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Isolation, characterization and function of the two cytochromes *c* of the yeast *Candida parapsilosis*

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Candida parapsilosis is a strictly aerobic yeast which possesses two respiratory chains with a peculiar organisation, different from that of plant mitochondria. Besides the classical electron transport pathway, mitochondria of *C. parapsilosis* develops an alternative pathway, which does not branch off at the ubiquinone level, but merges at the complex IV level. Two pools of cytochromes *c* were distinguished by their spectrometric and potentiometric properties: (i) sequential cytochrome *c* reduction was promoted by two substrates, PMS ($E_m = 70$ mV) and TMPD ($E_m = 280$ mV). TMPD promoted the reduction of a cytochrome *c* with maxima at 551.9 and 417.3 nm for the α and the Soret bands, respectively, whereas cytochrome *c* reducible by PMS exhibited maxima at 549.7 and 419.9 nm; (ii) two midpoint redox potentials were resolved at 180 mV and 280 mV, respectively. The two cytochromes *c* were copurified by ion-exchange chromatography on Amberlite; after this step, the two cytochromes *c* can always be differentiated by TMPD and PMS, these reductants promoting different absorption bands. The two cytochromes *c* were separated by reverse-phase HPLC; this last purification step resolved two proteins with the same relative molecular mass of 13600 but a different amino-acid composition. Comparison of N-terminal sequences revealed differences between the two proteins. It was hypothesized that one cytochrome *c* is implicated in the functioning of the main chain and the other in that of the secondary pathway.

Introduction

Electron transfer reactions play a fundamental role in many essential metabolic processes. One of the protein electron carriers, the most extensively studied, is the soluble high-potential cytochrome *c* which can be readily isolated from both eukaryotic and prokaryotic sources. Cytochrome *c* has been extensively studied to characterize its structural, physical and enzymatic properties. It is a small basic protein composed of a single polypeptide chain wrapped around a covalently attached heme.

Depending on the organism, one or two species of cytochrome *c* have been found. In mammalian mitochondria, only one cytochrome *c* has been described which is able to act in two ways: (i) it transfers electrons from complex bc_1 to cytochrome *c* oxidase; (ii) it acts as an electron shuttle between the NADH cytochrome b_5 reductase localized on the external mitochondrial membrane and cytochrome *c* oxidase [1].

In the facultative aerobe yeast *Saccharomyces cerevisiae,* two isozymes of cytochrome *c* were described, iso-1 and iso-2-cytochrome *c* [2–4]. Both proteins were nuclear-encoded [5,6] and differ by 17 amino-acid substitutions and a four residue extension at the amino terminus resulting in an overall 84% amino acid sequence identity [7]. The relative proportions of the two iso-cytochromes *c* are strongly dependent on growth conditions (carbon source and degree of aeration). Iso-1-cytochrome *c* is the predominant isozyme, accounting for approx. 95% of the total cytochrome *c* under normal physiological conditions (aerobic culture). Yeasts grown under catabolite-repressed (high glucose concentrations) or anaerobic conditions generally contain high proportions of iso-2-cytochrome *c*. Prezant and co-workers [8] hypothesized also that the iso-2-cytochrome *c* plays a role when cells make a transition from an anaerobic to an aerobic environment. However, in other yeasts, such as *Candida krusei* or *Schizosaccharomyces pombe*, only one cytochrome *c* was found [9,10].

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Abbreviations: BSA, bovine serum albumin; EGTA, ethylenebis (oxyethylenenitrolo)tetraacetic acid; PMS, phenazine methosulfate; TMPD, *N,N,N',N'*-tetramethyl-1,4-phenylendiammonium dichloride; SHAM, salicylic hydroxamic acid.

The yeast *Candida parapsilosis* CBS 7154 was isolated in the laboratory and definitively classified by restriction endonucleases analysis of mitochondrial DNA [11]. It is a strictly aerobe organism, only dependent on oxidative metabolism for growth (for review see Ref. 12). It is nevertheless able to grow on high glucose concentration and also on a glycerol medium supplemented with antimycin A or drugs acting at the mitochondrial protein synthesis level [13]. Besides its normal respiratory chain, *C. parapsilosis* develops a second electron transfer chain, which is antimycin A-insensitive and which allows the oxidation of cytoplasmic NAD(P)H. This electron transfer functions through a route different from the alternative pathways described in numerous plants and microorganisms [14]. This secondary chain involves two dehydrogenases specific for NADH and NADPH, respectively, located on the outer face of the inner mitochondrial membrane and a specific pool of quinone [15].

This paper reports the isolation, characterization and function of the two cytochromes *c* from the yeast *C. parapsilosis*, the existence of which was postulated in a previous paper [14].

Materials and Methods

Strain, culture conditions and preparation of mitochondria. C. parapsilosis CBS 7154 was from the laboratory [11]. Cells were grown at 28°C under vigorous aeration on a complete medium supplemented with 2% (w/v) glycerol, with or without antimycin A (2 mg/l) and harvested in the late exponential growth phase. Mitochondria were prepared after digestion of the yeast cell wall with snail (*Helix pomatia*) juice enzyme [16]. Oxygen uptake was followed polarographically at 28°C with a Clark electrode in a medium containing 0.65 M mannitol, 0.36 mM EGTA, 0.3% (w/v) bovine serum albumin and 10 mM Tris-maleate buffer (pH 6.7).

Isolation of cytochromes c. Cytochromes *c* were isolated by modifying the method of Sels et al. [2]. Cells (1 g dry weight corresponding approx. to 80 nmol of cytochrome *c*) were homogenized with 2 ml of 8.5% glycerol (v/v), 170 mg $Na_2S_2O_4$ and 1 ml ethyl acetate. The thick suspension was shaken overnight at room temperature under N_2 atmosphere. After dilution with 5 ml of 0.5 M NaCl, the suspension was centrifuged (10 min, $3000 \times g$). After a second washing with 0.5 M NaCl, the two supernatants were pooled and centrifuged for 20 min at $20000 \times g$. The resulting supernatant was dialysed against 5 mM K/Na_2 phosphate buffer (Sorensen buffer) (pH 7.0) containing 1 mM potassium ferricyanide. Then, cytochromes *c* were adsorbed in batches under agitation at 4°C on an Amberlite ion-exchanger (XE 64) previously treated with 4% sodium hypochlorite and equilibrated in 0.1 M Sorensen buffer (pH 7.0) (about 1 g of resin for 1 mmol of cytochrome *c*). For gradient chromatography, the resin containing adsorbed cytochrome *c* was layered onto the top of an Amberlite column. Cytochrome *c* was eluted using an NaCl gradient ranging from 0.1 to 0.4 M in 0.1 M Sorensen buffer (pH 7.0) with $2 \cdot 10^{-3}$ M dithiothreitol. Fractions containing cytochrome *c* (about 16 nmol) were pooled and dialysed against water (overnight) and then lyophilized. The lyophilizate of cytochrome *c* (four preparations were pooled at this step, corresponding to 64 nmol) was dissolved in water and loaded onto a 5-mm Vydac C_{18} column (0.4×25 cm). The column was developed at room temperature at a flow rate of 0.5 ml/min with a linear 60 min gradient from 20 to 35% solvent B_1, where primary the solvent (A) was aqueous 0.06% (v/v) trifluoroacetic acid and the secondary solvent (B_1) was acetonitrile/isopropanol (1:2) containing 0.06% trifluoroacetic acid (v/v). Detection was monitored at 280 nm.

Analytical methods. Amino-acid analyses of acid hydrolysates of protein (1 nmol) were performed according to Heinrikson and Meredith [17]. Samples were hydrolyzed in the presence of 6 M HCl for 24, 48 and 72 h. Phenylthiocarbamyl derivative amounts were determined by reverse-phase HPLC.

Cytochrome spectra and kinetics measurements. These were recorded at 20°C on a double-beam dual-wavelength Aminco Chance DW 2000 spectrometer.

Redox titrations. Redox titrations were performed by using the rapid scan spectrometer CD66 as previously described [18] except that a constant flow of dinitrogen instead of argon was used to maintain anaerobic conditions. Solutions of 0.5 M sodium dithionite and 0.5 M potassium ferricyanide were used as reductant and oxidant, respectively. Mediators were TMPD ($E_m = 280$ mV), diaminodurol ($E_m = 240$ mV), ferric chloride ($E_m = 170$ mV), duroquinone ($E_m = 5$ mV) and 2 hydroxy 1–4 naphthoquinone ($E_m = -150$ mV). Spectra were recorded with the CD 66 rapid scan spectrometer at redox equilibrium. Data were analysed on a HP 9000 computer according to Denis et al. [19]. Parameters of the generalized Nernst equation to be fitted on titration data of a given multicomponent redox system; EM(J), midpoint potential of component J with respect to the normal hydrogen electrode and for the pH value of the solution; DA(J), contribution of component J to the overall absorbance change of the solution, from fully oxidized to fully reduced, at the wavelength selected for monitoring the titration. The sign of this algebraic parameter points to a more absorbing reduced species when negative and the opposite when positive. The number of electrons involved in redox equilibria were fixed for the fitting, considering that in the case of hemes, the attached integer values could not fluctuate.

Electrophoresis. A modification of the discontinuous SDS system of Laemmli [20] described in Velours et al. [21] was used for slab electrophoresis. The slab gel was washed with methanol/H_2O/acetic acid (5:5:1) mixture and stained with the silver nitrate method [22].

Protein concentrations. Protein concentrations were determined by using the Lowry procedure [23]. Bovine serum albumin served as the standard protein.

Results

Evidence for two cytochromes c in C. parapsilosis mitochondria

The existence of two cytochromes *c* in the yeast *Candida parapsilosis,* related to the main respiratory chain and to the alternative pathway, respectively, was postulated from several lines of evidence: (i) addition of increasing concentrations of cyanide (up to 10 mM) to isolated mitochondria resulted in the progressive reduction of cytochrome *c*, while cytochrome *b* was fully reduced with 0.1 mM cyanide; (ii) sequential addition of NADH and ascorbate (+TMPD) resulted in the sequential reduction of cytochrome *c*, whatever the order of substrate addition [14]. Preliminary redox potential measurements, carried out on mitochondria, provided evidence for the presence of two cytochromes *c* (Guerin, M. and Ohnishi, T., unpublished results).

Experiments presented in Fig. 1 show that when using mitochondria isolated from cells grown in the presence of antimycin A and treated with increasing KCl concentrations, NADH oxidation became more sensitive to low cyanide concentration (0.1 mM). It had been shown that such KCl treatment removed cytochrome *c* from mitochondria [24]. Since the activity of the alternative pathway decreased during KCl treatment, we hypothesized that the cytochrome *c* involved in this pathway was preferentially lost.

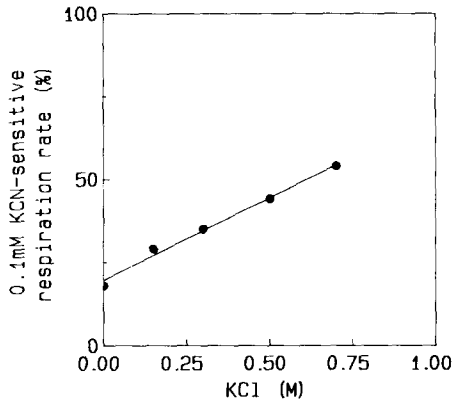

Fig. 1. Effect of KCl treatment on the 0.1 mM KCN-sensitivity of respiration. *C. parapsilosis* was grown in the presence of antimycin A. Mitochondria were incubated with increasing, KCl concentrations in 0.3 M mannitol buffer for 10 min and then centrifuged. The pellet was resuspended in the 0.6 M mannitol, 0.36 mM EGTA, 10 mM Tris/maleate, 0.3% BSA buffer (pH 6.7). Respiration was measured with 2 mM NADH as substrate.

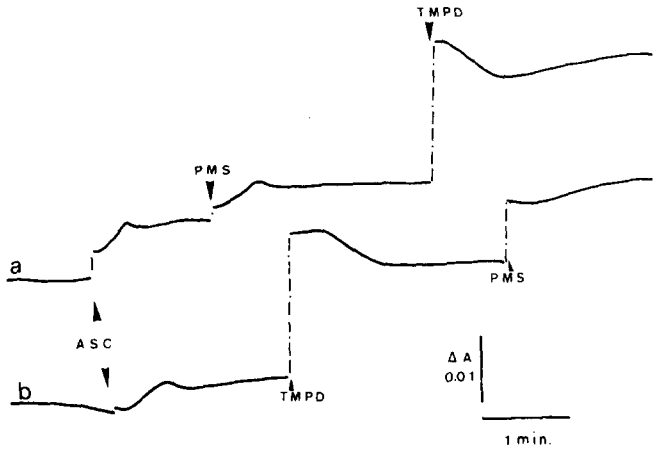

Fig. 2. Kinetics of cytochrome *c* reduction. *C. parapsilosis* was grown in the presence of antimycin A. Mitochondria (2.5 mg/ml) were suspended in a 0.65 M mannitol, 0.36 mM EGTA, 10 mM Tris/maleate buffer (pH 6.8), and oxidized at 0°C by shaking. Measurements were recorded at 550 nm with 540 nm as reference wavelength. Kinetics reduction were followed by successive additions of 2 mM ascorbate, 1.5 mM PMS, 0.7 mM TMPD (trace a) or 2 mM ascorbate, 0.7 mM TMPD, 1.5 mM PMS (trace b).

These results prompted us to investigate the spectral properties of these cytochromes, using either PMS (E_m = 70 mV) or TMPD (E_m = 280 mV) as electron donors, in the presence of ascorbate. Fig. 2 shows the reduction kinetics of cytochromes *c* of *C. parapsilosis*: ascorbate alone resulted in a very slight reduction whereas PMS addition instantaneously reduced part of cytochrome *c*, which cannot be enhanced by a second addition (not shown); subsequent addition of TMPD promoted an additional reduction. These sequential cytochrome *c* reductions were independent of the order of reductant addition. When added first, TMPD reduced part of cytochrome *c*, the remaining being reduced by PMS. When the same experiment was done on *S. cerevisiae* mitochondria, it was observed that ascorbate alone had no effect, and that addition of PMS or TMPD fully reduced cytochrome *c* (not shown).

Since different stationary states were observed in *C. parapsilosis,* depending on the electron donnor, spectrometric measurements were recorded between 400 and 580 nm (Fig. 3). Both steady states corresponded to two cytochromes *c* which can be distinguished by their absorption bands. The α and Soret bands are shown in Figs. 3A and 3B, respectively: curve a corresponds to the TMPD reduction and curve b to PMS reduction. PMS promoted the reduction of a cytochrome *c* with peaks at 549.7 and 419.6 nm, whereas the TMPD reducible cytochrome *c* had peaks at 551.9 and 417.3 nm. PMS addition resulted also in the formation of a Soret band at 442 nm, corresponding to the Soret band of reduced cytochrome 590 (the postu-

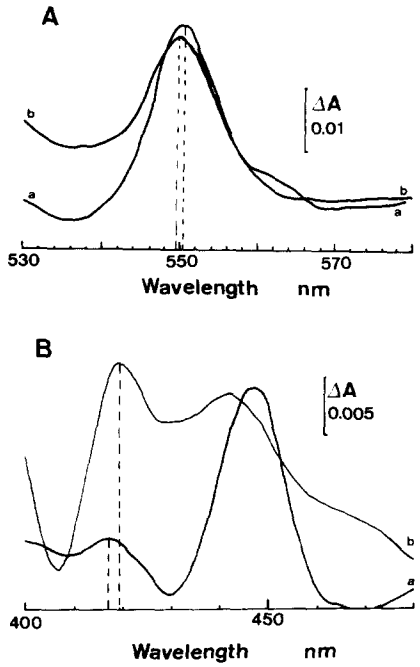

Fig. 3. Reduced-minus-oxidized difference spectra of *C. parapsilosis* mitochondria (stationary states). Experimental conditions were the same as in, Fig. 2. Mitochondria (3 mg/ml) were reduced in the presence of 2 mM ascorbate by 0.7 mM TMPD (trace a) or by 1.5 mM PMS (trace b). A: α bands; B: Soret bands.

lated alternative oxidase) [14] while TMPD addition resulted in the formation of a Soret band at 447 nm, corresponding to reduced cytochrome *c* oxidase.

In previous works it has been claimed that cytochrome *c* involved in the alternative pathway could deliver electrons either to cytochrome *c* oxidase or to the alternative oxidase, which is SHAM sensitive. We compared the reduction levels of cytochrome *c* when mitochondria were preincubated with 2 mM SHAM or 0.1 mM cyanide (Table I): (i) addition of SHAM increased by 20% the reduction level of PMS reducible cytochrome *c*, but the steady-state level obtained after TMPD addition was the same in the presence or in the absence of SHAM; (ii) in the presence of cyanide both cytochromes *c* were reduced after PMS addition. From these results we concluded that TMPD promoted reduction of the cytochrome *c* implicated in the main respiratory chain, whereas PMS promoted reduction of the cytochome *c* of the alternative pathway.

TABLE I

Effect of inhibitors on the cytochrome c reduction induced by TMPD or PMS

Spectra were recorded under the same conditions as in Fig. 3 with 2 mM SHAM or 0.1 mM KCN or without inhibitors (control). Values were expressed in variation of absorbance (%): % of reduction.

Experimental conditions	*A* (550–540 nm)	
	+PMS	+TMPD
Control	0.0168 (59%)	0.0285 (100%)
+SHAM	0.0205 (73%)	0.0282 (100%)
+KCN	0.0262 (99%)	0.0265 (100%)

Purification of the two cytochromes c

The existence of two hemes of the cytochrome *c* type being demonstrated by spectrometric experiments, it was important to know whether we had one protein carrying two hemes, as has already been described for some bacteria [25,26], or two different proteins.

Cells were grown on a glycerol medium supplemented with antimycin A to increase the alternative pathway contribution to the respiratory activity and harvested in the late exponential growth phase. Cytochromes *c* were extracted as described in the Material and Methods section. With the chromatographic technique used (Amberlite ion exchanger), allowing the separation of iso-1 and iso-2 cytochromes *c* of *S. cerevisiae,* we obtained a single elution peak of cytochrome *c* of *C. parapsilosis.* The peak was eluted at 0.1 M NaCl concentration for the oxidized cytochrome. This value is notably weaker than those obtained for iso-1 and iso-2 cytochromes *c* of *S. cerevisiae* (0.27 M and 0.32 M, respectively) and similar to the molarity (0.12 M) reported for horse heart cytochrome c [2]. At this step of purification, only one cytochrome *c* was detectable by electrophoresis; however, it was always possible, on this fraction, to differentiate two cytochromes *c* by reduction with TMPD (maxima at 550.2 and 418.2 nm) or PMS (maxima at 549.5 and 419.2 nm) (Results not shown). Separation of the two proteins was achieved in one step by reverse-phase HPLC on a C_{18} Vydac column eluted with a 60 min gradient of acetonitrile/isopropanol (1:2) in the presence of 0.06% trifluoroacetic acid (20% to 35%) (Fig. 4). Two protein peaks eluted at 47 and 52 min. These peaks corresponded to two cytochromes *c* (c_A and c_B) with a relative molecular mass of 13600 (Fig. 5). After this last step of purification, it was not possible to measure an oxidoreduction of cytochromes *c*.

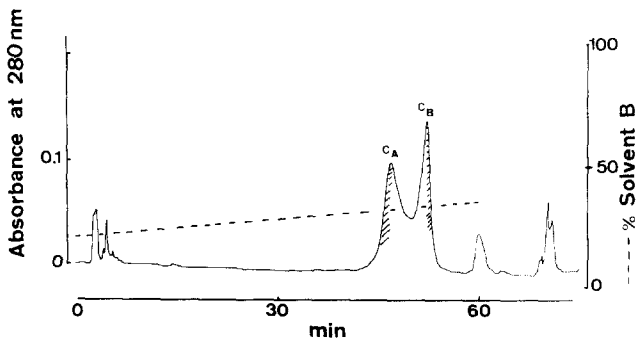

Fig. 4. Purification of cytochromes *c* by reverse-phase, HPLC. The enriched fractions obtained after Amberlite chromatography (7.5 nmol), were loaded onto a 5 mm Vydac, C_{18} column (0.46×25 cm). Flow rate was 0.5 ml/min. Gradient conditions and solvents are described in Materials and Methods.

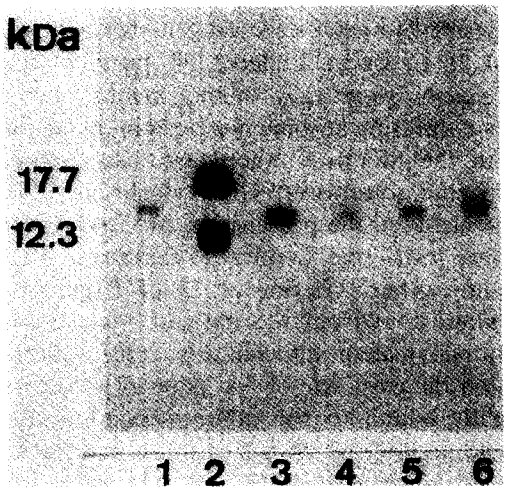

Fig. 5. SDS polyacrylamide gel electrophoresis of isolated cytochromes *c*. The fractions were dissociated and separated on a 15% polyacrylamide slab gel and silver stained. Lane 1: mix of the two cytochromes *c* before the last step of purification; lane 2: molecular weight markers (myoglobin and commercial yeast cytochrome *c*) (0.5 μg of each); lanes 3 and 4: cytochrome c_B; lanes 5 and 6: cytochrome c_A. The duplicate lanes for c_A and c_B correspond to two different experiments.

Amino-acid composition was performed on both fractions (Table II) from which some points can be evidenced: (i) a high content in proline was found in these two cytochromes *c*, as compared to the cytochromes *c* from other species; (ii) the amino-acid composition of cytochromes *c* from *C. parapsilosis* showed more similarities with that of *S. cerevisiae* than that of *C. krusei;* (iii) the two cytochromes *c* of *C. parapsilosis* exhibited the same differences as those found between the two cytochromes *c* of *S. cerevisiae* (iso-1 and iso-2).

Proteolytic cleavage of the two proteins with endoproteinase Arg-C was performed and the peptides were analyzed by reverse-phase HPLC. Although there were strong homologies between the two profiles, different peaks were found for the cytochrome c_A (not shown).

The N-terminal sequence of the two cytochromes *c* were determined (Fig. 6). The first 10 amino-acid residues were identical, but afterwards, some differences appeared between the two cytochromes *c*: lysine (+5), threonine (+8) and phenylalanine (+10) in c_B were replaced by proline (+5), serine (+8) and aspartic acid (+10) in c_A. Although amino-acid composition between cytochromes *c* from *C. parapsilosis* and from *C. krusei* was different, there was strong similarities between the N-terminal sequences.

Potentiometric properties of the isolated cytochromes c

Cytochromes *c* from *C. parapsilosis*, collected before the last separation step, were potentiometrically titrated at 20°C and pH 7.2. Titration curves corresponding to absorbance changes (at 550 nm) versus redox potential were analysed with respect to a generalized Nernst equation [19]. Fig. 7 illustrates such a titration with its theoretical representation. Whereas a simple observation of a curve would suggest the presence of a single component, two redox couples were resolved by the analysis procedure. Their midpoint

TABLE II

Amino-acid composition of the resolved cytochromes c

Amino-acid content of hydrolysates of cytochromes c_A and c_B of *C. parapsilosis* was determined after separation of the phenylthiocarbamyl derivatives by reverse-phase HPLC.
(a) Composition by amino acid analysis (average values of two experiments for c_A and of four experiments for c_B.
(b) Composition obtained by computation by using a molecular weight of 13000.
(c) Iso-1 cytochrome *c* of *S. cerevisiae* from Ref. 5; iso-2 cytochrome *c* of *S. cerevisiae* from Ref. 6; cytochrome *c* of *Schizosaccharomyces pombe* from Ref. 10; cytochrome *c* of *C. krusei* from Ref. 9.

	C. parapsilosis				*S. cerevisiae*		*S. pombe*	*C. krusei*
	nmol/100 (a)		mol/mol (b)		mol/mol (c)		mol/mol (c)	
	C_A	C_B	C_A	C_B	Iso-1	Iso-2		
Ala	6.6	6.3	7	7	7	8	11	12
Arg	2.9	2.8	3	3	3	3	4	4
Asp	4.7	4.5	5	5	11	12	10	8
Cys [a]	1	0.9	2	2	2	2	2	2
Glu	5.9	4.8	7	5	9	10	10	10
Gly	14.5	12.1	16	13	12	12	12	12
His	2.9	3.6	3	4	4	3	2	4
Ile	2.25	1.5	3	2	4	5	3	3
Leu	8	8.9	9	10	8	5	5	6
Lys	18	15.3	20	17	16	17	14	12
Met	nd	nd	nd	nd	2	3	1	3
Phe	5	5.5	5	6	4	4	6	4
Pro	8.6	10	10	11	4	6	6	7
Ser	6.1	5	7	6	4	5	3	6
Thr	7.2	7.2	8	8	8	8	9	7
Trp	nd	nd	nd	nd	1	1	1	1
Tyr	2.7	5	3	6	5	5	5	5
Val	6	6.6	7	7	3	3	4	3

[a] The quantity of cysteine was undervalued because analyses were done without heme removal.

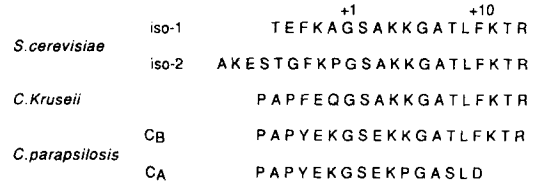

Fig. 6. Comparison of N-terminal sequences of cytochrome *c* from different yeast species. Automated sequence analyses of cytochromes c_A and c_B from *C. parapsilosis* were performed in an Applied Biosystem gas-phase sequenator (Model 475), equipped with an on-line analyzer (Model 120A). Phenylthiohydantoins were separated on a 5-mm C_{18} HPLC column (220×2.1 mm). N-terminal sequences of cytochromes *c* from *C. krusei* and *S. cerevisiae* (iso-1) were from Ref. 32 and *S. cerevisiae* (iso-2) from [7]. Position +1 was defined in function of the first amino-acid of horse cytochrome *c*.

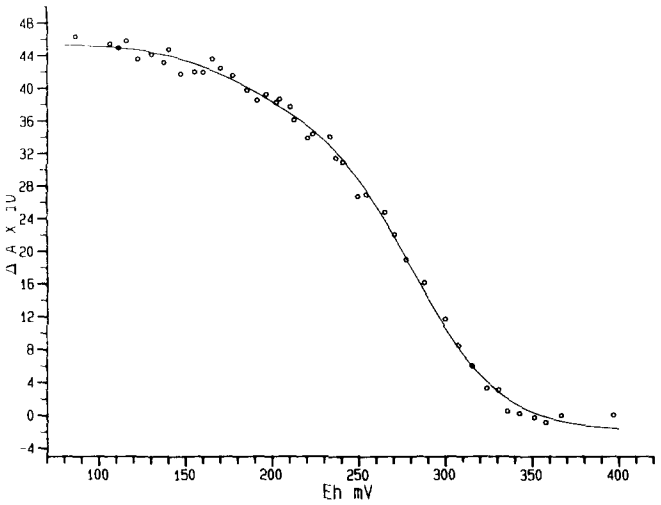

Fig. 7. Redox titration of isolated cytochromes *c* from *C. parapsilosis*. Sample: 24 nmol cytochromes *c* (199 mM final concentration) in 1 M phosphate respiratory buffer (pH 7.2) containing 3.36 mM TMPD, 16.6 mM diaminodurol and 8.3 mM $FeCl_3$. Temperature 20°C. Parameters of the fitted curve: AR = 45.45 $A \times 10^3$, EM(1) = 280.48 mV, DA(1) = −39.05 $A \times 10^3$, EM(2) = 180.80 mV, DA(2) = −8.37 $A \times 10^3$, the number of electrons involved in each couple being fixed to 1 (see Materials and Methods for the meaning of the parameters of the generalized Nernst relationship).

potentials are respectively 280 and 180 mV. The high midpoint potential cytochrome *c* was identified with the mitochondrial cytochrome *c* by titrating cytochrome *c* purified from *S. cerevisiae* (E_m = 285 mV) (data not shown), previously characterized in mitochondria [27]. The low midpoint potential cytochrome *c* was present in different amounts in the titrated samples (18 to 55%), which resulted in a higher uncertainty on its midpoint potential (180–220 mV) than the high midpoint potential cytochrome *c* (280–290 mV).

Discussion

In the prokaryotic kingdom, several cytochromes *c* have been identified in relation to the great diversity of their terminal electron transfer processes in comparison to those in mitochondria. Thereby, a classification system for cytochrome *c* has been proposed by Ambler [28,29]. Class I contains cytochromes showing sequence and structure homology with mitochondrial cytochromes *c*. The cytochromes c found mainly in photosynthetic bacteria, and having a very different structure, are placed in Class II. Class III contains homologous cytochromes with multiple haems and very low redox midpoint potentials. In this paper we were concerned only with Class I cytochromes *c*. Besides the situation of *S. cerevisiae*, where one haploid cell produces two iso-cytochromes *c* of different primary structure, other polymorphisms of cytochrome *c* have been described. In all these cases, polymorphism was tentatively ascribed to genetic heterogeneity of the starting material. In *C. krusei*, Lederer [30] purified from two strains, each genetically homogeneous, two cytochromes *c* differing at some positions in the amino-acid chain. In the literature, there are also examples of tissue-specific isoforms involving cytochrome *c*. Hennig [31] demonstrated the presence of two iso-cytochromes *c* in mouse testis, one identical to that present in adult tissues, the other differing at 13 of 104 amino-acid residues and found only during early mouse development. In mammalian mitochondria, some authors have postulated the existence of two pools of cytochrome *c*, one tightly bound to the inner mitochondrial membrane and another which is free in the intermembrane space and which acts as an electron carrier between cytochrome b_5 and cytochrome *c* oxidase [1].

Results presented in this work establish the existence of two functional cytochromes *c* in *C. parapsilosis* mitochondria. The purification of these cytochromes *c* provides evidence for two proteins having the same relative molecular mass of 13600, but a different amino-acid composition. According to these amino-acid compositions, these proteins show a greater similarity with cytochromes *c* from *S. cerevisiae* than with the one from *C. krusei*. However, when N-terminal sequences were compared, similarities were found between cytochromes *c* from *C. parapsilosis* and *C. krusei*. Cytochromes *c* from both these yeast species possess one amino-acid more than the one from *S. cerevisiae* (iso-1 cytochrome *c*). An 80% amino-acid sequence identity was found between the N-terminal sequence of cytochrome c_B from *C. parapsilosis* and the one from *C. krusei*. N-terminal sequences of both cytochromes *c* from *C. parapsilosis* differ by 3 amino-acid residues. However, when N-terminal sequences of cytochromes *c* from the 3 yeast species were compared, similarities were found from amino acid residue at position +1 to position +13.

The presence of two cytochromes *c* in *C. parapsilosis* suggested that they could have the same behaviour as the two cytochromes *c* from *S. cerevisiae* (iso-1 and iso-2). Nevertheless, the great difference between the two yeasts lies in the fact that iso-1 and iso-2 cytochrome *c* exhibit the same spectral properties, the same midpoint redox potential [2] and are only implicated in the classical respiratory chain; on the contrary, in *C. parapsilosis*, the two cytochromes *c* differ in physicochemical properties: (i) sequential reduction depending on the nature of substrate added, TMPD or PMS, enabled the visualization of different maxima in the α and Soret bands; (ii) 2 different midpoint redox potentials were resolved. The high midpoint potential cytochrome *c* (E_m = 280 mV) was unambiguously identified with mitochondrial cytochrome *c* through separated titration of the purified yeast enzyme. Surprisingly, the low midpoint potential cytochrome *c* exhibits

an E_m value rather similar to that of cytochrome c_1 form *S. cerevisiae* [27].

These two cytochromes *c* are involved in two different electron transfer pathways although they present strong similarities in amino acid composition. According to the results presented in this work, it appears that TMPD promotes the reduction of the cytochrome *c* implicated in the main respiratory chain, whereas the cytochrome *c* related to the alternative pathway is reduced by PMS. The problem is to understand why PMS does not reduced cytochrome *c* implicated in the main respiratory chain as it does in *S. cerevisiae* mitochondria. As an hypothesis, it is proposed that the alternative oxidase of *C. parapsilosis* has a midpoint potential lower than 280 mV, which is the mid point potential of the cytochrome *c* implicated in the main pathway. In this way, electrons flow preferentially through this oxidase, except when this latter is blocked with SHAM. Indeed, it was previously shown that it was possible to measure a P/O equal to 1.2 with NADH as substrate in the presence of antimycin A only when SHAM was added to mitochondria [15]. In *C. parapsilosis,* it is proposed that the secondary chain is working in parallel with the main one, with the possibility for electrons to pass through the cytochrome 590 or to branch the main pathway at the cytochrome *c* level.

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