Identification of the quinone species in cyanide-sensitive and cyanide-insensitive mitochondria of *Moniliella tomentosa*

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Moniliella tomentosa was investigated for the presence of different quinones that might be involved in the cyanide-sensitive and/or cyanide-insensitive electron-transport pathways. The naturally occurring quinone in *Moniliella tomentosa* was found to be ubiquinone-45. Other quinone species could not be detected. The concentration of ubiquinone-45 in mitochondria is not related to the presence or absence of the alternative oxidase activity.

The mechanism of cyanide-insensitive respiration is still puzzling. Various O_2 -reducing compounds have been postulated as possible candidates for the terminal step of the alternative respiratory pathway: an iron-sulphur protein (Bendall & Bonner, 1971), a quinol species that is autoxidizable and that interacts with the ubiquinone pool associated with the standard cytochrome pathway (Rich & Bonner, 1978), and a quinol oxidase (Huq & Palmer, 1978a; Rich, 1978).

The last two possibilities are supported by the finding that the major naturally occurring quinone in the mitochondria, or a related species, is indispensable for the activity of the alternative oxidase (Hanssens *et al.*, 1978; Huq & Palmer, 1978b). Moreover, cyanide-insensitive mitochondria were found to contain much more ubiquinone than did cyanide-sensitive mitochondria. This was only observed so far in *Neurospora crassa* by comparing wild-type with some *mi*-mutants, possessing a constitutive alternative oxidase (Drabikowska & Kruszewska, 1972; von Jagow *et al.*, 1973), but no other organisms were investigated with this purpose. From *Arum maculatum* mitochondria, a cyanide-resistant duroquinol oxidase was isolated (Huq & Palmer, 1978a; Rich, 1978), supporting the third hypothesis, but studies *in vitro* with menadiol and duroquinol revealed that both quinols are autoxidizable. The autoxidation of both quinols is inhibited by salicylhydroxamic acid and stimulated by purine nucleotides, by analogy with the mitochondrial alternative oxidase (Vanderleyden *et al.*, 1980).

Moreover, a prerequisite for an autoxidizable quinone pool, as mentioned above, is the presence of short-chain quinones in mitochondria, since long-

chain quinols are not or only slightly susceptible to autoxidation (Lang *et al.*, 1974). Authors who reported on the higher amounts of ubiquinone in cyanide-insensitive mitochondria than in cyanide-sensitive mitochondria did not discriminate between different possible homologues of ubiquinone. Only total concentrations were measured, with crude mitochondrial extracts.

The present paper reports on the identification and the amount of quinones present in cyanide-sensitive and cyanide-insensitive mitochondria of *Moniliella tomentosa*, whose respiratory properties have been described elsewhere (Vanderleyden *et al.*, 1979). These experiments revealed the presence of only one ubiquinone species, ubiquinone-45.

Experimental

Micro-organism and growth of cells

The organism used was the osmophilic obligate aerobic yeast-like fungus *Moniliella tomentosa* CBS 461.47. Cells were grown at 30°C in Erlenmeyer flasks on a reciprocal shaker. Cyanide-sensitive cells were obtained by growth for 20 h in medium containing 10% (w/v) glucose, 1% (w/v) Oxoid yeast extract and 0.1% (w/v) urea (GYU medium). Cyanide-insensitive cells were obtained by growth for 36 h in GYU medium, supplemented with 4 mg of chloramphenicol/ml or 0.5 µg of antimycin A/ml (Hanssens *et al.*, 1974) or with 6 mg of propan-1-ol/ml (Vanderleyden *et al.*, 1978).

Preparation of mitochondria

Mitochondria were prepared as previously described (Vanderleyden *et al.*, 1979).

Extraction of quinones

The method was described by Kröger & Klingenberg (1966), but was used with some modifications. Mitochondria were washed with a solution consisting of 10 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-EDTA and 0.1% (w/v) bovine serum albumin, and diluted with 0.15 M-KCl to contain about 40 mg of protein/ml. A 1 ml portion of this suspension was extracted with 16 ml of methanol/light petroleum (b.p. 40–60°C) (1:1, v/v). The mixture was shaken for 1 min in a glass-stoppered tube on a test-tube shaker. The suspension was centrifuged at 12000 *g* for 10 min. The light-petroleum layer was removed, and 8 ml of fresh light petroleum was added to the suspension. The extraction was repeated four times. The light-petroleum extracts were combined and evaporated *in vacuo*, and the residue was taken up in a minimum volume of iso-octane.

Column chromatography

The crude extracts were further purified by column chromatography in order to identify and determine the quinones. A silicic acid (Sigma SIL-LC) column (2.5 × 25 cm) was used; 60 g of silicic acid, suspended in iso-octane, was packed in the column and stabilized overnight with iso-octane, as described by Sottocasa & Crane (1965). The sample, which contained the extract of 40 mg of mitochondrial proteins, taken up in iso-octane, was pumped upwards in the column. The single components were then eluted by reversed flow with increasing concentrations of chloroform in iso-octane: 200 ml of iso-octane, 100 ml of chloroform/iso-octane (1:4, v/v), 200 ml of chloroform/iso-octane (1:1, v/v), 100 ml of chloroform/iso-octane (4:1, v/v), 200 ml of chloroform. The fractions (20 ml) collected from the column were evaporated *in vacuo* to dryness and dissolved in ethanol for t.l.c. and spectral analysis.

Thin-layer chromatography

Crude extracts were initially analysed on silica gel G-60 (Merck) plates. Chromatograms were developed with benzene. Ubiquinone-45 and ergosterol were used as standards. In order to detect spots of oxidizing compounds a leuco-Methylene Blue spray was used (Crane & Dilley, 1963). Ergosterol spots were detected under u.v. light.

Reversed-phase thin-layer chromatography

Appropriate fractions eluted from the silicic acid column were chromatographed on silica gel G-60 plates in the reversed-phase system, to ascertain which of the known ubiquinone homologues were present. The silica-gel plates were treated with paraffin and developed with acetone/water (19:1,

v/v) saturated with paraffin as described by Ramasarma & Jayaraman (1971). Chromatograms were run with known homologues of ubiquinone as controls. The spots were detected with leuco-Methylene Blue spray.

Spectral analysis

Spectra of the crude extracts and the column-chromatography fractions were obtained with a Perkin-Elmer 550 S spectrophotometer. The ubiquinone concentration was estimated from oxidized-minus-reduced spectra in ethanol, by using an absorption coefficient of $\epsilon_{280-289} = 8.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Kröger & Klingenberg, 1966). Reduction of ubiquinone was performed by addition of 2 μl of aq. 0.5% KBH₄ to 1 ml of sample, and oxidation by an overnight incubation of 3 ml of the ethanolic solution with 50 μl of a solution containing 0.2 mg of FeCl₃·6H₂O/ml of methanol. Ergosterol was determined spectrophotometrically at 282 nm by using an absorption coefficient of $\epsilon_{282} = 11.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Sober, 1970).

Protein determination

Mitochondrial protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Proteins were solubilized with 0.2% sodium deoxycholate before determination.

Materials

All chemicals used were of analytical grade. Iso-octane was dried over Na₂SO₄ before use. Ergosterol was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The different homologues of ubiquinone were generously given by Hoffman-La Roche, Basel, Switzerland.

Results

Identification of quinones in mitochondria of *M. tomentosa*

U.v.-spectral analysis of crude extracts of mitochondria from cyanide-sensitive and cyanide-insensitive cells indicated the presence of ubiquinone and ergosterol, the typical sterol of fungi (Goodwin, 1973), as the major components.

This conclusion was confirmed by chromatographic analysis of the crude extract. Fig. 1 shows the spectra of the single components of the mitochondrial extract of cyanide-insensitive cells, eluted from a silicic acid column. Fig. 1(a) shows the oxidized-minus-reduced spectrum of the first compound. This is identical with the spectrum of pure ubiquinone-45. Fig. 1(b) shows the spectrum of the second compound. This compound is not reducible with borohydride, and its spectrum is identical with that of pure ergosterol. The ubiquinone peak was eluted with chloroform/iso-octane (1:1, v/v) solvent

Fig. 1. Absorption spectra of fractions obtained by column chromatography of an extract of cyanide-insensitive mitochondria of *M. tomentosa*

A crude mitochondrial extract was fractionated on a silicic acid column by elution with increasing concentrations of chloroform in iso-octane, as indicated in the Experimental section. Fractions were evaporated and dissolved in ethanol. Samples were incubated overnight with FeCl_3 before spectral analysis, as indicated in the Experimental section. (a) Oxidized-minus-reduced spectrum of the first compound, eluted with the chloroform/iso-octane (1:1, v/v) solvent mixture; (b) absolute spectrum of the second compound, eluted with pure chloroform. The values above the traces are the wavelengths of the different peaks, shoulders and isosbestic points.

mixture, and ergosterol was eluted with chloroform. No other u.v.-detectable compounds were eluted from the column. The same results were found with mitochondria of cyanide-sensitive cells.

T.l.c. of the crude extracts of cyanide-sensitive and cyanide-insensitive mitochondria and the single components eluted from the silicic acid column show that the fractions obtained are pure, and that the crude extracts contain only two components, detectable under u.v. light or with the leuco-Methylene Blue spray. The R_F values of the spots correspond to the R_F value of ergosterol or the R_F value of ubiquinone-45. Ubiquinone-45 was further identified by reversed t.l.c., as different isoprenologues of ubiquinone can be resolved by this technique (Ramasarma & Jayaraman, 1971). Therefore fractions showing a typical ubiquinone spectrum were resolved on silica gel G-60 plates, coated with paraffin, together with different isoprenologues of ubiquinone (Fig. 2). The homologues of ubiquinone are very well separated, and only ubiquinone-45 is present in the mitochondrial fractions from cyanide-sensitive and cyanide-insensitive cells.

Quantitative determination of ubiquinone-45 and ergosterol in cyanide-sensitive and cyanide-insensitive mitochondria of M. tomentosa

Table 1 gives the concentration of ubiquinone-45 and ergosterol in different types of *M. tomentosa*

Fig. 2. Reversed-phase t.l.c. of the column fractions showing a typical ubiquinone spectrum

Fractions and appropriate standards were spotted on a silica gel G-60 plate, coated with paraffin, and developed with acetone/water (19:1, v/v) saturated with paraffin. The spots were detected with the leuco-Methylene Blue spray. Spot no. 1 is the ubiquinone-50 standard, spot no. 2 is the ubiquinone-45 standard, and spot no. 3 is the ubiquinone-40 standard. Spot no. 4 is a mixture of the three former standards. Spots 5 and 6 are the column fractions of cyanide-sensitive and cyanide-insensitive mitochondria respectively, showing a typical quinone spectrum as mentioned in Fig. 1(a).

mitochondria. Concentrations were measured spectrophotometrically on column-chromatographed fractions, as indicated in the Experimental section. There is little or no difference in ubiquinone-45 content between cyanide-sensitive and cyanide-insensitive mitochondria. The differences in ergosterol content are more pronounced.

Table 1. *Concentrations of ubiquinone-45 and ergosterol in mitochondria of M. tomentosa*

Concentrations of ubiquinone-45 and ergosterol were measured spectrophotometrically as indicated in the Experimental section. Determinations were done on fractions of mitochondrial extracts chromatographed on the silicic acid column.

Type of mitochondria	Ubiquinone-45 (nmol/mg of protein)	Ergosterol (nmol/mg of protein)
Cyanide-sensitive	3.5	120
Chloramphenicol-induced	4.3	43
Antimycin A-induced	3.0	158
Propan-1-ol-induced	1.9	35

Discussion

Studies *in vitro* with menadiol and duroquinol indicated that the autoxidation of these quinols resembles in some aspects the alternative oxidase of *M. tomentosa* (Vanderleyden *et al.*, 1980). The presence of similar quinones or short-chain ubiquinones (ubiquinone-0, ubiquinone-5 or ubiquinone-10) in cyanide-insensitive mitochondria would be an argument for an autoxidation mechanism *in vivo*. Short-chain ubiquinones are known in Nature, but in micro-trace concentration and without any known physiological function (Wan & Folkers, 1978).

Fractionation of lipid extracts of cyanide-sensitive and cyanide-insensitive mitochondria of *M. tomentosa* showed the presence of ubiquinone-45, but not any autoxidizable short-chain ubiquinol or any other autoxidizable quinol species such as vitamin K or tocopherol quinones. The absence of an autoxidizable quinol from cyanide-insensitive mitochondria of *M. tomentosa* argues strongly against an autoxidation mechanism *in vivo*, and favours an enzymic oxidation as a possible mechanism of the alternative oxidase. The enzymic oxidation has been suggested to be a quinol oxidase (Huq & Palmer, 1978a; Rich, 1978). Since ubiquinone-45 is the only quinone species present in *M. tomentosa*, it is not clear how reduced ubiquinone-45 discriminates between the two oxidizing pathways in cyanide-insensitive mitochondria. Huq & Palmer (1978b) proposed the operation of two distinct quinone pools in *Arum maculatum* mitochondria. In a first attempt, we estimated the total amount of ubiquinone-45 and ergosterol in mitochondria of *M. tomentosa*, having one or two respiratory pathways. Somewhat higher amounts of ubiquinone-45 were found in cyanide-insensitive mitochondria. However, when cyanide-insensitive respiration was induced by other means (antimycin A or propan-1-ol), amounts were lower in cyanide-

insensitive than in cyanide-sensitive mitochondria. Thus in *M. tomentosa* the presence of cyanide-insensitive respiration is not necessarily related to higher concentrations of ubiquinone. The changes in ergosterol concentration are more pronounced, but they also are not in line with the appearance of cyanide-insensitive respiration. With antimycin A the ergosterol concentration is somewhat higher, but with chloramphenicol or with propan-1-ol it is much lower. For chloramphenicol at least it is known that this compound may also inhibit processes other than protein synthesis, e.g. total lipid synthesis (Gordon *et al.*, 1972).

With *Neurospora crassa*, cyanide-insensitive mitochondria have been shown to contain a 5-fold higher concentration of ubiquinone than do cyanide-sensitive mitochondria (Drabikowska & Kruszewska, 1972; von Jagow *et al.*, 1973). These results, however, were obtained with mutants possessing a constitutive alternative oxidase. These mutants were shown to contain also higher concentrations of cytochrome *c*, compared with wild-type strains (Lambowitz *et al.*, 1972). However, it is widely accepted that cytochrome *c* is not involved in the alternative oxidase, indicating that events may occur in the mutants that are not directly related to cyanide-insensitive respiration.

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