QUINOL OXIDATION IN *ARUM MACULATUM* MITOCHONDRIA AND ITS APPLICATION TO THE ASSAY, SOLUBILISATION AND PARTIAL PURIFICATION OF THE ALTERNATIVE OXIDASE

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1. Introduction

The nature of the alternative oxidase which is present in a variety of higher plant mitochondria [1] has remained elusive. This stems from the fact that the oxidase is indistinct both in its spectrophotometric [2] and in its electron paramagnetic resonance parameters [3,4]. It can be shown, however, that the oxidase itself can accept electrons from the ubiquinone of the normal electron-transport pathway, which therefore forms the branchpoint for electron flow [5,6]. A further problem encountered in attempts at characterisation of the terminal components is the apparent lability of the oxidase [7] and the lack of an artificial donor which may be used for the direct assay of the alternative oxidase.

Investigations which utilise menadiol and ubiquinol-1 as possible donors to the alternative oxidase of *Arum maculatum* mitochondria are presented here. It is demonstrated that these quinols donate electrons to a point which is at or very close to the alternative oxygen-consuming step. Further, the oxidase may be detergent-solubilised and purified to some extent when these quinols are employed as donors, without rapid loss of activity. A preliminary communication of this work has already been presented [8].

Abbreviations: DBMIB, 2,5-dibromomethylisopropyl-*p*-benzoquinone; SHAM, salicylhydroxamic acid; EPR, electron paramagnetic resonance

2. Materials and methods

2.1. *Preparation of mitochondria*

Mitochondria were prepared by the method in [9]. In the case of *Arum maculatum* spadices, bovine serum albumin was increased to 0.5%, w/v, in the homogenisation medium and EDTA was increased to 2 mM in both homogenisation and resuspension media. Spadices were collected in Cambridgeshire, at a stage just prior to spathe opening. Potatoes were purchased locally. For experiments on solubilisation, *A. maculatum* mitochondria were frozen rapidly in liquid nitrogen and stored in the same until needed. No appreciable loss of alternative oxidase activity occurred over several weeks when the mitochondria were stored in this manner.

2.2. *Electron-transport assays*

Oxygen consumption was monitored with a Clark-type oxygen electrode. The mitochondria were diluted to a suitable protein concentration in 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, and 10 mM potassium phosphate buffer at pH 7.2, unless otherwise stated. Inhibitors were added before electron-donating substrate so that maximum inhibition was obtained.

2.3. *Preparation of reduced quinol substrates*

These were prepared from the corresponding quinone. A suitable amount of quinone was first dissolved in 50 ml diethyl ether and placed in a separating funnel. A 50 ml solution of 1 g sodium dithionite in 50 ml water was then added and the whole was

vigourously shaken until the quinone had been reduced to the colourless quinol. The aqueous layer was discarded and the ethereal layer was washed twice with saturated salt solution. After filtration through anhydrous sodium sulphate, the ether was dried off by vacuum dessication. The quinol was then dissolved in a suitable volume of 96% ethanol. In the case of the menadiol solution, the solution was kept under a flow of nitrogen to avoid autoxidation.

2.4. *Spectral measurements*

These were obtained with a Johnson Research Foundation split-beam spectrophotometer. Cytochromes and flavin were estimated from their reduced minus oxidised difference spectra and calculated with the extinction coefficients in [10].

Protein was estimated by the method in [11] with bovine serum albumin as a standard.

3. Results

3.1. *The interaction of quinols with the intact respiratory chain of plant mitochondria*

Both menadiol and ubiquinol-1 were rapidly oxidised by the intact respiratory chain of *Arum maculatum* mitochondria. Oxidation rates of ~300–700 and 300–600 nmol O_2 consumed/mg protein/min at 25°C were routinely observed with menadiol and ubiquinol-1 as substrates, respectively, in freshly prepared mitochondria. These oxidation rates were unaffected by the addition of KCN, antimycin A or DBMIB, and yet were inhibited $\leq$ 90% by 2 mM SHAM (table 1). Hence, it is concluded that both of these quinols donate electrons at or close to the alternative oxidase oxygen-consuming component.

As a control for these experiments, the effects of the inhibitors upon ubiquinol-1 oxidation by the fully cyanide-sensitive potato tuber mitochondria were investigated. It was found that both 1 mM KCN and 1 μg/ml antimycin A inhibited the ubiquinol-1 oxidase activity by 90–95%. This result indicated that the KCN- and antimycin A-resistant quinol oxidase of the *Arum maculatum* mitochondria was indeed due to the alternative oxidase which is associated with them. One interesting complication was that the ubiquinol-1 oxidase activity of the potato tuber mitochondria was rather SHAM-sensitive (inhibited 90% by 2 mM SHAM), although this may possibly be explained by the rather general competitive effects of hydroxamic

Table 1
Inhibitor sensitivities of the quinol oxidase activity of plant mitochondria

Inhibitor	Percentage inhibition of maximal rate		
	Arum maculatum		Potato tuber
	Ubiquinol-1→O_2	Menadiol→O_2	Ubiquinol-1→O_2
None	0	0	0
KCN (1 mM)	0	0	0
Antimycin A (1 μg/ml)	0	0	90
DBMIB (40 μM)	0	0	85
SHAM (2 mM)	88	89	91
SHAM (2 mM)+KCN (1 mM)	94	89	92

Mitochondria were resuspended to an appropriate protein concentration in a medium containing 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl and 10 mM potassium phosphate, at pH 7.2, for the ubiquinol-1 oxidase assay, or in a medium containing 50 mM 2-(*N*-morpholino)-ethane sulphonic acid at pH 6.0 for the menadiol oxidase assay. The inhibitor was always added before the quinol substrate to ensure that maximum inhibition was obtained. The uninhibited specific activities were: *A. maculatum* ubiquinol-1 oxidase, 390 nmol O_2 consumed/mg protein/min; *A. maculatum* menadiol oxidase, 520 nmol O_2 consumed/mg protein/min; potato tuber ubiquinol-1 oxidase, 119 nmol O_2 consumed/mg protein/min; all at 23°C. Initial concentrations of quinols were: ubiquinol-1, 250 μM; menadiol, 360 μM

acids on quinol binding sites (see section 4 and [12, 13]).

3.2. *The effects of detergent solubilisation on the alternative oxidase-mediated quinol oxidase activity of* Arum maculatum *mitochondria*

Figure 1 illustrates the inhibitory effects of potassium deoxycholate on a number of alternative oxidase-mediated substrate oxidations in *Arum maculatum* mitochondria. As the detergent concentration was increased, the maximal rates of cyanide-insensitive oxidation of both succinate and NADH decreased rapidly, which indicated either a dissociation or solubilisation of components so that they no longer interacted, or possibly an inhibition of one or more electron-transport functions. However, the cyanide-insensitive menadiol and ubiquinol-1 oxidase activities remained intact, a result which again indicated that both of the quinols were donating electrons at or extremely close to the alternative oxygen-consuming step. Even in the presence of detergent this quinol oxidase activity remained SHAM-sensitive and KCN- and antimycin A-insensitive. Centrifugation of the detergent-solubilised preparation at 100 000 × g_{av} for 1 h failed to precipitate any of this quinol oxidase activity, which indicated that the component had become solubilised. This detergent-solubilised preparation after centrifugation showed negligible rates of oxidation of succinate or malate. It was found that NADH was capable of promoting a slow rate of cyanide-insensitive oxygen consumption in the presence of detergent (fig.1). Since this rate was not hydroxamic acid-sensitive, however, it is assumed that it is caused by superoxide anion and hydrogen peroxide generation at sites other than the alternative oxidase [14].

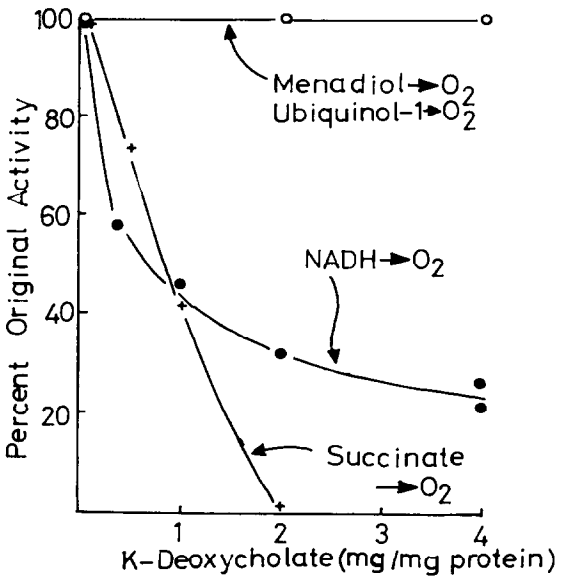

Fig.1. The inhibitory effects of deoxycholate on cyanide-insensitive substrate oxidations in *Arum maculatum* mitochondria. Mitochondria were resuspended in either 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl and 10 mM potassium phosphate, at pH 7.2 (succinate, NADH, ubiquinol-1), or in 50 mM 2-(*N*-morpholino)-ethane sulphonic acid, at pH 6.0 (menadiol). The appropriate amount of detergent was added, followed by 1 mM KCN to ensure that all electron flow was proceeding via the alternative oxidase. After incubation for 1 min at 23°C, substrate was added.

Cholate was found to be an unsuitable detergent for these experiments since extremely high concentrations were required to achieve comparable degrees of solubilisation. Triton X-100 also proved unsuitable because it inhibited the quinol oxidase activity.

The stability of the detergent-solubilised preparation was tested. No appreciable loss of either menadiol or ubiquinol oxidase activity occurred in 2 h aerobic incubation at 4°C, and hence a detergent-solubilised preparation of this type was considered a suitable method for further purification of the oxidase component.

3.3. *Partial purification of the quinol oxidase*

It was found that a selective solubilisation of the quinol oxidase could be obtained by careful control of the detergent : protein ratio. Hence, by utilising a detergent : protein ratio of 4:1, > 90% of the cytochrome oxidase pelleted after centrifugation at 100 000 × g_{av} for 1 h, whereas most of the quinol oxidase remained in the supernatant. The specific activity of the quinol oxidase could be increased 2–3-times by this method.

Analysis of such a preparation (table 2) revealed an almost complete absence of cytochromes aa_3, and a decrease in the amounts of the flavoprotein and of the *b*-type and *c*-type cytochromes. Further, it was possible to detect only a small activity of NADH oxidase and virtually no succinate oxidase. This was confirmed by the low activity of NADH dehydrogenase (assayed as NADH→menadiol→O_2, at pH 7.2) and the

Table 2
Analysis of a detergent-solubilised quinol oxidase preparation from *Arum maculatum* mitochondria

Assay	Whole mitochondria	Detergent-solubilised preparation
Menadiol→O_2 (nmol/mg/min)	590	1340
Succinate→O_2 (nmol/mg/min)	270	10
NADH→O_2 (nmol/mg/min)	640	30
Cytochrome aa_3 (nmol/mg)	0.045	0.005
Cytochromes *b* (nmol/mg)	0.24	0.11
Cytochromes *c* (nmol/mg)	0.15	0.085
Flavoprotein (nmol/mg)	0.31	0.075

A sample of *A. maculatum* mitochondria were diluted to ~10 mg protein/ml in 50 mM *N*-tris (hydroxymethyl) methylglycine at pH 8.0. A solution of 100 mg/ml sodium deoxycholate was then added to a final detergent : protein ratio of 1:4 and the whole was homogenised in a hand homogeniser. After centrifugation at 100 000 × g_{av} for 1 h, the clear yellow supernatant was pipetted away from the dark-red–brown pellet and assayed. Assays were as in section 2.2 and the legend to table 1

absence of succinate dehydrogenase (assayed as oxygen consumption in the presence of succinate and phenazine methosulphate). An EPR analysis of the preparation confirmed the lack of succinate dehydrogenase since no centre S-3 could be found. The only signals which could be detected in the preparation were a small amount of g=4.3 iron, a larger copper signal around g=2, and a small amount of a ferredoxin-type iron–sulphur signal around g=1.93. The iron and copper signals were not redox active. The ferredoxin was detectable at fairly high temperatures (>30 K) and is therefore thought to be a centre of the N-1 type.

The solubilised preparation offered an excellent opportunity to study the product of oxygen reduction by the oxidase in the absence of complicating parameters [14,15]. It was found that neither the oxygen consumption rate nor the stoichiometry of oxygen consumed/quinol oxidised could be altered by the addition of 1 mM KCN to inhibit residual catalase or by the addition of catalase to rapidly remove any hydrogen peroxide which might be formed. It is therefore concluded that the major final product of oxygen reduction by the alternative oxidase is water, and that the majority of the superoxide anion and hydrogen peroxide generation observed in the intact respiratory chain occurs at sites other than the alternative oxidase [14,15].

4. Discussion

It is clear from the inhibition studies that both ubiquinol-1 and menadiol donate electrons either directly or extremely close to the alternative oxygen-consuming component. Of interest was the finding that the quinol oxidase activity was not DBMIB-sensitive, a result which suggests that this inhibitor does not directly inhibit the alternative oxidase, in agreement with the suggestion in [16]. The finding that hydroxamic acids potently inhibited the quinol oxidase activity of both the cyanide-sensitive and the cyanide-insensitive mitochondria suggests that these inhibitors can prevent electron donation to both the alternative and cytochrome oxidase pathways. This result is rather difficult to explain if one assumes that hydroxamates are specific inhibitors of the alternative pathway. However, the result may be explicable if the mode of inhibition by hydroxamates is a general one of competition with quinols for their binding sites, as has been observed in peroxidase and tyrosinase [12,13].

Several major deductions may be made concerning the nature and mechanism of the oxidase from this work. For example, it is now clear that the oxidase activity is associated with a protein species and is not merely an autoxidisable quinone pool (c.f., discussion in [3,17]). Further the oxidase appears to be a quinol

oxidase which catalyses the oxidation of quinols by molecular oxygen to produce the corresponding quinone and water as the final products of the reaction. The activity may be distinguished from other similar enzyme activities, e.g., tyrosinase, by its unique hydroxamic acid-sensitivity and cyanide-insensitivity.

It was of interest to note that the oxidase was reasonably stable in the solubilised state and this has suggested that it may not be the oxidase itself, but rather the connection between the dehydrogenases and oxidase which is the unstable part of the system, when electron transport from respiratory substrate to molecular oxygen is measured (c.f. [7]). It has already been noted that electrons from respiratory substrates do not necessarily have access to the alternative oxidase [18–20] and hence a study of these phenomena may provide a useful insight into the structural organisation of components at this level.

The discovery of such donors which are capable of providing electrons so close to the alternative oxidase offers an invaluable tool for the further investigation of the oxidase itself. For example, it has allowed us to assay the oxidase protein in the absence of intact electron-transport chains from dehydrogenases to oxidase, and has provided a means of purification and ultimate characterisation of the oxidase component.

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