A HIGHLY PURIFIED PREPARATION OF CYTOCHROME P-450 FROM MICROSOMES OF ANAEROBICALLY GROWN YEAST

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SUMMARY: Cytochrome P-450 was purified from microsomes of anaerobically grown yeast to a specific content of 12-15 nmoles per mg of protein with a yield of 10-30 %. Upon sodium dodecylsulfate/polyacrylamide gel electrophoresis, the purified preparation yielded a major protein band having a molecular weight of about 51,000 together with a few faint bands. It was free from cytochrome b_5, NADH-cytochrome b_5 reductase, and NADPH-cytochrome c (P-450) reductase. In the oxidized state it exhibited a low-spin type absorption spectrum, and its reduced CO complex showed a Soret peak at 447-448 nm. It was reducible by NADPH in the presence of an NADPH-cytochrome c reductase preparation purified from yeast microsomes. Its conversion to the cytochrome P-420 form was much slower than that of hepatic cytochrome P-450.

Recent work in this laboratory has shown that the microsomal fraction from anaerobically grown cells of *Saccharomyces cerevisiae* contains an electron-transfer system which is analogous to that of liver microsomes (1-7). We have previously reported the purification of cytochrome P-450 from yeast microsomes to a specific content of 3-4 nmoles per mg of protein (1,4). The preparation obtained was, however, still of low purity and was not suitable for functional studies because the purification procedure involved a proteolytic enzyme treatment (Nagarse digestion). In this communication, we report that yeast cytochrome P-450 can be obtained in a much higher purity by a non-proteolytic procedure, which is a modification of the method of Imai and Sato (8,9) for purification of hepatic microsomal cytochrome P-450. Some properties of the purified yeast cytochrome P-450 preparation are also presented.

EXPERIMENTAL PROCEDURE

A wild-type strain of *S. cerevisiae* was grown anaerobically (2) and microsomes

Abbreviations used: DTT, dithiothreitol; $DodSO_4$, sodium dodecylsulfate.

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were prepared therefrom as described previously (3). All the purification steps were conducted at 2-4°. Microsomes (3.9 g of protein) were suspended (to a protei concentration of 15 mg/ml) in 0.1 M potassium phosphate buffer, pH 7.2, containing 20 % (w/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 1.0 % (w/v) sodium cholate. The suspension was allowed to stand for 60 min and then centrifuged at 65,000 × *g* for 120 min. The supernatant fluid was diluted 3-fold with 0.1 M potas sium phosphate buffer, pH 7.2, containing 20 % glycerol, 1 mM EDTA, and 1 mM DTT and subjected to ammonium sulfate fractionation. The precipitate formed between 35 and 60 % saturation was dissolved in 100 ml of 20 mM buffer[1] containing 1 mM EDTA, 1 mM DTT, and 0.5 % sodium cholate. The solution was dialyzed overnight against 5 litters of 10 mM buffer containing 1 mM EDTA, 1 mM DTT, and 0.3 % sodium cholate, and insoluble material formed was removed by centrifugation at 65,000 × *g* for 30 min. After adjusting the cholate concentration to 0.5 %, the dialyzate was applied to a column (2.0 × 15.5 cm) of 6-amino-*n*-hexyl Sepharose 4B (AH-Sepharose 4B, Pharmacia Fine Chemicals) equilibrated with 10 mM buffer containing 1 mM EDTA and 0.3 % sodium cholate. The column was washed with the equilibration buffer, and cytochrome P-450 was then eluted with 10 mM buffer containing 0.3 % sodium cholate and 0.1 % Emulgen 913 (Kao-Atlas Co., Tokyo). Fractions containing the cytochrome were pooled and adsorbed on a column (1.6 × 6.0 cm) of hydroxylapatite (Bio-Gel HT, Bio-Rad Laboratories) equilibrated with 10 mM buffer. After washing the column with 30 mM buffer containing 0.2 % Emulgen 913, cytochrome P-450 was eluted with 100 mM buffer containing 0.2 % Emulgen 913. Fractions rich in cyto-chrome P-450 were combined and diluted 10-fold with 20 % glycerol containing 0.2 % Emulgen 913. The diluted solution was applied to a CM-Sephadex C-50 column (0.85 × 3.0 cm) equilibrated with 10 mM buffer containing 0.2 % Emulgen 913. The column was washed successively with the equilibration buffer and a small amount of 40 mM buffer containing 0.2 % Emulgen 913. Cytochrome P-450 was then eluted slowly with 100 mM buffer containing 0.2 % Emulgen 913. The cytochrome was thereby obtained as a concentrated solution, which was used as the purified preparation.

Cytochrome P-450 and protein were determined by the methods of Omura and Sato (10) and of Lowry *et al.* (11), respectively. Sodium dodecylsulfate ($DodSO_4$)/poly-acrylamide gel electrophoresis was performed as described by Weber and Osborn (12) in the presence of 0.1 % $DodSO_4$ by using 10 % cross-linked gels.

RESULTS AND DISCUSSION

The purification procedure described above was similar to that described by Imai and Sato (8,9) for hepatic cytochrome P-450, but 6-amino-*n*-hexyl Sepharose 4B, rather than the 8-amino-*n*-octyl derivative, was used as hydrophobic adsorbent. Cytochrome P-450 preparation having specific contents of 12-15 nmoles per mg of protein could be obtained by this procedure from yeast microsomes with a yield of 10-30 %. The results of a typical purification experiment are summarized in Table I. The purified preparation was stable in the oxidized state and could be stored for at least one month at -80° without any changes in absorption spectra snd re-activity toward yeast NADPH-cytochrome *c* (P-450) reductase. Upon $DodSO_4$/poly-acrylamide gel electrophoresis, the preparation yielded one major protein band

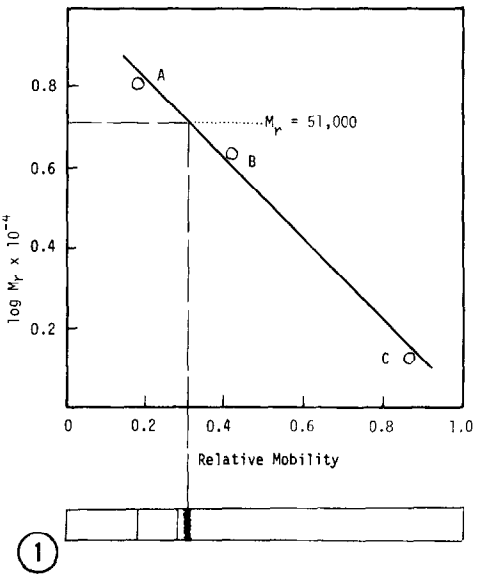
[1]Hereafter potassium phosphate buffers, pH 7.0, containing 20 % glycerol were used throughout the purification. They were simply called 20 mM buffer, *etc.*

Table I. A summary of purification of cytochrome P-450 from microsomes of anaerobically grown yeast. The purification procedure is described under EXPERIMENTAL PROCEDURE.

Step	Protein	Cytochrome P-450		
	(mg)	Total content (nmoles)	Specific content (nmoles/mg protein)	Yield (%)
Microsomes	3,870	202	0.052	100
$(NH_4)_2SO_4$ precipitate	722	169	0.234	83.7
AH-Sepharose 4B eluat	12.5	99.4	7.93	49.3
Hydroxylapatite eluate	6.99	85.7	12.3	42.5
CM-Sephadex eluate	4.51	66.5	14.7	33.0

together with a few minor bands. The molecular weight of the major band was estimated to be about 51,000 by comparing its electrophoretic mobility with those of several marker proteins (Fig. 1). This molecular weight predicts that the pure preparation should contain 19.6 nmoles of cytochrome P-450 per mg of protein; the specific contents actually obtained (12-15 nmoles/mg of protein), therefore, suggest that the purity of the preparations obtained in this study was 60-75 %. The preparations were, however, completely free from both NADH-cytochrome b_5 and NADPH-cytochrome c reductase activities when measured by the methods described previously (2).

The absorption spectra of the purified yeast cytochrome P-450 preparation (Fig. 2) are essentially identical with those reported for a preparation of lower purity obtained from the same source (1,4), but the values of spectral parameters determined (Table II) are slightly different from the previous ones (4). It should be noted that the spectrum of oxidized form (Soret peak at 418 nm and no absorption in the 650-nm region) is characteristic of a low-spin cytochrome P-450. As already reported (4), however, the Soret peak of the reduced CO compound of yeast cytochrome P-450 is situated at 447-448 nm. In this respect, therefore, yeast cytochrome P-450 resembles the cytochrome purified from liver microsomes of 3-methylcholanthrene-pretreated animals (13,14), although the latter preparation



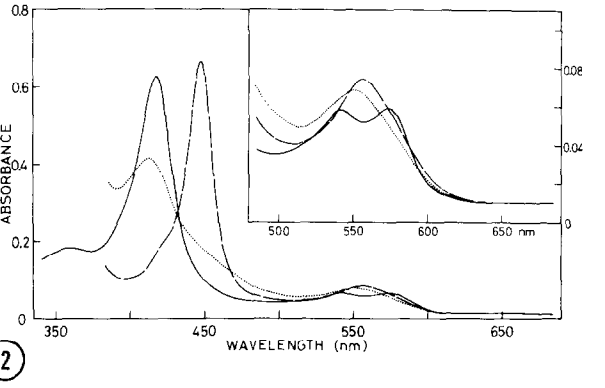


Fig. 1. Estimation of molecular weight of purified yeast cytochrome P-450 by $DodSO_4$/polyacrylamide gel electrophoresis. About 20 µg of the purified preparation was subjected to electrophoresis as described under EXPERIMENTAL PROCEDURE. The mobility of the main protein band was compared with those of three marker proteins, *i.e.* A, bovine serum albumin (M_r = 67,000); B, bovine pancreatic chymotrypsinogen (M_r = 25,000); C, horse-heart cytochrome *c* (M_r = 12,400).

Fig. 2. Absorption spectra of purified yeast cytochrome P-450. The cytochrome was dissolved in 0.1 M potassium phosphate buffer, pH 7.0, containing 20 % glycerol, and 0.2 % Emulgen 913. ———, Oxidized form; ····· , dithionite-reduced form; — — , CO compound of the reduced form.

exhibit a high-spin type absorption spectrum in the oxidized state. The absorption spectra shown in Fig. 2 further indicate that the purified yeast cytochrome P-450 preparation was devoid of both cytochromes P-420 and b_5.

Like cytochrome P-450 of liver microsomes (8,15), purified yeast cytochrome P-450 could be converted to a denatured form called cytochrome P-420, the reduced CO compound of which shows a Soret peak at about 420 nm, by treatment with mercurials, deoxycholate, organic solvents, or chaotropic salts. However, the rate of this conversion was much lower in yeast cytochrome P-450 than in hepatic cytochrome P-450. As shown in Fig. 3, complete conversion of the reduced CO compound of yeast cytochrome to the P-420 form in 1.0 M KSCN at room temperature was effected only after 150 min, suggesting that the structure in the vicinity of the

Table II. Spectral parameters of yeast cytochrome P-450

	Absorption peak (nm)	Millimolar absorbancy* ($mM^{-1}cm^{-1}$)
Oxidized	575	11
	540	11
	418	113
	360	35
Reduced	550	13
	412	71
Reduced CO complex	555	14
	447	117
Reduced CO-difference	448-500	92

*Values were calculated on the basis of protoheme content, which was determined by the method of Omura and Sato (10).

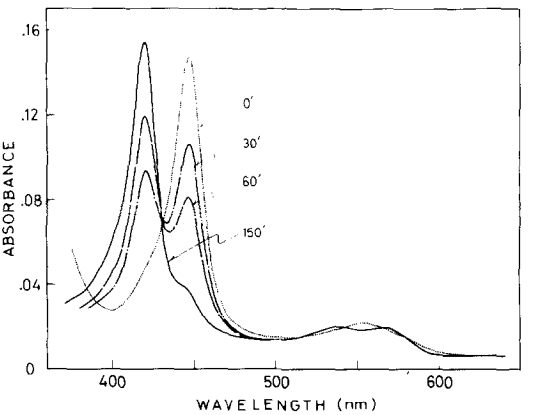


Fig. 3. Conversion of yeast cytochrome P-450 to cytochrome P-420 in 1.0 M KSCN at room temperature. The cytochrome dissolved in 10 mM potassium phosphate buffer, pH 7.0, containing 20 % glycerol and 0.1 % Emulgen 913 was bubbled with CO and then reduced with dithionite. KSCN was then added to the solution to a final concentration of 1.0 M. The absorption spectrum of the sample was recorded 0, 30, 60, and 150 min after the addition of KSCN.

heme in yeast cytochrome P-450 is more stable than that in hepatic cytochrome P-450.

Purified yeast cytochrome P-450 could be reduced by NADPH when supplemented with an NADPH-cytochrome *c* (P-450) reductase preparation purified from yeast microsomes (16, submitted to *Arch. Biochem. Biophys.*). The reduction rate was,

however, very low; the first-order rate constant was preliminarily determined to be 0.12 min^{-1} for the reduction in the presence of 1 unit of the reductase. This rate was much lower than that for the NADPH-dependent reduction of cytochrome P-450 in intact yeast microsomes (2). Our preliminary experiments, however, indicated that the reduction rate could be increased remarkably when lanosterol was added in the reaction mixture. It is, therefore, likely that the low reduction rate observed was due to the absence of a substrate in the purified cytochrome P-450 preparation, as suggested by the low-spin type absorption spectrum of the oxidized cytochrome preparation. It has been reported, on the other hand, that most cytochrome P-450 in intact yeast microsomes is in a high-spin state (4) and therefore thought to be substrate-bound. Furthermore, it was also observed that the low-spin type absorption spectrum of the purified preparation could be partially converted to a high-spin type by the addition of lanosterol. In any way, the purified cytochrome P-450 preparation obtained in the present study seemed to be catalytically active. The suitable reconstitution conditions as well as the significance of lanosterol as a substrate of yeast cytochrome P-450 are now being studied in this laboratory.

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REFERENCES

1. Yoshida, Y., and Kumaoka, H. (1972) *J. Biochem.*, 71, 915-918
2. Yoshida, Y., Kumaoka, H., and Sato, R. (1974) *J. Biochem.* 75, 1201-1210
3. Yoshida, Y., Kumaoka, H., and Sato, R. (1974) *J. Biochem.*, 75, 1211-1219
4. Yoshida, Y., and Kumaoka, H. (1975) *J. Biochem.*, 78, 785-794
5. Tamura, Y., Yoshida, Y., Sato, R., and Kumaoka, H. (1976) *Arch. Biochem. Biophys.*, 175, 284-294
6. Kubota, S., Yoshida, Y., and Kumaoka, H. (1977) *J. Biochem.*, 81, 178-195
7. Kubota, S., Yoshida, Y., Kumaoka, H., and Furumichi, A. (1977) *J. Biochem.*, 81, 197-205
8. Imai, Y., and Sato, R. (1974) *J. Biochem.*, 75, 689-697
9. Imai, Y., and Sato, R. (1974) *Biochem. Biophys. Res. Commun.*, 60, 8-14
10. Omura, T., and Sato, R. (1964) *J. Biol. Chem.*, 239, 2370-2378
11. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.*, 193, 265-272
12. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.*, 244, 4406-4412
13. Kawalek, J.C., Levin, W., Ryan, D., Thomas, P.E., and Lu, A.Y.H. (1975) *Mol. Pharmacol.*, 11, 847-878
14. Hashimoto, C., and Imai, Y. (1976) *Biochem. Biophys. Res. Commun.*, 68, 821-827
15. Imai, Y., and Sato, R. (1967) *Eur. J. Biochem.*, 1, 419-426
16. Aoyama, Y., Yoshida, Y., Kubota, S., and kumaoka, H. (1975) *Seikagaku* (in Japanese), 47, 506