CYTOCHROME BIOSYNTHESIS UNDER COPPER-LIMITED CONDITIONS IN *CANDIDA UTILIS*

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1. Introduction

Yeasts grown strictly under anaerobic conditions in the presence of a fermentable substrate lose their ability to respire and become devoid of the typical cytochrome system [1, 2]. The mitochondria of these cells differ in ultrastructure from normal mitochondria obtained in aerobically grown yeast and are termed pro-mitochondria [3, 4]. During aeration, the cells rapidly recover the cytochrome system and acquire typical mitochondria exhibiting normal respiratory activity. These changes in cell structure and function and the synthesis of cytochrome oxidase are initiated by oxygen [1, 2]. However, the cell in anaerobic conditions exhibits a change in all the cytochromes rather than a specific alteration of cytochrome oxidase. In contrast, when yeast is grown in a copper deficient medium, the synthesis of cytochrome oxidase is considered to be specifically inhibited with no change in the other cytochromes [5].

The present work describes the induction of cytochrome oxidase by copper and the behavior of the other cytochromes during the recovery phase of cytochrome oxidase.

2. Material and methods

Candida utilis was grown in copper free medium according to Galzy and Slonimski [6] using 1.5% ethanol as carbon source. The copper was extracted by zinc dibenzyldithiocarbamate, as described by Giorgio et al. [7] with minor modifications such as increase of chelator and a reduced time of copper extraction. Under such conditions, the synthesis of cytochrome oxidase is nearly completely inhibited. The growth medium used to obtain normal yeast from cytochrome deficient cells, was obtained by adding 110 μg of copper per liter of extracted medium (= copper supplemented medium). To study the induction of the synthesis of cytochrome oxidase, approximately 8 g of copper deficient cells were added to 7 l of copper sufficient medium. The cells were collected at various times from 0 to 16 hr, centrifuged, washed twice with distilled water, and suspended at a concentration of 5×10^9 cells/ml in 0.1 M phosphate buffer (pH 6.8) for spectroscopic examination. The direct effect of copper on the induction of cytochrome oxidase was determined by growing the copper deficient cells in the presence of increasing amounts of copper in the medium.

To study the presence of hypothetical apo-oxidase in copper deficient cells, cells were placed in non-growing conditions by omission of carbon source as follows: phosphate buffer 0.1 M, copper 110 μg per liter (pH 6.8) or in Galzy and Slonimski medium [6]. When added, chloramphenicol and cycloheximide were present in final concentrations of 4 mg/ml and 10 μg/ml, respectively.

In order to economize on cells and approximately to delineate the cytochromes, liquid nitrogen temperature (77 °K) difference spectra were obtained with a split beam spectrophotometer [8]. Under these conditions the ratio of the amounts of the cytochromes are sensitively and accurately identified. The reproducibility of the ratio method is illustrated by the plateau after 12 hr (fig. 2). The error of replicates is only a few percent. The observed scatter is 10 to 20% for "absolute" determinations as indicated in fig. 3, pos-

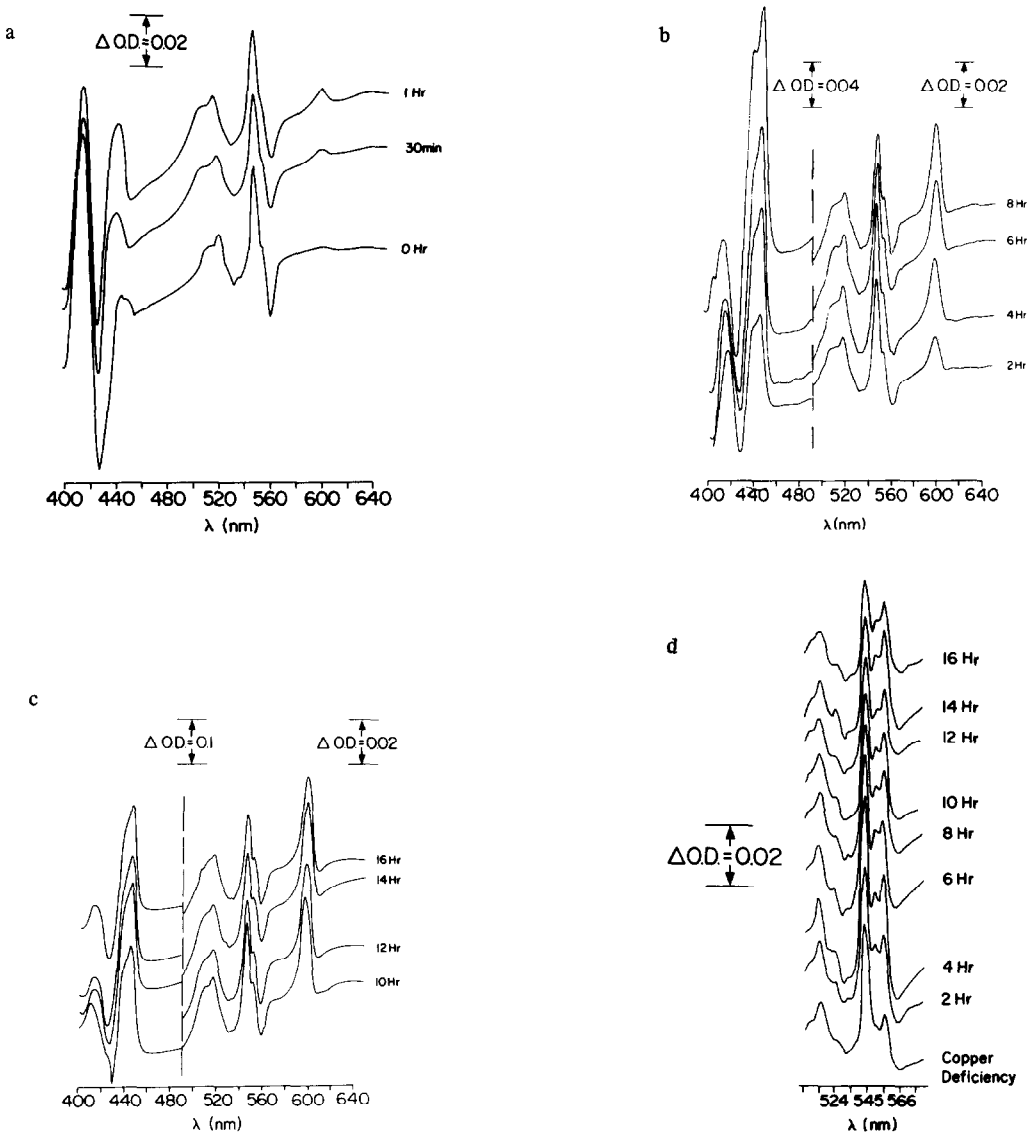

Fig. 1. Low temperature difference spectra (oxidized minus reduced) from copper deficient cells during the induction of cytochrome oxidase. Fig. 1a, 1b, 1c, difference spectra from zero time to complete recovery of cytochrome oxidase (16 hr). Oxidized spectra generated by hydrogen peroxide (see text) in the presence of antimycin A, reduced spectra generated by allowing to stand for 5 min without aeration. Fig. 1d, low temperature difference spectra for cytochrome *b* over the same time period.

sibly due to different rates of freezing in the successive experiments. Since constant cell numbers (5×10^9/ml) are used in these experiments, the scattering conditions for the successive stages of cell growth are the same.

The results appear to be equally consistent for the γ and the α bands of the cytochromes (again, see fig. 3).

Calculations of actual concentrations do not appear to be necessary for the purposes of this paper since the relative changes in the cytochrome components with time are of the greatest importance. There-

fore, the ordinates of the graphs are in terms of absorbancy change for the various cytochromes. The choice of wavelengths in this absorbancy difference measurement is important, cytochrome a_3 445–455, cytochrome *c* 545–540, cytochrome b_T 562–575 (fig. 1a, b, c), cytochrome b_K 558–575 (fig. 1d), cytochrome *a* 599–630 nm. The room temperature extinction coefficients for these pairs of wavelengths are available [9, 10, 11, 12] but the low temperature spectra involve an enhancement factor [13] which is ~ 30-fold for cytochrome *c* of baker's yeast. Thus, the characteristic level of cytochrome *c* before and following induction of the cytochrome oxidase is 12 nmoles/5 × 10^9 cells/ml.

The biochemical conditions for the spectroscopic assay were closely controlled. Excess substrate is washed away in two centrifugations and the cells are diluted to standard concentration of 5 × 10^9 cells/ml. Anaerobiosis was established in about 5 min. The steady state oxidized condition was obtained by the addition of 3 μl of 10% H_2O_2 (equivalent to several mM oxygen) sufficient catalase being present in the yeast cells to decompose the H_2O_2 rapidly to oxygen. In fig. 1a, b, c 10 μg/ml antimycin A was added in order to decrease the respiration rate and permit maximal oxidation of cytochromes. Since this treatment caused cytochrome *b* oxidation in anaerobiosis [14, 15] a second set of spectra were recorded (fig. 1d) without antimycin A and consequently at a higher respiration rate. Thus, in fig. 1d, the difference spectra represent the steady state oxidized minus anaerobic cells. Cytochromes which were 'nonfunctional', i.e., cannot be oxidized by cytochrome oxidase, would not be observed under these conditions. On the other hand, cytochrome oxidase which was not linked to cytochromes *b* or *c* would be observed in the difference spectrum.

3. Results

Cytochrome oxidase. Fig. 1 shows the successive difference spectra of cytochrome oxidase in copper deficient cells from ½ to 16 hr after adding copper. At *t* = 0 in the copper deficient cells, cytochrome *b* and *c* are present at roughly the level of the copper sufficient cells. The copper deficient cells synthesize cytochrome oxidase when transferred to copper sufficient media. Fig. 2 shows the growth curve under such conditions.

Rate of synthesis of cytochrome oxidase. Points from the spectra of fig. 1 are plotted in fig. 3. Considering first cytochrome a_3 + *a*, synthesis was constant over 8 hr when the enzyme level reached a plateau at 10 hr corresponding approximately to the end of the logarithmic phase (fig. 2). During the growth phase the relative amount of cytochrome *c* to cytochrome *a* falls rapidly in the first 2 hr and remains constant in the stationary phase. A maximal synthesis of cytochrome a_3 + *a* synthesis is reached in 10 hr. The rate of synthesis appears to be more or less constant from about 30 min to 4 hr, and then drops off.

Cytochrome c. A small but significant rise in the relative amount of cytochrome *c* begins at about 2 hr, reaches a maximum at 4 and falls to the initial level towards the end of the growth phase.

Cytochrome(s) b. Cytochrome(s) *b* can be assayed under two conditions, first in the presence of antimycin A, where cytochrome *b* (or more properly b_T [16]) becomes oxidized in anaerobiosis (fig. 1a, b, c) and reduced in aerobiosis, thus affording an absorbancy difference closely corresponding to the total cytochrome b_T in the preparation. As the points in fig. 3 show, this component decreases slightly and then recovers to its initial value. The usual estimation of cytochrome *b*, in the absence of antimycin A (fig.

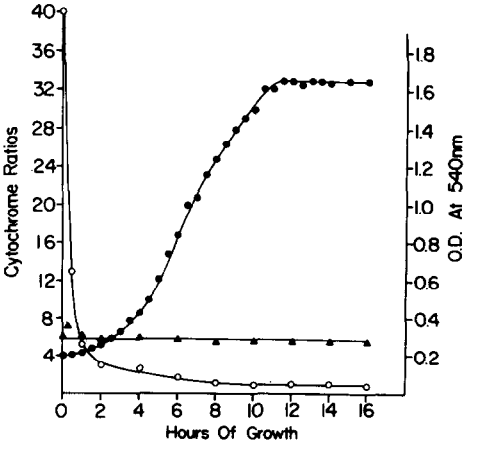

Fig. 2. —●—●— Growth of copper deficient cells after placing in media containing copper at time = 0. —○—○— Ratio cytochrome *c* to cytochrome *a*. —▲—▲— Ratio cytochrome a_3a to cytochrome *a* as the same cells.

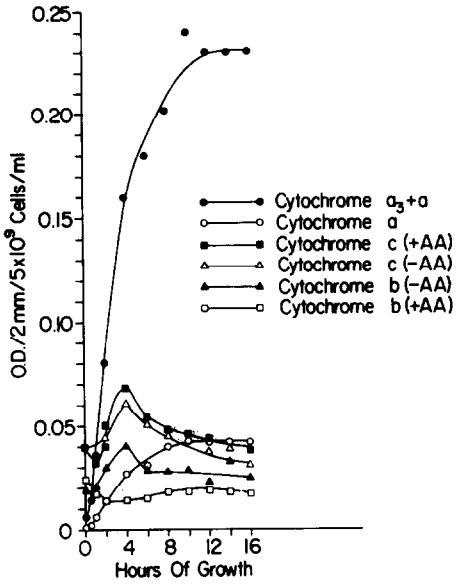

Fig. 3. Optical density of cytochrome $a, a_3 + a, b$ and c from copper deficient cells after placing in copper supplemented medium at time = 0.

1d and fig. 3) gives reduction in anoxia and in this case shows particularly cytochrome b_K [16]. This component rises and falls in concentration as did cytochrome *c*.

Other components such as Cu in cytochrome oxidase, flavoprotein, ubiquinone, etc. were not assayed.

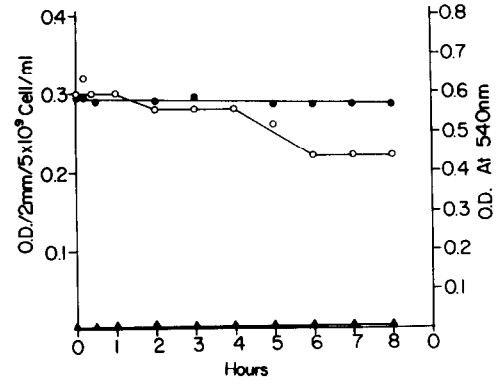

Fig. 4. Cell density —●—●—; cytochrome oxidase —▲—▲—; cytochrome *c* —○—○— of copper deficient cells placed in non-growing medium (see text) with copper at time = 0.

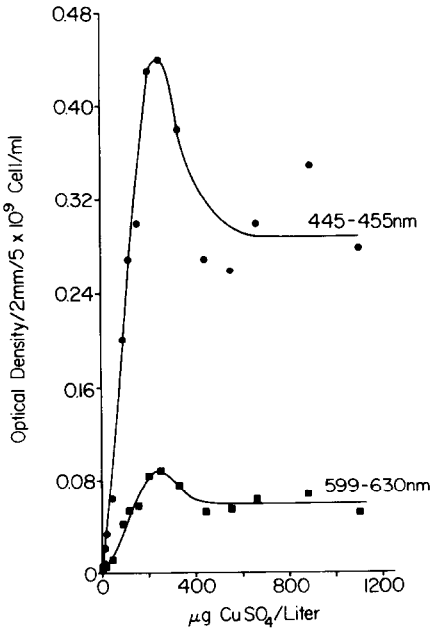

Fig. 5. The dependence of cytochrome oxidase content of cells on the concentration of copper in the medium.—●—●— cytochrome *a*, —■—■— cytochrome oxidase. The cells were harvested in each case after 36 hr incubation in copper containing media.

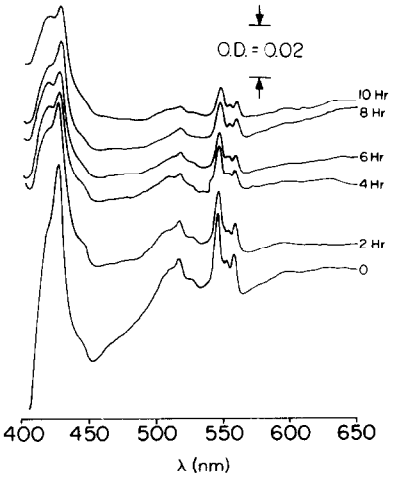

Fig. 6. Inhibition of cytochrome oxidase synthesis in copper supplemented (110 μg/ml) yeast cell. Chloramphenicol 4 mg/ml, cycloheximide 10 μg/ml.

Role of copper in the synthesis of cytochrome oxidase. When the cytochrome oxidase deficient cells are placed in media unsuitable for significant growth at 30° (vigorous aeration but no carbon source) the addition of copper causes no synthesis of cytochrome oxidase (fig. 4). A small decrease of cytochrome *c* is observed. On the other hand, when the copper deficient cells were supplemented with ethanol as a carbon source the amount of cytochrome oxidase formed at the end of 36 hr growth and assayed as in fig. 1 increases proportionally to the amount of copper added to the medium up to roughly 200 μg/l and reaches a maximum at 250 μg/l. Thereafter the cytochrome oxidase synthesis declines reaching a plateau above 600 μg/l. This is obviously a very complicated relationship and it is possible that other types of profiles could be obtained under different conditions of cultivation and different times of assay.

The role of cytoplasmic and mitochondrial protein synthesis. When the cytochrome oxidase deficient cells are grown in a copper supplemented medium (110 μg/l) in the presence of chloramphenicol and cycloheximide mitochondrial and cytoplasmic protein synthesis are respectively inhibited. Fig. 6 shows no detectable formation of cytochrome oxidase as evidenced by the absence of a band at 605 and the constancy of the shoulder at 445 nm as measured with respect to 455 nm. Over the 10 hr interval the absorption bands of cytochromes *c*, c_1 and *b* diminished to roughly half their initial value verifying the complete inhibition of protein synthesis. A more precipitous decrease of the Fp trough is also noted. These data indicate that de novo synthesis of protein(s) is necessary for the reconstitution of cytochrome oxidase.

4. Discussion

The biosynthesis of cytochrome oxidase induced by copper indicates: (a) the synthesis proceeds at a constant rate during logarithmic phase (fig. 3); (b) the synthesis of the cytochrome *a* and a_3 is synchronous and (c) the rate of synthesis of cytochrome oxidase is proportional to the amount of copper in the medium (fig. 5).

Several mechanisms by which copper could regulate the synthesis of cytochrome oxidase may be considered: (1) the apo-oxidase and the heme have been synthesized in the absence of copper and the complete enzyme is formed when copper is added; (2) copper is necessary for the synthesis of the apo-enzyme; (3) copper is necessary as the prosthetic group but is also required for the assembly of the complete enzyme. (2) suggests that copper is involved in the synthesis of the protein while (1) and (3) do not.

When the copper deficient cells are incubated in a non growing medium in the presence of copper there was no formation of the cytochrome oxidase. When the cells were placed under conditions appropriate for growth in the presence of copper readily detectable cytochrome oxidase synthesis occurred in 30 min. Since apo-enzyme heme recombinations are usually fast [17] the 30 min time lag indicates that synthesis is required for the formation of cytochrome oxidase as required by (2) and (3) above. This result is further supported by fig. 6 which demonstrates sensitivity of the cytochrome oxidase synthesis to chloramphenicol plus cycloheximide [18].

The total response of the cells to copper goes well beyond the levels which have been investigated in this paper and the conditions which we have employed can be identified as copper limited conditions. The remarkable synthesis that occurs in the presence of unusually high copper concentrations (300 μg/l) leads to a cytochrome *a* content well in excess of that usually observed in yeast cells 2.3 nmoles/5 × 10^9 cells/ml and 0.18 nmoles/mg protein in the mitochondria. No doubt complexities well above and beyond those considered here are involved in the phenomenon of cytochrome oxidase synthesis.

It is not yet possible to determine whether the small but significant synthesis of cytochrome *c* is related to the oxidase synthesis, but cytochrome *c* synthesis does not bear a precursor-product relationship to the cytochrome oxidase; the latter starts without a detectable delay prior to the maximum of the cytochrome *c* synthesis.

While these data do not solve the problems of cytochrome oxidase and membrane biosynthesis in yeast cells, this novel approach to a quantitation of the rate of membrane biosynthesis together with the related effects on the other cytochromes may eventually lead to a more definitive picture of the membrane function in mitochondria.

Acknowledgements

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