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Measurements of in Vivo Ubiquinone Reduction Levels in Plant Cells

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A method is described for the determination of in vivo ubiquinone (UQ) reduction levels in nongreen tissues by extraction and subsequent detection of ubiquinone-10 and ubiquinol-10 with high-performance liquid chromatography. In *Petunia hybrida* cell suspensions UQ reduction remained at a stable level of about 60%, despite the changing conditions during the batch culture (from excess sugar to starvation) and the concomitant variations in respiration. Also, in the presence of uncoupler, which causes a large increase in respiration via both the cytochrome pathway and the alternative pathway, UQ reduction levels stayed at 60%. In mitochondria isolated from these cells, activity of the alternative pathway was only observed at UQ reduction levels higher than 80%. It is proposed that in vivo the relationship between UQ reduction and the activity of the alternative oxidase is modulated by mechanisms such as thiol modifications and accumulation of organic acids. Accordingly, pyruvate concentration in *P. hybrida* cells increased in the presence of uncoupler.

With the introduction of the voltametric procedure for measuring the redox state of UQ (Moore et al., 1988; Dry et al., 1989), understanding of the regulation of plant mitochondrial respiration has increased tremendously. Differences in kinetics behavior of the two oxidizing pathways in plant mitochondria toward UQ reduction levels explain the proposed action of the alternative pathway as an "overflow" of the Cyt pathway (Bahr and Bonner, 1973a, 1973b), although the Cyt pathway may not always be saturated when the alternative pathway becomes engaged (Dry et al., 1989).

Several mechanisms activating the alternative oxidase and/or changing the relationship between oxidizing activity and UQ reduction levels have been described, including organic acid stimulation (Wagner et al., 1989; Lidén and Åkerlund, 1993; Millar et al., 1993) and regulation via the redox state of disulfide bonds in the oxidase (Umbach and Siedow, 1993; Umbach et al., 1994).

However, all of these results describe situations in isolated mitochondria with succinate, for the most part, as the only substrate. These conditions are certainly different from the in vivo situation, in which a mixture of internal and external substrates will be present, most of them probably in concentrations lower than those necessary to reach maximal activity of the dehydrogenases. Measurements of UQ reduction levels with substrates other than succinate or with multiple substrates have been performed, but external NADH, which is the substrate often giving the highest respiratory rate, sometimes disturbs the voltametric measurements and corrections have to be made (Day et al., 1991). Another disadvantage of the voltametric technique is that hydroxamates cannot be used; therefore, the relationship between respiration via the Cyt pathway and UQ reduction cannot be studied.

These problems can be overcome by determining UQ reduction levels in mitochondria with an extraction technique (Van den Bergen et al., 1994). After extraction UQ and UQH_2 can be detected by separation with HPLC, and the relative amounts of both compounds can be determined.

In this paper we describe how the same technique can also be successfully applied to whole plant cells. Most of the results were obtained with *Petunia hybrida* cell suspensions in batch culture, but it is also shown that it is possible to detect UQ and UQH_2 in other nongreen tissues, such as potato (*Solanum tuberosum* L.) and sweet potato (*Ipomoea batatas* L.) tuber tissue. The relationship between whole-cell respiration, in the absence or presence of respiratory inhibitors or uncoupler, and UQ reduction levels were measured in *P. hybrida* cell suspensions and compared with the situation observed in mitochondria isolated from these cells.

MATERIALS AND METHODS

Plant Material

Petunia hybrida Vilm (cv Rosy Morn) cell suspensions were grown in batch culture as described by Van Emmerik et al. (1992). Potato tubers (*Solanum tuberosum* L. cv Bintje) were obtained from the Proefboerderij Nederlandse Aardappelkeuringsdienst (Slootdorp, The Netherlands) and stored after harvest (in October) in a ventilated dark room at 8°C. Sweet potatoes (*Ipomoea batatas* L. white) were bought in local stores.

Isolation of Mitochondria

P. hybrida mitochondria were isolated by homogenizing 15-g fresh weight portions with a mortar and pestle at 4°C

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Abbreviations: BHAM, benzohydroxamate; S13, 5-chloro-3-*t*-butyl-2'-chloro-4'-nitrosalicylanilide; UQ, ubiquinone; UQ10, ubiquinone-10; UQH_2, ubiquinol; UQ10-H_2, ubiquinol-10.

with 15 mL of medium containing 0.4 M mannitol, 1 mM EDTA, 2 mM L-Cys, 0.2% BSA, 0.7% PVP-25, and 10 mM potassium phosphate buffer, pH 7.4. The homogenate was pressed through a double layer of cheesecloth and centrifuged at 1000*g* for 5 min at 4°C. Centrifuge and washing steps were performed as described by Van Emmerik et al. (1992). Potato tuber mitochondria were isolated as described previously (Hemrika-Wagner et al., 1982). Sweet potato mitochondria were isolated according to the method of Van den Bergen et al. (1991).

Measurements of Respiration

Respiration of cells and mitochondria was measured at 25°C using a Yellow Springs Instrument (Yellow Springs, OH) oxygen monitor model 53 and a 5-mL (cells) or 1-mL (mitochondria) reaction vessel. Mitochondrial respiration was measured in a reaction medium containing 0.4 M mannitol, 10 mM potassium phosphate buffer, 0.2% BSA, pH 7.1, with succinate (20 mM), malate (20 mM), NADH (2 mM), or a mixture of those three as substrates. Pyruvate was added in a final concentration of 5 mM. For state 3 measurements 0.1 mM ADP was supplied. Respiration of cells was measured by transferring 5 mL of the cell suspension to the reaction vessel. The alternative pathway was inhibited with BHAM (2 and 13 mM for mitochondria and cells, respectively), and the Cyt pathway was inhibited with KCN (0.1 mM in mitochondria) or azide (0.35 mM in cells). Uncoupler S13 (kindly donated by Dr. P.C. Hamm, Monsanto, St. Louis, MO) was supplied in a final concentration of 0.4 μM (cells and mitochondria). Mitochondrial protein was determined by the Bradford (1976) method.

UQ Extractions

Mitochondria (1 mg of protein) were added to 1 mL of reaction medium. After the required substrates and/or inhibitors were added, the oxygen uptake rate was recorded until a steady state was reached. The reaction mixture was removed from the vessel with a syringe filled with 3 mL of 0.2 M $HClO_4$ in methanol (0°C). The mixture was then rapidly transferred to a tube with 3 mL of petroleum ether (boiling point 40–60°C) and vortexed for 1 min. After the mixture was centrifuged (1500*g*, 2 min) the upper petroleum ether phase was removed, transferred to a test tube, and evaporated to dryness under a flow of nitrogen. Another 3 mL of petroleum ether was added to the lower phases, and the vortex and centrifugation steps were repeated. The upper phase was added to the one previously obtained. After evaporation extracts could be stored for at least 1 d under nitrogen at −20°C. Immediately before use, the extracted UQ/ubiquinol was resuspended with a glass rod in 100 μL of nitrogen-purged ethanol and analyzed in an HPLC (a Gilson [Villiers le Bel, France] pump system with a Perkin-Elmer model 811 detector) with a reversed-phase Lichrosorb (Chrompack, Bergen op Zoom, The Netherlands) 10 RP 18 column (size 4.6 mm, internal diameter, × 250 mm). The column was equilibrated with nitrogen-purged ethanol:methanol (3:2, v/v), and this mixture was used as the mobile phase. The flow rate was 1 mL/min. Detection of the quinones was performed at 290 nm. The amounts of UQ10 and UQ10-H_2 were calculated from the peak areas (as determined by weighing). Commercially obtained UQ10 (Sigma) was used as a standard. Calibration of the peaks was obtained with an oxidized and a reduced extract. An oxidized extract was obtained when the extract was evaporated to dryness under reduced pressure by rotary evaporation (during evaporation and ventilation the extract is completely oxidized), and a reduced extract was obtained when the mitochondria (or the standard UQ10) were reduced with dithionite before extraction. Methanol, ethanol, and petroleum ether were of analytical grade.

Whole-cell extracts from *P. hybrida* cell suspensions were obtained by rapidly separating approximately 5 g of cells, from which the steady-state oxygen uptake was measured, from the culture medium with a funnel and cheesecloth and transferring the cells to a mortar filled with liquid nitrogen. Frozen cells were ground to a fine powder, and 10 mL of 0.2 M $HClO_4$ in methanol (0°C) was added. From that step on the procedure followed the one described for the mitochondria with one exception. Instead of 3-mL aliquots, 10 mL of petroleum ether were added. For potato tuber and sweet potato, the same procedure as for *P. hybrida* cells was used with the exception that 20 g fresh weight were used, which were ground in 20 mL of 0.2 mM $HClO_4$ and mixed with 20 mL of petroleum ether.

Cyt *c* Oxidase Determinations

Cells were ground with a mortar and pestle first in liquid nitrogen and, after thawing, in 0.1 M potassium phosphate buffer (pH 7.2) and supplemented with 0.1% Triton X-100. Mitochondria were diluted 5-fold in 0.1 M potassium phosphate buffer (pH 7.4) with 0.1% Triton X-100. Both preparations were centrifuged at 3000*g* for 10 min. The supernatants were used for the spectrophotometrical assay. Cyt *c* oxidase activity (EC 1.9.3.1) was measured at 550 nm in the presence of 0.03 mM reduced Cyt *c* in 10 mM phosphate buffer (pH 7.2). Cyt *c* (from horse heart, Boehringer-Mannheim) was reduced with sodium dithionite, and excess dithionite was removed by applying the Cyt *c*/sodium dithionite mixture to a Dowex column (type 1 × 8, mesh width 100–200 openings per inch) and eluting with distilled water. Activities (measured at 25°C) were calculated as the first-order rate constant k (g^{-1} fresh weight min^{-1}) (Van Emmerik et al., 1994).

Determination of Pyruvate Concentration in the Cells

Cells were ground with a mortar and pestle first in liquid nitrogen and, after thawing, in 0.67 N $HClO_4$ in 0.01 M potassium phosphate buffer. After the sample was centrifuged for 5 min at 10,000*g*, the supernatant was adjusted to pH 3.5 with 10 N KOH, and another centrifugation precipitated the $KClO_4$ that was formed. The resulting supernatant was used as the extract for the determination of pyruvate. The reaction mixture contained 0.4 M triethanolamine (pH 7.6), 0.02 M EDTA, 0.15 mM NADH, and 1 mL of extract in a total volume of 3 mL (Brinkman and Van der

Meer, 1975). Pyruvate content was measured via the extinction changes at 340 nm after the addition of 10 μL of lactate dehydrogenase (specific activity 500 units mg^{-1}, Boehringer Mannheim).

RESULTS AND DISCUSSION

UQ Extractions from Intact Tissues

A first prerequisite for the successful determination of UQ reduction levels in vivo is that in addition to mitochondrial UQ/ubiquinol no other products are extracted that conceal the UQ peaks in the HPLC chromatogram. Schindler et al. (1984), who extracted UQ from a number of plant tissues, stated that a purification step by adsorption HPLC is necessary to separate UQ from other prenylquinones. Such a step would be extremely undesirable, because of the increased risk of oxidation of the ubiquinol during this extra step. As shown in Figure 1, A and B, it is apparently not necessary to purify the extracts from white (dark grown) *P. hybrida* cells. The HPLC chromatogram (detection at 290 nm) shows that in cells, as in mitochondria isolated from these cells, the UQ and ubiquinol peaks are easily detected. Chromatography of commercially obtained UQ10 and UQ10-H_2 (obtained by reduction with dithionite) showed that the observed peaks are derived from UQ10 and UQ10-H_2, respectively (data not shown). When a known amount of UQ10 was added to petunia cells before extraction, a recovery of 85% was found (±7%, $n = 3$).

In potato tubers (Fig. 1, C and D) UQ and ubiquinol are also easily recognized in the whole-cell extract, with UQ10 again being the only UQ present, but here an additional peak is visible in the cell extract. The peak, however, does not interfere in the determination of the relative amounts of UQ and ubiquinol. Sweet potato extracts show UQ10 and UQ10-H_2 only as the major peaks (Fig. 1, E and F).

Although a purification step is apparently not necessary in these nongreen tissues, a leaf extract from *Silene vulgaris* revealed an enormous number of peaks that made it impossible to determine accurately the relative heights of the UQ/ubiquinol peaks (data not shown).

Yields of UQ Extracted from *P. hybrida* Cells and Isolated Mitochondria

To draw conclusions concerning the relationship between the reduction state and the respiratory rate in whole cells, it has to be established that all UQ that is isolated from the whole cells has a mitochondrial origin and exclusively represents the mitochondrial pool. Therefore, we compared the relative amount of UQ extracted from *P. hybrida* cells with the mitochondrial yield (based on Cyt oxidase activities in cells and isolated mitochondria).

In Table I, results of two experiments are shown that demonstrate that the mitochondrial yield is ±20% based both on Cyt oxidase activity and on the amount of UQ extracted. Therefore, it is likely that all of the UQ extracted from the cells originates from the mitochondria. Both in mitochondrial extracts and in whole-cell extracts Cyt *c* reduction was negligible in the presence of 0.1 mM KCN, indicating that nonenzymatic Cyt *c* oxidation did not occur (data not shown).

Manipulation of the in Vivo UQ Reduction Levels in *P. hybrida* Cells

In Table II, reduction states of UQ extracted from *P. hybrida* cells under various conditions are shown. Upon addition of azide, an inhibitor of the Cyt pathway, reduction of UQ extracted from intact cells increases from 58% in the control cells to 73%. When azide was combined with BHAM, an inhibitor of the alternative pathway, 77% reduction was found. In cells incubated anaerobically for 10 min,

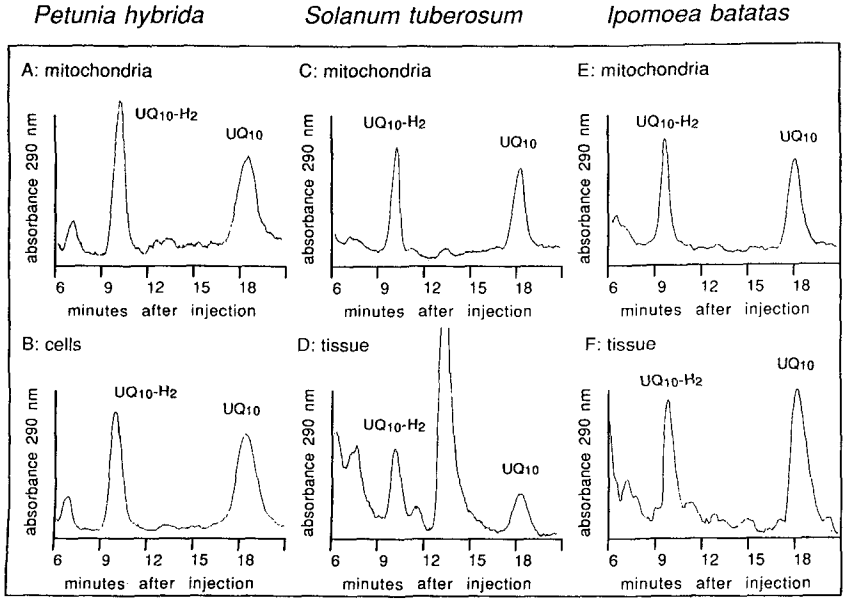

Figure 1. HPLC chromatograms of UQ and ubiquinol extracted from *P. hybrida* (A and B), potato tuber (C and D), and sweet potato (E and F). Extracts from cells (B, D, and F) are compared with extracts from mitochondria (A, C, and E). Extracts were prepared from approximately 1 mg of mitochondrial protein and approximately 5 g (fresh weight) of cells. A_{290} is in arbitrary units.

Table I. *Mitochondrial yield based on Cyt oxidase activities in P. hybrida cells and mitochondria isolated from these cells compared with the amount of extracted UQ (nmol/g fresh weight) in cells or mitochondria*

Data are from two separate experiments.

Experiment	Cyt *c* Oxidase			Total UQ Extracted		
	Cells	Mitochondria	Yield	Cells	Mitochondria	Yield
	k g^{-1}fresh wt min^{-1}		%	*nmol/g fresh wt*		%
1	39.1	8.6	22.0	0.91	0.22	24.2
2	26.3	5.5	20.9	1.30	0.27	20.8

a similar high UQ reduction level was observed. In cells that were killed by increasing the temperature to 80°C for 5 min, the UQ pool became oxidized. These results show that the reduction of UQ that was extracted from the whole cell varies with the treatment and, therefore, gives an indication of the in vivo situation.

UQ Reduction in *P. hybrida* Cells during a Batch Cycle

When studying whole-cell respiration, plant cell suspension cultures are an ideal tool, because they are homogeneous, consisting of undifferentiated cells, and oxygen uptake measurements and the effects of inhibitors are not confounded by diffusion problems. These cells go through a cycle of high supply of respiratory substrate at the beginning of the culture when the cells are diluted in fresh medium with excess Glc to a situation of Glc starvation. In *P. hybrida* cell suspensions, respiration varies by a factor of more than 2 in the various growth phases (Van Emmerik et al., 1992), which makes these cell cultures an ideal tool to investigate how these changing conditions are reflected in the reduction state of the UQ pool.

Figure 2A shows the well-known course of growth and sugar consumption during a batch cycle of *P. hybrida* cells. Growth starts immediately after subculturing and stops when the sugar is exhausted from the medium. An increase in respiration occurs following transfer of the cells to fresh medium. After 1 to 2 d respiration declines and returns to the level observed before inoculation (Fig. 2B).

During such a batch culture when in vivo UQ reduction levels are determined, the UQ pool appears to be about 60% reduced during the entire growth cycle, despite the large differences in respiration rate (Fig. 2B). Also, when sugar is exhausted from the medium (d 8) and substrate supply to the mitochondria can be expected to decrease, 60% of the UQ is still reduced. Only after a prolonged period of starvation does the reduction level decrease to about 25%. However, addition of inhibitors of the respiratory pathways (BHAM and azide) does not enhance these values (data not shown). Probably in these cells not all of the mitochondria are functioning (Couée et al., 1992), and dead cells might be present in the suspension.

To relate the 60% in vivo reduction of the UQ pool in whole cells to the energy state of the cells, a comparison has to be made of the relationship between respiratory rates and UQ reduction levels in isolated mitochondria from these cells. To do this, mitochondria from *P. hybrida* cells were isolated, and the relationship between UQ reduction and oxygen uptake rates was determined for respiration via the Cyt pathway (in the presence of BHAM and ADP, state 3) and the alternative pathway (in the presence of KCN). Respiratory rates were varied by additions of combinations of substrates.

In voltametric measurements in the absence of substrate, the UQ pool is taken to be 0% reduced (Dry et al., 1989). However, the UQ extraction procedure shows that, when *P. hybrida* mitochondria were incubated in the absence of any substrate, a 40% reduction of UQ was found (Fig. 3). Such nonoxidizable pools have been observed before in plant mitochondria (Van den Bergen et al., 1994; Ribas-Carbo et al., 1995) as well as in animal mitochondria (Jørgensen et al., 1985), but the function is not yet clear. Cyt respiration increases with increasing UQ reduction levels, but in the presence of KCN, oxygen uptake is always accompanied by very high UQ reduction levels (Fig. 3). Whereas in isolated mitochondria levels up to almost 100% can be reached in the presence of KCN, in vivo reduction levels do not exceed ±80% (Table II). Incomplete inhibition of the respiratory inhibitors in vivo may explain this difference, but also when the cells have been kept under anaerobiosis before extraction, reduction levels do not reach higher values. When whole-cell extractions are performed in the presence of dithionite, almost 100% reduc-

Table II. *Oxygen uptake rates (μmol O_2 h^{-1} g^{-1} dry weight, measured at 25°C) and UQ reduction levels (reduced UQ [UQ_{red}] as percentage of total UQ) in P. hybrida cell suspensions in the exponential growth phase (values obtained during d 4, 5, and 6 of batch culture)*

Data are mean values ± SD of separate determinations. The number of determinations is shown in parentheses. n.d., Not determined.

Addition	O_2 Uptake	Percent UQ_{red}
Control	145 ± 17.0 (7)	58 ± 6.1 (6)
0.35 mM azide	137 ± 11.2 (4)	73 ± 1.9 (3)
0.35 mM azide + 13 mM BHAM	31 ± 9.1 (6)	77 ± 2.9 (4)
10 min anaerobiosis	n.d.	75 ± 1.5 (3)
5 min 80°C	0 (2)	17 (2)

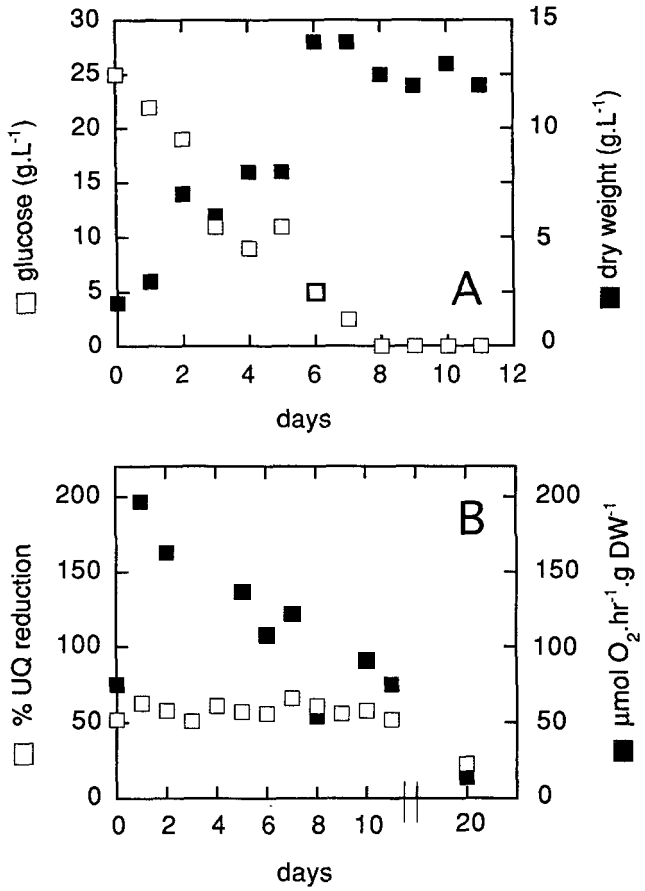

Figure 2. A, Growth (■, expressed as dry weight) and Glc concentration (□) of *P. hybrida* cells during a batch cycle. B, Total respiration (■, in µmol oxygen h^{-1} g^{-1} dry weight [DW]) and reduction of UQ (□, expressed as a percentage of UQ reduced of total UQ present) extracted from *P. hybrida* cells in batch culture. Each point is the mean value of one to three separate experiments.

tion is obtained (data not shown). There has been speculation concerning the role of distinct ubiquinol pools as antioxidants in the mitochondria (Landi et al., 1984), but such a hypothesis does not explain the discrepancy in size of this redox-inactive UQ pool in mitochondria and whole cells.

Based on the mitochondrial data, one would expect that in whole cells, in which UQ reduction levels remain approximately 60%, the alternative oxidase does not contribute significantly to oxygen uptake. Although this is the case during the late linear and stationary growth phase, some inhibition by added hydroxamates is generally observed during the first 2 d after inoculation, a result that at first sight is incompatible with the mitochondrial data (Van Emmerik et al., 1992).

Even more confusing in this respect are the data presented in Table III. In *P. hybrida* cells, as described before in other tissues (Lambers, 1985), a large increase in total respiration is observed upon addition of uncoupler to the cells. This increase is caused by an increase in Cyt pathway activity, but the alternative pathway also contributes to total uncoupled oxygen uptake (Table III). At first sight, this is easily explained. The effect of uncoupler addition in vivo is not only the relief of adenylate control but, also connected with that event, an acceleration of the glycolytic rate (so-called "Pasteur effect"), resulting in vivo in an increased substrate supply that may be high enough to increase UQ reduction up to levels at which the alternative pathway becomes engaged. To our surprise, however, UQ reduction levels in the presence of uncoupler were not different from the levels observed in the absence of uncoupler, despite the large engagement of the alternative pathway (Table III).

These results can only be explained by the assumption that upon addition of uncoupler in vivo the alternative pathway becomes engaged at lower UQ reduction levels than in the absence of uncoupler. In other words, the kinetics of the alternative oxidase toward reduced UQ has changed. Organic acids and especially pyruvate play a role in these changes (Umbach et al., 1994), and accumulation of pyruvate in the presence of uncoupler has been reported (Wiskich and Dry, 1985). Therefore, we measured pyruvate levels in these *P. hybrida* cells and, indeed, an increase was found when the cells were incubated in the presence of uncoupler: control cells (exponential growth phase) contained 100 nmol of pyruvate g^{-1} fresh weight (±9.7, $n = 3$), whereas after incubation in the presence of 0.4 µM S13 for 20 min, pyruvate content in these cells was increased to 166 nmol g^{-1} fresh weight (±33.8, $n = 3$).

In mitochondria isolated from these cells, organic acids stimulate only the alternative oxidase activity when NADH is the substrate; the addition of pyruvate, which is a very poor substrate itself, doubles NADH oxidation via the alternative pathway (Fig. 3), but no effect on succinate oxidation was visible (data not shown). Even in the presence of organic acids, substantial alternative pathway ac-

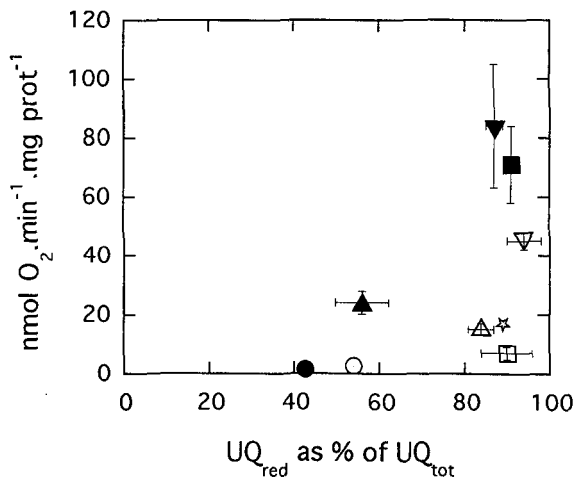

Figure 3. Relationship between UQ reduction and respiratory rates of mitochondria isolated from *P. hybrida* cells. Cyt respiration (in the presence of BHAM and ADP, closed symbols) or respiration in the presence of KCN (open symbols) was measured with the following substrates: ○, ●, no substrate; △, ▲, succinate; □, ■, NADH; ☆, NADH plus pyruvate; ▽, ▼, NADH plus succinate plus malate. Each point is the mean value of two to five separate experiments. Bars represent SD.

Table III. *Oxygen uptake rates (μmol O_2 h^{-1} g^{-1} dry weight, measured at 25°C) and UQ reduction levels (reduced UQ [UQ_{red}] as percent of total UQ) in P. hybrida cell suspensions in the exponential growth phase (values obtained at d 5–7 of batch culture)*

Data are given as mean values ± SD of two to seven separate determinations. The number of determinations is shown in parentheses.

Condition	O_2 Uptake	% UQ_{red}
Control	167 ± 29 (7)	61 ± 6 (5)
13 mM BHAM	169 ± 22 (6)	58 ± 2 (4)
0.4 μM S13	290 ± 28 (7)	60 ± 2 (5)
13 mM BHAM + 0.4 μM S13	228 ± 21 (4)	59 ± 1 (3)

tivity in isolated mitochondria is observed only at UQ reduction levels above 80%, and the addition of uncoupler did not affect the relation between UQ reduction and KCN-resistant respiratory rates (data not shown).

In vivo, however, in the presence of uncoupler, considerable alternative pathway activity could be observed at 60% reduction (Table III). Immunoblotting of the alternative oxidase from isolated *P. hybrida* mitochondria reveals that a considerable amount of the enzyme is in the oxidized, low-activity state (A. Wagner and M. Wagner, unpublished data). Umbach et al. (1994) reported that organic acids acted only on the activated form of the oxidase. If one assumes that in vivo the oxidase might be more reduced than in isolated mitochondria, this explains the discrepancy between the in vitro (mitochondria) and in vivo (cells) kinetics. In accordance with this assumption is the observation that in these mitochondria KCN resistance is rather low (less than 50%), whereas total cell respiration is almost 100% resistant to Cyt pathway inhibitors (Table II). All of these observations lead to the important conclusion that the kinetics properties of the alternative oxidase in isolated mitochondria might not necessarily represent the situation in vivo. It would be very interesting to determine the activation state of the alternative oxidase directly, without the step of isolating mitochondria, during which the in vivo activation state might change.

Addition of pyruvate to intact suspension cells in the exponential growth phase did not change the respiratory rates, and no inhibition by hydroxamates was observed (data not shown). Apart from the question of whether pyruvate is sufficiently taken up by the cells, a lack of inhibition does not necessarily indicate a lack of engagement of the alternative pathway. Because the Cyt pathway may not be saturated when engagement of the alternative pathway takes place, addition of BHAM may simply divert electrons from one pathway to the other (Umbach et al., 1994; Van den Bergen et al., 1994). It is clear that the classical "Bahr and Bonner" titrations, still widely used in determining the extent of engagement of the alternative pathway, may not give a correct indication of the engagement of the alternative pathway, because they are based on the assumption that the Cyt pathway is fully saturated when the alternative pathway is engaged. It is essential that new procedures be developed to estimate the contribution of the alternative pathway in plant respiration, which is necessary for calculations of energy yield. The measurement of the differential oxygen isotope discrimination between Cyt oxidase and the alternative oxidase (Guy et al., 1989; Robinson et al., 1992) seems to be a promising approach.

The data presented in this paper show that in *P. hybrida* cell suspensions the UQ reduction levels in vivo are maintained at a rather stable level and never exceed 60%. In this respect, it is crucial to know how accurately these levels can be determined. When duplicate extractions are performed from cells in one batch culture, the variation in reduction levels is less than 5%. There may be small differences under various conditions, but whereas in vitro (isolated mitochondria) engagement of the alternative pathway always coincides with high (>80%) UQ reduction levels, in vivo engagement can be observed at lower reduction levels (60%), which apparently is achieved by a change in the kinetics of the alternative oxidase in which thiol modifications and accumulating organic acids may play a crucial role.

The physiological significance of such a regulation mechanism might be that the alternative pathway helps to maintain UQ reduction at a stable level. Purvis and Shewfelt (1993) suggested that high UQ reduction levels would favor the production of oxygen-derived free radicals. Activation of the alternative pathway in situations of excessive substrate supply to the mitochondria (as in the presence of uncoupler) would help to prevent such dangerous situations.

How organic acids act on the alternative oxidase and especially what the in vivo relation is with the redox state of the disulfide bonds of the alternative oxidase will be a subject of further studies.

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The authors wish to dedicate this paper to the late Dr. W.M. Wagner.

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LITERATURE CITED

Bahr JT, Bonner WD (1973a) Cyanide-insensitive respiration. I. The steady state of skunk cabbage spadix and bean hypocotyl mitochondria. J Biol Chem **248:** 3441–3445

Bahr JT, Bonner WD (1973b) Cyanide-insensitive respiration. II. Control of the alternative pathway. J Biol Chem **248:** 3446–3450

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brinkman FG, Van der Meer LJ (1975) Dehydrogenases in the potato tuber (*Solanum tuberosum*). Identity, coenzyme-specificity and isoenzyme composition of malic enzyme, malate dehydrogenase and lactate dehydrogenase. Z Pflanzenphysiol **75:** 322–331

Couée I, Jan M, Carde JP, Brouquisse R, Raymond P, Pradet A (1992) Effects of glucose starvation on mitochondrial subpopulations in the meristemic and submeristemic regions of maize root. Plant Physiol **100:** 1891–1900

Day DA, Dry IB, Soole KL, Wiskich JT, Moore AL (1991) Regulation of alternative pathway activity in plant mitochondria. Deviations from Q-pool behavior during oxidation of NADH and quinols. Plant Physiol **95:** 948–953

Dry IB, Moore AL, Day DA, Wiskich JT (1989) Regulation of alternative pathway activity in plant mitochondria: nonlinear relationships between electron flux and the redox poise of the quinone pool. Arch Biochem Biophys **273:** 148–157

Guy RD, Berry JA, Fogel ML, Hoering TC (1989) Differential fractionation of oxygen isotopes by cyanide-resistant and cyanide-sensitive respiration in plants. Planta **177:** 483–491

Hemrika-Wagner AM, Kreuk KCM, Van der Plas LHW (1982) Influence of growth temperature on respiratory characteristics of mitochondria from callus-forming potato tuber discs. Plant Physiol **70:** 602–605

Jørgensen BM, Rasmussen HN, Rasmussen UF (1985) Ubiquinone reduction pattern in pigeon heart mitochondria. Identification of three distinct ubiquinone pools. Biochem J **229:** 621–629

Lambers H (1985) Respiration in intact plants and tissues: its regulation and dependence on environmental factors, metabolism and invaded organisms. *In* R Douce, DA Day eds, Higher Plant Cell Respiration, Encyclopedia of Plant Physiology, New Series, Vol 18. Springer Verlag, Berlin, pp 418–473

Landi L, Cabrini L, Sechi AM, Pasquali P (1984) Antioxidative effects of ubiquinones on mitochondrial membranes. Biochem J **222:** 463–466

Lidén AC, Åkerlund HE (1993) Induction and activation of the alternative oxidase of potato tuber mitochondria. Physiol Plant **87:** 134–141

Millar AH, Wiskich JT, Whelan J, Day DA (1993) Organic acid activation of the alternative oxidase of plant mitochondria. FEBS Lett **329:** 259–262

Moore AL, Dry IB, Wiskich JT (1988) Measurements of the redox state of the ubiquinone pool in plant mitochondria. FEBS Lett **235:** 76–80

Purvis AC, Shewfelt RL (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? Physiol Plant **88:** 712–718

Ribas-Carbo M, Wiskich JT, Berry JA, Siedow JN (1995) Ubiquinone redox behavior in plant mitochondria during electron transport. Arch Biochem Biophys **317:** 156–160

Robinson SA, Yakir D, Ribas-Carbo M, Giles L, Osmond CB, Siedow JN, Berry JA (1992) Measurements of the engagement of cyanide-resistant respiration in the Crassulacean acid metabolism plant *Kalanchoë daigremontiana* with the use of on-line oxygen isotope discrimination. Plant Physiol **100:** 1087–1091

Schindler S, Lichtenthaler HK, Dizengremel P, Rustin P, Lance C (1984) Distribution and significance of different ubiquinone homologues in purified mitochondria and in intact plant tissue. *In* PA Siegenthaler, W Eichenberger, eds, Structure, Function and Metabolism of Plant Lipids. Elsevier, Amsterdam, The Netherlands, pp 267–272

Umbach AL, Siedow JN (1993) Covalent and noncovalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. Plant Physiol **103:** 845–854

Umbach AL, Wiskich JT, Siedow JN (1994) Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox state in soybean seedling mitochondria. FEBS Lett **348:** 181–184

Van den Bergen CWM, Krab K, Wagner AM, Van der Plas LHW (1991) Valinomycin: a very effective inhibitor of the cyanide-insensitive alternative pathway in plant mitochondria. Biochim Biophys Acta **1058:** 203–208

Van den Bergen CWM, Wagner AM, Krab K, Moore AL (1994) The relationship between electron flux and the redox poise of the quinone pool in plant mitochondria. Interplay between quinol-oxidizing and quinone-reducing pathways. Eur J Biochem **226:** 1071–1078

Van Emmerik WAM, Van der Plas LHW, Wagner AM (1994) Characteristics of the respiratory peak in batch cultures of *Petunia hybrida*. Physiol Plant **90:** 459–466

Van Emmerik WAM, Wagner AM, Van der Plas LHW (1992) A quantitative comparison of respiration in cells and mitochondria from *Petunia hybrida* suspension culture. J Plant Physiol **139:** 390–396

Wagner AM, Kraak MHS, Van Emmerik WAM, Van der Plas LHW (1989) Respiration of plant mitochondria with various substrates: alternative pathway with NADH and TCA cycle derived substrates. Plant Physiol Biochem **27:** 837–845

Wiskich JT, Dry IB (1985) The tricarboxylic acid cycle in plant mitochondria: its operation and regulation. *In* R Douce, DA Day eds, Higher Plant Cell Respiration, Encyclopedia of Plant Physiology, New Series, Vol 18. Springer Verlag, Berlin, pp 281–313