ISOLATION OF A CYANIDE-RESISTANT DUROQUINOL OXIDASE FROM *ARUM MACULATUM* MITOCHONDRIA

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1. Introduction

Cyanide-resistant respiration in higher plant mitochondria is generally held to oxidize reduced quinone [1] via other redox components such as a flavoprotein, iron–sulphur protein or a protein possibly associated with another metal. We present here some preliminary results of attempts to isolate, purify and characterize a protein or phospholipid–protein complex that would mediate the cyanide-resistant, benzhydroxamic acid-sensitive oxidation of reduced quinone. We have shown [2] that duroquinol can be oxidized by a cyanide-resistant, benzhydroxamic acid-sensitive pathway in higher plant mitochondria. Using this as the assay for the cyanide-resistant oxidase in *Arum maculatum* spadix mitochondria we have developed a method to isolate a fraction, following solubilization with Lubrol, differential centrifugation and DEAE-cellulose column chromatography, with ~18-times the specific activity in the mitochondria and with a high (40%) yield. This fraction has been analysed and has been shown to be free of any cytochromes or electron paramagnetic resonance (EPR)-detectable iron–sulphur species and to contain a fluorescent species, which is probably a flavoprotein, and significant levels of copper.

2. Materials and methods

2.1. *Materials*

Arum maculatum spadices were collected from the wild. Salicylhydroxamic acid (SHAM) was obtained from Aldrich Chemical Co. Ltd., duroquinol from K and K Chemicals Inc. and DE23 cellulose anion exchanger from Whatman. Lubrol was a generous gift from ICI Ltd.

2.2. *Mitochondrial preparations*

Mitochondria were isolated from *Arum maculatum* spadix tissue by the method in [3] and stored in liquid nitrogen (77 K) before solubilization.

2.3. *Protein determination*

Protein was determined by the Lowry method [4] after solubilization with 0.8 (w/v) deoxycholate.

2.4. *Oxygen uptake*

Oxygen uptake was measured polarographically using a Clark-type oxygen electrode. The reaction medium was 0.3 M sucrose, 10 mM *N*-[(trishydroxymethyl)methyl]-2-aminoethane sulphonic acid, 5 mM KH_2PO_4 and 5 mM $MgCl_2$, at pH 7.2. The reaction volume was 1 ml.

2.5. *Metal analysis*

Metal analysis was carried out by two methods:

(i) By neutron activation (using the In-Core-Irradiation system at the University of London Reactor Centre at Silwood Park, Ascot) as in [5] using 0.1–0.2 mg protein/sample;

(ii) By plasma atomic emission (using an Appl. Res. Labs. 29 000B quantometer with a modified sample injection technique using a simple pneumatic nebulizer) as in [6].

2.6. *EPR analysis*

EPR analysis was carried out by the method in [3]

at 20 K on a Varian E4 spectrometer, using 20 mg/ml protein.

2.7. *NADH dehydrogenase assay*

NADH dehydrogenase was assayed using potassium ferricyanide as the acceptor for the NADH dehydrogenase [7]. Reduction of potassium ferricyanide was measured in an Aminco DW2 spectrophotometer in the dual beam mode at 420–490 nm using 1 ml reaction mixture containing 1 mM potassium ferricyanide, 0.5 mM NADH and 0.1–0.5 mg protein.

2.8. *Cytochrome c oxidase assay*

Cytochrome *c* oxidase activity was measured by the rate of oxygen uptake in 1 ml reaction medium in the presence of 25 mM ascorbate, 0.5 mM *NNN'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) and 0.005 mM cytochrome *c*, using 0.1–0.5 mg protein.

2.9. *Duroquinol oxidase assay*

The duroquinol oxidase activity was taken to be the component of the rate of oxygen uptake, using 1 mM duroquinol, that was sensitive to 1 mM SHAM. The oxidase was measured using 0.1–0.5 mg protein in 1 ml reaction medium.

2.10. *Cytochrome spectra*

Dithionite-reduced difference absorbance spectra of cytochromes were measured spectrophotometrically using an Aminco DW2 spectrophotometer. The concentrations of cytochromes per mg protein were calculated using extinction coefficients ($\epsilon_{mM^{-1}cm^{-1}}$) of 16 for cytochrome aa_3 at 602 nm and 20 for cytochrome *b* at 560 nm [8].

2.11. *Fluorescence spectra*

Flavoprotein fluorescence was measured in a Perkin Elmer MPF-3 fluorescence spectrophotometer.

2.12. *Isolation of oxidase fraction*

All steps were performed at 0–4°C. Mitochondria were suspended in 10 mM potassium phosphate buffer (pH 7.5) at 10 mg protein/ml. A typical isolation would start with 800 mg protein. To this suspension was added 10% Lubrol to final conc. 0.5% (w/v), stirred for 10 min then centrifuged at 100 000 × *g* for 1 h. The resulting supernatant had a clearly distinguishable denser layer at the bottom comprising ~15% of the total volume. This lower layer was removed with a Pasteur pipette and discarded, along with the pellet. The remainder of the supernatant was applied to a column (4 cm^2 × 30 cm) of DEAE-cellulose pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.5), containing 0.5% (w/v) Lubrol. All subsequent buffers used on the columns contained 0.5% (w/v) Lubrol. The column was washed with 40 ml buffer then the SHAM-sensitive duroquinol oxidase fraction was eluted with 100 mM potassium phosphate buffer (pH 7.5) and collected in ~60 ml. This fraction was then diluted to 10 mM potassium phosphate (pH 7.5) and applied to a second DEAE-cellulose column (2 cm^2 × 45 cm). The column was washed with 20 ml 10 mM potassium phosphate buffer (pH 7.5), then eluted with 200 ml of an ionic gradient from 10–100 mM potassium phosphate. Fractions of 10 ml were collected and assayed for the cyanide-resistant, SHAM-sensitive duroquinol oxidase. A total elution volume of ~50 ml was needed to remove the enzyme.

3. Results

The solubilization and purification of cyanide-resistant duroquinol oxidase was carried out within 10 h as prolonged procedures would result in considerable inactivation of the enzyme. The results of a good purification are summarized in table 1, showing the distribution of various marker enzymes and cytochromes during the purification procedure. A high recovery of the activity was achieved (40%) with complete loss of all the cytochromes and also of ascorbate/TMPD cytochrome *c* oxidase and NADH/$Fe(CN)_6$ reductase activity. However, there was evidence for some catalase activity remaining. The specific activity of the cyanide-resistant, SHAM-sensitive oxidase was increased nearly 18-fold as can be seen in table 2.

The isolated fraction was submitted to spectrophotometric and EPR analysis and had no dithionite-reducible difference absorption spectra in the cytochrome *c*, *b* or *a* regions, nor any dithionite or duroquinol-reducible EPR spectra in the $g = 2$, $g = 4$ or $g = 6$ regions.

The isolated enzyme fraction had a yellow colour

Table 1
Distribution of the cyanide-resistant oxidase, cytochrome oxidase, NADH dehydrogenase, cytochrome *b* and cytochrome a/a_3 during the isolation and purification procedure

Fraction	Cyanide-resistant oxidase	Cytochrome oxidase	NADH dehydrogenase	Cytochromes	
				a/a_3	*b*
Mitochondria	100	100	100	100	100
Pellet	8	37	23	100	35
Bottom layer	3	58	45	0	64
Supernatant	65	1.5	30	0	14
1st DEAE eluant	50	0	0.5	0	0.1
2nd DEAE eluant	40	0	0	0	0

Experimental procedures are in section 2. The figures indicate % total activity or concentration calculated as: (activity or concentration/mg protein) × total protein in each fraction

with a visible spectrum showing absorption peaks which were unchanged in the presence of dithionite at wavelengths of 423 nm, 448 nm and 477 nm. These are tentatively ascribed to contaminating carotenoids, after discussion with Dr P. R. Rich (Cambridge).

Fluorescence measurements of the active enzyme fraction showed an emission spectrum with peaks at 462 nm, 520 nm and 678 nm, with an excitation wavelength of 440 nm. The emission at 520 nm increased greatly as the enzyme activity decayed and was totally removed when the preparation was reduced with sodium dithionite. The fluorescence characteristics are consistent with the presence of a flavoprotein in the isolated enzyme fraction.

The isolated oxidase fraction was submitted to metal analysis using plasma atomic emission and neutron activation and the results are shown in table 3. Copper is the only metal, of those analyzed, present in significant amounts in the fraction containing the enzyme.

Table 2
Specific activity of the cyanide-resistant, SHAM-sensitive duroquinol oxidase during the isolation and purification procedure

Fraction	Specific activity (nmol O_2/min/mg protein)
Mitochondria	303
Supernatant	321
1st DEAE eluant	1987
2nd DEAE eluant	5412

Assay conditions are in section 2

4. Discussion

A method of isolating the cyanide-resistant oxidase from *Arum maculatum* spadix mitochondria has been developed. This method produces an 18-fold increase in specific activity with up to 40% recovery of the cyanide-resistant, benzhydroxamic

Table 3
Copper, zinc and manganese content of the isolated oxidase fraction

Technique	Fraction	Metal content (μg)		
		Copper	Zinc	Manganese
(A) Plasma atomic emission	Blank	0.02	0.02	n.d.
	Oxidase	0.13	0.02	n.d.
	Metal/mg protein	0.22	0.00	n.d.
				n.d.
(B) Neutron activation	Blank	0.124	n.d.	0.006
	Oxidase	0.189	n.d.	0.007
	Metal/mg protein	0.37	n.d.	0.001

n.d., not done

Assay methods are in section 2. The two different techniques of metal analysis were carried out on separate enzyme preparations. The blank in (A) was 10 mM potassium phosphate buffer + 0.5% Lubrol. The blank in (B) was the same buffer, taken off the DEAE cellulose column, which contains some copper, however, the amount of copper in the enzyme fraction is significantly greater than in the blank

acid-sensitive oxidase, but the activity decreases with time (it can be retained by storing at 77 K).

Initial analyses to determine the possible redox components of this oxidase indicate the absence of any detectable cytochrome spectra or EPR-visible species such as an iron–sulphur protein, thus providing direct evidence confirming that neither a cytochrome nor an EPR-detectable iron–sulphur species is the oxidase [9,10]. The possible presence of a flavoprotein with quenched fluorescence in the oxidase fraction is consistent with the involvement of such a component in the alternative pathway [11]. The presence of copper is consistent with the suggestion that a metallo-enzyme is involved. This possibility is attractive since water is the first detectable product formed by the oxidase [12]. However, it has not been possible, as yet, to determine whether the flavoprotein or copper actively mediate the electron flux from quinol to oxygen because the copper is not detectable using EPR analysis and the fluorescence is quenched in the active oxidase. It is, of course, not essential to postulate the involvement of any redox components since the oxidase may directly catalyze the autoxidation of quinol [10], although it it difficult to see how the autoxidation of quinol would produce water as the end product.

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