O_2 mg^{-1} protein min^{-1} (Table I), although the levels of reduction of the ubiquinone pool by succinate and NADH are comparable (Day et al., 1991). These differences do not exist in the presence of pyruvate when the alternative pathway contributes 39 and 47% to total respiration and succinate or NADH, respectively, are used as substrates. Thus, an additional factor must be contributing to the higher alternative pathway rate seen with succinate as the electron donor in the absence of pyruvate. The most likely possibility is that the oxidation of succinate leads to the formation of some pyruvate (via NAD malic enzyme), lowering the effective K_m of the alternative pathway for reduced ubiquinone when succinate is the substrate, relative to that when external NADH is used and no pyruvate is formed (Day et al., 1994; M. Ribas-Carbo and A.M. Lennon, unpublished data).

Under state 3 conditions, when the reduction state of the ubiquinone pool is low (Moore et al., 1988, 1991), the activity of the alternative pathway would be expected to be at or near zero. Although we have directly demonstrated this to be true in the absence of added pyruvate using the O_2 isotope fractionation system (Table II), in the presence of pyruvate, the alternative pathway is found to contribute to the total respiration rate. The alternative pathway contributes only about 10 to 12% to the total respiratory rate, but it is significant that the alternative pathway can be engaged at all under state 3 conditions when the main pathway is not saturated. This directly contradicts the long-standing paradigm that the alternative pathway becomes engaged only when the Cyt pathway is at or near saturation (Bahr and Bonner, 1973; Lambers, 1980; Moore and Siedow, 1991).

Equally critical for the traditional notion that the alternative pathway does not compete with an unsaturated Cyt pathway for electrons is the observation that in the presence of pyruvate inhibition of the alternative pathway by SHAM led to no inhibition of O_2 uptake (Tables III and IV). This result has significant ramifications for future O_2 electrode studies, particularly those involving measurements of respiration in intact tissues. Previously, the inhibition of O_2 uptake observed following the addition of saturating levels of SHAM was regarded as a reasonable estimation of the level of engagement of the alternative pathway in the absence of the inhibitor (Møller et al., 1988). Such is clearly not the case. In the presence of pyruvate, electrons can partition onto the alternative pathway, in either state 3 or state 4, under conditions where the Cyt pathway is not saturated and subsequent addition of an inhibitor of the alternative pathway leads to a redistribution of electron flux between the two pathways (Fig. 3; Tables I, III, and IV). These data are in agreement with those of Wilson (1988), who demonstrated a switching of electrons from the alternative pathway to the Cyt pathway in mung bean mitochondria, and, more recently, those of Hoefnagel et al. (1995), who compared O_2 and ferricyanide reduction rates in conjunction with inhibitor additions in soybean cotyledon mitochondria.

The effect of α-keto acids such as pyruvate on the alternative pathway is an important aspect of the regulation of electron flux between the two pathways that will need to be taken into account in all future studies that involve the use of the O_2 electrode.

An equally important outcome of this study is the demonstration of the utility of the application of the modified O_2 isotope fractionation system for measuring the partitioning of electron flow through the Cyt and alternative pathways in isolated mitochondria. The results of this study clearly demonstrate both the general applicability of the O_2 isotope fractionation system to the study of plant respiration and its clear advantage over the O_2 electrode in allowing the determination of the partitioning of electron

flow through the Cyt and alternative pathways in the absence of added inhibitors. These results provide the baseline data needed for the subsequent application of the related gas-phase GC-MS O_2 isotope fractionation system (Robinson et al., 1992, 1995) to the study of plant respiration and the role of the alternative pathway in intact tissues.

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