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Biochimica et Biophysica Acta 1188 (1994) 205–212

BBA Biochimica et Biophysica Acta

The reaction of the plant mitochondrial cyanide-resistant alternative oxidase with oxygen

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Received 24 January 1994; revised 31 May 1994

Abstract

The dependence of electron flux through the cyanide-resistant respiratory pathway on the redox poise of the ubiquinone pool and oxygen concentration was studied in purified mitochondria isolated from green and etiolated soybean (*Glycine max* L. Merr. cv. Ransom) cotyledons at different ages (4 and 10 days after planting), soybean roots and mung bean (*Vigna radiata* L.R. Vilcz) hypocotyls. In soybean, the K_m of the alternative oxidase with respect to oxygen was found to vary between values of 10 and 20 μM. These are generally higher than values of the K_m for oxygen of the alternative oxidase reported previously (0.5 to 2.0 μM). In addition, the value of the K_m for oxygen varied with the redox poise of the ubiquinone pool, measured voltametrically; the more reduced the quinone pool, the larger the observed K_m. These results are at variance with the behavior expected of the kinetic model developed by Siedow and Moore (1993; Biochim. Biophys. Acta 1142, 165–174) which predicts that the K_m for oxygen should decrease as the quinone pool becomes more reduced. A modified kinetic model is developed that incorporates an additional reaction step involving activation of the four-electron reduced oxidase into the earlier kinetic model. The modified model successfully simulates the dependence of the alternative oxidase activity on both ubiquinone pool redox poise and oxygen concentration.

Keywords: Alternative oxidase; Cyanide-resistant respiration; Oxygen dependence; Ubiquinone pool (Q-pool); Electron transfer

1. Introduction

The resistance of plant respiration to inhibition by cyanide has long been recognized and is associated with the operation of an alternative electron transport pathway to oxygen [1–3]. Storey initially demonstrated that the branch point of electrons from the main respiratory chain to the alternative pathway was at the level of the ubiquinone pool [4]. Since then several mechanistic models of the partitioning of electron flow between the main and alternative pathways have been proposed. Bahr and Bonner [5] initially suggested that the alternative pathway would only become engaged when electron flow through the cytochrome pathway was either saturated or inhibited. They attributed this to the redox potential of the alternative oxidase, which they suggested was less than that of the ubiquinone pool. De Troostemberg and Nyns [6], using the 'Q-pool' kinetic scheme of Kroger and Klingenberg [7], proposed a model where electrons were partitioned between both pathways, depending on the relative kinetic constants governing the oxidation of reduced ubiquinone (UQ_r) by each of the two quinol oxidases. This model, however, could not explain why there is often observed to be no apparent electron flux through the alternative pathway even when there is a high capacity of the pathway present [5].

The development of a voltametric technique that permits simultaneous measurement of the redox poise

Abbreviations: E_o, oxidized alternative oxidase; E_r, two-electron reduced alternative oxidase; E_{rr}, four-electron reduced alternative oxidase; E^*_{rr}, activated four-electron reduced alternative oxidase; E_t, total alternative oxidase; UQ, oxidized ubiquinone; UQ_r, reduced ubiquinone; Q_r, fractional reduction of the endogenous quinone pool; Q_t, total ubiquinone.

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SSDI 0005-2728(94)00117-0

of ubiquinone and the rate of oxygen uptake in isolated mitochondria [8] has led to a better understanding of the kinetics of electron transport between the ubiquinone pool and the alternative pathway. Dry et al. [9] used this technique to demonstrate that electron flux through the alternative pathway displayed a non-linear relationship with respect to the redox poise of the ubiquinone pool. Almost no activity was observed until the reduction state of the ubiquinone pool reached about 40%. At higher levels of ubiquinone reduction, the alternative pathway activity increased, but in a distinctly nonlinear fashion with respect to UQ_r. While the results of Dry et al. [9] were qualitatively consistent with the Bahr-Bonner concept of regulation of the alternative pathway, attempts to provide a quantitative fit of the data to a Bahr-Bonner kinetic model were unsuccessful. Recently, Siedow and Moore [3,10] have developed a kinetic model for the alternative oxidase based on the assumption that reaction with two molecules of reduced quinone would be needed to fully reduce the alternative oxidase. This two-step reduction model gave a reasonable fit to the relationship between electron transport through the alternative pathway and the redox poise of the ubiquinone pool [10]. This model also predicted that the affinity of the alternative oxidase for oxygen would depend on the redox state of the ubiquinone pool, such that the affinity for oxygen should decrease as the ubiquinone pool become more oxidized. This latter behavior was used to explain why the kinetic model did not completely fit the observed data at low values of UQ_r [10].

In this paper we report measurements of the K_m for oxygen of the alternative oxidase. We find that these values are higher (10 to 20 μM) than previously reported and that the affinity of the alternative oxidase for oxygen decreases as the ubiquinone pool becomes more reduced, the opposite of that predicted by the Siedow/Moore model. To accommodate these results, we have developed a modification of the two-step reduction model that incorporates an activation step of the fully reduced enzyme before it reacts with oxygen.

2. Materials and methods

2.1. Plant material

Seeds of soybean (*Glycine max* L. cv. Ransom) and mung bean (*Vigna radiata* L.) were treated with 10% Chlorox for 10 min and swelled in distilled water for 2 h with continuous bubbling of air.

For light-grown material, seeds were planted in a mixture of sand and perlite 1:1 and grown in a growth chamber at 27/23°C on a 14:10 h (L/D) regime at 1000 μmol photons $m^{-2} s^{-1}$. Plants were watered twice a day and the cotyledons harvested for mitochondrial purification at 4 or 10 days after planting, depending on the experiment.

For dark-grown tissue, soybean or mung bean seeds were planted in vermiculite and grown in the dark at 24°C. They were watered once a day and etiolated cotyledons (soybean) or hypocotyls (mung bean) were harvested for mitochondrial purification at 4 or 10 days after planting for soybean and at 10 days after planting for mung bean.

Root mitochondria were isolated from light-grown soybean plants at 10 days after planting.

2.2. Mitochondrial purification

Mitochondria, from green or etiolated soybean cotyledons, soybean roots or mung bean hypocotyls were purified on Percoll gradients following the procedure of Day et al. [11] with minor modifications [12].

2.3. Assay procedures

Oxygen consumption by isolated mitochondria was measured polarographically in a reaction medium containing 0.3 M sucrose, 5 mM KH_2PO_4, 10 mM TES, 10 mM KCl and 2 mM $MgSO_4$ (pH 7.2), either in a 2.1 ml reaction chamber housing a Rank Brothers oxygen electrode and a combination of glassy carbon, platinum and reference electrodes for the voltametric measurements of ubiquinone redox status [8] or in a 1.7 ml Gilson oxygen electrode chamber [12].

Mitochondria (0.1–0.5 mg protein) were incubated with 1.0 μM ubiquinone-1 and 0.15 mM ATP. Respiration was initiated with 5 mM succinate and succinate dehydrogenase was activated by a single state 3/state 4 transition, initiated with 150 μM ADP. The cytochrome pathway was subsequently inhibited with 2.0 μM myxothiazol. Successive inhibition of succinate dehydrogenase activity was obtained by addition of aliquots of 0.5 mM malonate [8]. Measurements of the cytochrome pathway were carried out as described above except that 1.0 mM salicylhydroxamic acid replaced myxothiazol in the reaction medium. The level of UQ_r was considered equal to 0 before succinate was added and was taken to be 1.0 after obtaining anaerobiosis [8]. Protein was estimated by the method of Lowry et al. [14] using BSA as a standard.

2.4. K_m Determination

For determination of the K_m for oxygen, alternative oxidase activity was initiated as described above, and rates of oxygen uptake were sequentially measured as the instantaneous slope of the oxygen electrode trace at different oxygen concentrations until all the oxygen was consumed. In order to measure the oxygen affinity of the alternative oxidase at different reduction states

of ubiquinone, a single addition of 1 mM or 4 mM malonate was made immediately after inhibition of the cytochrome pathway with 2.0 μM myxothiazol.

2.5. Immunoblotting

SDS-PAGE gels were modified from the system of Laemmli [18]. The procedure described by Berthold and Siedow [19] was followed without the inclusion of urea and using 100 mM DTT as the sample buffer reductant. Samples were denaturated in sample buffer by heating for 5 min at 90°C. High molecular mass standards from Sigma were used as molecular mass markers. Protein transfer from the SDS gels to nitrocellulose and subsequent incubation with antibodies was as described in Harlow and Lane [20]. The monoclonal antibody AOA, against the alternative oxidase protein from *Sauromatum guttatum* [21], was used at a dilution of 1:500. Visualization was with the ECL chemiluminescent reagent system (Amersham). For densitometry, films were scanned using the Stratagene Eagle Eye™ Still Video System and subsequently analyzed with NIH Image 1.45 software using 20-frame summing to reduce background.

2.6. Computer simulations

All computer simulations were carried out using the non-linear data regression analysis curve fitting program, ENZFITTER (R.J. Leatherbarrow, Elsevier BioSoft, Cambridge). The K_m for oxygen of the alternative pathway was obtained from the best fit of the oxygen dependence of alternative pathway activity to the standard Michaelis-Menten equation.

Simulations presented in Figs. 3 and 4 are generally not the statistical best fits achieved in each case, but the optimum fit for both sets of experiments: alternative pathway rate versus ubiquinone redox poise and versus oxygen concentration. The first kinetic constant obtained was k_4, which was directly calculated from the fit of the oxygen titration curve obtained following the addition of myxothiazol in the absence of malonate. This k_4 value was then held constant in subsequent simulations. After fixing k_4, a fit for the quinone titration curve was obtained and the resulting kinetic constants were used as initial estimates to obtain a fit for the oxygen titration. Successively, we alternated fits until a single set of kinetic constants was obtained that successfully simulated both sets of data. Once they were obtained, we used the kinetic model to predict the oxygen dependence at the Q_r values obtained with the voltametric technique at different malonate concentrations (Fig. 3, dotted lines). The oxygen concentration used for the simulations of the quinone titration was 240 μM and E_t was taken as a relative value with $E_t = 1.0$ arbitrarily set as the specific activity of the alternative oxidase from mitochondria purified from thermogenic *Arum maculatum* spadices (823 nmol O_2 (mg protein)$^{-1}$ min^{-1}) [10].

3. Results and discussion

3.1. Oxygen affinity of the alternative oxidase

With isolated mitochondria, the K_m for oxygen of the alternative oxidase has been reported to be quite low (0.5 to 2.0 μM) [22–24], although such studies are limited in number and go back a number of years. Moreover, no primary data of the K_m value of the alternative oxidase for oxygen using isolated mitochondria were presented in any of these reports. Such a low K_m for oxygen was used as a primary reason for suggesting that the alternative oxidase could not contain a flavoprotein [25]. Although the K_m value for oxygen of the alternative oxidase has been found to be much higher in intact tissues (10 to 25 μM), the difference has been attributed to diffusion [26] and substrate limitations [15].

Measurements of the K_m value of the alternative oxidase for oxygen in isolated soybean cotyledon mitochondria gave values that differed considerably from the values cited previously (Table 1). The K_m for oxygen varied between 10 μM in older (10-day-old)

Table 1
Oxygen uptake (Valt), oxygen affinity (K_m) and ubiquinone reduction state of the alternative pathway in isolated mitochondria

Source	Organ		Age (days)	Valt (nmol O_2 min^{-1} (mg protein)$^{-1}$)	K_m O_2 (μM)	Q_r
Soybean	Cotyledons	(G)	10	82	13.4	0.96
			4	45	21.8	0.77
		(E)	10	26	12.3	0.80
			4	55	18.2	0.67
	Roots	(G)	10	32	9.9	0.86
Mung bean	Hypocotyls	(E)	10	45	25.2	-

Mitochondria were isolated from 10 and 4-day-old green (G) and etiolated (E) soybean cotyledon, 10-day-old soybean roots and 10-day-old etiolated mung bean hypocotyls as described in Section 2.

cotyledons or roots and 20 μM for mitochondria isolated from young (4-day-old) cotyledons (Table 1). Mung bean hypocotyl mitochondria also showed a low affinity for oxygen (K_m = 25 μM), contrary to the value of 0.5 μM reported by Ikuma et al. [24].

At low oxygen concentrations, the oxygen electrode has the potential problem of oxygen leakage into the chamber. We employed several strategies to ensure that the observed values of the K_m for oxygen of the alternative oxidase were correct. First of all, we determined the leakage rate of the chamber at low oxygen concentrations by decreasing the oxygen concentration in the cuvette after purging with nitrogen. The rate of oxygen leakage into the chamber at 15 μM oxygen was found to be 1.0 nmol O_2 min^{-1}. This rate represents less than 5% of the rate of oxygen uptake by the mitochondria at the same oxygen concentration. If this leakage had a significant effect on the oxygen uptake rate at low oxygen, the measured K_m would vary depending on the mitochondrial oxygen uptake rate, the lower the rate the higher the observed K_m. To test for this, we varied the amount of added mitochondria over a 5-fold range to change the absolute rate of oxygen uptake in our assays, and we obtained the same K_m value in each case (data not shown). In addition, all the points associated with the K_m determinations gave linear Eadie-Hofstee plots, suggesting no problems existed relating to changes in substrate availability during the course of taking the measurements [15]. It is also well established that cytochrome *c* oxidase has a very low K_m for oxygen (0.1–0.15 μM) [16,17]. We compared K_m values for the cytochrome and alternative oxidases at similar absolute rates of oxygen uptake (Fig. 1A and B). We observed that the K_m for cytochrome oxidase was much lower than that for the

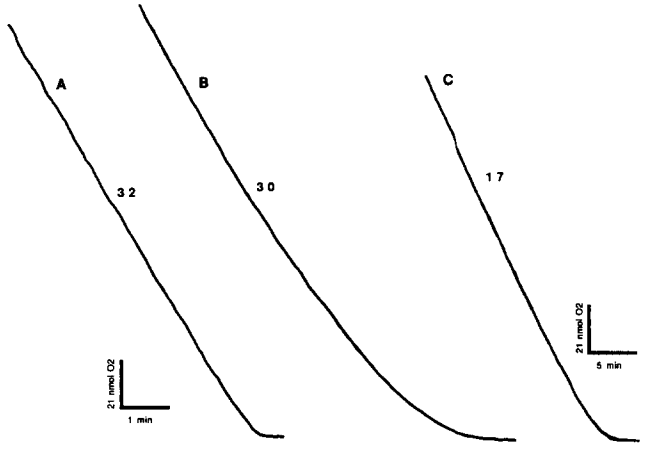

Fig. 1. Oxygen uptake traces for the cytochrome (A), and alternative (B) pathways in the absence of malonate, and the alternative pathway in the presence of 3.0 mM malonate (C) in mitochondria isolated from 10-day-old soybean roots. Rates are in nmol O_2 min^{-1} and were measured as described in Section 2. The amount of mitochondria used was 0.23, 1.15, and 0.58 mg protein, respectively.

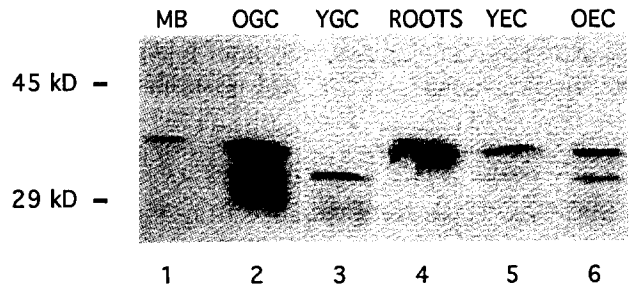

Fig. 2. Immunoblots of the alternative oxidase protein in isolated mitochondria. Blots were developed with the AOA monoclonal antibody and mitochondria isolated from: 1, 10-day-old mung bean hypocotyls; 2, 10-day-old green soybean cotyledons; 3, 4-day-old green soybean cotyledons; 4, 10-day-old soybean roots; 5, 4-day-old etiolated soybean cotyledons; 6, 10-day-old etiolated soybean cotyledons. The gel was loaded with 25 μg (lanes 1–4) and 35 μg (lanes 5–6) of protein. Molecular mass (kDa) markers are shown on the left.

alternative oxidase. Although we were not able to ascertain the exact value of the K_m for oxygen of cytochrome oxidase, the differences between the two oxidases were clear.

To further establish the validity of the O_2 electrode technique for measuring the oxygen K_m, the redox poise of the ubiquinone pool was measured using the voltametric method [8], and it was found to remain constant until just before the reaction reached anaerobiosis (data not shown). To avoid potential problems with changing concentrations of reduced ubiquinone, all rate measurements were taken above 5 μM oxygen, which is within the range over which the level of ubiquinone pool reduction remained unchanged. The constancy of the redox state of the ubiquinone pool over the course of the reaction is also consistent with the observation that linear transformations were obtained when the rate of oxygen uptake versus oxygen concentration was plotted using the method of Eadie-Hofstee [15]. Were the level of ubiquinone reduction changing appreciably during the course of the reaction, linear Eadie-Hofstee plots would not have resulted [15]. All these controls indicate that the oxygen electrode does provide a reliable technique for measuring oxygen K_ms under our experimental conditions.

Fig. 2 shows an immunoblot of mitochondria isolated from different tissues using the AOA monoclonal antibody [21] against the alternative oxidase. These immunoblots, in agreement with previously described ones using similar material [27,28], show two different molecular weight bands in mitochondria isolated from 10-day-old soybean cotyledons, from either green (Fig. 2, lane 2) or etiolated (Fig. 2, lane 6) tissue. Ten-day-old root (Fig. 2, lane 4) and mung bean hypocotyl mitochondria (Fig. 2, lane 1) show the presence of only the upper band. Likewise, only one band appears in 4-day-old soybean cotyledon mitochondria, but in this case, mitochondria from green and etiolated cotyledons show

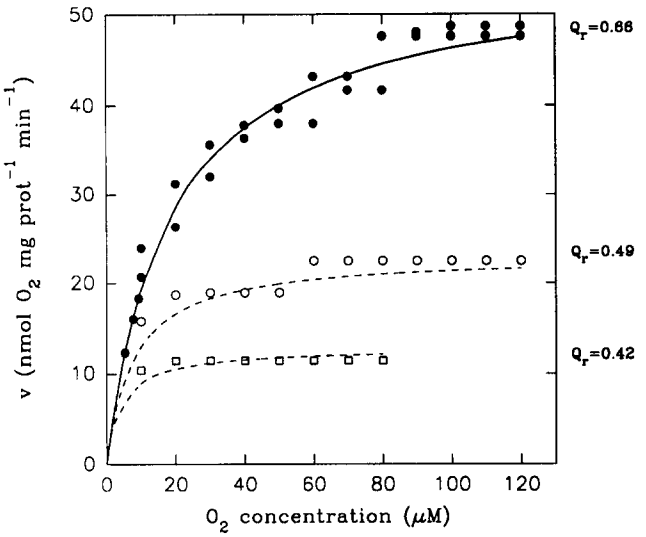

Fig. 3. The dependence of alternative pathway activity on oxygen concentration at different ubiquinone redox poises in isolated 4-day-old etiolated soybean cotyledon mitochondria. The alternative pathway activity was measured using succinate (5 mM) as an electron donor and different ubiquinone redox levels were obtained by addition of 0 mM, 1.0 mM or 4.0 mM malonate. The solid line represents a fit with the revised model (Eq. 8) using the following kinetic constants: $k_1 = 5704$, $k_{-1} = 79569$, $k_2 = 63689$, $k_{-2} = 45831$, $k_3 = 1092$, $k_4 = 45$, $Q_t = 0.665$ and $E_t = 0.066$.

different patterns. Young etiolated mitochondria have only the upper band (Fig. 2, lane 5) while young green mitochondria show only the lower band (Fig. 2, lane 3). A similar developmental pattern of expression of alternative oxidase molecular weight subspecies has been reported previously in soybean cotyledon mitochondria [27,28]. When the results in Fig. 2 are compared with the oxygen K_ms associated with mitochondria isolated from each of the tissues (Table 1), there does not appear to be any relationship between the particular molecular weight species present and the oxygen K_m. For example, young etiolated soybean cotyledon (K_m = 18.2 μM), 10-day-old soybean root (K_m = 10 μM) and mung bean hypocotyl (K_m = 25 μM) mitochondria each showed only the single high molecular weight subspecies but had different affinities for oxygen.

The observed affinity of the alternative oxidase for oxygen was also found to be dependent on the redox state of the ubiquinone pool. Figs. 1C and 3 show the effect of malonate, which inhibits succinate dehydrogenase, on the affinity of the alternative oxidase for oxygen. There is a clear relationship between Q_r and the affinity of the alternative oxidase for oxygen; as Q_r decreases, the value of the oxygen K_m also decreases (Fig. 3). The same relationship was found with mitochondria isolated from soybean roots and mung bean hypocotyls. Although the exact K_m values for oxygen obtained in the presence of malonate could not be accurately assessed with the oxygen electrode technique, differences between high (−malonate) and low (+malonate) K_ms are very clear (Fig. 3). The effect of malonate further indicates that oxygen leakage is not likely affecting the measured K_m value. One and 4.0 mM malonate inhibited the specific activity of the alternative pathway by 54 and 75%, respectively, but the K_m was observed to decrease markedly. This is the opposite of that expected if oxygen leakage were contributing significantly to the observed value of K_m. The effects of malonate also suggested that the variations in K_m observed with different mitochondria might be associated with differences in the extent of reduction of the ubiquinone pool. As shown in Table 1, differences in ubiquinone redox poise, measured voltammetrically, do not account for the K_m differences observed among the various mitochondrial types. However, another possible explanation for the variature in K_m could be the existence of different amounts of total ubiquinone in mitochondria isolated from different tissues and/or plant sources, since the actual substrate of the oxidase is reduced ubiquinone. Further experimentation would be needed to clarify this aspect.

Given limitations in the use of the voltametric technique with certain substrates and/or inhibitors [13], this relationship between oxygen K_m and the redox state of the ubiquinone pool may prove useful as a monitor of the quinone pool redox poise in isolated mitochondria under different experimental conditions. Given the marked dependence of the oxygen K_m of the alternative oxidase on the redox poise of the ubiquinone pool, it is also possible that the lower values of oxygen K_m reported previously [22–24] resulted from the mitochondrial succinate dehydrogenase not being maximally activated, leading to a lower level of reduction of the ubiquinone pool than we obtained. The dependence of the oxygen K_m of the alternative oxidase on the level of ubiquinone pool reduction also confounds in vivo measurements of the oxygen K_m, because the redox state of the ubiquinone pool may vary considerably depending upon the energetic state of the tissue under any specific set of conditions.

3.2. Alternative oxidase kinetic model

Application of the voltametric method for measuring the redox poise of the ubiquinone pool has facilitated kinetic studies of the engagement of the alternative pathway [8]. Using the relationship between quinone redox state and alternative pathway activity, Siedow and Moore developed a kinetic model for the regulation of electron flow to the alternative pathway which reflected the fact that the oxidase brings about a four-electron reduction of oxygen to water [3,10]. This model envisioned that the reduction of the alternative oxidase by reduced ubiquinone followed a two-step pathway, leading to a four-electron reduced enzyme

that subsequently reacted with molecular oxygen to give two molecules of water, as outlined below:

$$E_o + UQ_r \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_r + UQ \quad (1)$$

$$E_r + UQ_r \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_{rr} + UQ \quad (2)$$

$$E_{rr} + O_2 \xrightarrow{k_3} E_o + 2H_2O \quad (3)$$

UQ and UQ_r represent the oxidized and reduced forms of ubiquinone, respectively, and E_o, E_r and E_{rr} represent the oxidized, two-electron reduced and four-electron reduced forms of the alternative oxidase.

This model provided a better simulation of the observed dependence of alternative oxidase activity on the redox poise of ubiquinone than that provided by the original Bahr-Bonner model [3,10]. One testable prediction of this model was that the apparent K_m for oxygen should decrease as the ubiquinone pool becomes more reduced and the enzyme accumulates in the E_{rr} form. However, the observed behavior of the K_m of the alternative oxidase for oxygen is the opposite of that predicted; as Q_r decreases, so does the K_m for oxygen (Fig. 3).

In order to reconcile this discrepancy between the kinetic model and the observed data, it was necessary to modify the model. Taking the two-step reduction component of the model as being basically correct, the model of Siedow and Moore [10] was modified by inserting an additional reaction step following formation of the four-electron reduced enzyme (E_{rr}). This step involves the irreversible conversion of E_{rr} from an unreactive form to an activated form that can then react with oxygen (E_{rr}^*). The modified two-step kinetic scheme is described by the following sequence of reactions:

$$E_o + UQ_r \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_r + UQ \quad (4)$$

$$E_r + UQ_r \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_{rr} + UQ \quad (5)$$

$$E_{rr} \xrightarrow{k_3} E_{rr}^* \quad (6)$$

$$E_{rr}^* + O_2 \xrightarrow{k_4} E_o + 2H_2O \quad (7)$$

The steady-state rate equation for this reaction series is defined as:

$$v = \left\{k_1k_2k_3k_4O_2(Q_r)^2E_t\right\} / \left\{k_1k_2k_3Q_r^2 + k_4O_2 \times \left[k_1k_2Q_r^2 + Q_r(k_3(k_1 + k_2)) + (Q_t - Q_r) \times (k_1k_{-2}Q_r + k_{-1}k_3) + k_{-1}k_{-2}(Q_t - Q_r)^2\right]\right\} \quad (8)$$

In order to test this model, both a quinone titration and measurements of the oxygen affinity at different

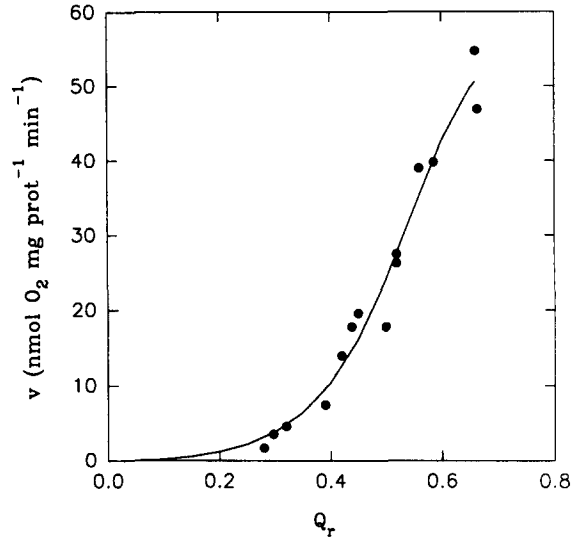

Fig. 4. The dependence of oxygen uptake by the alternative oxidase in 4-day-old etiolated soybean cotyledon mitochondria on the redox poise of the ubiquinone pool. Measurements of quinone redox poise and oxygen uptake were taken as described in Section 2. The solid line represents a fit using the revised kinetic model (Eq. 8) and the same kinetic constants used for the fit of the dependence of alternative oxidase activity on oxygen concentration shown in Fig. 3.

malonate concentrations were carried out using a single set of isolated mitochondria. The solid lines in Figs. 3 and 4 show the simulations obtained for both sets of titrations using 4-day-old etiolated soybean cotyledon mitochondria. As is seen, a single set of kinetic constants yield a good simulation of both the quinone titration (Fig. 4) and the oxygen titrations in the absence of malonate (Fig. 3). Furthermore, the revised model correctly predicts the general effect of oxygen on the alternative oxidase activity at different ubiquinone pool redox levels (Fig. 3, dotted lines). However with the addition of malonate, we found the same problem seen with cytochrome oxidase. Even though we could clearly observe a decrease in the K_m, accurate measurements at these low values of K_m using the oxygen electrode technique were not possible.

With the oxygen titrations, the values of Q_r used to fit the three curves were derived experimentally from voltametric measurements of the redox state of the UQ-pool on a separate, but identical reaction mixture. Equally good fits to analogous oxygen and quinone titrations using one set of kinetic constants were obtained with soybean mitochondria isolated from 4-day-old and 10-day-old green cotyledons (Table 2). Although successful fitting of a kinetic mechanism does not serve as proof of the validity of that mechanism, the revised two-step model presented in this paper is strengthened by its ability to successfully approximate independent sets of kinetic measurements with a single set of kinetic constants (Figs. 3 and 4, solid lines) and

Table 2
Derived rate constants and calculated redox potential differences between the mitochondrial Q-pool and the alternative oxidase in isolated soybean mitochondria

	Green	Etiolated	
	4-day	10-day	4-day
k	$1.4 \cdot 10^4$	$2.5 \cdot 10^4$	$5.0 \cdot 10^3$
k_{-1}	$5.4 \cdot 10^5$	$2.9 \cdot 10^6$	$8.0 \cdot 10^4$
ΔE_1 (mV)	−47	−61	−35
k_2	$9.7 \cdot 10^4$	$1.0 \cdot 10^6$	$6.4 \cdot 10^4$
k_{-2}	$8.2 \cdot 10^4$	$2.5 \cdot 10^5$	$4.6 \cdot 10^4$
ΔE_2 (mV)	+2	+18	+4
k_3	950	846	1100
k_4	37.5	61	45
Q_t	0.77	0.96	0.66
E_t	0.045	0.10	0.066

The kinetic constants were derived from the fits with the revised model (Eqn. 8) using data obtained from oxygen and quinone titrations on a single set of isolated mitochondria. The ΔE values are the redox potential differences ($n = 2e^-$) between the alternative oxidase and the ubiquinone pool for the first (ΔE_1) and the second (ΔE_2) reduction steps. The value of E_t is normalized to a value of 823 nmol oxygen min^{-1} (mg protein)$^{-1}$ and Q_t represents the maximum level of quinone reduction obtained with the alternative pathway following addition of myxothiazol, as described by Siedow and Moore [10]. Units for the rate constants are M^{-1} $time^{-1}$, except for k_3, which is $time^{-1}$.

predict the general effect of the redox state of the ubiquinone pool on the affinity of the alternative oxidase for oxygen (Fig. 3, dotted lines).

The kinetic constants obtained for the fits in Figs. 3 and 4 were $k_1 = 5704$, $k_{-1} = 79569$, $k_2 = 63689$, $k_{-2} = 45831$, $k_4 = 45$ (M^{-1} $time^{-1}$), $k_3 = 1092$ ($time^{-1}$), $E_t = 0.066$ mg mitochondrial $protein^{-1}$, and $Q_t = 0.66$. These kinetic constants should be taken as relative values that are directly proportional to the actual rate constants [10]. From these derived rate constants, the redox potential difference between the alternative oxidase and the ubiquinone pool for each of the two reduction steps can be calculated. With 4-day-old etiolated cotyledon mitochondria, the first reduction reaction is thermodynamically unfavorable ($\Delta E = -35$ mV), while the second reduction is slightly favored energetically ($\Delta E = +4$ mV). Interestingly, the redox potential difference of the first reduction step is similar to that predicted by Bahr and Bonner [5]. Table 2 shows derived kinetic constants and calculated redox potential differences for the different sets of mitochondria studied. All of the fits indicate that the first reduction step is energetically unfavored. The second reduction step was more variable but was energetically favorable in all three cotyledon mitochondria. Overall, the redox potential difference (ΔE) for the four-electron reduction of the alternative oxidase by reduced ubiquinone only varied between −31 and −45 mV for the three systems studied.

We have tested several other kinetic models to attempt to fit the data shown in Figs. 3 and 4. If the second reduction step in the model of Siedow and Moore [10] is made irreversible, the dependence of the oxygen K_m on the redox state of ubiquinone is consistent with that seen in Fig. 3. Similarly, making the step converting E_{rr} to E^*_{rr} reversible, gave the correct qualitative relationship between the oxygen K_m and ubiquinone reduction. However, neither of these mechanisms allowed the simultaneous fitting of both the oxygen and quinone titrations to a single set of kinetic constants. As seen previously [10], attempts to include oxygen in the reaction mechanism prior to formation of the four-electron reduced enzyme did not give rise to reasonable fits to the observed data.

The revised kinetic model for the alternative pathway defined here, although still a model, does help to advance our understanding of the regulation of the alternative pathway. The ability of this model to fit both quinone and oxygen titration results further supports the validity of the concept of a two-step reduction mechanism for the alternative oxidase during its catalytic cycle [10]. In addition, the revised model postulates the formation of an intermediate, activated form of the reduced oxidase prior to its reaction with oxygen. This activated form could be a consequence of a conformational change in the oxidase protein, although further investigation will be needed.

Attempts to purify the alternative oxidase have not been totally successful, although its topology has been described [29,30], antibodies against the alternative oxidase have been developed [21,31] and cDNAs encoding the alternative oxidase protein have been sequenced [32–35]. All these advances should aid in the purification of the alternative oxidase, which is ultimately needed to determine the nature of the electron transfer species associated with the protein, as well as allow a direct measure of the activated state postulated here.

Acknowledgements

The authors would like to acknowledge Dr. A.L. Umbach for carrying out the immunoblot (Fig. 2) and for her helpful suggestions regarding the manuscript, Drs. D.A. Day and J.T. Wiskich for their helpful discussion and also Dr. T.E. Elthon for his generous gift of the AOA antibody. This work was supported by grants from the National Science Foundation (DCB90-19735) to J.N.S., the Spanish Ministry of Education and Science to M.R.-C., Comision Interministerial para la Ciencia y Tecnologia (AGR89-580) to J.A.-B. and M.R.-C. and from the DOE National Institute for Global Environmental Change (W/GEC 92-010A) to J.A.B. This is Carnegie Institution of Washington, Department of Plant Biology Publication No. 1192.

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