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IDENTIFICATION OF THREE DISTINCT SPECTRAL SPECIES OF YEAST MITOCHONDRIAL CYTOCHROME *b* USING A COMBINATION OF RESPIRATORY INHIBITORS *

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Appropriate combination of specific inhibitors of electron transport in the cytochrome bc_1 segment of the respiratory chain of *Saccharomyces cerevisiae* allows the rapid resolution of three spectral forms of mitochondrial cytochrome *b*. (1) Addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to aerobic yeast submitochondrial particles preincubated with cyanide and mucidin in the presence of NADH reveals cytochrome *b*-561.5. (2) Addition of funiculosin to aerobic yeast submitochondrial particles preincubated with cyanide, mucidin and *n*-heptylhydroxyquinoline *N*-oxide in the presence of NADH reveals cytochrome *b*-558 independently of cytochrome *b*-561.5 and cytochrome *b*-565. (3) Specific resolution of cytochrome *b*-565 can be obtained either by addition of mucidin to aerobic submitochondrial particles preincubated with cyanide, DCMU and NADH, or by addition of antimycin plus an oxygen pulse to NADH-reduced particles, preincubated with cyanide, in the presence of ascorbate plus TMPD, or by addition of antimycin A in the presence of oxidized TMPD to aerobically NADH-reduced particles.

Introduction

The existence of at least three spectral species of mitochondrial cytochrome *b* has been demonstrated in various organisms (for reviews see Ref. 1–6). A major band of absorption with a maximum at 562 nm (561.5 nm in yeast) was identified as the classical cytochrome b_K readily reduced by succinate [7,8]. Two other minor absorption peaks at 566 nm (565 nm in yeast) and 558 nm were attributed to a single cytochrome (b_T) reducible by succinate in the presence of ATP in respiring heart submitochondrial particles [9–11]. Alternatively, these two absorption bands were attributed to two different cytochrome *b* species in rat liver [13,14] and yeast [15,16]. Specific reduction of cytochrome b_T can be elicited by addition of antimycin plus oxygen to anaerobic or to cyanide-inhibited heart mitochondria [9–12].

We have previously shown that the addition of DCMU, antimycin A, mucidin, HpHOQnO or funiculosin together with a pulse of oxygen to yeast submitochondrial particles which either have reached anaerobiosis or which have been supplied with cyanide stabilizes an oxidant-induced extra reduction of cytochrome *b* [17,18]. These so-called 'extra reductions' measured in the presence of antimycin A, HpHOQnO or DCMU involved cytochrome *b*-561.5 as well as cytochrome *b*-565. This was not the case with mucidin where the major spectral contribution of extra-reduced cytochrome *b* was provided by cytochrome *b*-565. In the presence of funiculosin the broadness of the peak of extra-reduced cytochrome *b* indicated an unusually large contribution of cytochrome *b*-558 compared to that observed with all other inhibitors. tested.

From these observations, we have elaborated a set

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Abbreviations: HpHOQnO, *n*-heptylhydroxyquinoline *N*-oxide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron).

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of experimental conditions which permit us to identify specifically and independently the three spectral forms of cytochrome *b* of *Saccharomyces cerevisiae*. These results support the view that the absorption bands at 558 and 565 nm belong to two different spectral forms of cytochrome *b* which can be at least partly reduced independently of each other.

Material and Methods

Yeast submitochondrial particles were prepared from the wild-type strain KL14-4A as previously described [17]. The spectroscopic measurements were carried out at 25°C in a reaction medium containing 250 mM sucrose, 20 mM Tris-HCl, pH 7.5, and 1 mM EDTA (disodium salt), using an Aminco DW2 spectrophotometer as fully described in the figure legends.

Antimycin A was purchased from Boehringer (Mannheim), HpHOQnO from Sigma (St. Louis, MO). DCMU was a gift provided by E.I. du Pont de Nemours and Co. (Wilmington, DE) and funiculosin was generously given by Sandoz, A.G. (Basel). Mucidin was kindly given by Dr. J. Subik (Bratislava).

All the inhibitors were dissolved in methanol and added to the reaction mixture so that the final concentration of methanol did not exceed 0.5% (v/v).

Results and Discussion

Reduction of cytochrome b-561.5

The difference spectrum A of Fig. 1 shows that the addition of DCMU to yeast submitochondrial particles incubated with cyanide and mucidin, under aerobic conditions in the presence of NADH, induces the extra reduction of a cytochrome *b* absorbing at 561.5 nm. The contribution of cytochrome *b*-565 to this absorption band has been nullified by preincubation of the particles in the sample and reference cuvettes in the presence of mucidin which induces specifically the reduction of cytochrome *b*-565 in yeast [18].

Reduction of cytochrome b-558

Addition of funiculosin to aerobic cyanide-poisoned, NADH-reduced submitochondrial particles in which cytochromes *b*-561.5 and *b*-565 have been previously extra reduced by HpHOQnO and mucidin

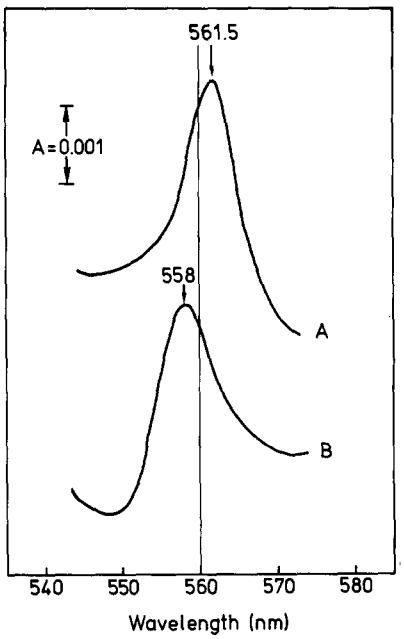

Fig. 1. (A) Absorption spectrum of cytochrome *b*-561.5. Yeast mitochondrial particles (2 mg protein/ml) in both the sample and reference cuvettes in the medium described in Material and Methods were supplemented with 2 mM NaCN, 2 μM mucidin and 5 mM NADH. Then 200 μM DCMU was added to the sample cuvette and the spectrum was immediately measured. (B) Absorption spectrum of cytochrome *b*-558. Mitochondrial particles (2 mg protein/ml) in both the sample and reference cuvettes were supplemented with 2 mM NaCN, 0.4 μg mucidin, 5 μM HpHOQnO and 5 mM NADH. Then 8 μM funiculosin was added to the sample cuvette and the spectrum was recorded 3–5 min later.

[18], respectively, induces a specific reduction of the short-wavelength cytochrome *b*-558 (spectrum B of Fig. 1). No contribution from a long-wavelength cytochrome *b* form is observed at 565 nm.

Reduction of cytochrome b-565

Several different ways to reduce specifically cytochrome *b*-565 by using inibitors of the cytochrome bc_1 segment were suggested from our previous studies [17,18]. The extra reduction of cytochrome *b*-565 of yeast can be observed by addition of mucidin to cyanide-poisoned, NADH-reduced particles preincubated with DCMU to rule out any contribution of cytochrome *b*-561 (Fig. 2, spectrum A).

Another method can be used where cytochromes *c* and aa_3 of the submitochondrial particles are fully

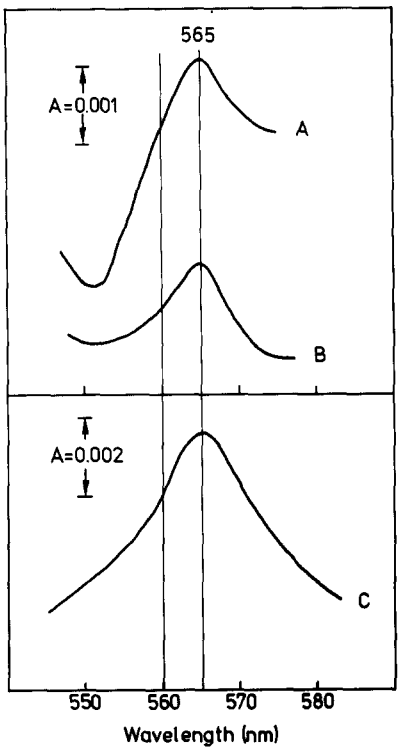

Fig. 2. Absorption spectrum of cytochrome *b*-565. (A) Yeast mitochondrial particles (2 mg protein/ml) in both the sample and reference cuvettes in the reaction medium were supplemented with 2 mM NaCN, 100 μM diuron and 5 mM NADH. Then 0.4 μg mucidin was added to the sample cuvette and the spectrum was measured immediately. (B) Yeast particles (2 mg protein/ml) in both the sample and reference cuvettes were supplemented with 5 mM ascorbate, 0.2 mM TMPD, 2 mM NaCN and 5 mM NADH. Then 0.5 μg antimycin A was added to the sample cuvette and the spectrum was measured immediately. (C) Yeast particles (3 mg protein/ml) in the sample cuvette were reduced by addition of 5 mM NADH, 1 μg antimycin A and 1 mM TMPD. 5 mM NADH were added to the particles of the reference cuvette. The spectrum was immediately measured (before anaerobiosis was reached in both cuvettes).

reduced in both cuvettes by ascorbate plus TMPD in the presence of cyanide [19]. NADH is then added to the two cuvettes to reduce cytochrome *b*-561.5. Finally, the addition of antimycin A to the sample cuvette extra reduces the cytochrome *b*-565 only (Fig. 2, spectrum B).

Specific reduction of cytochrome *b*-565 can also be observed very easily in aerobically NADH-reduced particles to which are successively added, antimycin A and the artificial redox mediator TMPD, while in the reference cuvette the particles are aerobically reduced by NADH only, in the absence of inhibitor. TMPD establishes a by-pass over the antimycin A-sensitive site of the respiratory chain [19] leading to reduction of cytochromes cc_1 and aa_3 and to specific reoxidation of cytochrome *b*-561.5 which together with cytochrome *b*-565 had been previously reduced in the presence of antimycin A. As a result only a very symetric peak of absorbance of reduced cytochrome *b*-565 is observed (Fig. 2C).

In conclusion, appropriate combination of the specific inhibitors of electron transfer in the cytochrome bc_1 segment allows the detection of three distinct forms of mitochondrial cytochrome *b* in the yeast *S. cerevisiae*. To our knowledge, it is the first time that the spectral band of cytochrome *b*-558 can be demonstrated without contamination of the band of cytochrome *b*-565 in this yeast. This is due to an unexpected property of funiculosin which among the cytochrome bc_1 inhibitors has been the least studied so far. Whether this cytochrome *b*-558 belongs to complex II or III [21] of the respiratory chain cannot presently be assessed.

The methods we propose are rapid but qualitative at the present stage. They can be used for identification of the different spectral forms of cytochrome *b* in the various yeast mutant strains modified in their cytochrome *b* which are now available [18,22–28].

The above data also illustrate clearly the conclusion obtained in earlier reports [17,18,29] that the modes of action of DCMU, HpHOQnO, mucidin, funiculosin and antimycin A on the yeast cytochrome bc_1 complex are not identical. They are in line with the genetic data which distinguish at least five different genetic loci for drug resistance in distinct exons of the split gene of mitochondrial DNA [24–28].

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