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Biochemical and Biophysical Research Communications 338 (2005) 605–609
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A cytosolic cytochrome b_5-like protein in yeast cell accelerating the electron transfer from NADPH to cytochrome *c* catalyzed by Old Yellow Enzyme

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Received 11 July 2005
Available online 15 September 2005

Abstract

A 410-nm absorbing species which enhanced the reduction rate of cytochrome *c* by Old Yellow Enzyme (OYE) with NADPH was found in *Saccharomyces cerevisiae*. It was solubilized together with OYE by the treatment of yeast cells with 10% ethyl acetate. The purified species showed visible absorption spectra in both oxidized and reduced forms, which were the same as those of the yeast microsomal cytochrome b_5. At least 14 amino acid residues of the N-terminal region coincided with those of yeast microsomal b_5, but the protein had a lower molecular weight determined to be 12,600 by SDS–PAGE and 9775 by mass spectrometry. The cytochrome b_5-like protein enhanced the reduction rate of cytochrome *c* by OYE, and a plot of the reduction rates against its concentration showed a sigmoidal curve with an inflexion point at 6×10^{-8} M of the protein.

Keywords: Cytochrome b_5; Cytochrome b_5-like protein; Old Yellow Enzyme; Electron transfer; *Saccharomyces cerevisiae*; MALDI-TOF/MS

Old Yellow Enzyme (OYE) was discovered by Warburg and Christian in 1932 [1]. However, the physiological role of the enzyme has long been uncertain. Massey's group reported reductase activities of OYE toward cyclopentadiene and a series of its derivatives [2]. We reported the mode of one-electron transfer from NADPH to menadione or dyestuffs by OYE [3]. Since these are not natural products, we have been searching for natural acceptors other than oxygen or cytochrome *c* which is not a physiological acceptor. In the course of the preparation of OYE, we had noticed that some species with absorbance at 410 nm was coeluted with OYE from ion-exchange chromatography at relatively low ionic strength. After removal of OYE by ultrafiltration at higher ionic strength, we realized that the filtrate showed absorbance at 410 nm and enhanced the rate of cytochrome *c* reduction by OYE with NADPH. In the present work, the 410-nm absorbing species was purified and its properties were investigated.

Materials and methods

OYE was purified according to the procedure of Abramovitz and Massey [4]. Preparative gel electrophoresis was carried out with an NA-1800 type instrument (column diameter, 34 mm) from Nihon Eido, Tokyo, Japan [5,6]. Spectroscopy was performed with a Hitachi U-3210 spectrophotometer.

DEAE–Sepharose CL-6B anion-exchange chromatography. Anion-exchange chromatography was done using a DEAE–Sepharose CL-6B column (5 mm × 50 mm, Amersham Bioscience, NJ, USA) equilibrated with 25 mM Tris–HCl (pH 7.3). The flow rate was 0.2 ml/min. Adsorbed substances were eluted by stepwise increase of sodium chloride concentration. The eluates were monitored at 280 and 410 nm.

POROS HQ/M anion-exchange chromatography. Fractions with high absorbances at 410 nm eluted from DEAE–Sepharose were collected and

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.09.033

desalted with an Ultrafree centrifugal filter (MWCO 5000, Millipore, MA, USA). The solution was applied to a POROS HQ/M (4.6 mm × 100 mm, Applied Biosystems, CA, USA). The flow rate was 1 ml/min, and adsorbed substances were eluted with a linear gradient of sodium chloride from 0 to 0.6 M for 30 min.

TSKgel G2000SW$_{XL}$ gel permeation chromatography. A gel permeation chromatography was performed with a TSKgel G2000SW$_{XL}$ column (7.8 mm × 300 mm, Tosoh, Tokyo, Japan). The column was equilibrated with 50 mM sodium phosphate buffer at pH 6.0, containing 0.3 M sodium chloride. The flow rate was 0.1 ml/min, and eluents were monitored at 280 and 410 nm. All chromatography was done with a Shimadzu LC-10A HPLC system. Hitachi L-4000 and Gilson HM holochrome were used as monitors at 280 and 410 nm, respectively.

SDS–polyacrylamide gel electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli's method [7]. The acrylamide concentration of the gel was 15% (w/v) for separation. Precision plus protein standards (Bio-Rad Laboratories, CA, USA) were used as molecular weight markers. Proteins were detected with a silver staining kit (Daiichi Pure Chemicals, Tokyo, Japan).

Mass spectrometry. Mass spectrometry was carried out with a Voyager-DE PRO matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems, CA, USA). Samples were adsorbed on ZipTipC$_{18}$ (Millipore, MA, USA) and eluted by a matrix solution (10 mg/ml sinapinic acid, 0.05% trifluoroacetic acid, and 50% acetonitrile) on the MALDI plate. Measurements were done in a linear mode and the accelerating voltage was 25,000 V.

Amino acid sequence of the cytochrome b_5-like protein. Amino acid sequence analyses were performed with an Applied Biosystems sequencer 477A (Applied Biosystems, CA, USA).

Measurement of the cytochrome c reduction rate by OYE with NADPH. Although cytochrome *c* is not a physiological acceptor, the activity of OYE was measured as the NADPH-cytochrome *c* reductase activity. The reaction was carried out at 37 °C in a total volume of 700 μl of 0.1 M potassium phosphate buffer, pH 6.84, containing 67 nM OYE, 9.2 μM cytochrome *c*, 71 μM NADPH, and the cytochrome b_5-like protein at various concentrations. The reaction was initiated by the addition of NADPH. The increase of the absorbance of cytochrome *c* at 550 nm was followed.

Results and discussion

Preparation of a crude extract

Wet brewer's yeast (*Saccharomyces cerevisiae*) was obtained from a local factory and kept in a deep-freezer. According to the method of Lutz and Nelson [8] with a minor modification of using ethyl acetate instead of toluene, the frozen yeast (250 g) was treated with 25 ml ethyl acetate and 50 ml of 25 mM Tris–HCl (pH 7.3). After the addition of 10 μM phenylmethylsulfonyl fluoride (PMSF), the mixture was placed in a New Brunswick Series-25 incubator shaker (New Brunswick Scientific, NJ, USA) for 2 h at 25 °C with mild stirring. The mixture was centrifuged, and the clear supernatant obtained was dialyzed overnight against 10 mM Tris–HCl (pH 7.3). After the solution was freeze-dried, the resulting powder was used for an enzyme source.

Purification of the heme protein

The powder (350 mg) was suspended in 2.5 ml of 25 mM Tris containing 0.3 M glycine, and insoluble materials were removed by centrifugation. The supernatant had an absorbance shoulder at 410 nm. Glycerol was added to the sample solution at 20% (w/v), and the mixture was subjected to a preparative gel electrophoresis. The instrument was a Nihon Eido type NA-1800 with a column (34 mm in diameter). The gels were composed of condensing and separation layers of 4% and 15% (w/v) acrylamide, respectively. A buffer solution was used, based on the method of Davis [9]. The preparative electrophoresis was carried out at 4 °C with a constant current of 40 mA. Fractions with high absorbance at 410 nm were collected, and dialyzed against 25 mM Tris–HCl (pH 7.3), followed by concentration with an Ultrafree centrifugal filter. The concentrated solution was applied to the DEAE–Sepharose CL-6B anion-exchange chromatography. After charging the sample on the column equilibrated with 25 mM Tris–HCl (pH 7.3), the elution was carried out by stepwise increases in NaCl molarity. The elution pattern is shown in Fig. 1. The fractions eluted at 0.35 M NaCl showed absorption at 410 nm and enhanced the reduction rate of cytochrome *c* by OYE. The fractions were collected, concentrated with an Ultrafree centrifugal filter, and applied again to the ion-exchange chromatography. Fig. 2 shows visible

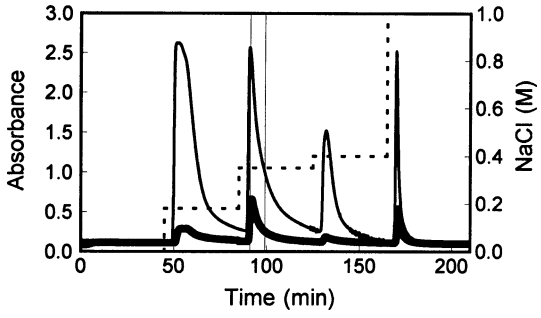

Fig. 1. DEAE–Sepharose CL-6B anion-exchange chromatography of the b_5-like protein. After NA-1800 preparative electrophoresis, a dialyzed and concentrated sample was charged on the DEAE–Sepharose CL-6B column (5 mm × 50 mm) equilibrated with 25 mM Tris–HCl (pH 7.3). The elution was carried out by stepwise increase in NaCl concentration (a broken line). The flow rate was 0.2 ml/min, and eluates were monitored at 280 nm (fine line) and 410 nm (bold line). The fractions between two axial lines were collected and used in the next step.

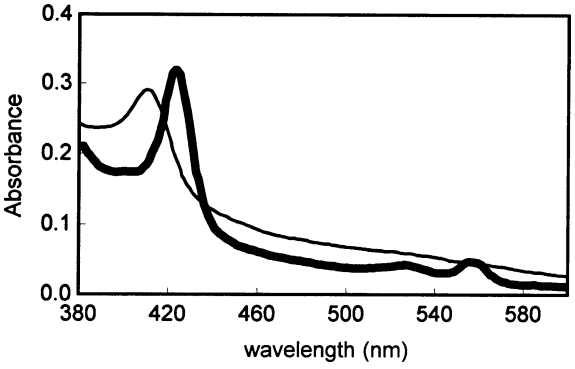

Fig. 2. Absorption spectra of oxidized and reduced forms of the b_5-like protein. The fine line denotes the oxidized form; the bold line denotes the reduced form obtained by the addition of dithionite.

Table 1
Absorption peaks in a visible region of the b_5-like protein

	α-band (nm)	β-band (nm)	γ-band (nm)
Oxidized form			411
Reduced form	555	525	421

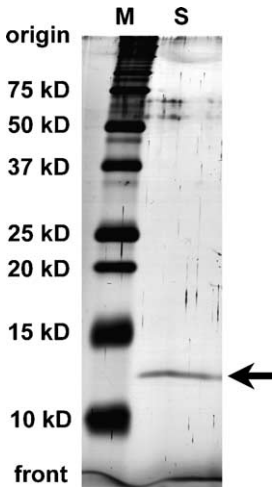

Fig. 3. SDS–polyacrylamide gel electrophoresis of the b_5-like protein at 15% acrylamide gel. The arrow in lane S indicates the purified b_5-like protein, and the bands in lane M are molecular weight standards.

absorption spectra of the oxidized and reduced forms of the solution obtained after re-chromatography. The oxidized form showed an absorption peak at 411 nm and a weak band between 530 and 560 nm. The reduced form had absorption peaks at 421 and 525, and an asymmetric broad α-band at 555 nm. These absorption peaks are summarized in Table 1. The spectral properties coincided with those of cytochrome b_5 in yeast [10,11].

For further purification, the POROS HQ/M anion-exchange column was used. The materials with absorbance at 410 nm were eluted with 0.3 M NaCl. Fractions with high absorbances at 410 nm were put through a TSK G2000SW$_{XL}$ column. The purified protein was subjected to SDS–PAGE (Fig. 3). Its molecular weight was estimated to be 12,600 by SDS–PAGE, and its mass spectrum showed the molecular weight to be 9775 (Fig. 4). In a low-mass region of the spectrum (Fig. 4, inset), moreover, we observed a signal ($m/z = 624$) derived from the heme moiety [12].

An amino acid sequence of the N-terminal region of the protein was determined as follows: **PKVYVYQEVX EHNG**. This sequence is essentially the same as that (PKVYSYQEVAEHNG) of cytochrome b_5 in baker's yeast [13,14].

Effect of the cytochrome b_5-like protein on cytochrome c reduction rate

A high concentration of glycine, as used in the NA-1800 preparative electrophoresis, stabilizes the superoxide anion and raises the rate of reduction of cytochrome *c*, while a high

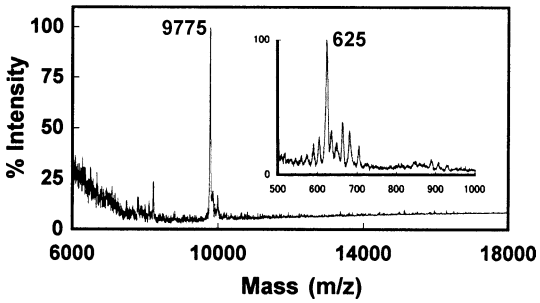

Fig. 4. MALDI-TOF mass spectrometry of b_5-like protein. The analysis was performed using sinapinic acid as a matrix in a linear mode with a Voyager-DE PRO. The inset is the mass spectrum of the same sample recorded in the low mass region demonstrating the presence of heme.

concentration of salt inhibits cytochrome *c* reduction. Taking these factors into consideration, the reaction was carried out in 0.1 M phosphate buffer at pH 6.84. Fig. 5A shows time courses of the reaction of cytochrome *c* reduction by OYE with NADPH in the presence of a variety of concentrations of the b_5-like protein. The concentrations were estimated

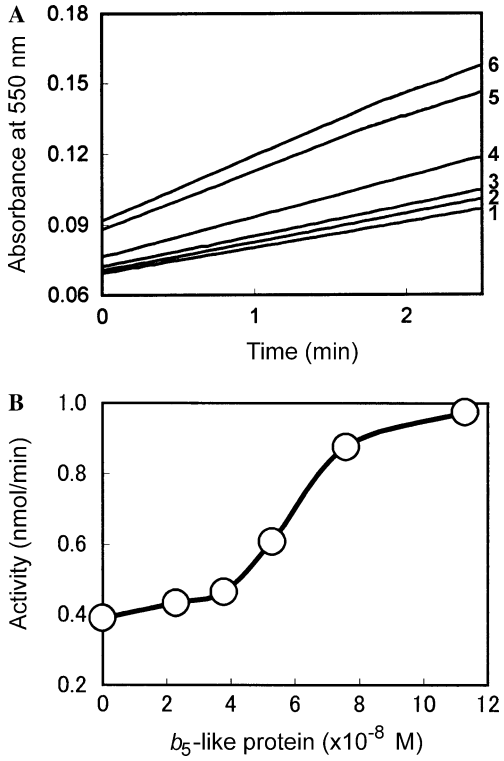

Fig. 5. The reduction of cytochrome *c* by OYE with NADPH enhanced by cytochrome b_5-like protein. (A) The time courses of reactions. Cytochrome *c* was used as an electron acceptor. The reaction was started by adding NADPH to the reaction mixture, and the increase of absorbance at 550 nm was followed at 37 °C. The concentrations used were: cytochrome *c*, 9.2 μM; NADPH, 71 μM; OYE, 67 nM in a total volume of 700 μl of 0.1 M potassium phosphate buffer, pH 6.84. The concentrations of b_5-like protein were as follows: 1, 0.0; 2, 2.3; 3, 3.8; 4, 5.3; 5, 7.6; 6, 11.3 ($\times 10^{-8}$ M). (B) The reduction rates against concentrations of b_5-like protein.

based on the molecular extinction coefficient of 117 $mM^{-1} cm^{-1}$ at 412 nm in the oxidized form [10]. Fig. 5B shows replots of the reduction rates against concentrations of b_5-like protein. The curve is sigmoidal, suggesting an allosteric effect [15]. In this case, however, OYE shows a certain activity of cytochrome *c* reducton without the b_5-like protein, and the reduction rate increases by adding the b_5-like protein. These results indicate that a complex of OYE with the b_5-like protein enhances the reduction rate. The complex formation between OYE and the b_5-like protein may be relatively tight, judging from the inflexion point of the sigmoidal curve at 6×10^{-8} M.

The physiological function of OYE is still unclear. Recently, Schaller and Weller [16] reported a bacterial 12-oxophytodienoate reductase showing good homology with the primary structure of OYE. In another report [17], Miura et al., described NEM reductase from *E. coli* with a primary structure similar to that of OYE.

On the other hand, cytochrome b_5 is known as a microsomal enzyme, which is involved in cytochrome P450-related hydroxylation and acts as a second electron donor [18]. Another function of cytochrome b_5 in yeast microsomes is to synthesize delta-9 fatty acids [19]. Cytochrome b_5 in yeast was solubilized using trypsin or detergent [10,11]. The anchoring domain of cytochrome b_5 to microsomal membrane was characterized [20]. A cytochrome b_5-like protein was reported with a different structure in its C-terminal region, rich in negative charges, in the rat mitochondrial outer membrane [21]. Lederer's group [22] reported lactate dehydrogenase, which was composed of cytochrome b_5 and flavoprotein moieties, and localized in yeast mitochondria. A recent report described cytochrome *b*-type NAD(P)H oxidoreductase which was ubiquitously expressed in human cells [23]. This oxidoreductase was localized in the perinuclear space and its function was proposed to be as an oxygen sensor [23]. Very recently, it was reported that OYE prevented actin from the damage by oxidative stress [24]. The cytochrome b_5-like protein may be involved in the system as an electron mediator.

The question of whether the cytochrome b_5-like protein presented here is a desirable processing product or an undesirable product of proteolysis remains to be clarified. Though we paid much attention to preventing proteolysis during the preparation, the isolation of the protein was reproduced in a series of experiments. Release of cytochrome b_5 from microsomes was reported to take place to a very low extent [25]. We are now attempting to disrupt yeast cells using an enzymatic treatment of cell wall. In preliminary experiments, the cytochrome b_5-like protein was obtained from a cytosol fraction thus prepared. The reduction of cytochrome *c* by OYE with NADPH depended on superoxide. Superoxide dismutase consistently inhibited the effect of the cytochrome b_5-like protein on the cytochrome *c* reduction rate. In the former study, we identified menadione and dyestuffs as an artificial acceptor of one electron from OYE [3]. These results strongly suggest that the one-electron transfer from OYE proceeds more smoothly than the two-electron transfer from OYE. Cytochrome b_5 is well known to mediate one electron transfer from the reduced flavoenzyme in the P-450-linked hydroxylation system [18]. The physiological electron acceptor(s) from OYE we have been searching for may be cytochrome b_5 or the b_5-like protein as stated herein.

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