Effect of Temperature on Rates of Alternative and Cytochrome Pathway Respiration and Their Relationship with the Redox Poise of the Quinone Pool

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We investigated the effect of short-term changes in temperature on alternative (Alt) and cytochrome (Cyt) pathway respiration, both in intact tissues and isolated mitochondria of 14-d-old cotyledons of soybean (Glycine max L. cv Stevens). We also established the extent to which temperature alters the interaction between the oxidizing pathways and the level of ubiquinone (UQ) reduction (UQr/UQt). No difference was found between the temperature coefficient of respiration (Q10; proportional change per 10° C) of Alt and Cyt pathway respiration in cotyledon slices (Q10 = 1.92 and 1.86, respectively). In isolated mitochondria, the Q10 of the fully activated Alt pathway (Q10 = 2.24–2.61) was always equal to, or higher than, that of Cyt c oxidase (COX) alone (Q10 = 2.08) and the complete Cyt pathway (Q10 = 2.40–2.55). This was true regardless of substrate or whether ADP was present. There was little difference in the Q10 of the Cyt pathway with or without ADP; however, the Q10 of COX was substantially lower in the presence of an uncoupler (Q10 = 1.61) than its absence (Q10 = 2.08). The kinetics of Alt and Cyt pathway activity in relation to UQr/UQt were not affected by temperature. For a given UQr/UQt activity are observed at any given UQr/UQt value, the proportion of maximum flux taking place was similar at all temperatures for both pathways (±ADP). However, the Q10 of the Alt and the Cyt pathways (+ADP) increased with increasing UQr/UQt. We conclude that the Alt pathway is not less temperature sensitive than the Cyt pathway or COX per se and that changes in the degree of control exerted by individual steps in the respiratory apparatus could result in changes in the Q10 of mitochondrial O₂ uptake.

Plant mitochondria possess a branched electron transport chain that contains two pathways: the cytochrome (Cyt) pathway (terminating at Cyt c oxidase; COX) and the alternative (Alt) pathway. Both the pathways obtain their electrons from reduced ubiquinone (UQr). The Alt pathway consists of a single-subunit quinol oxidase (Alt oxidase; AOX) which is activated by pyruvate (Millar et al., 1993, 1996); this increases the reactivity of the Alt pathway toward UQr (Umbach et al., 1994). In the presence of pyruvate, substantially higher rates of Alt pathway activity are observed at any given UQr/UQt value (e.g. Millar et al., 1998a). In addition, the AOX exists as a dimer and contains a regulatory sulfhydryl-disulfide system; the sulfhydryl linkages between paired subunits must be reduced for maximal activity (Umbach and Siedow, 1993). As a result, additions of the reducing agent dithiothreitol (DTT) increase Alt pathway activity in some tissues (Umbach and Siedow, 1993). Electron transport from UQr to O₂ via the Cyt pathway results in the movement of protons across the inner mitochondrial membrane, thereby building a proton-motive force that drives ATP synthesis. Flux via the Cyt pathway is rapid so long as there is an abundant supply of ADP; whenever the turnover of ATP to ADP is limited, flux via the Cyt pathway will become adenylate restricted. In contrast, no protons are translocated when electrons pass directly from UQr to O₂ via the Alt pathway. The Alt pathway is thus not subject to adenylate control per se. However, high rates of Alt pathway activity reduce the amount of ATP produced per unit O₂ consumed (Millar et al., 1998a).

Several positive roles for the Alt pathway in plant metabolism have been suggested. It may allow carbon flux through the TCA cycle when ADP supply limits Cyt pathway activity, thereby providing carbon skeletons for other cellular processes (Lambers and Steingrover, 1978). Moreover, the Alt pathway may protect against harmful reactive O₂ generation when the UQ pool is highly reduced (Purvis and Shewfelt, 1993; Wagner and Krab, 1995), as may occur when Cyt pathway activity is limited either by adenylates or by exposure to inhibitors such as nitric oxide (Millar and Day, 1996, 1997). Several authors have also proposed that the Alt pathway may maintain flux through the mitochondrial electron transport system in the cold (Kiener and Bramlage, 1981; Smakman and Hofstra, 1982; McNulty and Cummins, 1987; Rychter et al., 1988; Stewart et al., 1990a, 1990b; Vanlerbergh and McIntosh, 1992; Purvis and Sheffield, 1993) and illness including diabetes, heart disease, and cancer. The Alt pathway may be crucial in maintaining normal cellular function.

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Shewfelt, 1993; Ribas Carbo et al., 2000) and in doing so reduce the production of reaction O₂ species (Burris et al., 1995). This ability to maintain flux in the cold could be due to the Alt pathway being less temperature sensitive than the Cyt pathway after short-term changes in temperature (i.e. a lower temperature coefficient of respiration (Q₁₀; proportional change in respiration per 10°C change in temperature) for the Alt pathway than the Cyt pathway; Kiener and Bramlage, 1981; McNulty and Cummins, 1987; Stewart et al., 1990a) and/or to the de novo synthesis of AOX protein after long-term exposure to the cold (Stewart et al., 1990a, 1990b; Vanlerberghe and McIntosh, 1992; González Meler et al., 1999; Ribas Carbo et al., 2000). Whereas increases in AOX protein content invariably occur when plants are exposed to low temperatures, not all studies have reported lower Q₁₀ values for the Alt pathway. Weger and Guy (1991) reported that the Alt pathway of Picea glauca roots is highly sensitive to changes in temperature. Moreover, a recent study using the ¹⁸Ο fractionation method found that the Q₁₀ of the Alt pathway is not lower than the Q₁₀ of the Cyt pathway in mung bean (Vigna radiata) leaves and hypocotyls or soybean (Glycine max L. Merr. cv Stevens) cotyledons (González Meler et al., 1999). Another study found that cold-induced increases in AOX protein and activity are not related to chilling tolerance in maize (Ribas Carbo et al., 2000). Clearly, the temperature sensitivity of the Alt pathway and its role in chilling tolerance has not been fully elucidated.

Many of the past studies that investigated the temperature sensitivity of the Alt and Cyt pathways used respiratory inhibitors to estimate Alt pathway activity at different temperatures (e.g. Kiener and Bramlage, 1981; Smakman and Hofstra, 1982; McNulty and Cummins, 1987; Weger and Guy, 1991). However, the use of respiratory inhibitors to measure flux via the Alt and Cyt pathways is now considered unreliable (Day et al., 1996). As a result, past estimates of the Q₁₀ (proportional change in respiration per 10°C) of the Alt pathway in intact tissues (except González Meler et al., 1999) may be incorrect. Moreover, the results of studies on the temperature sensitivity of respiration in isolated mitochondria (e.g. Kiener and Bramlage, 1981; Lin and Markhart, 1990; Stewart et al., 1990a) need to be treated with caution, as activators of the Alt pathway (pyruvate and DTT) were not present in the assay media.

Although Q₁₀ values of respiration are commonly around 2.0, values as low as 1.4 and as high as 4.0 have been reported (Azcón-Bieto, 1992; Atkin et al., 2000a). Respiratory Q₁₀ values differ among species (e.g. Larigauderie and Körner, 1995) and are influenced by the metabolic state of the tissue and the growth environment (Atkin et al., 2000a, 2000b, 2000c). For example, Q₁₀ values are higher in plants with high concentrations of soluble carbohydrates (Wager, 1941; Breeze and Elston, 1978; Berry and Raison, 1981; Azcón-Bieto and Osmond, 1983) and in tissues where the demand for energy is increased when tissues are coping with environmental stress (Atkin et al., 2000c). Moreover, leaf respiration is also more temperature sensitive in darkness than in the light (Atkin et al., 2000b). The cause of this variability in respiratory Q₁₀ values has not been established. Nevertheless, it seems likely that variations in Q₁₀ values depend on the availability of respiratory substrate, degree of adenylate control, and/or the temperature sensitivity of respiratory enzymes per se (Atkin et al., 2000a). Variations in the flux via the Alt and Cyt pathways, as well as the degree of adenylate control may thus play an important role in determining the overall temperature sensitivity of plant respiration.

Our study investigates the effect of short-term changes in temperature on respiration in intact tissues and mitochondria isolated from 14-d-old soybean cotyledons. There were two components to our study. First, we determined whether the temperature sensitivity of the Alt and Cyt pathways differs in intact tissues and isolated mitochondria when the AOX is fully activated. To avoid the complicating effects of temperature on dehydrogenase activity and/or other components of the mitochondrial electron transport chain, we also determined the Q₁₀ value of the Alt pathway and COX per se (i.e. independent of limitations imposed by dehydrogenase activity) in isolated mitochondria. Second, we established the extent to which the UQ₉/UQ dependence of Alt pathway and Cyt pathway activity is affected by changes in temperature.

RESULTS

Temperature Sensitivity of Respiration

The effect of temperature on respiration in cotyledon slices is shown in Table I. Cotyledon respiration was temperature sensitive, both in the absence and presence of inhibitors (KCN or salicylhydroxamic acid [SHAM]). When calculated over the entire 10°C to 25°C range, uninhibited respiration, respiration in the presence of KCN, and respiration in the presence of SHAM exhibited Q₁₀ values of 1.98, 1.86, and 1.92, respectively (calculated from the slope of log₁₀ respiration versus temperature plots). There was therefore little difference in the temperature sensitivity of the KCN-resistant, Alt pathway and SHAM-resistant, Cyt pathway. The lack of inhibition by KCN (Table I) suggests that the capacity of the Alt pathway was equal to or greater than the control respiration rates. Residual respiration (i.e. respiration in the presence of SHAM and KCN) was temperature insensitive (Table I). Although the cause of the high residual rates (Table I) is unclear, it is unlikely that they are mitochondria in nature. Soybean cotyledons contain substantial amounts of lipid that can be degraded by beta-oxidation; there is an oxidation step that takes...
place in the glyoxysomes in which FADH$_2$ is oxidized directly by O$_2$ to produce H$_2$O$_2$, which is broken down by catalase. Respiratory inhibitors do not inhibit this process. Inhibitor-insensitive O$_2$-dependent peroxidation of fatty acids by lipoxygenase may also contribute to the high residual rate.

Figure 1 shows the effect of temperature on respiration in isolated mitochondria. Using succinate as a respiratory substrate, O$_2$ consumption via the Alt pathway (in the presence of DTT, ±5 mM pyruvate) was substantially lower than that of respiration via the Cyt pathway, both in the presence or absence of ADP (Fig. 1). All three parameters [Alt pathway and Cyt pathway (± ADP) respiration] were temperature sensitive across the 10°C to 25°C range (Fig. 1). Stimulation of Alt pathway activity by pyruvate increased with increasing temperature.

The fact that Cyt pathway activity was higher than Alt pathway activity in isolated mitochondria (Fig. 1) contrasts with the higher rates of KCN-resistant respiration than SHAM-resistant respiration in intact tissues (Table I). The most likely explanation for this discrepancy was that respiration was ADP- and substrate-limited in the intact tissues but not in isolated mitochondria (where saturating levels of ADP and substrate were supplied). Limitations in ADP supply in intact tissues will have a greater detrimental effect on Cyt pathway activity than Alt pathway activity, due to electron flux from UQ to O$_2$ via the Cyt pathway being coupled to proton translocation and ATP synthesis. No protons are translocated when electrons are transported from UQ to O$_2$ via the AOX. Evidence of substrate limitation in intact tissues come from the high rates of respiration in the presence of KCN (Table I). Whereas KCN inhibits electron transport via the Cyt pathway in both intact tissues and isolated mitochondria, this inhibition does not always result in reductions in O$_2$ consumption in intact tissues. This is because KCN severely reduces the synthesis of ATP with the result that cellular ATP to ADP ratios decline. Low ATP to ADP ratios can stimulate the rate of glycolysis and, hence, the rates of substrate supply to the mitochondria (Lambers et al., 1996). Overall respiratory flux can therefore be higher in intact tissues in the presence of KCN than in its absence whenever the Alt pathway capacity is high. Because KCN-resistant respiration and uninhibited respiration rates were similar (Table I), it seems likely that O$_2$ uptake in intact tissues was limited by the provision of substrate to the respiratory system. In contrast to KCN, SHAM does not decrease the ATP to ADP ratio or increase the rate of substrate supply in intact tissues. We, therefore, interpret the relatively low rates of SHAM-resistant respiration rates (Table I) to mean that the Cyt pathway was substrate (and ADP) limited in intact tissues.

To determine the temperature sensitivity (i.e. the $Q_{10}$ of each parameter shown in Figure 1, we plotted the respiration rates on a log 10 scale against temperature (not shown). In all cases, the resultant plots were linear. A single $Q_{10}$ value therefore applied to the entire 10°C to 25°C range for each pathway in the isolated mitochondria. The $Q_{10}$ of the Alt pathway

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Uninhibited Respiration</th>
<th>KCN Resistance</th>
<th>SHAM Resistance</th>
<th>Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>7.9 ± 0.7</td>
<td>8.1 ± 1.4</td>
<td>4.1 ± 0.7</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>15.0</td>
<td>14.3 ± 0.7</td>
<td>15.3 ± 3.3</td>
<td>8.9 ± 0.7</td>
<td>5.6 ± 0.8</td>
</tr>
<tr>
<td>20.0</td>
<td>19.0 ± 1.8</td>
<td>19.9 ± 2.3</td>
<td>9.6 ± 0.7</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>25.0</td>
<td>22.6 ± 1.4</td>
<td>20.9 ± 2.7</td>
<td>11.8 ± 0.9</td>
<td>5.1 ± 0.6</td>
</tr>
</tbody>
</table>

Table I. Effect of temperature on respiration rates in sliced soybean cotyledons (±SE, n = 4)

Respiration measured in the absence of inhibitors and in the presence of 1 mM KCN, 30 mM SHAM, or both KCN plus SHAM (residual).
was greater in the presence of pyruvate than in its absence (Table II). Although the fully activated Alt pathway was slightly more temperature sensitive than Cyt pathway respiration in the presence of ADP, there was little difference between the $Q_{10}$ value of the fully activated Alt pathway and that exhibited by Cyt pathway respiration in the absence of ADP (Table II).

To further assess the temperature sensitivity of the Alt pathway and COX independent of limitations caused by dehydrogenase activity, we determined the effect of temperature on Alt pathway and COX activity using nonenzymatic electron donors (ubiquinol-1 [UQ$_1$H$_2$] and 2,3,5,6-tetramethyl-p-phenylenediamine dihydrochloride [TMPD]-ascorbate, respectively). Alt pathway assays were conducted in the presence of DTT and pyruvate. At 25°C, rates of UQ$_1$H$_2$-dependent O$_2$ consumption via the Alt pathway (Fig. 2A) were similar to those exhibited by succinate-dependent O$_2$ consumption via the Alt pathway when pyruvate was present (Fig. 1). However, TMPD-ascorbate resulted in far higher rates of O$_2$ consumption via COX (Fig. 2A) compared with when succinate was used a respiratory substrate (Fig. 1). The $Q_{10}$ value of the Alt pathway (in the presence of pyruvate) appeared to be lower with UQ$_1$H$_2$ as an electron source (Table II) than with succinate (Table II). Similarly, the $Q_{10}$ was lower for TMPD-ascorbate-dependent COX activity (Table II) than that for succinate-dependent O$_2$ consumption via the Cyt pathway in the presence of ADP (Table II). The stimulatory effect of carbonyl cyanide-m-chlorophenylhydrazone (CCCP) on COX activity (using TMPD-ascorbate as an electron donor) decreased with increasing temperature (Fig. 2B). As a result, the $Q_{10}$ of uncoupled respiration was substantially lower than that of the coupled state (Table II). Taken together, these results demonstrate that in isolated mitochondria the Alt pathway is not less temperature sensitive than the Cyt pathway, regardless of the source of the respiratory substrate (i.e. succinate for both pathways or TMPD-ascorbate for COX and UQ$_1$H$_2$ for AOX).

### Table II. Temperature sensitivity of respiration in isolated mitochondria in soybean cotyledons

<table>
<thead>
<tr>
<th>Respiratory Pathway</th>
<th>Substrates and Assay Components</th>
<th>$Q_{10}$ Value</th>
<th>Source Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alt pathway</td>
<td>Suc + DTT + Pyr</td>
<td>2.61</td>
<td>Fig. 1</td>
</tr>
<tr>
<td></td>
<td>Suc + DTT - Pyr</td>
<td>2.25</td>
<td>Fig. 1</td>
</tr>
<tr>
<td></td>
<td>UQ$_1$H$_2$ + DTT + Pyr</td>
<td>2.24</td>
<td>Fig. 2</td>
</tr>
<tr>
<td>Cyt Pathway</td>
<td>Suc + ADP</td>
<td>2.40</td>
<td>Fig. 1</td>
</tr>
<tr>
<td></td>
<td>Suc - ADP</td>
<td>2.55</td>
<td>Fig. 1</td>
</tr>
<tr>
<td>COX</td>
<td>TMPD-ascorbate</td>
<td>2.08</td>
<td>Fig. 2</td>
</tr>
<tr>
<td></td>
<td>TMPD-ascorbate + CCCP</td>
<td>1.61</td>
<td>Fig. 2</td>
</tr>
</tbody>
</table>

UQ Redox Poise and Temperature

The experimental results above provide estimates of the $Q_{10}$ of the Alt pathway, the full Cyt pathway and COX alone, either at UQ$_i$/UQ$_r$ values that differ (Fig. 1) or near-saturating UQ reduction levels (Fig. 2A). To assess whether the $Q_{10}$ values of the Cyt pathway and Alt pathway (in the presence of pyruvate) depend on the UQ$_i$/UQ$_r$ value, we titrated succinate dehydrogenase with malonate (Fig. 3). Regardless of UQ$_i$/UQ$_r$ value, low temperatures reduced Cyt pathway activity (both in the presence and absence of ADP) and Alt pathway activity (Fig. 3). However, for any given parameter, the UQ$_i$ to UQ$_r$ ratio was relatively similar at each temperature when respiration was measured in the absence of malonate.

![Figure 2. Respiration of soybean cotyledon mitochondria at several temperatures, using artificial electron donors. Rates of respiration via COX (○) and AOX (●). COX activity was determined in the presence of octyl gallate (OG) and using TMPD-ascorbate as an electron donor. Alt pathway activity was assessed in the presence of antimycin A and myxothiazol, and using UQ$_1$H$_2$ as an electron donor. To calculate $Q_{10}$ values, respiration rates were also plotted on a log$_{10}$ scale versus temperature (not shown), with the slopes used to calculate the $Q_{10}$ values. The $r^2$ values for the slopes of the log$_{10}$ respiration versus temperature plots (used to calculate $Q_{10}$ values) were 0.89 and 0.79 for the Alt pathway and COX, respectively. B, The effect of uncoupler (CCCP) on COX activity, expressed as a proportion of the values shown in A. Values represent the mean of three to five replicate mitochondrial isolations (±SE).](https://www.plantphysiol.org/embargo/doi/10.1104/pp.014769)
(i.e. at maximum O₂ uptake rate), being 0.35 to 0.5 for state 3, 0.8 to 0.95 for state 4, and 0.8 to 0.9 for the Alt pathway. Cyt pathway respiration rates were always higher in the presence of ADP than in its absence, regardless of the UQₐ/UQₜ value.

To assess whether temperature altered the overall relationship between respiration rates and UQₐ/UQₜ values, we expressed the data shown in Figure 3 as a ratio of the maximum rates (using succinate as a substrate) for each temperature (i.e. V/Vₘₐₓ, Fig. 4). Figure 4 shows that changes in temperature had little affect on the relative rates of respiration obtained at each temperature. This suggests that the ability of each pathway to compete for a given amount of reduced UQ remains unaltered by changes in temperature.

To assess whether the Q₁₀ of the Alt pathway and/or Cyt pathway varies with UQₐ/UQₜ value, we...
fitted regression equations to the data shown in Figure 3 to predict respiration rates at set UQr/UQt values and temperature. These regression equations where then used to determine the temperature sensitivity of respiration at a set UQr/UQt value. Figure 5 suggests that the Q10 values for the Cyt pathway and the Alt pathway vary with UQr/UQt. The temperature sensitivity of the Cyt pathway in the absence of ADP was constant at all levels of Q reduction. In contrast, both the Alt pathway and the Cyt pathway in the presence of ADP appeared to become more temperature sensitive at high levels of UQ reduction. The calculated Alt pathway and Cyt pathway (+ADP) Q10 values remained UQr/UQt-dependent even when calculated using the highest and lowest 95% confidence intervals for the data shown in Figure 3 (data not shown). We are therefore confident that despite variability among the respiration rates of separate mitochondrial preparations (particularly at 25°C; Fig. 3), the Q10 did increase with increasing UQr/UQt. One result of this is that whereas the Cyt pathway respiration in the absence of ADP was more temperature sensitive than Cyt pathway respiration in the presence of ADP at low UQr/UQt values, there was little difference in the Q10 values in the presence and absence of ADP at high UQr/UQt (Fig. 5). It is important that the Q10 of the Alt pathway remained higher than that of the Cyt pathway in the presence of ADP at all UQr/UQt values (Fig. 5). The Q10 of the Alt pathway was also higher than that of the Cyt pathway in the absence of ADP for all except the lowest levels of UQ reduction (Fig. 5).

Data obtained in the absence of malonate represent the intersection between the kinetics of the oxidizing pathways (i.e. Cyt pathway ± ADP or Alt pathway) and the reducing pathway (succinate dehydrogenase) (Fig. 3). For any given temperature, a curve fitted through these intersections represents the activity of succinate dehydrogenase at different UQr/UQt values. When these points were plotted separately, it was clear that succinate dehydrogenase activity decreased with decreasing temperature (Fig. 6). To further illustrate this, we titrated the Cyt pathway (in the presence of ADP) with KCN at the following temperatures: 25°C, 20°C, 15°C, and 10°C. When these data were combined with the uninhibited Cyt pathway (±ADP) data, the kinetics of succinate dehydrogenase in the face of increasing UQr/UQt values at each temperature could be observed (Fig. 6). When coupled to oxidation via the Cyt pathway, succinate dehydrogenase activity was clearly temperature dependent at all UQr/UQt levels, decreasing by approximately 50% for each 10°C decline in temperature (i.e. the Q10 of succinate dehydrogenase was approximately two).

**DISCUSSION**

**Temperature Sensitivity of the Alt and Cyt Pathways**

It has been suggested that the Alt pathway plays a role in protecting plant cells against the generation of high levels of superoxide and H2O2 in cold-stressed tissues (Purvis and Shewfelt, 1993). Support for this suggestion comes from the fact that long-term exposure to low temperatures increases AOX protein levels in tobacco leaves (Vanlerbergh and McIntosh, 1992) and mung bean hypocotyls and leaves (González Meler et al., 1999). Moreover, the relative partitioning of electrons via the Alt pathway is increased after long-term cold acclimation in some species (González Meler et al., 1999). However, despite the
clear role increases in Alt pathway activity may play during acclimation to long-term changes in temperature, our results suggest that during short-term exposure to low temperature the Alt pathway is not able to reduce the risk of oxidative damage. Rather than being less temperature sensitive than the Cyt pathway (see Kiener and Bramlage, 1981; Smakman and Hofstra, 1982; McNulty and Cummins, 1987; Stewart et al., 1990a), the $Q_{10}$ of the Alt pathway was either similar to or greater than that exhibited by the Cyt pathway (Table II). Other studies have also reported that the short-term $Q_{10}$ of the Alt pathway is not less than that of the Cyt pathway (Weger and Guy, 1991), including a recent study that used the $^{18}$O-discrimination method (González Meler et al., 1999). It seems likely, therefore, that the Alt pathway does not ameliorate the detrimental effects of short-term exposure to low temperature.

Why was the Alt pathway more temperature sensitive in our study than in previous studies? One explanation is that the $Q_{10}$ for the Alt pathway reported by Stewart et al. (1990a) was an underestimate of the actual $Q_{10}$, as neither pyruvate or DTT were present in their reaction media. In our study pyruvate stimulated Alt pathway activity by approximately 25% at 25°C but had little effect at 10°C (Fig. 1). As a result, the $Q_{10}$ of the Alt pathway was higher in the presence of pyruvate than in its absence. We did not directly assess the effect of DTT on the $Q_{10}$ of the Alt pathway. However, comparison of rates in the presence of DTT (Fig. 1) and those in its absence (Fig. 3), both in the presence of pyruvate, indicate that DTT had little effect on the $Q_{10}$ of the Alt pathway in mitochondria isolated from our 14-d-old soybean cotyledons. Whereas this suggests that the AOX was fully reduced in our soybean cotyledon mitochondria, it is still important to include DTT in mitochondrial assay media whenever maximum AOX activity is being assessed as the reduced state of AOX is likely to vary among species, tissues and/or environmental treatments.

Distribution of Control within the Respiratory Apparatus and the $Q_{10}$

Our study suggests that changes in the degree of control exerted by individual steps in the respiratory apparatus could result in changes in the $Q_{10}$ of mitochondrial O$_2$ uptake. For example, the $Q_{10}$ of the COX was substantially lower in the presence of an uncoupler (i.e. when control over electron flow to O$_2$ resides in the electron transport chain itself and/or the entry of reduced TMPD into the membrane) than in its absence (i.e. when electron flow is limited by phosphorylation; Table II). Similarly, the $Q_{10}$ of the Alt and Cyt pathways (+ADP) appear to increase with increasing $UQ_r/UQ_t$ (Fig. 5). At low $UQ_r/UQ_t$, a greater proportion of control over electron flow is likely to be exerted by the UQ reducing pathways (e.g. succinate dehydrogenase) than by the UQ oxidizing pathways (e.g. Alt and Cyt pathways). In contrast, control over electron flow would shift toward the UQ oxidizing pathways at high $UQ_r/UQ_t$ values.

In contrast to the apparent $UQ_r/UQ_t$ dependence of the $Q_{10}$ values exhibited by the Alt pathway and Cyt pathway respiration in the presence of ADP, $Q_{10}$ values of Cyt pathway activity in the absence of ADP were clearly $UQ_r/UQ_t$-independent (Fig. 5). In the absence of ADP, the control exerted by the components of the Cyt path is near zero, with control being mostly exerted by the leak of protons across the inner mitochondrial membrane (Diolez et al., 1993). In contrast, control is distributed among several steps in the presence of ADP, including the dehydrogenase, proton leak, the ATPase, CIII, Cyt c, and COX (Douce and Neuburger, 1989). The overriding importance of the proton leak in controlling flux via the Cyt pathway in the absence of ADP suggests the $Q_{10}$ value of Cyt pathway respiration in the absence of ADP reflects primarily the $Q_{10}$ value of the proton leak. Moreover, the lack of change in the $Q_{10}$ of the Cyt pathway respiration in the absence of ADP suggests that the temperature sensitivity of the proton leak is independent of $UQ_r/UQ_t$.

Our study highlights the importance of ensuring that the $UQ_r/UQ_t$ is similar for the Alt and Cyt pathways when assessing their temperature sensitivity in isolated mitochondria. If we had only measured the $Q_{10}$ of the two pathways using succinate or other non-saturating substrates such as malate, then the $UQ_r/UQ_t$ value we would have overestimated the difference in $Q_{10}$ values exhibited by the Alt pathway and Cyt pathway in the presence of ADP. In the absence of inhibitors, both pathways would be competing for electrons from the UQ pool, with a common $UQ_r/UQ_t$ value being available for both terminal oxidase pathways. It is thus misleading to only compare the $Q_{10}$ of the Alt and Cyt pathways without ensuring that the comparison is made at a common $UQ_r/UQ_t$ value.

In addition to not altering the ability of each pathway to compete for a given amount of reduced UQ, changes in temperature also appear to have not altered the balance between the activity of the reducing and oxidizing pathways that determine the level of UQ reduction in mitochondria provided with succinate as a substrate. Decreases in temperature reduced flux via the UQ reducing (succinate dehydrogenase; Fig. 6) and oxidizing pathways (Fig. 3) to a similar extent. As a result, $UQ_r/UQ_t$ remained unaltered in the absence of malonate for each pathway, regardless of temperature (Fig. 3). If temperature had had a greater impact on either the reducing or oxidizing pathways, then $UQ_r/UQ_t$ values in the absence of malonate would not have been the same at each temperature.
Our conclusion that the \( Q_{10} \) of the Alt and Cyt (+ADP) pathways is UQ\(_r\)/UQ\(_t\)-dependent was supported by the fact that the \( Q_{10} \) remained UQ\(_r\)/UQ\(_t\)-dependent when the analysis was repeated using 95% confidence interval values for the data shown in Figure 3 (see “Materials and Methods” and “Results”). One reason for why the \( Q_{10} \) increases with increasing UQ reduction might be that high UQ\(_r\)/UQ\(_t\) activates the oxidizing pathways and that the degree of activation is greatest at high temperatures. Indeed, the AOX is activated by high UQ\(_r\)/UQ\(_t\) values (Hoefnagel and Wiskich, 1998). However, it is not known if this degree of activation increases with increasing temperature. Moreover, the extent to which the Cyt pathway (+ADP) is activated by high UQ\(_r\)/UQ\(_t\) at different temperatures is not known.

Effect of Temperature on the Regulation of Respiratory Flux

Respiratory flux can be controlled by the availability of substrate supply, the degree to which respiration is regulated by adenylates and/or the maximum capacity of respiratory enzymes (Atkin et al., 2000a). Changes in temperature may alter the degree to which each factor regulates the overall rate of \( O_2 \) consumption. At moderate temperatures (e.g., 25°C), limitations in the amount of active AOX may limit \( O_2 \) consumption by the Alt pathway. However, in plant tissues with high Cyt pathway capacity \( O_2 \) consumption is unlikely to be strongly limited by enzymatic capacity, when measured at 25°C. Rather, respiratory flux at moderate temperatures is controlled largely via adenylate regulation of respiratory enzymes in glycolysis (phosphofructokinase and pyruvate kinase) and the mitochondrial electron transport chain (Day and Lambers, 1983). Changes in substrate supply also play a role in some situations (Lambers et al., 1996). Wiskich and Dry (1985) found that the activity of isolated mitochondria typically exceeds actual measured rates of intact tissues. Similarly, several studies where the activity of enzymes of glycolysis, the TCA cycle and the electron transport chain have been altered in transgenic plants have led to, at best, only modest changes in respiratory rates, when measured at moderate temperatures (e.g. Gottlob-McHugh et al., 1992; Millar et al., 1998b). Moreover, whereas glycolytic flux is regulated by the activity of two key enzymes, phosphofructokinase and pyruvate kinase, changes in the rate of respiration likely occur primarily via adenylate (i.e. ATP to ADP ratio) regulation of these enzymes with little control being exerted by the enzymatic capacity per se (Thomas et al., 1997). Respiratory flux is also likely to be regulated in part via changes in the activation state of pyruvate dehydrogenase complex (PDC). PDC is strongly regulated, with its activity being determined by the concentration of substrates, products, NADH and ATP, and other factors (Moore et al., 1993). The actual amount of PDC protein is unlikely to control respiratory flux at moderate temperatures. To our knowledge, no study has investigated the extent to which capacities of enzymes control respiratory flux at low temperatures. Nevertheless we can get some insight by measuring the \( Q_{10} \) values of intact tissues and those of isolated mitochondria provided with saturating substrate and abundant ADP (i.e. not adenylate or substrate limited). If the \( Q_{10} \) value of substrate/ADP-saturated, enzyme-limited systems is substantially greater than that of intact tissues, then exposure to low temperatures may result in a transition from control of respiration by extramitochondrial reactions (e.g. by the ratio of ATP to ADP) to control exerted by the capacity of respiratory enzymes. \( Q_{10} \) values are greater in substrate-saturated, enzyme-limited isolated mitochondria than in substrate-unsaturated, intact shoot segments at measuring temperatures less than 10°C to 15°C in wheat and rye but are similar at higher temperatures (Pomeroy and Andrews, 1975). This suggests that respiration might be partly controlled by the capacity of mitochondrial processes at low temperatures.

CONCLUSIONS

Our measurements demonstrate that the temperature sensitivity of the Alt pathway is not less than that of COX alone, or the complete Cyt pathway. Moreover, we have shown that the kinetics of Alt pathway and Cyt path activity in relation to the UQ pool are temperature independent and that the \( Q_{10} \) of Alt pathway and the Cyt pathway (in the presence of ADP) increase with increasing levels of Q reduction. Our results also demonstrate that the \( Q_{10} \) of \( O_2 \) consumption can be highly variable, depending on the level of reduction of the UQ pool, degree of adenylate control of the Cyt pathway and the activation state of the Alt pathway. This may partly explain why \( Q_{10} \) values of plant respiration vary so markedly in nature.

MATERIALS AND METHODS

Plant Culture and Reagents

All work was conducted using 14-d-old cotyledons harvested from soybean (Glycine max L. Merr. cv Stevens) seedlings propagated in trays of vermiculite. For the isolated mitochondria studies, plants were grown under glasshouse conditions. In vivo respiration measurements were conducted using controlled-environment grown seedlings (constant 25°C, 14-h day, 60% relative humidity, 300 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\)). Percoll was purchased from Pharmacia Biochemicals Inc. (Uppsala), whereas UQ\(_1\) was prepared by Dr A.D. Ward (University of Adelaide, Australia). All other chemicals were purchased from Sigma (St. Louis).
In Vivo Measurements

In vivo measurements of dark respiration of the cotyledons were determined using a Rank Brothers Clark-type O₂ electrode (Cambridge, UK). KCN and SHAM were used to inhibit COX and Alt pathway, respectively. In preliminary studies very high rates of respiration remained in the presence of KCN and SHAM, regardless of the concentrations of KCN or SHAM used, or the solvent in which SHAM was dissolved (including dimethyl sulfoxide). We therefore decided to slice the cotyledons into 2 mm thick slices using a sharp razor blade to minimize residual respiration. One millimolar KCN (1.0 mM stock in 20 mM HEPES, pH 5.8) and 30 mM SHAM (from a 1.0 mM stock in methoxyl ethanol) were used to inhibit the Cyt and Alt pathways, respectively. Although substantial residual respiration remained in the presence of both inhibitors (see Table I), the absolute rates of residual respiration were less than one-half that compared with unsliced cotyledons (data not shown). During slicing, cotyledons were submerged in a buffer (2 mM CaCl₂, 10 mM HEPES, and 10 mM MES, pH 7.2). Slices were left in the buffer and darkness for 30 min to overcome transient postillumination and wounding effects (Axéon et al., 1983). O₂ consumption was then measured in darkness with the sliced cotyledons submerged in a fresh volume of the same buffer.

Mitochondrial Assays and Temperature Treatments

Mitochondria were prepared according to Day et al. (1985). O₂ consumption was measured using a Rank Brothers electrode in a standard reaction medium (0.3 mM Suc, 10 mM TES buffer, 5 mM KH₂PO₄, and 2 mM MgCl₂, pH 7.2). O₂ consumption was measured at several temperatures, typically in the 10°C to 25°C range unless otherwise stated. Air-saturated, temperature-equilibrated water was used to calibrate the electrode at each temperature. For each temperature treatment the pH of the standard reaction medium was adjusted to 7.2 using a pH meter calibrated using buffers at the same temperature. Unless otherwise stated, all Alt pathway activity assays included 5 mM pyruvate (Millar et al., 1993). DTT (5 mM) was also used to fully reduce the AOX, where indicated. The protein content of samples was estimated according to the method of Lowry et al. (1951).

Alt Pathway and COX Activity

To assess the temperature dependence of Alt pathway activity, independent of limitations imposed by dehydrogenase activity, 400 μM UQ₃H₂ (an analog of ubiquinol; Hoefnagel et al., 1997) was provided as electron sources in the standard reaction medium, in the presence of antimycin A (5 μM) and KCN (1 mM). Antimycin A (5 μM) was added to inhibit transhydrogenase activity from UQ₁H₂ to UQ₁₀ (Hoefnagel and Wiskich, 1998). The temperature dependency of COX activity, independent of dehydrogenase activity, was determined using TMPD in the presence of ascorbic acid as an electron donor. The reaction medium contained 5 mM TMPD and 20 mM ascorbic acid. KCN (1 mM) was added to subtract residual activity at the end of each assay. The effect of the uncoupler CCCP on TMPD-ascorbate dependent COX activity was also assessed. These latter experiments were conducted in the presence of 2 μM OG, an inhibitor of the Alt pathway.

Malonate Titrations and UQ Reduction Measurements

Malonate (0.5–20 mM) was used to titrate succinate (10 mM) oxidation for Cyt (± ADP) and Alt pathway respiration according to Dry et al. (1989). At each stage of the malonate titration, we measured the redox state of UQ using glassy carbon and platinum electrodes (Bioanalytical Systems, West Lafayette, IN) and a non-cycling UQ Electrode Potentiostat (ARN Electronics, Adelaide, Australia) according to the method of Moore et al. (1988). Before adding succinate, mitochondria were pre-incubated in 1.0 mM UQ₁, and 0.5 mM ATP for 3 min. OG (2 μM) and myxothiazol (6 μM) were used to inhibit Alt pathway and the Cyt pathway, respectively. Fully oxidized levels of UQ were taken as the voltametric reading before succinate was added. To obtain a fully reduced UQ reading, we added 2 mM NADH at the end of each experiment in the presence of OG and myxothiazol. The direct effects of NADH, OG, and myxothiazol on the UQ electrode were determined in the absence of mitochondria and subtracted from the final fully reduced UQ signal where necessary.

Calculations

The temperature sensitivity of respiration was assessed by calculating Q₁₀ values (i.e. the proportional change in respiration per 10°C change in temperature). Q₁₀ values were calculated using the equation $Q_{10} = \left(\frac{10^{(\text{regression slope})}}{10}\right)^{\frac{\Delta T}{10}}$ where the regression slope is the slope of a log₁₀ respiration rate versus temperature plot. In all our measurements, the regression slope was linear in the 5°C to 30°C range; consequently, a single Q₁₀ value could be applied across this temperature range.

To assess the temperature sensitivity of respiration at any given UQ reduction level, we first fitted second order regression equations to the respiration-UQₙ/UQₚ curves generated from the malonate titrations (SigmaPlot Version 5, Jandel Scientific, San Rafael, CA). To obtain a respiration rate at a high UQₙ/UQₚ value for Cyt pathway in the presence of ADP, both the respiration rate and UQ reduction level were measured in the presence of NADH (plus OG, minus myxothiazol). Again, the direct effect of NADH on the UQ electrode was taken into account when determining the level of UQ reduction. For each temperature, respiration rates for Cyt pathway (± ADP) and the Alt pathway for five UQₙ/UQₚ values (0.01, 0.2, 0.4, 0.5, 0.6, 0.8, and 1.0) were then estimated from 2nd order regression equations fitted to the flux versus UQₙ/UQₚ. Log₁₀ transformed values of respiration were then plotted against temperature, with the slopes then used to calculate the Q₁₀ value at the seven UQₙ/UQₚ values. To assess the robustness of these calculated Q₁₀ values, we repeated the above
calculations using the highest and lowest 95% confidence interval values fitted to the respiration-UQ, UQt plots.

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