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The Partially Reduced Species Present in Purified Cytochrome Oxidase from Baker's Yeast Is Cytochrome *a*

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Abstract

Cytochrome oxidase purified from baker's yeast submitochondrial particles is found to exist in a partially reduced state in the resting enzyme. Studies utilizing optical and EPR spectroscopy indicate that the "inactive" fraction contains a reduced low-spin heme, cytochrome *a*, possibly indicating a block of electron transfer from cytochrome *a* to cytochrome a_3. There is no apparent reduction of either the EPR-detectable copper or the species associated with the 830 nm band. Oxidative titrations of the resting-state yeast cytochrome oxidase indicate that the reduction potential of the species titrating is higher than that of ferricyanide. This "inactive" cytochrome oxidase is not the result of the isolation procedure, but seems to represent a species which is present in the intact yeast.

Key Words: Cytochrome oxidase; baker's yeast, submitochondrial particles; cytochrome a; EPR.

Introduction

Recent years have seen a large amount of work devoted to the study of baker's yeast cytochrome oxidase (EC 1.9.3.1). Several soluble preparations of the yeast enzyme, exhibiting a wide range of properties and specific activities, have been reported (Mason *et al.*, 1973; Phan and Mahler, 1976; Rubin and Tzagoloff, 1973a, b; Sekuzu *et al.*, 1967; Shakespeare and Mahler, 1971). Much of the recent work has focused attention on the biosynthesis of cytochrome oxidase. One major objective of these studies has been to resolve the separate roles played by the mitochondrial and nuclear systems of gene

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expression in the biogenesis of the multisubunit cytochrome oxidase complex. In order to accomplish this, a knowledge of the subunit composition of the enzyme is required. Among the various purified preparations of the enzyme reported, there is general agreement that the intact yeast enzyme consists of seven subunits. Three large, hydrophobic subunits are synthesized in the mitochondrion whereas the four smaller subunits originate in the cytoplasm (Mason and Schatz, 1973; Rubin and Tzagoloff, 1973b).

Most of the above studies have assumed that their isolated, purified cytochrome oxidase represented a single, homogeneous species. The results of this study suggest that a significant fraction of the purified yeast cytochrome oxidase is present in an "inactive," reduced state.

Materials and Methods

Materials

Several sources of commercially grown baker's yeast were used in the study. Fleischmann's yeast was purchased from Standard Brands, Inc., Red Star Yeast from Universal Food Co., and Budweiser yeast from Anheuser-Busch, Inc.

All chemicals were of analytical reagent grade. Cytochrome c (Type III), phenylmethane sulfonylfluoride, and cholic acid were obtained from Sigma. Cholic acid was recrystallized as described by Mason *et al.* (1973). Enzyme-grade ammonium sulfate was from Mann.

Methods

Yeast submitochondrial particles were prepared according to the procedure of Mason *et al.* (1973) using passage through a Manton-Gaulin homogenizer to break the yeast cells. Yeast cytochrome oxidase was isolated and purified from the resulting submitochondrial particles by combining steps associated with preparations described previously by Kuboyama *et al.* (1972) using heart muscle and Mason *et al.* (1973) using baker's yeast.

Enzyme activity was assayed at 25°C according to the spectrophotometric procedure of Smith and Conrad (1956). The activities are expressed as first-order rate constants (min^{-1}). The concentration of cytochrome oxidase was determined spectrophotometrically on a heme *a* basis using values of 21.2 mM^{-1} cm^{-1} for the absorbance of reduced heme *a* at 603 nm (Horie and Morrison, 1964) or 10.6 mM^{-1} cm^{-1} ($603_{red} - 603_{ox}$) (Lemberg, 1969).

Protein content was determined using the method of Lowry *et al.* (1951). Spheroplasts were prepared by the procedure of Fujimoto *et al.* (1974) Room-temperature spectrophotometric measurements were made on a Cary model 17 spectrophotometer and low-temperature redox difference spectra of

Table I. Absorbance Ratios of Purified Yeast Cytochrome Oxidase

Ratio	Preparation	
	Yeast[a]	aa_3[b]
$\gamma_{red}/424_{red}$	2.32–2.08	>2.0
γ_{red}/γ_{ox}	1.16–1.26	1.25–1.30
γ_{red}/α_{ox}	4.6–4.9	5.0–5.1
$A_{603_{red}}/A_{603_{ox}}$	1.5–1.75	2.0–2.2

[a]The range of values obtained from six different preparations.
[b]Values suggested by Lemberg (1969).

yeast submitochondrial particles were recorded on an Aminco DW-2 spectrophotometer (Bonner, 1961; Chance, 1957). EPR[4] spectra were recorded on a Varian E-6 spectrometer run under the following conditions: 2mW power at 10°K with a modulation amplitude of 20 G.

Results

The general properties of the baker's yeast cytochrome oxidase used in these studies were similar to those reported previously. The specific activity calculated on either a protein (1400 min^{-1} · mg $protein^{-1}$) or heme *a* (280 min^{-1} · nmol heme a^{-1}) basis was comparable to the value reported for other yeast preparations (Sekuzu *et al.*, 1967, Rubin and Tzagoloff, 1973a, b; Phan and Mahler, 1976). In spite of the high specific activity of the cytochrome oxidase preparation, the ratio of nanomoles heme *a* to milligrams protein consistently fell in the range of 5 to 7. Previous reports of cytochrome oxidase from baker's yeast have given values that range from 7 (Sekuzu *et al.*, 1967) to 14 (Phan and Mahler, 1976).

The absorption spectra of the yeast cytochrome oxidase used in these studies closely resembled those of previous preparations from both baker's yeast (Sekuzu *et al.*, 1967; Mason *et al.*, 1973) and heart muscle (Babcock *et al.*, 1976; Hartzell and Beinert, 1975). Peaks associated with the α and Soret bands appeared in the oxidized form at 599–600 and 422–424 nm respectively. In the reduced form, these two peaks were shifted to 603 nm (α) and 444 nm (γ). Isosbestic points were observed at 617, 584, and 458 nm. The absorbance ratios associated with the two states are shown in Table I. With the exception of the ratio of A_{603}(red):A_{603}(ox) (= α_{red}:α_{ox}) the values are similar to those described by Lemberg (1969).

Attempts to improve upon the preparation with regard to the value of the ratio α_{red}:α_{ox} met with no success. Most procedures gave little or no increase in

[4]EPR ± electron paramagnetic resonance.

the observed ratio (1.5–1.75) and usually resulted in decreased specific activity. In addition, baker's yeast cytochrome oxidase purified according to the procedures of either Mason *et al.* (1973) or Rubin and Tzagoloff (1973a, b) invariably gave products with values of α_{red}:α_{ox} less than 2.0. Different brands of commercial yeast all gave the same result.

The absorption spectrum of oxidized cytochrome oxidase purified from heart muscle shows an absorbance at the α band maximum (599 nm) which is no greater than the absorbance of the plateau found between 500–540 nm (Babcock *et al.*, 1976; Hartzell and Beinert, 1975). In all yeast preparations we have examined, the absorbance of the α band is 15 to 30% higher than the absorbance at 520 nm (Fig. 1). This suggests that the low ratio of ca. 1.7 (for α_{red}:α_{ox}) may be due to the partial reduction of yeast cytochrome oxidase in the resting state. This was confirmed by observing the change in the absorption spectrum of purified yeast cytochrome oxidase upon the addition of excess potassium ferricyanide (Fig. 1). In this particular preparation the absorption of the α band decreased approximately 22% and shifted from 600 to 597 nm; the absorbance at 600 nm now became the same as that at 520 nm. Reduction of the ferricyanide-oxidized cytochrome oxidase with excess dithionite gave a spectrum typical of the fully reduced protein, and the ratio of A_{603}(red):A_{603}(Fe(CN)$_6$ox) was 2.04 compared with an observed ratio of 1.58 with this sample in resting-state enzyme. The dithionite-reduced cytochrome oxidase could be reoxidized by aerating the sample (2 to 5 min), but only to the level of oxidation present in the resting state prior to the addition

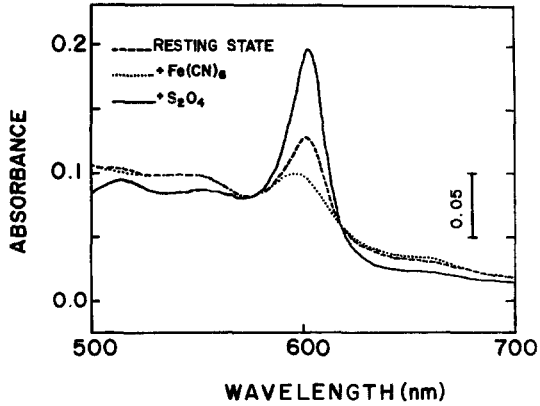

Fig. 1. Effect of potassium ferricyanide on the yeast cytochrome oxidase absorption spectrum in the 500–700 nm region. The enzyme (9.3 nmol heme *a* per ml) was dissolved in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.5% Tween 80. The dashed line shows the enzyme in the resting state; dotted line, oxidized with excess ferricyanide; solid line, reduced with excess dithionite. The path length was 1.0 cm. All spectra were recorded at 18°C.

of ferricyanide. Partial reduction could be maintained through multiple cycles of such reduction/oxidation treatments, which indicates that this phenomenon is not due to the presence of endogenous reductants in the preparation. Further, the presence of added cytochrome *c* had no effect on the level of oxidation reached by aeration (c.f. Yonetani, 1960).

Using the changes in absorbance at 603 nm between ferricyanide-oxidized, resting-state, and dithionite-reduced enzyme, it was calculated that the α band at 603 nm was approximately 30% reduced in this preparation. This percentage is derived from the ratio of the absorbance of the α-band in the resting minus ferricyanide-oxidized states versus the dithionite-reduced minus ferricyanide-oxidized states (Fig. 1). The results from over 20 different preparations have shown the yeast enzyme to be always 20–35% reduced as isolated. In addition, it should be noted that no absorbance change was observed in the region of the weak band located at approximately 830 nm upon addition of excess ferricyanide. This latter transition is generally assigned to one of the two copper atoms found in cytochrome oxidase (Lemberg, 1969).

Having ascertained the heterogeneous nature of the yeast cytochrome oxidase preparation, it was of interest to determine which component(s) in the enzyme was affected. This was established from EPR spectra recorded on the enzyme before and after treatment with ferricyanide (Fig. 2). The EPR of the

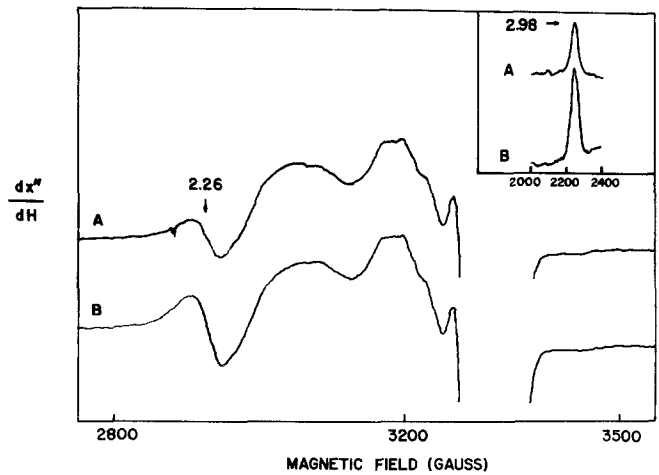

Fig. 2. Effect of potassium ferricyanide on yeast cytochrome oxidase EPR spectrum. The enzyme (48 nmol heme *a*) was dissolved in 0.335 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.5% Tween 80. (A) EPR spectrum of the resting-state yeast cytochrome oxidase. (B) EPR spectrum of the enzyme after addition of 55 nmol of ferricyanide. The insert shows the EPR signal obtained at low magnetic field ($g = 2.98$) with the same sample. EPR signals were measured as described under "Materials and Methods."

low-spin ferric heme in yeast cytochrome oxidase has not been reported previously and exhibits g values slightly different from those of the heart enzyme, 2.98, 2.26, and 1.50 for the former compared to 3.03, 2.21, and 1.45 for the latter. Addition of ferricyanide led to a 20–35% increase in the intensity of these low-spin ferric heme resonances commonly ascribed to cytochrome *a* (Fig. 2). The EPR signal due to the "visible" copper ($g \simeq 2.0$) and the small signal associated with a high-spin ferric heme ($g \sim 6$) were both unchanged upon addition of excess ferricyanide.

At attempt to perform a stoichiometric oxidative titration of the reduced cytochrome *a* using potassium ferricyanide as an oxidant revealed that complete oxidation required approximately one mole of ferricyanide per total heme *a* present (Fig. 3). Identical results were obtained monitoring the fractional change in either the amplitude of the low-spin heme EPR signal at $g \simeq 2.98$ (×) or the absorbance change at 603 nm (○). Because the total heme a was 25–30% reduced in the resting state in this preparation, complete oxidation required about a 3–4-fold excess of ferricyanide added over the heme *a* actually oxidized. It thus seems that the reduction potential of the reduced fraction of cytochrome *a* is substantially more positive than that of the normal species which should undergo complete oxidation with stoichiometric levels of ferricyanide given current estimates of its midpoint reduction

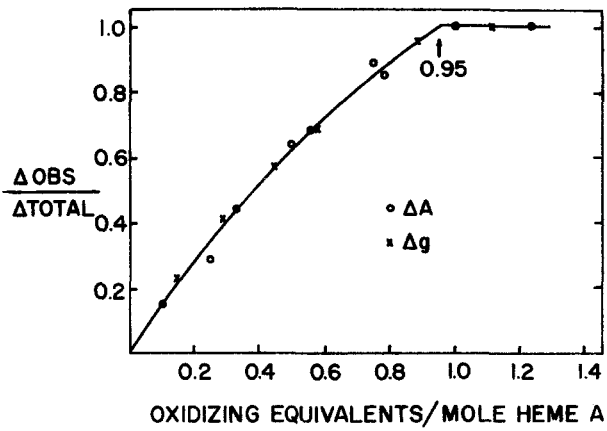

Fig. 3. Oxidative titration of yeast cytochrome oxidase with potassium ferricyanide. The enzyme was dissolved in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.5% Tween 80 to a concentration of 12.4 nmol heme *a* per ml (optical) and 112 nmol heme *a* per ml (EPR). The fractional changes were derived from the absorbance change at 603 nm (○) and from the change in intensity of the EPR resonance at $g \simeq 2.98$ (×) upon the addition of a known amount of ferricyanide. The abscissa refers to the number of moles of ferricyanide added per total moles of heme *a* present in the sample. EPR signals were measured as described under "Materials and Methods."

potential (Wikstrom *et al.*, 1976; Erecinska and Wilson, 1978). Based on the results in Fig. 3, we estimate the potential of this center to be about +440 mV, which might explain why this particular species fails to reduce the other redox centers in the enzyme.

We also attempted to establish whether the reduced fraction was the result of the isolation procedure or an intrinsic feature of the yeast enzyme. Yeast submitochondrial particles were isolated and repeatedly washed with 0.1 M sodium phosphate buffer, pH 7.4, to remove the large amount of endogenous reductant found to be present after breaking open the yeast cells. The absence of endogenous reductant was verified by the lack of any measurable oxygen uptake by washed submitochondrial particles in the absence of an added electron donor after 10 min; four cycles of washing were generally required to reach this state. The "fractional reduction" of cytochrome oxidase in the washed submitochondrial particles was determined using redox difference spectra obtained at liquid-nitrogen temperatures. The change in absorbance (603 nm minus 630 nm) of resting-state submitochondiral particles *minus* those treated with excess ferricyanide was used to determine the amount of "reduced cytochrome oxidase" present in the resting state. The results indicated there was an appreciable amount of reduced cytochrome oxidase (15–25%) present in washed submitochondrial particles. These values correspond to the fractional reduction found in the purified enzyme. Experiments using washed submitochondrial particles isolated from heart muscle showed less than 5% of the cytochrome oxidase present in the reduced state under the same conditions.

To exclude the possibility that the heterogeneity was brought about during isolation of the submitochondrial particles, intact yeast mitochondria were isolated following the enzymatic procedure described by Fujimoto *et al.* (1974). In this procedure, no mechanical disruption is required at any point, but the results were similar to those obtained using the standard isolation procedure. Almost 20% of the cytochrome oxidase was present in the reduced state in the resulting mitochondria even after extensive washing to remove endogenous reductants. Spheroplasts or SMP's from laboratory-grown yeast (D274–10B and a strain of Red Star yeast isolated from commercial sources) also showed the presence of this partially reduced species, and addition of the protease inhibitor phenylmethane sulfonylfluoride during isolation failed to remove this component.

Discussion

We have shown that a rather large portion of the yeast cytochrome oxidase present in our preparation may be in an "inactive" form. Furthermore close examination of previously reported yeast preparations reveals that

this partially reduced cytochrome oxidase may be more prevalent than was realized. The preparation of Sekuzu *et al.* (1967) was reported to "be not completely oxidized without the addition of chemical oxidant." This property was not noted by later workers. However, the ratio of $A_{603}(ox):A_{520}(ox)$ in spectra of the preparations reported by Shakespeare and Mahler (1971), Rubin and Tzagoloff (1973a, b), and Mason *et al.* (1973) is clearly greater than 1.0, consistent with partial reduction of some of the heme.

A similar effect has been observed in yeast enzyme prepared at Ithaca (D.C. Wharton, personal communication, 1977) and Farmington (R.O. Poyton, personal communication, 1978) and an enzyme isolated from *Candida* prepared in Seattle (B. Mackler, personal communication, 1979).

The data presented here show that the partial reduction is, at least in part, a consequence of a modification of cytochrome *a* leading to a substantial increase in its reduction potential. Whether this is sufficient to eliminate electron transfer to cytochrome a_3 (and thereby to oxygen) or whether there are additional modifications (possibly affecting cytochrome a_3) which result in a kinetic block between these two centers remains to be established.

A related observation was recently reported in certain beef heart preparations by Vanneste *et al.* (1974) who interpreted the decline in specific activity of heart muscle cytochrome oxidase during purification as due to the blockage of electron transfer between cytochrome *a* and cytochrome a_3. However, all their measurements were made by observing the redox level of cytochrome *a* in the aerobic steady state during turnover. Similar observations on the preferential reduction of cytochrome *a* in isolated cytochrome oxidase have been reported (Smith, 1955; Yonetani, 1960; Sekuzu *et al.*, 1960), but, as with Vanneste *et al.* (1974), all these results were obtained during steady-state turnover of the enzyme. With yeast cytochrome oxidase, partial reduction of cytochrome *a* appears in the absence of any added reductant. The possibility of some endogenous reductant being present is unlikely. Multiple cycles of reduction/oxidation (± cytochrome *c*) using added dithionite consistently result in the enzyme reaching the same final state of reduction. Further, attempts to remove "endogenous reductant" by gel filtration or extensive dialysis have all failed.

Vanneste *et al.* (1974) suggested that while inactive oxidase was present in their preparations, it eventually oxidizes, possibly via intermolecular electron transfer between active and inactive species. In all other cases we know of, resting-state cytochrome oxidase, in the absence of any reductant, exists in the fully oxidized state. The yeast enzyme therefore represents a more extreme example of this sort of inactive oxidase since the cytochrome *a* not only remains reduced in the absence of any reducing substrate but also seems incapable of interacting with any active species. Whatever the exact nature of the partially reduced species, future workers using yeast cyto-

chrome oxidase should recognize that they are working with a heterogeneous population of molecules.

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