Kinetic analysis of the mitochondrial quinol-oxidizing enzymes during development of thermogenesis in *Arum maculatum* L

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The dependence of the rate of oxygen uptake upon the ubiquinone (Q)-pool reduction level in mitochondria isolated during the development of thermogenesis of *Arum maculatum* spadices has been investigated. At the α -stage of development, the respiratory rate was linearly dependent upon the reduction level of the Q-pool (Qr) both under state-3 and -4 conditions. Progression through the β/γ to the δ -stage resulted in a non-linear dependence of the state-4 rate on Qr. In the δ -stage of development, both state-3 and -4 respiratory rates were linearly dependent upon Qr due to a shift in the engagement of the alternative oxidase to lower levels of Qr. Western blot analysis revealed that increased alternative oxidase activity could be correlated with expression of a 35 kDa protein. Respiratory control was only observed with mitochondria in the α -stage of development. At the β/γ -stage of

development, the addition of ADP resulted in a significant oxidation of the Q-pool which was accompanied by a decrease in the respiratory rate. This was due either to decreased contribution of the alternative pathway to the overall respiratory rate under state 3 or by deactivation of succinate dehydrogenase activity by ADP. Cold-storage of the spadices at the β -stage of development led to increased activity of both the cytochrome pathway and succinate dehydrogenase, without any change in alternative oxidase activity. Results are discussed in terms of how changes in the activation level of the alternative oxidase and succinate dehydrogenase influence the activity and engagement of the quinol-oxidizing pathways during the development of thermogenesis in *A. maculatum*.

INTRODUCTION

It is well-established that plant mitochondria possess a branched respiratory chain due to the presence, in addition to cytochrome *c* oxidase, of a cyanide- and antimycin A-resistant alternative oxidase [1–5]. The alternative oxidase is an integral membrane protein, the active site of which is located on the matrix side of the inner membrane [3,4]. It branches from the main respiratory chain at the level of the ubiquinone (Q) pool, is non-protonmotive and its activity results in the net reduction of oxygen to water [1–5]. Recently, comparison of all known plant alternative oxidase sequences revealed a conserved double iron-binding motif similar to that found in di-iron-centre proteins such as methane mono-oxygenase [6,7]. A model of the active site of the alternative oxidase has been proposed, based upon the presence of these motifs, which is analogous to that observed in methane mono-oxygenase, suggesting that the alternative oxidase may be the newest member of the oxo-bridged di-iron-centre proteins [6,7].

The contribution of the alternative pathway to the overall rate of respiration can vary dramatically being very low in mitochondria isolated from potatoes while in mitochondria from thermogenic tissues such as *Arum maculatum* virtually all respiratory flux is via the alternative oxidase [1]. Electron transfer via the alternative oxidase depends on the extent to which the Q-pool is reduced and in mitochondria from non-thermogenic tissues, such as soybean cotyledons, significant activity is not observed until 35–40% of the quinone pool is reduced [8,9]. After this point activity increases disproportionately as a function of quinone reduction. Such non-linear dependence of the rate of electron transfer on the quinone reduction level has been

postulated to be due to engagement of the alternative pathway and a number of kinetic models have been proposed to account for such behaviour [1,9–12].

Additional factors that may also play a major role in regulating alternative pathway activity (other than the reduction state of the Q-pool), include the amount of alternative oxidase protein present, its redox status and degree of aggregation and total amount of mitochondrial Q [3–5]. In non-thermogenic tissues observed alternative pathway activity strongly correlates with the presence of a protein of molecular mass 35 kDa, whereas in thermogenic tissues, such as *Sauromatum guttatum*, the large amount of flux through the alternative oxidase correlates positively with the expression of proteins at 36 and 35 kDa [13,14]. The interconvertible state of the redox linkage at Cys-172 from oxidized to reduced is also considered to play a regulatory role *in vivo* since the reduced form of the enzyme is primarily associated with activity [15]. A number of carboxylic acids such as pyruvate [16], succinate and malate [17,18] can act as allosteric activators of the protein, lowering the quinone reduction value at which the alternative oxidase is engaged, possibly by altering the protein's affinity for quinol [19,20]. It is suggested that fluctuations in pyruvate levels during development may provide a fine-tuning mechanism for regulation of alternative oxidase activity *in vivo* [3–5,20].

In most developing plant systems the dependence of the respiratory rate (under state-4 conditions) on the redox poise of the Q-pool becomes distinctly non-linear upon the onset of alternative pathway activity [8,9,11,21]. However, in mitochondria isolated from thermogenic tissues the relationship between these two parameters is distinctly linear [9] even under state-4 conditions.

Abbreviations used: SHAM, salicylhydroxamic acid; Q, ubiquinone; Qr, reduced ubiquinone; Qt, total reducible ubiquinone; AOA, alternative oxidase all antibodies; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Vo, oxygen consumption.

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In an attempt to determine whether the kinetics of the quinol-oxidizing pathways are developmentally regulated we have investigated the kinetic properties of these pathways in *A. maculatum* mitochondria isolated at various stages of development of thermogenesis. The development of thermogenesis involves the spadices of *A. maculatum* passing through a recognizable sequence of developmental stages (which have been termed α - δ , see refs. [22] and [23]) which results in a rapid rise in respiration which ultimately is responsible for a period of heat production during pollination. At the α -stage of development, the respiratory rate under state-3 and -4 conditions was linearly dependent upon Q-redox levels since engagement of the alternative oxidase was not apparent until a substantial proportion of the Q-pool had been reduced. Progression through the β/γ -stage of development resulted in the state-4 rate becoming initially non-linear with respect to reduced ubiquinone (Qr) levels, as the alternative oxidase became engaged at lower Qr values. In the δ -stage of development, both state-3 and -4 respiratory rates were indistinguishable and linearly dependent upon Qr redox levels. Western blot analysis of mitochondrial samples at the same stages of development revealed that increased alternative oxidase activity could be correlated with expression of a 35 kDa protein. Cold storage of the spadices, prior to mitochondrial isolation, resulted in significant increases in respiratory activity for both pathways without any change in the protein expression pattern. Results are discussed in terms of the regulation of the quinone-reducing and quinol-oxidizing pathways during the development of thermogenesis in *A. maculatum*.

EXPERIMENTAL

Inflorescences of the spadix of *A. maculatum*, at varying stages of development including the α , β/γ and δ stages [22,23], were collected from plants growing in local Sussex woods and were used immediately for mitochondrial isolation following rinsing in cold distilled water. In the case of cold-storage, inflorescences were stored overnight at 4 °C prior to mitochondrial isolation.

Mitochondria from *A. maculatum* spadices were isolated and purified on Percoll gradients as described previously by Proudlove et al. [24] as modified by Moore et al. [25].

Changes in the steady-state redox poise of the Q-pool and the rate of oxygen consumption were continuously monitored voltammetrically in 2.2 ml of reaction medium containing 0.3 M mannitol, 1 mM MgCl₂, 5 mM KH₂PO₄, 10 mM KCl, and 20 mM Mops (pH 7.2) in a specially constructed cell (University of Sussex) housing a Rank oxygen electrode and glassy carbon and platinum electrodes connected to an Ag/AgCl reference electrode by an agar bridge as previously described [26].

Mitochondria (0.1–0.6 mg of protein) were incubated with 1 μ M ubiquinone-1 (Q-1) and respiration initiated by the addition of 5 mM succinate. Succinate dehydrogenase was activated either by the addition of 0.5 mM ATP or following a state-3/4 transition. Succinate oxidation was progressively inhibited by addition of aliquots of 1 M malonate either in the presence or absence of 2 μ M antimycin A. Alternative oxidase activity was inhibited by preincubating mitochondria with 1 mM salicylhydroxamic acid (SHAM) for 1–2 min prior to addition of Q-1. The initial addition of SHAM (which interacts with the electrode system) resulted in a full-scale deflection of the chart recorder. Once a steady-state reading had been achieved (1–2 min) an offset control was used to reset the recorder and the experiment initiated by the addition of Q-1 and succinate. Fully oxidized levels of the mitochondrial Q-pool were taken from the position of the trace following addition of Q-1 and mitochondrial protein

but prior to addition of substrate, whereas fully reduced levels were taken as the trace deflection following anaerobiosis.

Protein was estimated according to the procedure of Bradford [27] using BSA as standard.

One-dimensional SDS/PAGE gels were run as described by Laemmli [28]. Samples were denatured in sample buffer which excluded mercaptoethanol by heating for 5 min at 95 °C. Molecular-mass markers were obtained from Amersham International. The blotting procedure was as described by Harlow and Lane [29]. The monoclonal antibody AOA, raised against the alternative oxidase protein from *S. guttatum* [30], was used at a dilution of 1:14000 and the peroxidase-linked secondary antibody was used at a dilution of 1:1000. The enhanced chemiluminescent reagent system was used to detect the alternative oxidase proteins. Gels were scanned using a Pharmacia Image Master scanning densitometer.

All chemicals were of the highest purity commercially available. Q-1 was a gift from Dr. T. Wiggins (Zeneca plc, U.K.).

RESULTS

Figure 1 summarizes the dependency of the succinate-dependent respiratory rate on the redox poise of the Q-pool (fraction of reduced ubiquinone, Qr) in mitochondria isolated from *A. maculatum* spadices at varying stages of development. Mitochondria were isolated from the spadices at increasing stages of thermogenesis (α - δ) and malonate titrations were performed under state-3 and state-4 conditions and in the presence of antimycin A. In mitochondria isolated from young spadices (Figure 1A; α -stage) the respiratory rate is directly proportional to the redox state of the Q-pool under state-3 conditions. In state 4, a slight upward deviation from linearity is apparent probably due to engagement of the alternative oxidase since this pathway becomes engaged only at high reduction levels of the Q-pool (Qr $\sim$ 0.7), comparable with that observed with other plant mitochondria [11,21,26]. Figures 1(B) and 1(C) show that the kinetics of the oxidizing pathways change during the development of thermogenesis in *A. maculatum*. At the β/γ -stage (Figure 1B), the dependence of the rate of electron transport on the redox state of the Q-pool under state 3 is comparable with that observed in Figure 1(A). However, in the absence of ADP, the relationship between these two parameters is distinctly non-linear and it is apparent from Figure 1(B) that this is due to engagement of the alternative pathway at a lower Q-pool reduction level than observed in Figure 1(A) (see Figure 1B; $\circ$). Figure 1(C) shows that when *A. maculatum* mitochondria become highly cyanide-resistant, the state-3 and state-4 titration curves are both linear and indistinguishable from each other, a result supported by the finding that such mitochondria do not demonstrate respiratory control. At this stage of development of thermogenesis, the majority of respiratory flux is mediated via the alternative oxidase and in the presence of antimycin A the shape of the curve is virtually identical to that observed under state-3 or -4 conditions (Figure 1C). Such linear dependencies are reminiscent of the classical Q-pool behaviour observed by Kroger and Klingenberg [31,32] using mammalian submitochondrial particles and Moore et al. [26] with potato mitochondria (which lack an alternative oxidase).

Any non-linearity in the dependence of the state-4 respiratory rate on the redox poise of the Q-pool is generally attributed to engagement of the alternative pathway. Figure 2 illustrates that such an assumption is correct since pre-incubation with SHAM (which inhibits alternative pathway activity), prior to a malonate titration, results in a linear dependency between these two

Figure 1 Dependency of the state-3 and -4 respiratory rate upon the redox poise of the Q-pool in *A. maculatum* mitochondria

The redox state of the Q-pool was poised at various levels by the addition of aliquots of malonate (0–1.3 mM). Oxygen consumption (V_o) and Q_r/Q_t ratios were measured in the presence of 0.5 mM ATP and 5 mM succinate in mitochondria isolated from *A. maculatum* spadices at three stages of development. State 4 was measured following a state-3/4 transition and state 3 initiated by the addition of 1 mM ADP. Antimycin A was added to a final concentration of 3.4 mg/mg of mitochondrial protein. (A) α -stage; (B) β/γ -stage; (C) δ -stage.

parameters (Figure 2A). Previous attempts to titrate alternative pathway activity using the Q-electrode have been unsuccessful due to interaction between SHAM and the electrode. This was overcome by pre-incubation with the inhibitor prior to the addition of Q-1. At the β/γ -stage of development, dependency of the state-4 rate on Q_r is markedly non-linear when both oxidizing enzymes are operative (Figure 1B) but linear when only the cytochrome pathway is functioning (Figure 2A). Figure 2(B) also shows that progression of development of thermogenesis from the α - to the δ -stage results in a decrease in the value of Q_r at which significant alternative oxidase activity is observed (from $Q_r = 0.7$ in the α -stage to approx. 0.25–0.3 at the δ -stage). The increase in respiratory rate observed in the absence of malonate (the maximal rate at each stage) when progressing from the α - to the δ -stage is probably the direct consequence of the alternative oxidase kinetic curve shifting to lower values of Q_r , since

Figure 2 Dependency of the alternative and cytochrome pathway activity on the redox poise of the Q-pool

Malonate titrations were performed on mitochondria isolated at the α -stage, the β/γ -stage and the δ -stage in the presence of 5 mM succinate and 0.5 mM ATP and either (A) 1.35 mM SHAM or (B) antimycin A (3.4 mg of antimycin A/mg of mitochondrial protein).

succinate dehydrogenase activity would be expected to be higher at lower values of Q_r (see ref. [11]). Figure 2(A) indicates that developmental increases in engagement of the alternative pathway are not accompanied by any change in cytochrome pathway activity. Interestingly Figures 1(B) and 2 indicate that at the β/γ -stage of development the state-4 rate (in the absence of malonate) is higher than the state-3 rate. Although the data presented in Figure 2(B) show that alternative pathway activity contributes much more to the overall respiratory rate under state-4 conditions (because Q_r is higher in state 4), Figure 2(A) indicates that the kinetics of the quinol-oxidizing pathways cannot explain why a lower respiratory rate is observed as the Q-pool becomes more reduced. One possible explanation is that the kinetics of the reducing pathway (succinate dehydrogenase) differ between states 3 and 4.

Data in the literature suggest that the relationship between alternative pathway activity and the redox poise of the Q-pool can be dramatically altered by the presence of pyruvate [5,16,33]. In such cases there is a stimulation of alternative oxidase activity at low levels of Q_r similar to that observed in Figure 1 [19]. Table 1 shows the effect of pyruvate upon alternative pathway activity during development of *A. maculatum* spadix mitochondria. It is apparent from Table 1 that a marked increase in the rate of oxygen consumption, upon addition of pyruvate, is only observed at the α/β -stage of development (succinate respiration is stimulated by 32–36%, whereas respiration on external NADH is stimulated by 53–70%). There is no such large stimulation of respiratory activity with either succinate or NADH at the δ -stage of development. The data in Table 1 also show that stimulation

Table 1 The effect of pyruvate on alternative oxidase activity in mitochondria isolated at various stages of development of thermogenesis in *A. maculatum* spadices

Mitochondrial respiratory activity (V_o) and redox poise of the Q-pool (Q_r/Q_t) were measured, at two stages of development (α/β - and δ -stages), in the presence of 0.5 mM ATP and 3.4 mg of antimycin A/mg of mitochondrial protein following addition of either 5 mM succinate or 2 mM NADH. Pyruvate was added to a final concentration of 5 mM. Values of V_o are expressed in nmol of $O_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ and V_o and Q_r/Q_t are the averages of four separate experiments at the two stages of development of thermogenesis.

Stage	Addition	V_o (succinate)	Q_r/Q_t	V_o (NADH)	Q_r/Q_t
α/β	—	110	0.74	160	0.98
	Pyruvate	150	0.67	272	0.95
δ	—	279	0.26	917	0.95
	Pyruvate	279	0.26	917	0.89

Figure 3 Immunoblots of *A. maculatum* mitochondria using antibodies raised against the alternative oxidase

Lines indicate estimated molecular masses of the standards in kDa. Lanes α , β , β/γ , δ : refer to developmental stages of the spadices. The β -stage sample was isolated from tissue that had been cold-stressed overnight at 4 °C. Each lane was loaded with 14 μg of mitochondrial protein with reductant omitted from the sample preparation. (A) Immunoblot following a 30 min exposure and (B) following a 15 s exposure. Rates refer to succinate-dependent respiratory activity (in the presence of antimycin A) in nmol of $O_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$. The immunoblot illustrated in (B) was scanned in a densitometer and the values obtained (in arbitrary units and with background subtracted) for each lane for the upper (37 kDa) and lower band (35/36 kDa) are as indicated.

of the respiratory rate by pyruvate is only associated with a small decrease in the value of Q_r , much smaller than the shifts in the level of Q_r observed during the development of thermogenesis (Figures 1 and 2). It can therefore be concluded that although the rate of oxygen consumption by the alternative pathway can be stimulated, at specific stages of development of thermogenesis, by the addition of pyruvate this effect is not totally responsible for all of the changes observed in this report.

Figure 3 shows that when mitochondria, isolated at the various stages of development, were probed with alternative oxidase antibodies, the immunoblots indicated that not only is the alternative oxidase protein constitutively expressed at all stages but primarily exists in the reduced state (35 kDa corresponding to its active form [15]) with only a small proportion of the protein being in its oxidized (66 kDa being relatively non-active) state

Figure 4 Reversed respiratory control in *A. maculatum* mitochondria

Oxygen uptake and Q-reduction were measured in the presence of 5 mM succinate, 0.5 mM ATP, and either 1 mM ADP or 1 mM CCCP. The state-3 ($\blacktriangle$) and -4 ($\circ$) values obtained from the respiratory trace depicted in (A) were replotted on to the V_o/Q_r curve obtained in Figure 1(B) and shown as insert (B). The rates on the oxygen uptake trace are in nmol of $O_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ and on the Q_r trace as percentage of Q_t .

(Figure 3A). Figure 3(B) (decreased exposure time) reveals, however, that as the spadices mature, increases in alternative oxidase activity can be correlated not only with the developmental stage of thermogenesis but also, more significantly, with the increased appearance of a 35/36 kDa band. Densitometer measurements (Figure 3B) indicate that during progression from the α - to the δ -stage of development the 37 kDa band remains relatively constant whereas the 35/36 kDa band increases 20-fold.

Figure 4(A) shows a typical respiratory trace and steady-state changes in the redox poise of the Q-pool of mitochondria, isolated at the β/γ -stage of development, oxidizing succinate as a substrate. Upon addition of ADP, following prior activation of succinate dehydrogenase by ATP, there is a significant oxidation of the Q-pool (Q_r decreases from 0.54 to 0.37) which is accompanied by a decrease in the respiratory rate such that the state-4 rate is actually higher than the state-3 rate; this phenomenon we have termed 'reversed respiratory control'. When the respiratory rate and corresponding Q_r value are plotted on to the titration curves depicted in Figure 1(B), it can be seen that such values readily fit the titration curves (Figure 4B), demonstrating this result to be a genuine effect. It should be noted from Figure 4(B), however, that the ADP-dependent oxygen consumption (V_o) and Q_r values observed in Figure 4(A) do not fit on to the titration curve obtained in the presence of an uncoupler (Figure 4B). Similar results (reversed respiratory control) have also been observed with potato mitochondria which do not possess any

Figure 5 The effect of cold-storage on the dependence of the respiratory rate on the redox poise of the Q-pool

Malonate titration of succinate oxidation in mitochondria isolated at the β -stage of development in which the spadices are either unstressed (○) or cold-temperature-stressed (●) overnight at 4 °C. (A) State 4; (B) state 4 + antimycin A (3.4 mg of antimycin A/mg of mitochondrial protein) and (C) state 4 + 1 mM SHAM.

alternative oxidase activity (G. R. Leach and A. L. Moore, unpublished work) upon partial inhibition of succinate dehydrogenase activity to approx. 50% by the addition of malonate. Such results suggest that the phenomenon of reversed respiratory control depends on the properties of succinate dehydrogenase, whose activity and characteristics are dependent upon the tissue used for mitochondrial isolation.

It is well-documented that alternative pathway activity, in some plant species, can be induced by exposure to low temperatures [34]. Figure 5 (β -stage) shows that when mitochondria are isolated from spadices that have been stored at low temperature (overnight) there is a considerable increase in the overall rate of V_o particularly in the presence of antimycin A. Figure 5(A) shows that there is a dramatic change in state-4 kinetics, resulting in higher rates of respiration at all values of Q_r . However, when the cytochrome pathway (Figure 5B) is inhibited by antimycin A it is very apparent from Figure 5(B) that the kinetics of the alternative pathway are not affected by

cold treatment (the kinetic curves overlap). When the alternative oxidase activity is inhibited by SHAM (Figure 5C), however, cytochrome pathway activity is increased following cold-shock treatment (the kinetic curves shift to lower values of Q_r). Such results strongly suggest that cold treatment increases both cytochrome pathway (probably by increasing enzyme amounts) and succinate dehydrogenase activity. It is not considered to be a consequence of uncoupling since the state-3 rates also increase after cold treatment (from 150 to 460 nmol of $O_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$). Interestingly the only component that is not affected by such cold treatment is the alternative oxidase itself. The increased oxygen uptake rates observed in Figure 5(B) being solely explained on the basis of an increase in succinate dehydrogenase activity. This conclusion is strengthened by the immunoblots depicted in Figure 3(B), which reveal that there is no major increase in alternative oxidase protein in the cold-shocked samples (β -stage) and furthermore protein levels follow the overall pattern of development of thermogenesis as the level of expression of the alternative oxidase (following cold treatment) is intermediate between the α - and γ -stages.

DISCUSSION

In the present paper we have investigated the relationship between the kinetics of the mitochondrial quinol-oxidizing enzymes and the development of thermogenesis of *A. maculatum* spadices. In the early stages of development (α -stage), the alternative oxidase is present but active only at high values of Q_r . This results in a very slight contribution to the overall respiratory rate even under state-4 conditions, and hence it is very difficult to detect deviations from linearity, even in state 4 (Figure 1A). At later stages of development (β/γ -stages) the kinetic properties of the alternative oxidase change such that its apparent affinity for quinol is increased; as a result the alternative oxidase is engaged at lower reduction levels of Q_r and the dependency of the respiratory rate upon Q_r becomes markedly non-linear under state-4 conditions (Figure 1B). Finally in the δ -stage the apparent affinity for quinol is increased such that the alternative oxidase is active at the lowest Q_r range and thus its activity dominates oxygen uptake both in states 3 and 4 (Figure 1C). This results in a linear kinetic relationship between oxygen uptake and Q_r in both respiratory states.

Titration in the presence of SHAM (Figure 2A) indicate that the kinetic properties of the cytochrome pathway do not change during development. Furthermore Figure 2B reveals that the change in kinetics is due not only to an increase in alternative pathway activity [from 130 (α -stage) to 200 nmol $O_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (δ -stage)] but more importantly to changes in the level of Q_r at which the alternative oxidase becomes engaged (from 0.8 to 0.5 during the α to δ transition). Additionally the titrations depicted in Figures 1 and 2 show that during the progression from the α - to the δ -stage alternative oxidase activity becomes an increasingly major contributor to the overall respiratory rate not only under state-4 conditions (from 7 to 62%) but also under state-3 conditions (from 0 to 67%). Because of the clear correlation between the non-linearity of the state-4 kinetics with the shifting of the alternative oxidase kinetics to lower Q_r , and because of the linear kinetics observed in all stages in the presence of SHAM, it follows that the non-linearity observed in Figure 1(B), and seen with other plant mitochondria when both pathways are operative [1–5], is indeed due to engagement of the alternative oxidase at high Q_r reduction levels. In mitochondria isolated at the δ -stage (Figure 1C) virtually all of the observed flux is via the alternative pathway (Figure 2B) and under these conditions respiratory activity is relatively insensitive to ADP and antimycin A.

According to the kinetic models [1,8–12], increased alternative pathway activity at lower Q_r values can be accounted for by an apparent increased affinity of the alternative oxidase for quinol or a decreased affinity for quinone.

It has recently been postulated that pyruvate acts as an allosteric effector of the alternative oxidase [5,16], primarily by increasing the apparent affinity of the reduced form of the alternative oxidase for ubiquinol [20,33]. Table 1 shows that only in the earlier stages of development (α - to γ -stages) is there a major stimulation of alternative oxidase activity upon the addition of pyruvate. Despite the respiratory stimulation being considerable (70% in the case of NADH) it is only associated with a small oxidation of the Q-pool. At the δ -stage of development, the addition of pyruvate has no effect either on the respiratory rate or the Q redox poise. Perhaps endogenous pyruvate levels are higher in the later stages of thermogenesis and hence the addition of pyruvate at this stage of development does not result in any noticeable stimulation of respiration. Furthermore the decreased Q_r value at which the alternative oxidase is engaged may be due to an increase in pyruvate levels during development. If the increases in alternative pathway activity were, however, solely due to pyruvate acting as an allosteric effector then its addition at the α -stage should result in similar respiratory rates and Q_r values of engagement to that observed at the δ -stage. Since this is not the case additional factors need to be considered. A possible explanation for the observed changes in kinetics is a developmental-dependent change in the structure of the alternative oxidase resulting in the more favourable engagement of the oxidase at lower Q_r values. Figure 3 shows that during development there is a change in the type of reduced species expressed. The lower-molecular-mass form of the two species becomes more predominant and it is interesting to speculate that the increase in alternative pathway activity can be correlated with the appearance of this protein species.

Figure 4 shows that the addition of ADP to mitochondria isolated at the β/γ -stage of development results in an inhibition of the oxygen uptake rate and a net oxidation of the Q-pool in contrast to that normally observed either at the α -stage of development or in mitochondria isolated from non-thermogenic tissues (i.e. respiratory control). Figure 4(B) shows that the state-3 and -4 oxygen and Q_r values readily fit on to the titration curves depicted in Figure 1(B). A possible explanation for such an observation is that addition of ADP results in deactivation of succinate dehydrogenase [28]. As a result of this, the steady state is reached at lower Q_r , and at a lower rate of oxygen uptake. The actual change in rate depends on the change in cytochrome kinetics going from state 4 to state 3, the degree of inactivation of succinate dehydrogenase, and the precise shape of the kinetic curves. When Q_r shifts from the range where the alternative oxidase is active to the range where it is not (as can easily occur in β/γ -stage mitochondria), the relatively high state-4 rate is likely to be larger than the resulting state-3 rate. However, the phenomenon is primarily due to dehydrogenase inactivation and is not directly due to changes in alternative oxidase activity. Figure 4(B) supports this explanation since malonate titration of succinate oxidation, in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), results in a higher respiratory rate than that of states 3 and 4. In addition the rate in the presence of CCCP at any given Q reduction value is higher than that of the state-3 rate, suggesting that ADP is having an inhibitory effect upon succinate dehydrogenase. As previously indicated reversed respiratory control can also be observed in potato mitochondria when succinate dehydrogenase has been inhibited by approx. 50% ($Q_r/Q_t = 0.45$, where Q_t is the total reducible ubiquinone). In this case decreases in alternative

oxidase activity cannot account for the phenomenon as there is no detectable alternative pathway activity in potato mitochondria that have been isolated from fresh tubers, suggesting that an inhibition of the dehydrogenase by ADP is the most plausible explanation. Such results underline the importance of gaining a fuller understanding of the influence of development upon the activity of the reducing pathways and the effect such changes have on the rate of respiration (and hence energy production and carbon turnover).

The cold shock samples also fit the general pattern of development of thermogenesis yet the overall rate of oxygen consumption has nearly trebled (Figures 5A–5C). The shapes of the curves reveal that the apparent affinity of the alternative oxidase for quinol has not been altered but the increased activity of both cytochrome pathway and succinate dehydrogenase suggests that the overall turnover of the respiratory chain has been increased. Figure 3(B) (lane β) reveals that there is no major increase in the expression of the lower-molecular-mass species (35–36 kDa) when compared with that observed at the β/γ -stage. A comparison of Figures 1 and 2 with Figure 5 clearly shows that the changes in kinetics induced by cold treatment are not the same as those observed during development. It is interesting to speculate whether the increased activity of succinate dehydrogenase deduced from Figure 5(B) is due to activation of the dehydrogenase. Activation of succinate dehydrogenase upon cold-storage may either be the result of large increases in the levels of glutamate or decreases in the levels of oxaloacetate. Indeed a number of respiratory dehydrogenases, such as glucose-6-phosphate, lactate and isocitrate dehydrogenases, have been shown to increase in activity following exposure to low temperatures [35,36]. Obviously further experiments are required to substantiate such a suggestion. Nevertheless such results suggest it is becoming increasingly important to have kinetic information on both the oxidizing and the reducing enzymes and to consider the interplay between them in order to gain a fuller understanding of how respiratory activity is regulated in developing plant systems [7] and *in vivo*.

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