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The antimycin-A-insensitive respiratory pathway of *Candida parapsilosis*: evidence for a second quinone involved specifically in its functioning

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The involvement of a quinone in the antimycin A-insensitive electron transfer from NADH-dehydrogenase to cytochrome *c* via the alternative respiratory chain of *Candida parapsilosis*, by-passing complex II, has been studied. After a partial extraction of quinones, the residual respiration was fully antimycin-A-sensitive, but reincorporation of the organic extract partially restored an antimycin A-insensitive respiration. Analysis of quinone content by HPLC, after purification by thin-layer chromatography, evidenced another quinone species in a very low amount. Myxothiazol and stigmatellin were shown to inhibit the alternative pathway but at a higher concentration than required to inhibit the classical pathway. Cytochrome spectra analysis showed that, in the presence of high myxothiazol concentrations, cytochromes *c* and aa_3 were not reduced, while they were in the presence of antimycin A. It is suggested that the secondary pathway of *C. parapsilosis* involved a specific quinone pool which can be displaced from its binding site by high concentrations of myxothiazol or analoguous compounds.

Introduction

For several years, we have been interested in the study of a laboratory strain of *Candida parapsilosis*, the strain CBS 7154 belonging to the form I of the subgroup I [1]; indeed, this yeast species is divided in several groups, some of them supporting pathogenicity [2].

This strictly aerobic yeast possesses interesting features, both at the genetic level, since its mitochondrial DNA is linear [1,3], and at the oxidative metabolic level. Indeed, although it lacks a fermentative pathway [4], *C. parapsilosis* is able to grow on high concentrations of glucose and also on a non-fermentative carbon source supplemented with antimycin A or drugs acting at the level of mitoribosomal protein synthesis [5]. This feature is due to the peculiar organisation of its respiratory chain; for, in addition to the classical respiratory chain, *C. parapsilosis* possesses a second electron transfer pathway, which is different from the alternative pathways described in plants and microorganisms (for review, see Ref. 6) and yeasts as *Saccharomyces cerevisiae* [7] or *Candida utilis* [8].

Oxidation of exogenous NAD(P)H is a general property of mitochondria from plants and fungi [9], but electrons are transferred to molecular oxygen via different routes: In *S. cerevisiae*, as in plant mitochondria, they use an NADH : ubiquinone oxidoreductase located on the outer face of the inner membrane, and give rise to only 2 mol of ATP for each mol of NAD(P)H oxidized [7]; in this yeast, all the electrons are transferred to complex IV, whilst in plant mitochondria, some of them are transferred to an alternative pathway, antimycin-A-insensitive, which branches from the quinone pool to an alternative oxidase (for review see Ref. 6). On the other hand, in addition to the oxidoreduction chain of the inner membrane, intact mitochondria from numerous origins contain in the outer membrane an NADH-dehydrogenase which catalyses electron transfer from NADH to complex IV using cytochrome *c* to shuttle electrons between the outer and the inner membrane [10–12].

In *C. parapsilosis*, in addition to the NADH ubiquinone : oxidoreductase, entry of electrons occurs through two dehydrogenases, specific for NADH and NADPH, respectively, which are amytal- and mersalyl-

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sensitive, and located on the external side of the inner membrane [13]. This alternative pathway interacts with the main respiratory chain after the bc_1 complex [14,15]. Two pools of cytochrome *c* have been identified, which can be distinguished by their electron donor [15,16], their molecular weight and their amino-acid composition (Camougrand, unpublished data) and their redox potential (Guérin, M. and Ohnishi, T., unpublished results). Electrons are transferred either to cytochrome aa_3 or to the alternative oxidase, cytochrome 590, which is sensitive to salicylhydroxamates and to high concentrations of cyanide [15].

These data prompted us to inquire about the intermediate step between NAD(P)H dehydrogenases and the cytochrome *c* of the alternative pathway (cytochrome $c_{alt.}$); indeed, since it is antimycin-A-insensitive, are quinones involved in this electron transfer pathway? The current study provides some evidence for possible involvement of a peculiar quinone associated with the secondary pathway of *C. parapsilosis*, and which is different from that implicated in the classical 'Q-cycle'. Preliminary data have already been published [16].

Materials and Methods

Strain. Candida parapsilosis (CBS 7154) was from the laboratory [1].

Subcellular fractionation. Cells were grown aerobically at 28°C on a complete medium (pH 4.5) added with 2% glycerol as carbon source and supplemented or not with antimycin A (2 mg/l).

Cells were harvested in logarithmic growth phase and mitochondria were isolated either from protoplasts prepared enzymatically or from glass-bead-broken cells according to Guérin et al. [17].

Protein concentration was measured by the biuret method using bovine serum albumin as standard.

Cytochrome spectra. Spectra were recorded on a double-beam dual-wavelength spectrophotometer, SLM Aminco DW 2000.

Determination of ATP/O. Mitochondrial oxygen consumption was measured at 27°C in the basal medium (0.65 mannitol/0.36 mM EGTA/10 mM Tris-maleate (pH 6.7)/0.3% BSA/3 mM NADH), using a Clark electrode connected to a microcomputer giving an on-line display of rate values. ATP synthesis was initiated by addition of 2 mM ADP and 3 mM [^{32}P]phosphate. After acid extraction, the rate of [^{32}P]ATP synthesis was measured as previously described in Ref. 18.

Extraction and reincorporation of quinones. Q-depleted mitochondria were prepared according to Nordling et al. [19] by extracting lyophilised mitochondrial particles with dry pentane.

Reincorporation of quinones was done according to Nordling et al. [19]. After extraction and reincorporation, the various preparations were suspended in the basal medium and immediately subjected to the assays.

In some experiments, exogenous quinones in methanolic solutions were added to the mitochondrial suspensions [12].

Analysis of quinones. Quinones were extracted from mitochondria according to Takada et al. [20]; the crude organic extract was then purified by thin-layer chromatography on silica-gel plates (Merck Kieselgel $60F_{254}$) developed in benzene as solvent. Quinones were visualised under UV light at 254 nm and eluted from the silica-gel with diethyl ether. Then they were separated by high-performance liquid chromatography according to Takada et al. [20] or to Reed and Ragan [21], i.e., without $NaClO_4$. Synthetic ubiquinones UQ_{10} and UQ_7, and coenzyme Q_6 from *S. cerevisiae* were used as standards. Each peak was collected and analyzed by UV spectrophotometry.

Results

From experiments carried out on growing cells [14] as well as on isolated mitochondria [15], it has been proposed that, in *C. parapsilosis* CBS 7154, the branching of the alternative pathway occurred after the bc_1 complex.

To confirm this proposal, measurements of ATP/O ratios with NADH as substrate were carried out. On one hand, we compared mitochondria isolated from cells grown in the absence (control) or in the presence of antimycin A (antimycin A mitochondria). On the other hand, oxidative phosphorylations were measured in the presence of antimycin A and SHAM, the former inhibitor acting upstream of cytochrome *c* on the main respiratory chain and the latter blocking the alternative route downstream of cytochrome $c_{alt.}$ [15]. From the results reported in Table I, several lines of evidence can be drawn: (i) In absence of inhibitor, $P/O = 1.1$ and 0.6 for control mitochondria (in which two phosphorylation sites are functioning) and antimycin-A mitochondria, respectively. (ii) In the presence of antimycin A, $P/O = 0.76$ for control mitochondria. (iii) In the presence of both inhibitors antimycin A and SHAM, a weak electron flux occurred through the complex IV, leading to a proton efflux coupled to phosphorylation in both types of mitochondrion. Under these conditions, where only the third phosphorylation site was effective, the P/O values were higher than 1 ($P/O = 1.2$ to 1.4) and larger than that of the control experiment. This enhanced value of P/O had already been observed in mitochondria of *S. cerevisiae* when the electron flux was limited, and was explained as a modification in the stoechiometry of the proton pump [22].

From these results it was evident that the second respiratory chain of *C. parapsilosis* joins the main one at the cytochrome *c* level, from which electrons are

TABLE I

Measurements of oxidative phosphorylations in the presence of inhibitors of the respiratory chains

Mitochondria were isolated from cells grown in the absence (1) or in the presence (2) of antimycin A and enzymatically prepared. They were suspended (1 mg/ml) in the basal medium added with 2 mM ADP and with either 0.2 μg/mg antimycin A or 0.2 mM KCN or 2 mM SHAM. Respiration was started by 2.6 mM NADH and phosphorylation by 3 mM [^{32}P]phosphate (1000 cpm/nmol). Respiration rate was expressed in ngatom O $min^{-1} mg^{-1}$ and rate of ATP synthesis in nmol $min^{-1} mg^{-1}$.

Addition	Respiration rate		Rate of ATP synthesis		*P/O*	
	1	2	1	2	1	2
Control NADH	260	60	270	38	1.1	0.6
+ antimycin A	94	–	71	–	0.76	–
+ KCN	63	54	0	0	0	0
+ SHAM + antimycin A	13	30	18	34	1.4 [a]	1.15 [a]

[a] Average of three experiments on a range of protein concentrations: value ± 0.05.

delivered either to the alternative oxidase (non-phosphorylating) or to the third phosphorylation site.

Other exogenous substrates

It was previously shown that other dehydrogenases specific for exogenous substrates, such as NADPH and glycerol-3-*P* could deliver electrons to the secondary pathway of *C. parapsilosis*. However, as can be seen in Table II, the relative distribution of electrons between the two pathways was dependent on the substrate used: whereas with NADH as substrate the antimycin-A-insensitive pathway represented 15–18% of the total respiratory rate, its participation was equal to about 50% and 30% for NADPH and glycerol-3-*P*, respectively. It should be noted that, in all cases, the sum of the respiratory rates measured in each of the pathways was equal to the respiratory rate measured in the absence of inhibitors, whatever the substrate used.

The relative antimycin-A-insensitivity of the glycerol-3-*P* oxidation prompted us to compare utilisation of this substrate by mitochondria from *S. cerevisiae*, which is fully antimycin-A-sensitive, and *C. parapsilosis*. From these experiments, it appeared that: (i) Oxidation of this substrate was insensitive to amytal in *C. parapsilosis* (Table II) as in *S. cerevisiae* (not shown), which ruled out the possible formation (and oxidation) of external NADH due to the functioning of the glycerol-3-*P* dehydrogenase in *C. parapsilosis*. (ii) Measurements of oxidative phosphorylations gave rise to *P/O* equal to 0.39 and 0.84 for *C. parapsilosis* and *S. cerevisiae* respectively, indicating a dissipation of the electron flux in the former mitochondria. These data suggested that, in *C. parapsilosis*, similarly to NAD(P)H, there could exist two mitochondrial glycerol-3-*P* dehydrogenases which can donate reducing equivalents to both respiratory pathways.

Extraction and reincorporation of quinones

To examine the potential role of quinone in the electron transfer between NAD(P)H dehydrogenases and cytochrome c_{alt}, mitochondria were progressively depleted from endogenous quinone. After each extraction the organic extract was reincorporated, and at each step the respiration rate and the antimycin A-sensitivity were measured. From results reported in Table III it can be seen that: (i) lyophilisation of mitochondria decreased the oxidation rate but did not affect functioning of antimycin A insensitive pathway; (ii) after one pentane extraction, 60% of respiratory capacity was lost, but the remaining respiration was fully inhibited by antimycin A; (iii) reincorporation of the pentane extract led to 63% recovery of initial respiration rate and, above all, this oxidation was partly antimycin-A-

TABLE II

Relative partition of electron fluxes between main and secondary respiratory pathways

Mitochondria from cells grown under normal conditions and enzymatically prepared were purified on a discontinuous sucrose gradient. 1 mg protein was suspended in 3 ml of respiration buffer adjusted to pH 6.7 for NADH and glycerol-3-*P*, and to pH 5.3 for NADPH. Additions: 2.6 mM amytal; 0.2 μg/mg antimycin A; 0.15 μg (1) or 2 μg (2) of myxothiazol. Parentheses represent the % of respiration.

Addition	Substrate: NADH	NADPH	Glycerol-3-*P*
None	226 (100%)	83 (100%)	80 (100%)
Amytal	190 (84%)	36 (43%)	80 (100%)
Antimycin A	41 (18%)	52 (62%)	25 (31%)
Myxothiazol (1)	40 (18%)	42 (50%)	23 (29%)
Myxothiazol (2)	5 (2%)	8 (10%)	5 (6%)

TABLE III

Extraction and reincorporation of quinones

Cells were grown without antimycin A and mitochondria were prepared following the glass beads method. They were lyophilised and then treated with pentane, either once (first extraction) or twice (second extraction). Organic extract (supernatant 1 or 2) was then reincorporated. Mitochondria were then suspended in respiration buffer and respiration initiated with 3 mM NADH, in the presence or in the absence of 0.2 μg/mg antimycin A. Reproduced with permission of Guérin et al. [16].

Treatment	Respiration rate	% of residual respiration	% of antimycin sensitivity
Control	273	–	65
Lyophilisation	219	100	53
1st extrn.	92	42	100
Ex I + surn. I	140	63	64
2nd extrn.	41	19	100
Ex II + surn. II	115	52	50

insensitive again; (iv) after two pentane extractions, 80% of respiration was lost, but reincorporation of organic extract led to a respiration recovery partly antimycin-A-insensitive.

Analysis of quinone content

The organic extract of mitochondria was first purified by thin-layer chromatography and vizualised under UV light. Under the major band of quinone and close to it, a very slight band was detected (Fig. 1, inset). Both bands (Fig. 1A), or only the second band (Fig. 1B) were eluted from the plate and then analyzed by HPLC: in Fig. 1A, only a major peak corresponding to ubiquinine$_9$ was depicted; in Fig. 1B, two peaks appeared, one corresponding to a residual amount of Q_9 and another peak, Q_x. Both quinones were collected, mixed with UQ_6, UQ_7 and UQ_{10} (Fig. 1C): Q_x did not comigrate with UQ_6 or UQ_7, but was between them.

To permit spectrophotometric study, quinones were separated according to Reed and Ragan [21], and both exhibited a redox spectrum (Guérin et al., unpublished results).

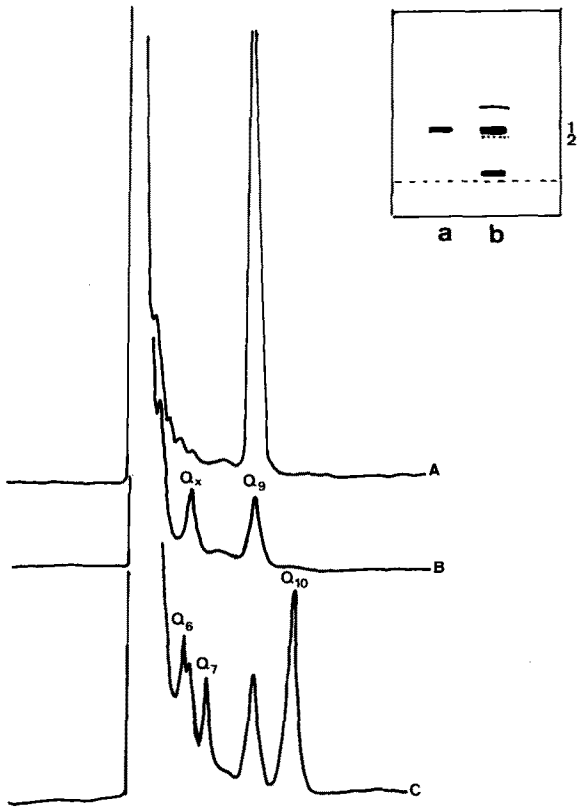

Fig. 1. Analysis by HPLC of the quinones of *C. parapsilosis*. After purification by thin-layer chromatographiy on silicagel plate developed in benzene (inset), quinones were separated by HPLC; elution buffer: ethanol/methanol (3/2); flow rate: 0.5 ml min^{-1}. (A) Band 1+2 (see inset). (B) Band 2. (C) Both quinones mixed with control quinones. Inset: lane a = UQ_{10}; lane b = organic extract of *C. parapsilosis*.

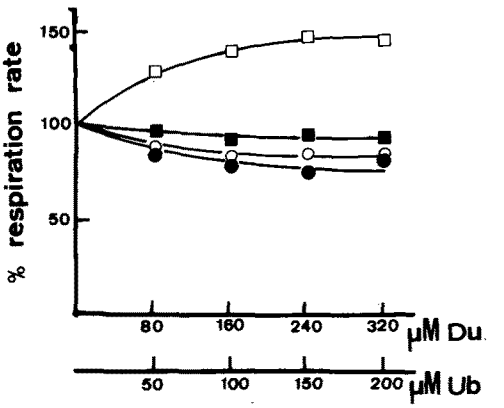

Fig. 2. Effect of duroquinone (Du) and ubiquinone$_{10}$ (Ub) on the NADH oxidation rate. Reduced duroquinone (■□) or ubiquinone (● ○) were added to mitochondria suspended in the respiration buffer (1 mg/ml) in the presence of (0.2 µg/mg) antimycin A (■●) or 2.6 mM amytal (□ ○), then respiration was started by addition of 2.6 mM NADH.

Effect of exogenous quinones on NADH oxidation

The effect of addition of reduced ubiquinone$_{50}$ or duroquinone on NADH oxidation was measured on control mitochondria in the presence of amytal or of antimycin A. It can be seen in Fig. 2 that both quinones acted differentially on the two pathways: addition of ubiquinone$_{10}$ slightly inhibited the alternative route, as it did the main one; in contrast, duroquinone enhanced the oxidation rate through the classical respiratory chain but not through the secondary pathway.

Effect of inhibitors of the bc_1 complex acting at centre 'o'

Inhibitors of the bc_1 complex can be classified into one of the two groups, depending on which of the two pathways of cytochrome *b* reduction they block. In mammalian mitochondria, it has been shown that antimycin A blocks the pathway of *b* reduction through centre 'i', in the terminology of the Q cycle [23], by binding to a site close to the haem of *b*-562 [24,25] and destabilising ubisemiquinone [26]. Myxothiazol acts at centre 'o' by binding proximal to the haem of *b*-566 [27] and apparently blocking oxidation of ubiquinol at a site on the iron-sulphur protein of the bc_1 complex [28].

We have previously demonstrated that the alternative pathway of *C. parapsilosis* is insensitive to inhibitors acting at center 'i' but electrons can still be transferred to cytochrome *c* through a quinone pool. Experiments were therefore carried out with compounds acting at centre 'o', such as myxothiazol and stigmatellin.

Myxothiazol is known to exhibit one high-affinity binding site to the bc_1 complex of mitochondria from beef heart [29] or yeast [30]. As a control experiment, a first titration was done on the oxidation rate of a substrate using the internal NADH dehydrogenase, such as 2-oxoglutarate (Fig. 3, curve a). The inhibition curve was monophasic with a half-inhibition constant equal to

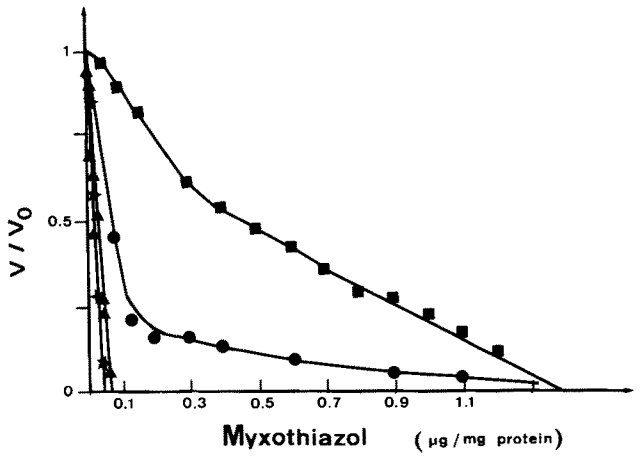

Fig. 3. Myxothiazol titration of oxygen consumption. Respiration of intact or pentane-extracted mitochondria was progressively inhibited by sequential addition of myxothiazol (v). (a) Oxidation of 2-oxoglutarate ★———★; (b) Oxidation of NADH ■———■; (c) Oxidation of NADH in the presence of antimycin A ●———●; (d) Oxidation of NADH by pentane-extracted mitochondria ▲———▲. Actual respiration rates (v_0) (ng atom O $min^{-1} mg^{-1}$): (a) 30; (b) 260; (c) 50; (d) 40.

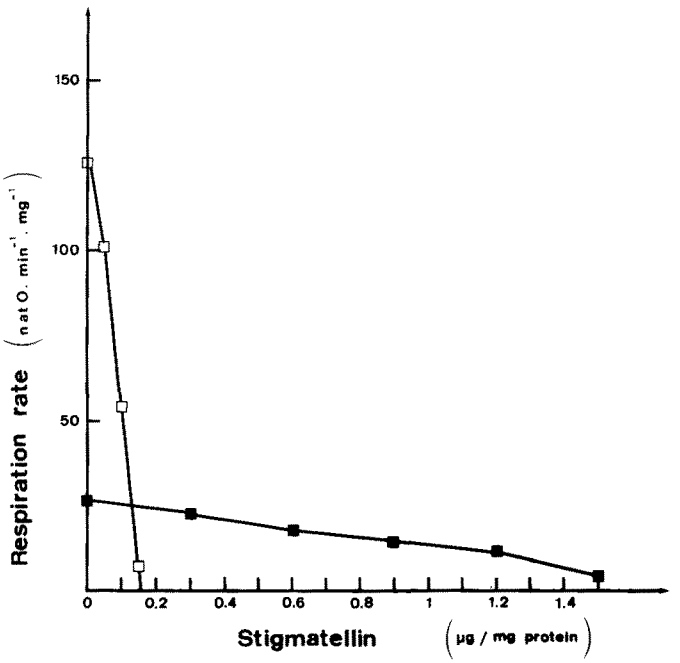

Fig. 4. Effect of stigmatellin on NADH oxidation. Mitochondria (1 mg/ml) were preincubated with antimycin A (■) or amytal (□). The stigmatellin, on a range of concentration, was added to mitochondria before NADH.

0.02 µg/mg protein, close to that measured with rat liver mitochondria (not shown).

In contrast, when exogenous NADH was the substrate, the inhibition curve profile was dependent on the functioning of one or two respiratory chains. (i) When electrons were transferred through both routes, the inhibition curve was biphasic (Fig. 3, curve b), with two half-inhibition constants equal to 0.06 and 0.6 µg/mg protein, respectively. (ii) When the experiment was carried out in the presence of antimycin A (Fig. 3, curve c), only the second class of inhibitory site was effective ($K_{1/2}$ = 0.06 µg/mg protein). (iii) When measurements were done on pentane-treated mitochondria, only the first class of inhibitory site was evidenced (Fig. 3, curve d), with a half-inhibition constant close to that measured with 2-oxoglutarate ($K_{1/2}$ = 0.03 µg/mg protein). Same results were obtained with NADPH or glycerol-3-*P* as substrates (Table II).

To confirm these data, the effect of stigmatellin [31], another inhibitor of centre 'o', was assayed on NADH oxidation in the presence of amytal or antimycin A, leading to electron transfer through the main or the secondary pathway, respectively. As shown in Fig. 4, and similar to that observed with myxothiazol, two titration curves were obtained, depending on the electron-transfer route. As reported by Tierbach et al. [30], the half-inhibitory concentration of stigmatellin determined for the first class of sites was close to that measured with myxothiazol.

In a second set of experiments cytochrome spectra were recorded with NADH as substrate, on intact or pentane-extracted mitochondria, in the presence of high concentrations of antimycin A or myxothiazol (Fig. 5).

In the presence of antimycin A, a part of cytochromes *c* and aa_3 were reduced in intact mitochondria but not in pentane-extracted mitochondria, showing that the component allowing the electron transfer between the dehydrogenase and cytochrome *c* was lost. In contrast, in the presence of myxothiazol, no difference was observed between the two types of mitochondrial preparation, and in both cases only cytochromes *b* were reduced,

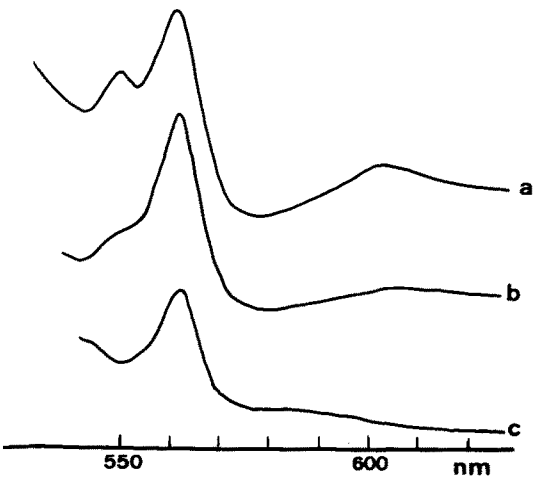

Fig. 5. Cytochrome spectra of intact or pentane-extracted mitochondria in the presence of inhibitors of bc_1 complex. Cytochrome spectra were recorded with intact (a, b) or pentane-extracted mitochondria (c), NADH reduced versus oxidised. (a) In the presence of antimycin A; (b) in the presence of myxothiazol; (c) in the presence of antimycin A or myxothiazol.

confirming that this inhibitor blocked both respiratory chains.

From these results, it appeared that the second pathway of *C. parapsilosis* was sensitive to inhibitors which normally act at centre 'o', but the concentrations needed suggested that they displaced the quinone involved in this pathway either from a specific Q-binding protein or from another site of the iron-sulphur protein.

Discussion

From previous experiments carried out either at the cellular level [14] or at the mitochondrial level [15], it was proposed that *C. parapsilosis* develops a peculiar alternative pathway which parallels the main respiratory chain. Electrons originated from external NAD(P)H or glycerol-3-*P* oxidation are partially transferred via this route until cytochrome $c_{alt.}$ from which they are transferred either to cytochrome *c* oxidase or to the alternative oxidase (for a review see Ref. 16). Experiments reported in the first part of this work confirm this organisation: even in the presence of two inhibitors, the first acting upstream of cytochrome *c* of the main respiratory chain (antimycin A) and the second downstream from the cytochrome *c* of the alternative pathway (SHAM), a weak electron flux was coupled to phosphorylation at the third phosphorylation site.

Since the second chain was antimycin-A-insensitive, it was of particular interest to determine whether quinones were involved in the electron transfer between external dehydrogenases and cytochrome *c*.

Several lines of evidence presented in this report suggest such an involvement of quinones in that chain: (i) partial extraction of quinones preferentially slowed down the alternative pathway (see Table III), since the residual respiration was fully antimycin-A-sensitive. (ii) Reincorporation of organic extract partly restored an oxygen consumption which was relatively antimycin-A-insensitive. (iii) Analysis of the organic extract by HPLC showed the presence of a second quinone species, in a very low amount relative to the coenzyme Q_9, the major quinone.

This rapid loss of the alternative pathway activity after a partial extraction of ubiquinone by pentane has already been described for *Arum maculatum* spadix mitochondria [32]. It has been suggested that this is due to the presence of two pools of quinone in these mitochondria, associated with the cyanide-sensitive and -resistant respiratory pathways. In higher plants, however, the quinone pool(s) are oxidised by a route that bypasses complex IV, involving a quinol oxidase [33]. In *C. parapsilosis* the problem was different since electrons are transferred from NADH dehydrogenase to complex IV, bypassing the bc_1 complex. On one hand, antimycin A, although blocking cytochrome *b* oxidation, allowed electron transfer to cytochrome *c* in intact mitochondria, whereas myxothiazol, at high concentrations, fully inhibited both pathways.

These results led us to consider the possibility that they may be two distinct pools of quinone in *C. parapsilosis*. The observation that duroquinone, which reacts with the electron-transfer chain by the same mechanism as the natural quinones [34,35], seems to have no effect on the alternative pathway, whereas it stimulates the NADH oxidation rate through the classical route, suggests the presence of a specific type of quinone, presumably with a redox potential different from that of the classical chain. Evidence in favour of this proposal arises from the result that the second quinone evidenced by HPLC is not a UQ_n. The sensitivity of the second respiratory pathway to high concentrations of myxothiazol or stigmatellin could account for the participation of a specific Q-binding protein, different from the subunit VII of the bc_1 complex [36]. This dual sensitivity to inhibitors has already been described for photosynthetic systems where stigmatellin was ascribed as having two different inhibition sites, both involving, presumably, plastoquinone-binding sites [37].

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