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Substrate Kinetics of the Plant Mitochondrial Alternative Oxidase and the Effects of Pyruvate[1]

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The kinetics of alternative oxidase (AOX) of *Arum italicum* spadices and soybean (*Glycine max* L.) cotyledons were studied both with intact mitochondria and with a solubilized, partially purified enzyme. Ubiquinone analogs were screened for their suitability as substrates and ubiquinol-1 was found to be most suitable. The kinetics of ubiquinol-1 oxidation via AOX in both systems followed Michaelis-Menten kinetics, suggesting that the reaction is limited by a single-step substrate reaction. The kinetics are quite different from those previously described, in which the redox state of ubiquinone-10 was monitored and an increase in substrate was accompanied by a decrease in product. The difference between the systems is discussed. Pyruvate is a potent activator of the enzyme and its presence is essential for maximum activity. The addition of pyruvate to the solubilized enzyme increased the maximum initial velocity from 6.2 ± 1.3 to 16.9 ± 2.8 μmol O_2 mg^{-1} protein min^{-1} but had little effect on the Michaelis constant for ubiquinol-1, an analog of ubiquinol, which changed from 116 ± 73 to 157 ± 68 μM. It is concluded that pyruvate (and presumably other keto acids) increases the activity of AOX but does not increase its affinity for its substrate. In agreement with this is the finding that removal of pyruvate (using lactate dehydrogenase and NADH) leads to an 80 to 90% decrease in the reaction rate, suggesting that pyruvate is important in the mechanism of reaction of AOX. The removal of pyruvate from the enzyme required turnover, suggesting that pyruvate is bound to the enzyme and is released during turnover.

The AOX of the inner mitochondrial membrane of plants catalyzes the direct oxidation of $Q_{10}H_2$ by O_2 (bypassing the Cyt path) and is in itself nonphosphorylating. It can be activated by keto acids, most notably pyruvate (Millar et al., 1993; Day et al., 1994). The suggestion that succinate and malate act as activators (Hemrika-Wagner et al., 1986; Wagner et al., 1989, 1995) results from pyruvate production during their metabolism (Millar et al., 1996). Furthermore, activity is regulated by the reduction state of the enzyme (Umbach and Siedow, 1993; Umbach et al., 1994), which can exist as an inactive covalently bound oxidized dimer or as a reduced active monomer (Umbach and Siedow, 1993). The enzyme can be reduced in vivo by those Krebs-cycle substrates (e.g. citrate) that can reduce NADP, and it has been suggested that thioredoxin may be involved in transferring reducing equivalents to the enzyme (Vanlerberghe et al., 1995).

Traditionally, substrate kinetics of enzymes have been studied using a (partially) purified enzyme and measuring the initial rate of activity at various substrate concentrations in the absence of any product. Thus far this has not been possible with AOX, because the solubilized enzyme has proven to be very unstable, with rapid loss of activity (Berthold and Siedow, 1993; Zhang et al., 1996), and because its natural substrate $Q_{10}H_2$ is very hydrophobic. Therefore, steady-state kinetics have been studied in isolated mitochondria using a voltametric device that monitors the level of reduction of the quinone pool. However, in that system an increase in substrate concentration is accompanied by a decrease in product concentration, not allowing the study of substrate kinetics independently of changing product levels.

This method of measuring the reduction level of the ubiquinone pool within isolated mitochondria oxidizing succinate (or malate) showed a nonlinear kinetic relationship between substrate ($Q_{10}H_2$) concentration and AOX activity (Dry et al., 1989). Whereas the Cyt pathway was fully active at 20% reduction of the Q pool, AOX did not show significant activity unless the Q pool was reduced by 40% (Dry et al., 1989). Some empirical models were developed to describe the relation between the reduction level of the Q pool and AOX activity (Moore and Siedow, 1991; Siedow and Moore, 1993; James et al., 1994; Van Den Bergen et al., 1994). Pyruvate increased the activity of AOX at all levels of Q reduction and it was suggested that pyruvate increased the affinity of the enzyme for $Q_{10}H_2$ (Umbach et al., 1994). After activation of the enzyme with pyruvate, competition with the Cyt pathway for electrons could be demonstrated; this was not apparent in the absence of pyruvate (Hoefnagel et al., 1995a; Ribas Carbo et al., 1995).

The kinetic relationship between the reduction level of the Q pool and the activity of AOX during succinate oxidation using the voltametric device was found to be different for mitochondria from thermogenic and nonthermogenic tissue (Siedow and Moore, 1993). However, it became clear that succinate oxidation could not maintain the Q pool fully reduced in *Arum maculatum* mitochondria, as it

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Abbreviations: AOX, alternative oxidase; CMC, critical micelle concentration; EDT-20, *N,N',N'*-polyoxyethylene(10)-*N*-tallow-1,3-diamino-propane; LDH, lactate dehydrogenase (EC 1.1.1.28); Q, ubiquinone-10; Q_1, ubiquinone-1; Q_1H_2, ubiquinol-1; $Q_{10}H_2$, ubiquinol.

did in nonthermogenic mitochondria (Hoefnagel and Wiskich, 1996). NADH was used as a substrate in the presence of pyruvate (to activate AOX) to show that the kinetic relationship between the reduction level of the Q pool and AOX activity was similar in mitochondria from both thermogenic and nonthermogenic tissues (Hoefnagel and Wiskich, 1996).

Study of the enzyme kinetics in the traditional way has become possible now that it has been found that pyruvate stabilizes the partially purified AOX, which retains activity for weeks (Zhang et al., 1996), and by using less hydrophobic quinol analogs as substrates. We have studied the substrate kinetics of AOX using Q_1H_2, an analog of $Q_{10}H_2$, both with intact mitochondria and with the solubilized, partially purified enzyme. The oxidation of Q_1H_2 by AOX can be described using simple Michaelis-Menten kinetics for a single substrate and these initial rate kinetics are quite different from the more complex steady-state kinetics described thus far. Furthermore, the effect of pyruvate was to increase V_{max} without altering the affinity for the substrate, and the results suggest that pyruvate is important in the mechanism of reaction of AOX.

MATERIALS AND METHODS

Arum italicum flower spadices were collected in the Royal Botanic Gardens in Adelaide (Australia) in the late γ- and early δ-stage (as defined by James and Beevers, 1950). Soybean (*Glycine max* L.) plants were grown in a greenhouse at 25°C in trays of vermiculite and after 12 to 14 d the cotyledons were harvested. Mitochondria were isolated essentially according to the method Day et al. (1985) and stored at −80°C.

Assays

The mitochondria were assayed at 25°C in 1 mL of reaction medium (0.3 M Suc, 25 mM Tes, 10 mM potassium phosphate, and 2 mM magnesium sulfate, pH 7.0) and the rates of oxidation of quinol analogs were measured using an O_2 electrode. For analysis of the substrate kinetics the initial rates (first 40–60 s) of quinol oxidation were used. The Cyt pathway was inhibited with a combination of myxothiazol (6 μM) and antimycin A (2 μM); the latter was added to avoid the reduction of endogenous Q_{10} at the bc_1 complex (Zweck et al., 1989). Nonspecific O_2 uptake was determined in the presence of myxothiazol, antimycin A, and an inhibitor of AOX, either 30 μM *n*-propyl gallate or 1 μM *n*-octyl gallate (Siedow and Bickett, 1981; Hoefnagel et al., 1995b), and the residual rate was subtracted from all other rate measurements. This nonspecific O_2 uptake was never greater than 10% of the total rate and was not affected by either pyruvate or oxidized quinone analogs. The partially purified enzyme was extracted with and stored in deoxy-3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (0.3%), but it has been shown that the addition of another detergent (0.025% EDT-20) in the assay increased the activity 3- to 4-fold. At pH 7.0 autoxidation of the quinols was marginally increased by the addition of EDT-20, was insensitive to gallates, and never exceeded 10% of the rate of oxidation by AOX.

AOX of *A. italicum* spadix mitochondria was solubilized and partially purified according to the method of Zhang et al. (1996); however, after purification using a DEAE-Sepharose column the preparation was not further purified using a Q-Sepharose column. The enzyme was purified in the presence of pyruvate to avoid loss of activity (Zhang et al., 1996) and, in experiments in which the effects of pyruvate were tested, pyruvate was removed by passing the extract through a PD10 desalting column (Sephadex G-25M, Pharmacia).

Mitochondrial protein was determined according to the method of Lowry et al. (1951), and the protein content of the partially purified AOX preparation was determined according to the method of Peterson (1977), both with BSA as a standard.

For the substrate kinetics of AOX the initial rates of oxidation were determined (during the first 40–60 s) and were stable and repeatable. The kinetic parameters K_m and V_{max} were estimated by nonlinear regression using the SigmaPlot program (Jandel Scientific, San Rafael, CA). All of the results presented in this paper have been repeated at least once, but mostly they have been repeated more than twice and typical results are shown.

Solubility of the (Hydro)quinone Analogs

The (hydro)quinone analogs used in this study have limited solubility in water and can form micelles at rather low concentrations, thereby decreasing their effective concentration in the medium. Therefore, the CMC of these hydroquinone analogs was estimated under conditions of the kinetic experiments. First, light scattering was determined at 400 nm, at which they have a very low absorbance. Q_1H_2 showed little absorbance up to 350 μM, above which micelle formation resulted in a significant increase in absorbance (Fig. 1A). In the presence of 0.025% EDT-20 there was no significant absorbance up to 650 μM (Fig. 1A). Second, the effective concentration of Q_1H_2 in the medium was tested by determining the substrate kinetics of the oxidation of Q_1H_2 by H_2O_2 catalyzed by peroxidase (EC 1.11.1.7), which was expected to have a low affinity for Q_1H_2.

Peroxidase activity was determined in a 3-mL cuvette at a dual wavelength (290–305 nm), measuring Q_1 formation using a spectrophotometer (model DW2, Aminco, Silver Spring, MD) equipped with a rapid-stirring device. After 100 s to allow micelles to form, the reaction was started with H_2O_2. The relationship between Q_1H_2 concentration and reaction rate was linear up to 600 μM (Fig. 1B). Therefore, there appears to be no decrease in the effective concentration of Q_1H_2 in the medium below 600 μM.

Decylubiquinone and Q_1 were prepared by Dr. D.A. Ward (Department of Organic Chemistry, University of Adelaide) and, like the other quinone analogs, were reduced according to the method of Rich (1981). All other chemicals were obtained from Sigma.

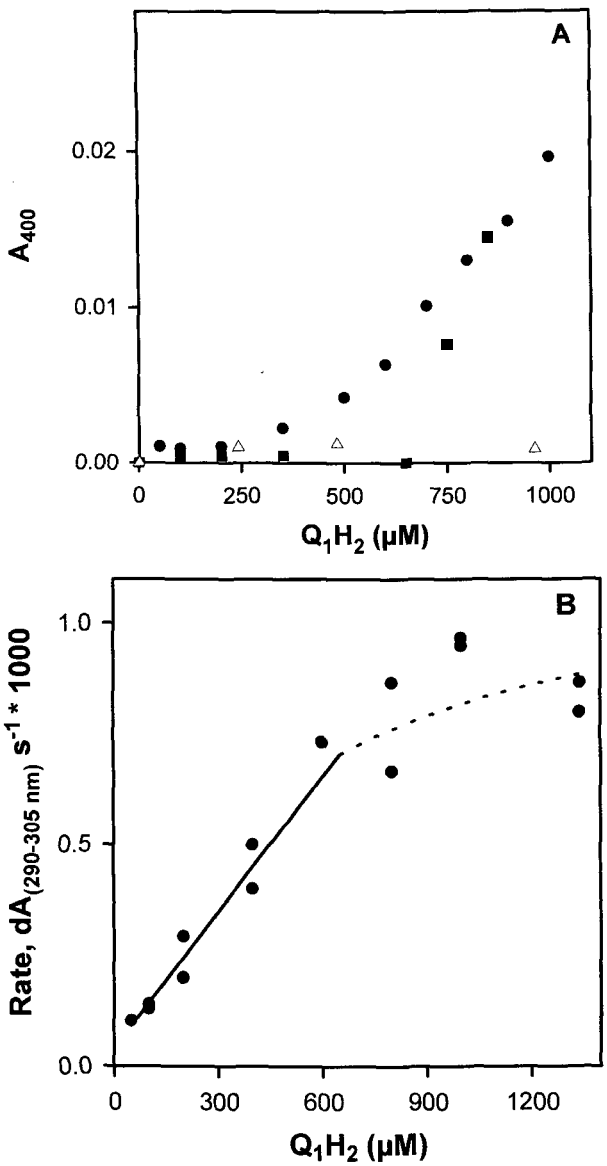

Figure 1. A, Light scattering of Q_1H_2. The absorbance of Q_1H_2 (from a 45-mM stock solution in DMSO) in reaction buffer with (■) and without (●) 0.025% EDT-20 or in ethanol (△) was measured in a 1-mL cuvette in a spectrophotometer at 400 nm. Values are corrected for the absorbance by the same amounts of DMSO without Q_1H_2 in identical media. B, The rate of Q_1H_2 oxidation by H_2O_2 catalyzed by peroxidase. Q_1H_2 was added to the 2.5-mL reaction medium from a 50-mM stock solution in DMSO. To this 0.5 unit of horseradish peroxidase (EC 1.11.1.7) was added, and after 100 s the reaction was started with 2.5 mM H_2O_2. The formation of Q_1 was monitored in a 3-mL cuvette in a spectrophotometer with a rapid-mixing device at the wavelength pair 290 to 305 nm.

RESULTS

Partially Purified Enzyme Preparation

The solubilized and partially purified AOX preparation oxidized Q_1H_2 at a rate of 60 μmol mg^{-1} protein min^{-1}, an activity that was fully sensitive to octyl gallate. From this and the estimated turnover number of AOX in mitochondrial membranes (186 s^{-1}; Hoefnagel et al., 1995b) it can be calculated that the preparation contains at least 20% pure, active enzyme; this may be higher if the turnover of the enzyme out of its membrane environment and with a quinol analog is lower. One of the contaminating proteins had NADH-Q_1 oxidoreductase activity. NADH on its own was slowly oxidized by the extracted enzyme, but the addition of Q_1 increased the rate dramatically (Fig. 2). This activity was activated by pyruvate and completely inhibited by octyl gallate, and it appears that Q_1 was reduced by the NADH dehydrogenase and reoxidized by AOX.

The rate of reduction of Q_1 (33 μM) was determined (Zhu and Beattie, 1988) in the presence of octyl gallate and was found to be 168 μmol mg^{-1} protein min^{-1}, 2 to 3 times faster than the maximum rate of Q_1H_2 oxidation by AOX. The rate of NADH oxidation in the absence of added Q_1 was also sensitive to octyl gallate and was probably due to some Q_{10} being co-extracted with the enzyme.

The presence of NADH dehydrogenase activity was used advantageously in some experiments to keep the added Q_1H_2 reduced and to maintain a constant substrate level.

Pyruvate Is Required for Full Activity

Day et al. (1994) showed that in mitochondria isolated from soybean roots the activation of AOX by pyruvate was reversible, because the removal of pyruvate by addition of LDH in the presence of NADH reduced the activity by 93%.

We obtained a similar result with a solubilized, partially purified preparation of AOX (Fig. 3). Although free pyruvate had been removed by passing the enzyme preparation through a PD-10 column, it still had significant activity that was decreased by LDH and restored by the addition of pyruvate (Fig. 3). The recovery of the rate by pyruvate was not complete (however still 82%), which was to be ex-

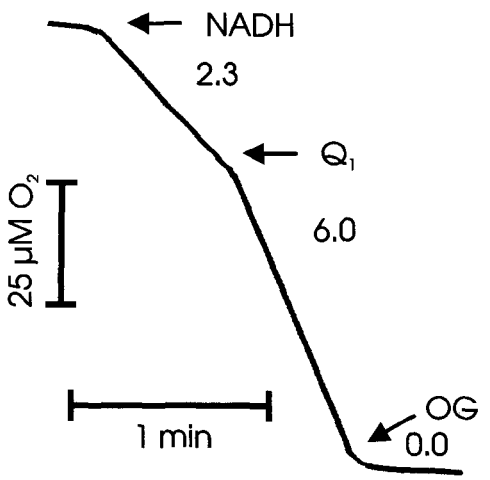

Figure 2. NADH oxidation by the solubilized and partially purified AOX preparation. The enzyme (8.4 μg of protein), prepared from *A. italicum* spadices, was suspended in reaction medium (1-mL final volume) with 2 μM antimycin A, 6 μM myxothiazol, and 5 mM pyruvate. Where indicated, the following additions were made: 1 mM NADH, 175 μM Q_1, and 1 μM octyl gallate (OG). Rates are indicated in micromoles of O_2 per milligram of protein per minute.

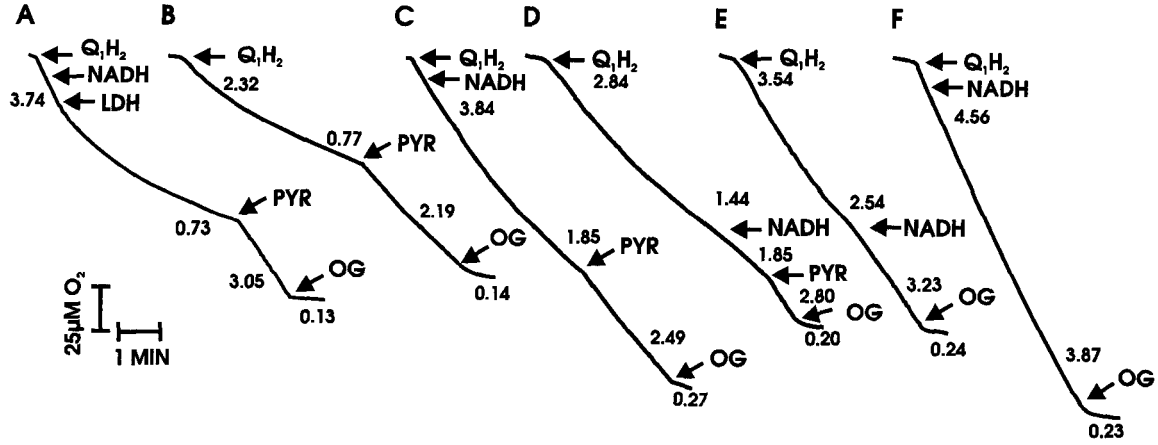

Figure 3. Effect of removing pyruvate on the activity of the solubilized AOX preparation. The rate of Q_1H_2 oxidation by the partially purified AOX (56 μg of protein) was determined in a 1-mL standard reaction medium with 2 μM antimycin A and 3 μM myxothiazol by measuring the O_2 consumption with a polarographic electrode. Where indicated, the following additions were made: 1 mM Q_1H_2, 10 mM pyruvate (PYR), 0.5 μM octyl gallate (OG), 2 mM NADH, 0.25 units LDH (EC 1.1.1.28). In trace B the enzyme was incubated in an anaerobic reaction medium in the presence of LDH and NADH for 7 min and re-aerated before Q_1H_2 was added. In traces E and F pyruvate was added before Q_1H_2 was added. Rates are indicated in micromoles of O_2 per milligram of protein per minute.

pected, because some enzyme activity was also lost while oxidizing Q_1H_2 in the presence of NADH and pyruvate (15% in 4 min; Fig. 3F). This activity loss may be due to protease activity, as suggested for other partially purified AOX preparations (Berthold and Siedow, 1993).

It was hypothesized that after the removal of free pyruvate some pyruvate was still bound to the enzyme, accounting for the activity, and that this bound pyruvate was released during the 7 min of reaction (Fig. 3A). Therefore, the preparation was incubated with LDH and NADH for the same period (Fig. 3B), under anoxic conditions to prevent turnover, before assay of the Q_1H_2 oxidation in an O_2 electrode. Although some activity (38%) had been lost, the kinetics of the subsequent decrease in activity were similar to that of enzyme preparations that had not been preincubated with LDH and NADH. This pattern of inhibition (Fig. 3) suggests that the pyruvate that had been bound to the enzyme was released during turnover. Without the addition of LDH the loss of activity in the absence of added pyruvate was much slower than in the presence of LDH (Fig. 3, A and C). As mentioned above, NADH-Q oxidoreductase activity maintained the level of reduced substrate (see above). Therefore, when NADH was not present the rate decreased faster than when it was added, both in the presence and absence of pyruvate (Fig. 3, D and E), undoubtedly due to the decrease in Q_1H_2 concentration during the experiment. Adding NADH (in the presence of pyruvate) restored the rate (Fig. 3, D and E).

These results show that the solubilized AOX has little or no activity when pyruvate is completely removed (Fig. 3A) and may indicate that pyruvate is important in the mechanism of reaction of AOX. Therefore, kinetic studies of the enzyme should not be performed in the absence of (added) pyruvate.

Oxidation of Various Quinol Analogs by AOX

When a Q electrode (Moore et al., 1988) was used to study the substrate dependence of AOX activity in mitochondria, only the relative Q reduction level was obtained and not the actual $Q_{10}H_2$ concentration. Therefore, various water-soluble quinol analogs, which can be added to the medium at a known concentration, were tested for their suitability to determine the substrate dependence of AOX, both with whole mitochondria and with the solubilized and partially purified enzyme.

Q_0H_2, which was the most hydrophilic and had the highest redox potential of the quinols tested, was not oxidized at all. Decylubiquinol was oxidized by AOX but had a low solubility (CMC < 100 μM) and did not approach saturation of the enzyme (data not shown). In fact, a linear relation between concentration and oxidation rate was found up to the CMC. Duroquinol was a good substrate for AOX and gave fast rates both with intact mitochondria and with solubilized enzyme, but it did not come close enough to saturation to determine substrate kinetics. Q_1H_2 gave considerably faster rates than duroquinol, and AOX activity did approach saturation (Fig. 4). Therefore, Q_1H_2 was used to study the substrate kinetics of AOX (see below).

The presence of the detergent EDT-20 with the solubilized enzyme during the assay increased the rate of oxidation of the quinol analogs considerably, and this increase was fully sensitive to octyl gallate (data not shown). However, the stability of the enzyme was decreased in the presence of EDT-20 but only in the absence of pyruvate, again demonstrating that pyruvate can protect against loss of activity, as was previously reported (Zhang et al., 1996).

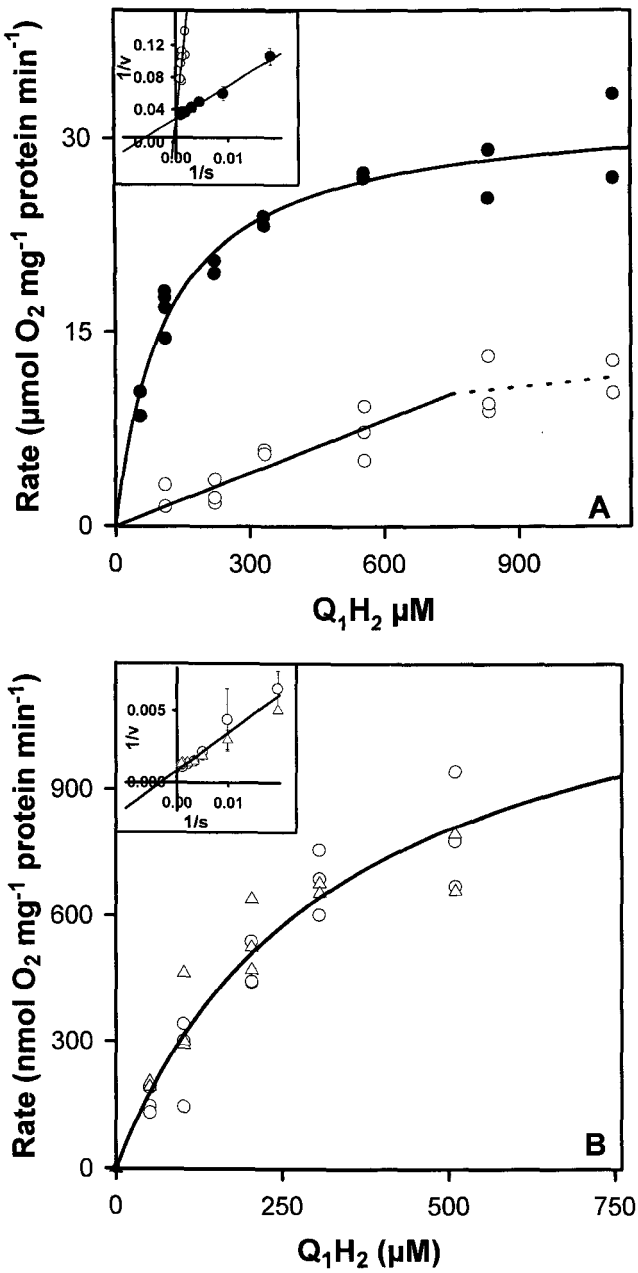

Figure 4. Kinetics of Q_1H_2 oxidation by AOX. A, Solubilized enzyme. The enzyme (1.68 μg protein mL^{-1}) was suspended in standard reaction medium (1-mL final volume) containing 5 mM pyruvate, 3 μM myxothiazol, and 2 μM antimycin A, with (●) and without 0.025% EDT-20 (○). B, Purified *A. italicum* spadix mitochondria (87 μg of protein in a 1-mL final volume) were suspended in standard reaction medium with 5 mM pyruvate, 6 μM myxothiazol, and 2 μM antimycin A. Rates of Q_1H_2 oxidation in the presence (△) and absence (○) of 5 mM DTT. The estimated Michaelis-Menten kinetics are represented by the lines. Inset, Reciprocal plot of the data using averages with error bars (1/s in micromolar Q_1H_2 and 1/v in micromolar O_2 per milligram of protein per minute).

pH Dependence of the Enzyme

The optimum pH for oxidation of all three quinol analogs by the solubilized enzyme was about 7.0 to 7.5, and there was a quinol-type independent pK of about 6.5 (Fig. 5), which most likely reflected the pK of the protein that had to be deprotonated to react with the quinol, as has been demonstrated for Cyt reductase (Rich, 1984). The pH dependence of duroquinol oxidation in the absence of pyruvate is in agreement with the results of Elthon and McIntosh (1986).

The Kinetics of AOX Activity with Q_1H_2

In the presence of EDT-20 (0.025%) and pyruvate the substrate (Q_1H_2) dependence of the enzyme followed Michaelis-Menten kinetics (Fig. 4A; K_m = 115.3 ± 16.3 μM; V_{max} = 32.3 ± 1.6 μmol O_2 mg^{-1} protein min^{-1}). In the absence of EDT-20 the rate of oxidation of Q_1H_2 by the solubilized enzyme showed a linear relationship with substrate concentration up to between 600 and 800 μM (Fig. 4A). This confirms that up to 600 μM the effective concentration of Q_1H_2 was not affected by micelle formation (see "Materials and Methods"). It has to be noted that micelle formation is time dependent; as shown in Figure 4A initial rates (40–60 s) were used and micelles may not have formed yet. Therefore, the effective concentration may have been linear with the added concentration above the CMC.

The difference between the kinetics in the presence and absence of added EDT-20 is undoubtedly the result of

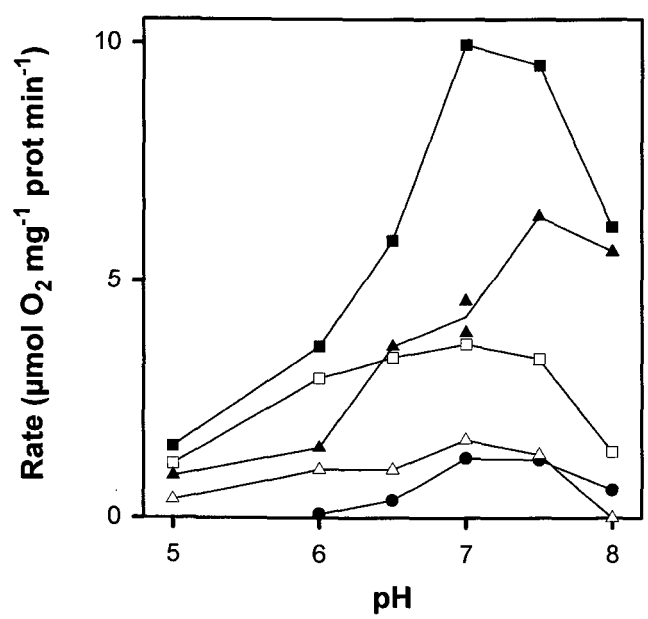

Figure 5. pH profile of quinol oxidation by the solubilized AOX. The rates of oxidation of decylubiquinol (500 μM; circles), duroquinol (900 μM; squares), and Q_1H_2 (900 μM; triangles) were determined in 1 mL of standard reaction medium with a 40-μL AOX preparation (final concentration 3.12 μg protein mL^{-1}) with 6 μM myxothiazol and 2 μM antimycin A in the presence (closed symbols) and absence (open symbols) of 5 mM pyruvate. The octyl gallate (0.1 μM) insensitive rate was subtracted.

differential partitioning of Q_1H_2 between the detergent and the water phase (i.e. the concentration of Q_1H_2 in the detergent phase will be higher than in the water phase). Therefore, the apparent K_m is not the true K_m of the enzyme for its substrate. Analysis of the data showed that the K_m in the absence of EDT-20 was about 20 times higher than in its presence, with little change in V_{max} (Fig. 4A, inset), indicating that EDT-20 indeed increased the effective concentration of Q_1H_2.

Intact mitochondria are a more complex system with which to study enzyme kinetics than the partially purified enzyme. One of the factors that may affect the kinetics is the presence of oxidized ubiquinone (Q_{10}) in the mitochondrial membrane. Note that during the experiments antimycin A was present to avoid reduction of this via the transhydrogenase (Zweck et al., 1989). In intact *A. italicum* spadix mitochondria, the substrate dependence of Q_1H_2 oxidation via AOX could also be described using Michaelis-Menten kinetics (Fig. 4B). Although Q_1H_2 did not fully saturate the enzyme at 500 μM, estimates of the kinetic parameters could be made (K_m = 331 ± 88 μM; V_{max} = 1341 ± 189 nmol O_2 mg^{-1} protein min^{-1}). Again, there is a different partitioning of Q_1H_2 between the water phase and, in this case, the mitochondrial membrane, and therefore the K_m is an apparent K_m.

V_{max} Determination in *A. maculatum* Mitochondria by Combining Q_1H_2 and NADH as Substrates

Accurate determination of V_{max} and K_m using subsaturating substrate concentrations is difficult, given their mutual interdependence and the need for high substrate concentrations for confident and convincing estimation of V_{max}. However, if V_{max} can be established otherwise, the K_m can be determined from the kinetic power (V_{max}/K_m), which can be determined accurately even with subsaturating concentrations. To estimate the V_{max} of AOX of intact *A. italicum* mitochondria, we used a combination of natural substrates and Q_1H_2.

Of the natural substrates NADH gave the fastest rate; a combination of NADH with succinate and malate did not give a rate faster than NADH alone (result not shown). In agreement with these results, the activity of the NADH dehydrogenase (measured as the rate of NADH-Q_1 reductase according to Zhu and Beattie [1988]) was 4 times faster than the rate of NADH oxidation via AOX (result not shown). Moreover, we showed earlier that after inhibition of the Cyt chain, NADH maintained the Q pool at a level about 95% reduced in the absence of pyruvate and 90% in its presence (Hoefnagel and Wiskich, 1996) during oxidation via AOX. In other words, NADH on its own is sufficient to elicit the maximum flux through the alternative pathway in these mitochondria. But even under these conditions the activity of AOX may still be limited by the total amount of Q in these mitochondria, because even if the Q pool is fully reduced, the amount of substrate may be too low to saturate the enzyme if the K_m is too high relative to the Q-pool size.

Adding NADH to *A. italicum* mitochondria oxidizing Q_1H_2 via AOX increased O_2 uptake rates by 34 to 40% (Table I). Apparently, added Q_1H_2 was not saturating, just as the Michaelis-Menten kinetics seemed to indicate. Conversely, Q_1H_2 addition to mitochondria oxidizing NADH did not (or only marginally) increase the rate (0–10%, Table I) of O_2 uptake. The maximal activity measured with both NADH and Q_1H_2 (1408 nmol O_2 mg^{-1} protein min^{-1}; Table I) was similar to the V_{max} calculated from the Q_1H_2 data (1341 nmol O_2 mg^{-1} protein min^{-1}; Fig. 4B) and thus corroborated this number.

Pyruvate Increased V_{max} but Not the Substrate Affinity

It had been suggested that the activation of AOX was the result of an increased affinity for its substrate (Umbach et al., 1994). Therefore, we were interested in the effect of pyruvate on substrate kinetics. During purification of the enzyme pyruvate was always present to minimize loss of activity (Zhang et al., 1996). Pyruvate was removed using a desalting column before the effects of pyruvate on the enzyme kinetics could be studied. If assayed after removal of free pyruvate, the initial rates of Q_1H_2 oxidation by the enzyme preparation were about 70% lower in the absence of added pyruvate than those obtained when pyruvate was added (Fig. 6A). The kinetics show clearly that pyruvate did not increase the affinity for Q_1H_2 (K_m increased from 116 ± 73 to 157 ± 68 μM) but did increase V_{max} (from 6.2 ± 1.3 to 16.9 ± 2.8 μmol O_2 mg^{-1} protein min^{-1}).

In intact *A. italicum* mitochondria pyruvate did not increase the substrate affinity of AOX but did increase V_{max} (Fig. 6B). Average K_m and V_{max} values were 196 ± 84 μM and 156 ± 96 nmol O_2 mg^{-1} protein min^{-1}, respectively, in the absence of pyruvate and 404 ± 93 μM and 658 ± 470 nmol O_2 mg^{-1} protein min^{-1}, respectively, in the presence of pyruvate (four independent preparations). Again, it has to be noted that intact mitochondria are a more complex system (with the presence of oxidized ubiquinone in the membrane) to study enzyme kinetics on than the solubilized enzyme. Still, the data show clearly that pyruvate increased V_{max} but did not increase the substrate affinity.

It has been shown that the enzyme can exist as an inactive oxidized dimer that has to be reduced to restore activity (Umbach et al., 1994; Day et al., 1995; Vanlerberghe et al., 1995); therefore, the effects of DTT on kinetics were also studied. In the absence of pyruvate, DTT did not affect the

Table I. *AOX activity of A. italicum mitochondria oxidizing NADH and Q_1H_2*

Rates were determined with 15 μg of mitochondrial protein in 1 mL of reaction medium with 5 mM DTT, 5 mM pyruvate, 2 μM antimycin A, and 6 μM myxothiazol.

Sequential Additions	Rate
	nmol O_2 mg^{-1} protein min^{-1}
Q_1H_2 (1 mM)	1075
NADH (2 mM)	1408
Octyl gallate (1 μM)	90
NADH (2 mM)	1382
Q_1H_2 (1 mM)	1485
Octyl gallate (1 μM)	102

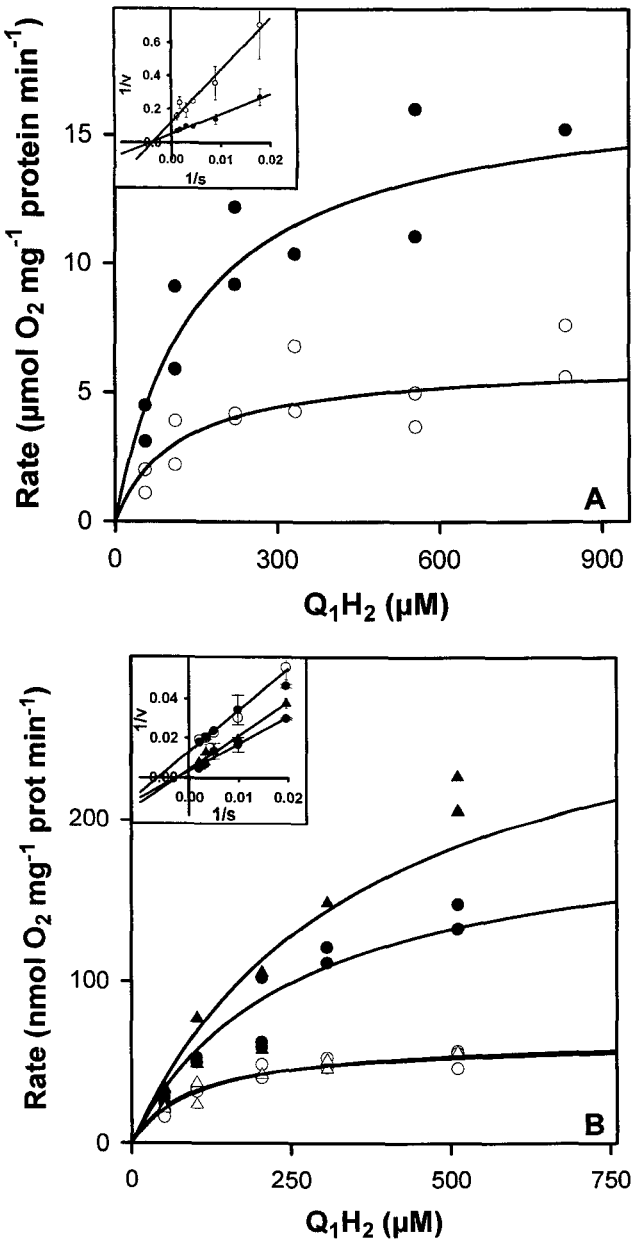

Figure 6. Effects of pyruvate and DTT on the substrate kinetics of AOX. A, Solubilized enzyme; as in Figure 4A after removal of pyruvate from the enzyme preparation using a PD-10 column. The assay was performed with 2.3 mg protein mL^{-1} in the presence of 0.025% EDT-20. Rates in the presence (closed symbols) and absence (open symbols) of 5 mM pyruvate. B, Intact mitochondria; rates of Q_1H_2 oxidation in the presence (triangles) and absence (circles) of 5 mM DTT and in the presence (closed symbols) and absence of 5 mM pyruvate (open symbols). The mitochondria (279 mg of mitochondrial protein in a 1-mL final volume) were suspended in standard reaction medium to which 3 μM myxothiazol and 2 μM antimycin A were added. The estimated Michaelis-Menten kinetics are represented by the lines. Insets, Reciprocal plot of the data using averages with error bars (1/s in micromolar Q_1H_2 and 1/v in micromolar O_2 per milligram of protein per minute).

oxidation rate in any of the mitochondrial preparations, i.e. those with soybean cotyledon mitochondria (data not shown) or those with *A. italicum* mitochondria (Fig. 6B). In the presence of pyruvate all soybean mitochondria responded to DTT (data not shown). In the presence of pyruvate, some *A. italicum* mitochondrial preparations responded to DTT and both V_{max} and K_m increased upon DTT addition (from 206 ± 14 to 314 ± 36 nmol O_2 mg^{-1} protein min^{-1} and from 296 ± 27 to 363 ± 108 μM, respectively; Fig. 6B). However, several preparations of *A. italicum* mitochondria showed no response to DTT (Fig. 4B), probably indicating that AOX is not completely reduced in all *A. italicum* mitochondrial preparations. In agreement with these results, analysis of the mitochondria using SDS-PAGE and western analysis with an antibody against AOX (Umbach et al., 1994) showed that the AOX in isolated *A. maculatum* spadix mitochondria was completely or almost completely reduced, whereas the AOX of soybean cotyledon mitochondria was only partly reduced (data not shown).

DISCUSSION

Thus far the dependence of AOX activity on its substrate has mainly been studied using an electrode system that measures only the relative level of reduction of the mitochondrial Q pool (Dry et al., 1989; Siedow and Moore, 1993). In that system it is not possible to determine dependence of the activity on the actual substrate concentration and, more importantly, the substrate concentration cannot be varied independently of the product concentration. Extracting ubiquinone from the mitochondrial membrane allowed determination of the actual $Q_{10}H_2$ concentration (Van den Bergen et al., 1994) but still does not solve the major problem of the steady-state kinetics, which is the decrease in product concentration accompanying an increase in substrate concentration. Therefore, we tested some water-soluble quinol analogs for their suitability to determine the substrate kinetics of AOX. Q_1H_2 was found to be the most suitable because it was relatively water soluble and is similar to the natural substrate, albeit having a shorter isoprenyl side chain. Apart from its aqueous solubility Q_1H_2 has the same properties (e.g. redox potential) as the natural substrate, $Q_{10}H_2$, because the groups on the reacting quinol ring are the same but with a shorter isoprenyl tail.

Initial Rate Kinetic Analysis Versus Steady-State Kinetics

Having an enzyme preparation that is stable in the presence of pyruvate (Zhang et al., 1996) and a suitable substrate, we were able to determine the substrate kinetics of AOX using initial rates at various substrate concentrations in the absence of the product of the reaction. The dependence of AOX activity on substrate concentration using Q_1H_2 did not show unusual kinetics, could be described using the Michaelis-Menten equation for a single substrate, and may indicate that only one step of Q_1H_2 binding limits the reaction rate. This not only applied to a partially purified enzyme preparation but also to intact mitochondria.

The initial rate kinetics described here are quite different from the steady-state kinetics of intact mitochondria oxidizing succinate or NADH described previously (Dry et al., 1989; Siedow and Moore, 1993; Van den Bergen et al., 1994). The latter monitors the redox poise of the Q pool and the substrate ($Q_{10}H_2$) concentration cannot be varied independently of redox state or oxidized Q_{10} concentration. The kinetics appeared more complex; one model to describe them involved two binding steps of quinol (Siedow and Moore, 1993). Another model describing AOX activity in the dependence of its substrate incorporated both substrate-oxidizing and substrate-reducing enzymes, used simple Michaelis-Menten kinetics, and included the product term (Van den Bergen et al., 1994). Our results support the latter model, using simple Michaelis-Menten kinetics for a single substrate, because in our system only one step of quinol binding appeared to be rate limiting.

The main difference between these analyses using a quinol analog and those using a Q electrode (or Q extraction) is the absence of the change in the product term. Possibly, the difference in kinetics between the two systems can be explained by this product term. Oxidized quinone may act as an inhibitor because of its effect on the redox potential of the substrate or on the Q-binding site. Because of the low solubility of oxidized Q_1, a reliable study of its effects on the reaction is not possible (see also below).

Our results are in agreement with the recently proposed model of the active site of AOX as a hydroxo-bridged binuclear iron center, with the capacity to accommodate only two electrons within the center between the fully oxidized and fully reduced states (Siedow et al., 1995). With such a reaction scheme it is likely that only one of the binding steps of quinol is rate limiting.

Pyruvate Involved in Reaction Mechanism

Removing pyruvate from the reaction media with LDH and NADH severely inhibited (85–95%) AOX activity in both intact mitochondria (Day et al., 1994) and the enzyme extract (Fig. 3). This inhibition was reversible because the subsequent addition of pyruvate recovered the activity. However, passing the enzyme extract through a desalting column to remove the free pyruvate did not cause as severe an inhibition. If the desalted extract was assayed in the absence of added pyruvate with Q_1H_2 as a substrate, the inhibition increased gradually with time; when LDH and NADH were present the onset of inhibition was more rapid. It is concluded that the desalted extract contained some pyruvate bound to the enzyme that is released during turnover. Pyruvate may be bound to the enzyme to form a thiohemiacetal, as suggested by Umbach and Siedow (1996). We would like to suggest that pyruvate (or any other effective acid) is a prerequisite for maintaining enzymic activity. Such a requirement would agree with the increase in V_{max} without changing substrate affinity. These results have important implications for our understanding of the activity of AOX and need to be incorporated into any proposed mechanism of action of the enzyme.

The Effect of Pyruvate on Substrate Kinetics

The activation of AOX by pyruvate (and other keto acids) was most clearly demonstrated by its effect on the relationship between activity and reduction level of the Q pool (Umbach et al., 1994). Given the effect of pyruvate on AOX activity in isolated mitochondria with respect to the redox state of the Q pool, where high activity was found at relatively low substrate concentrations, it is not surprising that Umbach et al. (1994) suggested that pyruvate increased the substrate affinity of AOX. However, using Q_1H_2 as a substrate, we have found that, in the purified enzyme and in intact mitochondria, the only effect of pyruvate is an increased V_{max}. We suggest that pyruvate has the same effect in mitochondria oxidizing the natural $Q_{10}H_2$.

The increase in V_{max} is in agreement with an involvement of pyruvate in the reaction mechanism, where saturating pyruvate allows all AOX to operate. This, however, does not yet explain the effect pyruvate has on the steady-state kinetics (Umbach et al., 1994; Hoefnagel and Wiskich, 1996), but some alternative explanations can be given. First, the steady-state Q-titration curve for soybean mitochondria can be converted to the pyruvate-activated curve by applying an increased V_{max} to the model of Van den Bergen et al. (1994), where both Q-oxidizing and Q-reducing reactions are considered (Millar et al., 1997). Second, pyruvate could have an effect on the redox potential of AOX, allowing a faster reaction rate at lower levels of reduction of the Q pool. Third, pyruvate could reduce the binding of the oxidized product to AOX, thereby minimizing inhibition at lower levels of reduction of the Q pool. With respect to this, we have added oxidized Q_1 to mitochondria oxidizing Q_1H_2, and the rate appeared to decrease. However, this result has to be treated with caution because the CMC of oxidized Q_1 is much lower than for Q_1H_2 and may have lowered the effective concentration of Q_1H_2 in the solution. This low CMC makes an analysis of the effects of oxidized Q_1 unreliable.

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