

# Partitioning of Electrons between the Cytochrome and Alternative Pathways in Intact Roots<sup>1</sup>

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To test the hypothesis that the cytochrome pathway is not invariably saturated when the alternative pathway is engaged, we titrated root respiration of several species with KCN (an inhibitor of the cytochrome pathway), both in the absence and presence of an inhibitor of the alternative pathway (salicylhydroxamic acid, SHAM). The slopes of the resultant KCN  $\rho$  plots ( $\rho_{\text{cyt}}$ ) were then used to determine whether the cytochrome pathway was saturated in each species. The species used were *Festuca ovina* ssp. *ovina* L., *Phaseolus vulgaris* L., and six *Poa* species (*Poa pratensis* L., *Poa compressa* L., *Poa trivialis* L., *Poa alpina* L., *Poa costiniana* Vick., and *Poa fawcettiae* Vick.). Although the cytochrome pathway was saturated in a number of species (i.e.  $\rho_{\text{cyt}}$  values were 1.0), several others exhibited  $\rho_{\text{cyt}}$  values of less than 0.5. Alternative pathway capacity correlated negatively with  $\rho_{\text{cyt}}$ , with  $\rho_{\text{cyt}}$  values of less than 1.0 occurring in tissues in which the alternative pathway capacity was greater than 25 to 30% of total respiration. The species that did not show full engagement of the cytochrome pathway rarely exhibited SHAM inhibition in the absence of KCN. We conclude that this lack of SHAM inhibition is not due to a lack of alternative pathway engagement but rather to the diversion of electrons from the alternative pathway to the unsaturated cytochrome path following the addition of SHAM.

Plant mitochondria possess a branched electron transport chain that contains two pathways: the Cyt pathway and the alternative pathway. Both the cyanide-sensitive, SHAM-resistant Cyt pathway and the cyanide-resistant, SHAM-sensitive alternative pathway obtain their electrons from  $Q_r$  (for a recent review, see Moore and Siedow, 1991). In contrast to the Cyt pathway, electron transport from  $Q_r$  to  $O_2$  via the alternative pathway does not lead to the synthesis of ATP. However, despite the potential importance of the alternative pathway in determining the total ATP yield in vivo, relatively little is known about the manner in which it is regulated in intact tissues and about its physiological significance.

It was assumed until recently that the alternative pathway is unable to compete with the Cyt pathway for  $Q_r$ . Rather, the alternative pathway was generally thought to become engaged only after the Cyt pathway is at, or near,

saturation (Douce and Neuberger, 1989; Lance, 1990; Day, 1992). For example, Dry et al. (1989) found that in soybean mitochondria a high degree of  $Q_r$  is needed to initiate significant alternative oxidase activity, a situation that can occur only when engagement of the Cyt pathway is very high. However, recent investigations of mitochondria have demonstrated the allosteric dependence of alternative pathway respiration on the presence of organic acids (Wagner et al., 1989; Lidén and Åkerlund, 1993; Millar et al., 1993; Umbach et al., 1994), in particular pyruvate (Millar et al., 1993), and on the extent to which the disulfide bonds linking the alternative oxidase subunits are reduced (Umbach and Siedow, 1993; Umbach et al., 1994).

The mechanisms by which these factors regulate alternative pathway respiration have yet to be thoroughly characterized. Nevertheless, recent work on soybean mitochondria has shown that pyruvate lowers the apparent  $K_m$  of the alternative oxidase for  $Q_r$  (Umbach et al., 1994), with the overall capacity of the alternative oxidase being governed by the presence of pyruvate (Day et al., 1994) and the redox state of the intramolecular disulfide bonds (Umbach et al., 1994). Furthermore, Hoefnagel et al. (1995) reported that, under state 4 conditions and in the presence of pyruvate, the alternative pathway can share electrons with the Cyt pathway in soybean mitochondria. The apparent lack of SHAM sensitivity in some intact tissues, despite substantial cyanide resistance (Atkin et al., 1993), may therefore be due not to a lack of alternative pathway engagement but rather to the diversion of electrons from the alternative pathway to the unsaturated Cyt pathway following addition of SHAM. This may be particularly important in leaves that commonly show high levels of  $V_{\text{alt}}$  but little SHAM sensitivity (Lambers et al., 1983; Atkin et al., 1992, 1993).

According to the inhibitor-titration technique (Theologis and Laties, 1978; Bingham and Farrar, 1987; Møller et al., 1988; Van der Werf et al., 1988), the engagement of the Cyt pathway can be determined by titrating respiration with increasing concentrations of KCN, an inhibitor of the Cyt pathway, first in the absence and then in the presence of SHAM, an inhibitor of the alternative pathway. The en-

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Abbreviations: coupled  $V_{\text{cyt}}$ , coupled Cyt pathway capacity;  $Q$ , ubiquinone;  $Q_r$ , reduced ubiquinone; SHAM, salicylhydroxamic acid;  $\rho_{\text{cyt}}$ , Cyt pathway engagement;  $v_{\text{alt}}$ , alternative pathway activity;  $V_{\text{alt}}$ , alternative pathway capacity;  $v_{\text{cyt}}$ , Cyt pathway activity;  $v_{\text{res}}$ , residual respiration.

agement of the Cyt pathway is then determined by plotting the rate of respiration in the absence of SHAM against the rate in the presence of SHAM for each concentration of KCN. This produces the so-called "KCN  $\rho_{\text{cyt}}$  plot." The slope of this plot to the left of the breakpoint ( $\rho_{\text{cyt}}$ ) provides an estimate of coupled Cyt pathway (i.e. in the absence of uncoupler and additional substrate) engagement (Bingham and Farrar, 1987). The breakpoint that may be seen in such plots is due to partial inhibition of the Cyt pathway by low KCN concentrations and diversion of those electrons down the alternative pathway (assuming it is not fully engaged). This diversion of electrons was thought to occur only during KCN titrations of the Cyt pathway (Theologis and Latics, 1978). However, the results of Wilson (1988) and Hoefnagel et al. (1995) suggest that electrons can also be diverted from the alternative pathway to the Cyt pathway during SHAM titrations. Both SHAM and KCN titrations can therefore be expected to exhibit breakpoints.

To our knowledge, no study has drawn attention to  $\rho_{\text{cyt}}$  values of less than 1.0, and hence it was assumed that the Cyt pathway is always fully engaged when the alternative pathway was operating (Lambers, 1990). The results from isolated mitochondria of Hoefnagel et al. (1995) demonstrate, however, that the Cyt pathway does not have to be saturated for the alternative pathway to be operating. If such conditions exist in intact tissues, then titrations with KCN should yield  $\rho_{\text{cyt}}$  values of less than 1.0.

In this study we assessed whether the Cyt pathway is fully engaged in intact roots of several plant species.  $\rho_{\text{cyt}}$  was assessed using information drawn from the inhibition of respiration by increasing concentrations of KCN, both in the absence and presence of SHAM (Møller et al., 1988). We report that in roots of several species the Cyt pathway is not fully saturated and that in such species  $\rho_{\text{cyt}}$  is negatively correlated with  $V_{\text{alt}}$ .

## MATERIALS AND METHODS

### Plant Material and Growth conditions

Several plant species differing in relative growth rates and biomass allocation patterns were chosen for this study. These included six *Poa* species (*Poa pratensis* L., *Poa compressa* L., *Poa trivialis* L., *Poa alpina* L., *Poa costiniana* Vick., and *Poa fawcettiae* Vick.), *Festuca ovina* ssp. *ovina* L., and *Phaseolus vulgaris* L. Seeds of each species were germinated on moistened filter paper and grown on sand (20°C constant temperature, 60% RH, 14-h daylength, 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  PPF) until the roots of each species were sufficiently long (approximately 3–14 d, depending on the species) to allow transfer to aerated nutrient solutions (Poorter and Remkes, 1990). This nutrient solution contained 2 mM  $\text{NO}_3^-$  [ $\text{KNO}_3$ ,  $\text{Ca}(\text{NO}_3)_2$  in a ratio of 1:1.3] as its nitrogen source. The nutrient solutions were changed twice weekly, and the pH was adjusted daily to 5.8. Individual plants of each species were suspended over the solutions (32-L tanks) in Styrofoam discs (two plants per disc for each species other than *Phaseolus vulgaris*, for which there was only one plant per disc). Growth conditions on the nutrient solutions were identical with those during germination.

### Respiratory Measurements

Dark respiration rates of intact, detached roots of each species were determined using a Clark-type  $\text{O}_2$  electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) mounted into the side of a light-tight chamber (50 mL for all species other than *Phaseolus vulgaris*, for which 100- and 200-mL cuvettes were used). Total rates of respiration were measured polarographically with the roots submerged in a buffered nutrient solution (10 mM HEPES and 10 mM Mes, pH 5.8) identical with that in which the plants were grown, except that iron, which chelates with SHAM, was excluded. Although the effect of buffering of the nutrient solutions was not determined for the above species, previous studies have found no effect on root respiratory rates of several species (Atkin, 1993; Collier et al., 1993; Atkin and Cummins, 1994).

Inhibition of the alternative and Cyt pathways in roots of all species was achieved via direct injection of SHAM (1.0 M stock in methoxyethanol) and KCN (1.0 M stock in 20 mM HEPES, pH 5.8) into the reaction vessels. Methoxyethanol alone had no effect on total root respiration in any of the investigated species.

To select the optimal concentration of SHAM to inhibit the alternative oxidase and to avoid potential side effects of SHAM on either peroxidase activity or  $v_{\text{cyt}}$  in roots of each species, we titrated with increasing concentrations of SHAM both in the absence and presence of 0.5 mM KCN (Møller et al., 1988). To prevent any  $\text{O}_2$  or substrate limitations during titration experiments, a different plant was used for each concentration of inhibitor used.

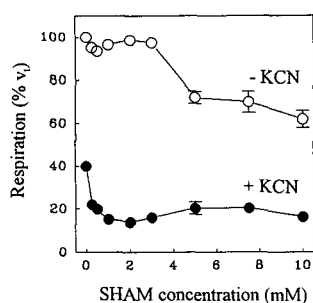
To assess the degree of  $\rho_{\text{cyt}}$ , we titrated roots of each species with KCN, both in the absence and presence of the optimal concentration of SHAM (Møller et al., 1988).

## RESULTS AND DISCUSSION

The optimum concentration of SHAM (i.e. that concentration required to fully inhibit the alternative pathway but not stimulate peroxidase-mediated  $\text{O}_2$  consumption and/or inhibit the Cyt pathway; Møller et al., 1988) differed between the selected species. The chosen concentrations of SHAM were: *P. pratensis*, 10 mM; *P. compressa*, 3 mM; *P. trivialis*, 3 mM; *P. alpina*, 2 mM; *P. costiniana*, 3 mM; *P. fawcettiae*, 7.5 mM; *F. ovina* ssp. *ovina*, 7 mM; and *Phaseolus vulgaris*, 20 mM. An example of the effect of increasing concentrations of SHAM (both in the absence and presence of KCN) on root respiration of *P. alpina* is shown in Figure 1. In this species, concentrations of SHAM higher than 3 mM appeared to inhibit the Cyt pathway.

The low degree of SHAM inhibition of root respiration in the selected species (Table I) suggests that there was little engagement of the alternative pathway (and therefore little  $v_{\text{alt}}$ ). However, for this conclusion to be valid, the Cyt pathway must have been fully saturated in all species, because  $v_{\text{alt}}$  will be underestimated if the Cyt pathway is less than 100% engaged. This is due to the ability of the unsaturated Cyt pathway to accept electrons from the alternative pathway upon inhibition by SHAM.

Was the Cyt pathway fully saturated in the selected species? The results of the KCN titrations suggest that that

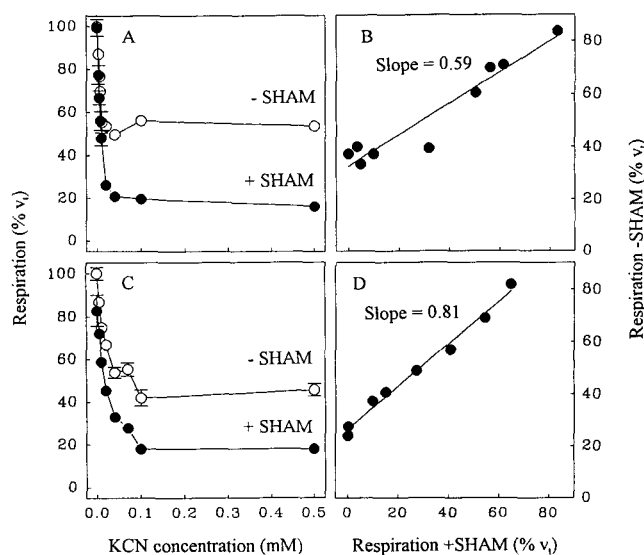


**Figure 1.** Effect of a range of SHAM concentrations on root respiration of *P. alpina*. Respiration in the absence (○) and presence (●) of 0.5 mM KCN ( $\pm$ SE,  $n = 4$ ). See Table I for total respiration ( $v_t$ ) values.

it was not. For example, in *F. ovina* and *P. alpina* increasing concentrations of KCN in the presence of SHAM inhibited respiration more than KCN alone (Fig. 2, A and C). As a result, the slopes of the resultant KCN  $\rho$  plots were less than unity in both species (*F. ovina*,  $\rho_{\text{cyt}} = 0.59$ , Fig. 2B; *P. alpina*,  $\rho_{\text{cyt}} = 0.81$ , Fig. 2D; Table I). The true rates of  $v_{\text{alt}}$  were therefore likely to be much greater than suggested by the low SHAM inhibition values (Table I).

Some species do, however, appear to exhibit fully saturated Cyt pathways. For example, previous studies have shown that the Cyt pathway is near saturation in the following tissues: roots of *Pisum sativum* (De Visser and Blacquièr, 1984), *Hordeum distichum* (Bingham and Farrar, 1987), *Carex acutiformis* (Van der Werf et al., 1988), *Triticum aestivum* (Collier et al., 1993), *Oxyria digyna* (Atkin, 1993), and *Dryas integrifolia* (Atkin, 1993), and leaves of dark-grown *Cichorium intybus* (O.K. Atkin and D.E. Collier unpublished data).

Increased partitioning of electrons from  $Q_r$  to the alternative oxidase may provide an explanation for the low  $\rho_{\text{cyt}}$  values observed in our study (Table I; Fig. 2). Based on the observation that pyruvate decreases the apparent  $K_m$  of the alternative oxidase for  $Q_r$ , Umbach et al. (1994) proposed that the alternative pathway could achieve substantial rates without saturation of the Cyt pathway in the presence of pyruvate. Subsequently, Hoefnagel et al. (1995) demonstrated that, in isolated soybean mitochondria, the alternative pathway does become engaged prior to saturation of the Cyt pathway in the presence of pyruvate. Pyruvate also



**Figure 2.** Effect of a range of KCN concentrations on root respiration of *F. ovina* ssp. *ovina* (A and B) and *P. alpina* (C and D). A and C, Respiration in the absence (○) and presence (●) of SHAM ( $\pm$ SE,  $n = 4$ ). See Table I for total respiration ( $v_t$ ) values. B and D, KCN  $\rho$  plots of the values shown in A and C.  $v_{\text{res}}$  was subtracted from the values in A and C before being plotted in B and D.

increases the level of KCN resistance (and therefore  $V_{\text{alt}}$  in isolated soybean mitochondria [Day et al., 1994]). Decreases in  $\rho_{\text{cyt}}$  therefore, appear to be associated with increases in both the activity and capacity of the alternative pathway in isolated mitochondria.

Although it was not possible to measure  $v_{\text{alt}}$  in the tissues exhibiting  $\rho_{\text{cyt}}$  values of less than 1, because of the diversion of electrons to the unsaturated Cyt pathway upon addition of SHAM, we were able to determine the  $V_{\text{alt}}$  (the rate of respiration in the presence of KCN, minus  $v_{\text{res}}$ ; Møller et al., 1988) in the selected tissues (Table I; Fig. 3).  $\rho_{\text{cyt}}$  was found to be negatively correlated with  $V_{\text{alt}}$  with  $\rho_{\text{cyt}}$  values of 1 occurring only in tissues in which  $V_{\text{alt}}$  was less than approximately 25 to 35% of total respiration (Fig. 3). A negative correlation may also exist between  $v_{\text{alt}}$  and  $\rho_{\text{cyt}}$  because no breakpoint was observed in the KCN

**Table I.** Respiration of intact roots of several plant species

Data concerning the respiratory properties of *F. ovina* were generously supplied by Ingeborg Scheurwater (Utrecht University).  $v_t$ , Total respiration.

Species	$v_t$	SHAM Inhibition ( $n = 4$ )	$V_{\text{alt}}$ ( $n = 4$ )	$\rho_{\text{cyt}}^c$	$v_{\text{res}}$
	nmol $O_2$ $g^{-1}$ fresh wt $s^{-1}$	% $v_t$	% $v_t$		% $v_t$
<i>F. ovina</i>	$2.9 \pm 0.1^a$ (36) <sup>b</sup>	$0.6 \pm 4.0^a$	$37.2 \pm 1.6^a$	0.59	$16.2 \pm 2.4^a$
<i>P. alpina</i>	$3.2 \pm 0.1$ (28)	$1.3 \pm 1.9$	$27.4 \pm 4.5$	0.81	$13.9 \pm 0.7$
<i>P. compressa</i>	$3.0 \pm 0.1$ (32)	$8.5 \pm 3.8$	$33.3 \pm 2.0$	0.64	$11.2 \pm 0.5$
<i>P. costiniana</i>	$2.8 \pm 0.1$ (32)	$-0.1 \pm 2.0$	$40.4 \pm 3.3$	0.41	$20.8 \pm 3.0$
<i>P. fawcettiae</i>	$2.5 \pm 0.1$ (28)	$0.0 \pm 2.3$	$35.5 \pm 1.5$	0.47	$19.2 \pm 2.5$
<i>P. pratensis</i>	$2.6 \pm 0.1$ (32)	$23.7 \pm 4.3$	$52.5 \pm 2.6$	0.46	$16.6 \pm 0.8$
<i>P. trivialis</i>	$3.4 \pm 0.1$ (36)	$11.7 \pm 2.8$	$28.1 \pm 2.2$	0.74	$11.3 \pm 1.9$
<i>Phaseolus vulgaris</i>	$3.0 \pm 0.1$ (60)	$4.0 \pm 2.3$	$45.1 \pm 4.2$	0.54	$11.7 \pm 1.9$

<sup>a</sup> Results are average values  $\pm$  SE.

<sup>b</sup> Number of replicates are shown in parentheses.

<sup>c</sup>  $\rho_{\text{cyt}}$  expressed as a proportion of  $V_{\text{cyt}}$ .

$\rho$  plots for the species listed in Table I (e.g. Fig. 2, B and D). The lack of a breakpoint suggests that the alternative pathway was near saturation. Thus, given the negative correlation between  $\rho_{\text{cyt}}$  and  $V_{\text{alt}}$  (Fig. 3), it seems likely that a similar negative correlation exists between  $\rho_{\text{cyt}}$  and  $v_{\text{alt}}$ . Increased alternative pathway respiration may, therefore, provide an explanation for the occurrence of  $\rho_{\text{cyt}}$  values of less than 1 (Table I; Fig. 3). Further work, using the  $^{16}\text{O}/^{18}\text{O}$ -isotope discrimination technique (Guy et al., 1989, 1992; Robinson et al., 1992), is needed to assess the degree to which in situ  $v_{\text{alt}}$  is coupled to  $V_{\text{alt}}$  and to confirm that low  $\rho_{\text{cyt}}$  values are correlated with high levels of in situ  $v_{\text{alt}}$ .

An important result in Figure 3 is that light treatment resulted in a decrease in  $\rho_{\text{cyt}}$  in *C. intybus* leaves. Atkin et al. (1993) previously reported that  $V_{\text{alt}}$  is induced by light treatment (as shown in Fig. 3), whereas SHAM sensitivity was not affected. This lack of SHAM inhibition in the light-grown plants, despite a high  $V_{\text{alt}}$  (Atkin et al., 1993), may have been due to a diversion of electrons from the alternative pathway to the unsaturated Cyt pathway upon the addition of SHAM. The value of  $\rho_{\text{cyt}}$  therefore appears to be influenced by environmental conditions, in addition to genetic characteristics.

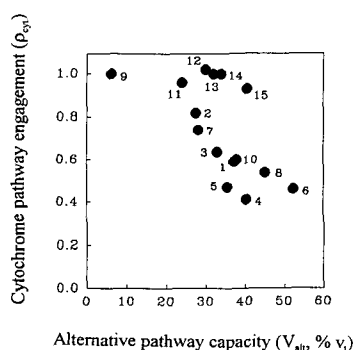
Other than Umbach et al. (1994) and Hoefnagel et al. (1995), the only other study that previously raised the possibility of sharing of electrons from  $Q_r$  between the alternative and Cyt pathways was that by Wilson (1988), who reported that  $v_{\text{cyt}}$  pathway activity increased upon inhibition of the alternative pathway in mung bean mitochondria. This suggests that the Cyt pathway was not fully engaged in the mitochondria used by Wilson (1988). Day (1992), using a similar approach to that of Wilson (1988), i.e. FeCN was used as an electron acceptor from the  $\text{Cyt}b/c_1$  complex, found no increase in  $v_{\text{cyt}}$  upon inhibition

of the alternative pathway in soybean cotyledon mitochondria. One explanation for these different results may be that  $\rho_{\text{cyt}}$  (and thus competition between the Cyt and alternative pathways) differed between the two mitochondrial preparations from the two species. In the study of Hoefnagel et al. (1995), sharing of  $Q_r$  electrons between the Cyt and alternative pathways in soybean cotyledon mitochondria was greater under state 4 conditions (in the absence of ADP) than under state 3 conditions (Hoefnagel et al., 1995). The degree of sharing therefore appears to differ between species and physiological states.

An important aspect of our results is that SHAM inhibition alone should no longer be used to assess the activity of the alternative pathway in vivo. Measurements of respiration in the presence of SHAM (minus  $v_{\text{res}}$ ) will yield overestimates of  $v_{\text{cyt}}$  and underestimates of  $v_{\text{alt}}$  when  $\rho_{\text{cyt}}$  is less than 1.0. Van der Bergen et al. (1994), in a paper that modeled the kinetic behavior of dehydrogenase input into Q and utilization of  $Q_r$  by the terminal oxidases, also concluded that results obtained with inhibitors of the alternative pathway could lead to an underestimate of the true engagement of the alternative pathway.

It is tempting to suggest that single additions of SHAM can be used to estimate  $v_{\text{alt}}$  and  $v_{\text{cyt}}$  when  $\rho_{\text{cyt}}$  is equal to 1.0. However, accurately distinguishing whether the Cyt pathway is saturated or unsaturated is problematic, given that the data generated by KCN titrations of intact tissues often lack precision. A further complication with the use of inhibitors in vivo is that we know little about the effect inhibitors have on the rate of substrate input into Q. It seems likely that activity of reducing enzymes may be altered when one or both of the terminal oxidases are inhibited. For example, Van der Bergen et al. (1994) indicated that the rate of succinate dehydrogenase activity is decreased at higher  $Q_r$  levels and in the presence of inhibitors. Given these concerns, we recommend that the  $^{16}\text{O}/^{18}\text{O}$ -isotope discrimination technique (Guy et al., 1989, 1992; Robinson et al., 1992) be used whenever possible to assess the level of  $v_{\text{alt}}$  in vivo, especially when titration analyses demonstrate that the Cyt pathway is not fully saturated.

In conclusion, our results indicate that the Cyt pathway is not saturated in intact tissues of many plant species and that in such tissues  $v_{\text{alt}}$  represents a far greater proportion of total respiration in intact tissues than previously thought. The degree of  $v_{\text{alt}}$  in intact tissues therefore needs to be reassessed, taking into account the fact that sharing of electrons from  $Q_r$  can occur between the alternative and Cyt pathways.



**Figure 3.** The relationship between  $\rho_{\text{cyt}}$  and  $V_{\text{alt}}$  in tissues of several plant species.  $V_{\text{alt}}$  values were derived from the y axis intercept of the KCN  $\rho$  plot for each species. The values are for roots, with the exception of dark- and light-grown *C. intybus*, for which leaf slices were used. The numbers designate individual plant tissues: 1, *F. ovina* ssp. *ovina*; 2, *P. alpina*; 3, *P. compressa*; 4, *P. costiniana*; 5, *P. fawcettiae*; 6, *P. pratensis*; 7, *P. trivialis*; 8, *P. vulgaris*; 9, dark-grown *C. intybus* (O.K. Atkin and D.E. Collier, unpublished data); 10, light-grown *C. intybus* (O.K. Atkin and D.E. Collier, unpublished data); 11, *H. distichum* (Bingham and Farrar, 1987); 12, *O. digyna* (Atkin, 1993); 13, *P. sativum* (De Visser and Blacquièrre, 1984); 14, *T. aestivum* (Collier et al., 1993); and 15, *C. acutiformis* (Van der Werf et al., 1988).

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ments and for providing the *F. ovina* SHAM and KCN titration data.

#### NOTE ADDED IN PROOF

Recent work by Millar et al. (1995) has demonstrated that in isolated mitochondria, the kinetics of Cyt pathway inhibition by KCN depend on the rate of flux through the pathway. The same was found for SHAM inhibition of the alternative pathway. Millar et al. (1995) observed that whenever the Cyt pathway was not fully engaged, inhibition by KCN yielded nonlinear  $\rho_{\text{Cyt}}$  plots. The fact that we observed linear  $\rho_{\text{Cyt}}$  plots with slopes of less than 1.0 in intact tissues, without any break point, suggests that the quinol-oxidizing and quinol-reducing pathway kinetics may differ in intact tissues and isolated mitochondria. For example, the kinetics of the dehydrogenases with respect to  $Q_1/Q_2$  may be far steeper in intact tissues than in isolated mitochondria when only succinate is oxidized. Nevertheless, the results of Millar et al. (1995) provide additional reasons for avoiding the use of inhibitors to assess  $v_{\text{alt}}$  in vivo.

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