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Expression of rat liver vitamin D_3 25-hydroxylase cDNA in *Saccharomyces cerevisiae*

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The cDNA coding for the precursor protein of rat liver mitochondrial vitamin D_3 25-hydroxylase, cytochrome $P450_{LMT25}$, was expressed under the control of the yeast alcohol dehydrogenase I promoter and terminator in *Saccharomyces cerevisiae* AH22 cells. The transformed yeast cells produced a $P450_{LMT25}$ protein with an almost similar apparent molecular weight as compared with that of the authentic mature enzyme. The expression level of the $P450_{LMT25}$ hemoprotein was about 5×10^4 molecules per cell as determined by reduced CO-difference spectra. The mitochondrial fraction prepared from the transformed yeast cells exhibited both 25-hydroxylase activity toward 1α-hydroxyvitamin D_3 and 27-hydroxylase activity toward 5β-cholestane-3α, 7α, 12α-triol in a reconstituted system containing bovine adrenodoxin and NADPH-adrenodoxin reductase.

Vitamin D_3 25-hydroxylase; Cytochrome P450; 5β-Cholestane-3α,7α,12α-triol 27-hydroxylase; Heterologous expression in yeast

1. INTRODUCTION

Vitamin D_3 ($V\text{-}D_3$) is converted in mammals into the active form through two sequential hydroxylation reactions. The initial hydroxylation at position 25 of $V\text{-}D_3$ occurs in liver microsomes and/or mitochondria, and the subsequent hydroxylation at position 1α of 25-hydroxylated $V\text{-}D_3$ is in kidney mitochondria [1]. Masumoto et al. [2] purified $V\text{-}D_3$ 25-hydroxylase, cytochrome $P450_{LMT25}$, from mitochondria of female rat livers on the basis of monitoring the corresponding enzymatic activity. Thereafter, Okuda et al. [3] reported that the purified 5β-cholestane-3α, 7α, 12α-triol (THC) 27-hydroxylase also showed 25-hydroxylase activity toward $V\text{-}D_3$. Moreover, Ohyama et al. [4,5] revealed that a cytochrome P450 species purified from mitochondria of rat livers catalyzed both 25-hydroxylation toward 1α-hydroxyvitamin D_3 ($1\alpha\text{-(OH)-}D_3$) and 27-hydroxylation toward THC. Recently, Usui et al. cloned the cDNA coding for rat liver mitochondrial $P450_{LMT25}$ [6] and expressed the cDNA in nonsteroidogenic COS7 cells, which showed both 25-hydroxylase activity toward $1\alpha\text{-(OH)-}D_3$ and 27-hydroxylase activity toward THC [7].

Many of the cloned microsomal P450 cDNAs were functionally expressed in the yeast *Saccharomyces cerevisiae* [8]. However, functional expression of a mitochondrial P450 species in the yeast has not succeeded yet. This article reports expression of the mitochondrial $P450_{LMT25}$ cDNA in the yeast, and enzymatic activities of $P450_{LMT25}$ produced in the yeast.

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2. MATERIALS AND METHODS

Restriction enzymes, a *Hin*dIII linker DNA and an M13 DEAZA sequencing kit were purchased from Takara Shuzo Co. (Kyoto, Japan). $1\alpha\text{-(OH)-}D_3$ was obtained from Duphar (Weesp, The Netherlands). [^{3}H]THC was synthesized from [^{3}H]cholic acid (Radiochemical Centre, Amersham, UK) according to the method described by Bergström and Krabisch [9]. The cDNA clone pLMT25 [6] for rat liver mitochondrial $P450_{LMT25}$, purified $P450_{LMT25}$ and anti-$P450_{LMT25}$ IG[2] were reported previously. *S. cerevisiae* AH22[ϱ^+] strain was used as a host, which was obtained by mating of AH22[ϱ^0] (a, *leu2, his4, can1*) [cir$^+$] given by Dr Y. Oshima (Osaka University, Osaka) with YAT[ϱ^+] (α, *leu2, lys10, cyh, kar1*) [cir^0] given by Dr A. Tohe (Hiroshima University, Hiroshima) followed by selection on a mininum nutrient agar plate. Recombinant DNA procedures were described elsewhere [10]. The modified yeast expression vector pAAH5N [11] was used for construction of an expression plasmid for $P450_{LMT25}$. A P450 hemoprotein in transformed yeast cells was measured by reduced CO-difference spectra [10]. Yeast cellular proteins were analyzed by Western immunoblotting using anti-rat $P450_{LMT25}$ IG and [^{125}I]anti-mouse IG F(ab')$_2$ fragment (Amersham Japan, Tokyo) as described [7]. Yeast spheroplasts were prepared [10], and then subjected to subcellular fractionation [12]. $P450_{LMT25}$-dependent monooxygenase activities toward $1\alpha\text{-(OH)-}D_3$ and THC were assayed as described before [2,3].

3. RESULTS

The expression plasmid pAC25 for the precursor protein of rat liver mitochondrial $P450_{LMT25}$ was constructed as shown in Fig. 1. Two *Eco*RI-*Sac*I fragments (0.32 kb and 1.85 kb) were prepared from pLMT25 and

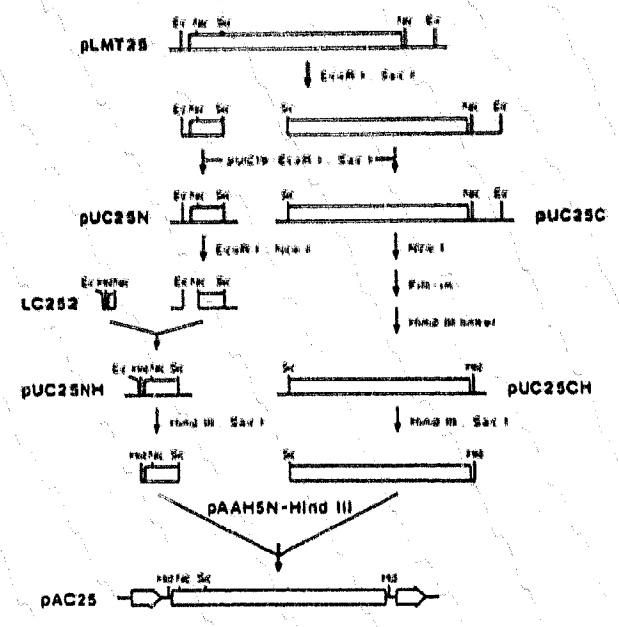

Fig. 1. The procedure for construction of the expression plasmid pAC25 for the precursor protein of rat mitochondrial $P450_{LMT25}$. The open box indicates the protein-coding region for rat $P450_{LMT25}$. The open arrows indicate yeast alcohol dehydrogenase I (ADH) promoter and terminator regions. Restriction sites indicated are: Ec, *Eco*RI; Nc, *Nco*I; Sc, *Sac*I; Hd, *Hind*III. The synthesized linker LC252;

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5'AATTCAAGCTTAAAAAAATGGCTGTGTTGAGCCGCATGAGACTGAGATGGGCGCTTCTGGACACTCGTGTGATGGGC3'
   3'GTTCGAATTTTTTTACCGACACAACTCGGCGTACTCTGACTCTACCCGCGAAGACCTGTGAGCACACTACCCGGTAC5'
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then inserted into the *Eco*RI-*Sac*I site of pUC19 to yield pUC25N and pUC25C, respectively. Replacement of the *Eco*RI-*Nco*I fragment of pUC25N with the synthesized *Eco*RI-*Nco*I linker DNA (LC252) resulted in the construction of pUC25NH. pUC25C was modified to yield pUC25CH, in which the original *Nco*I site in the 3'-flanking region was filled-in and ligated to a *Hin*dIII linker. From pUC25NH and pUC25CH, both *Hin*dIII-*Sac*I fragments (0.27 kb and 1.37 kb) were prepared, respectively, and doubly inserted into the *Hin*dIII site of pAAH5N to yield the expression plasmid pAC25. The structure of the constructed plasmid was confirmed by DNA sequencing.

S. cerevisiae AH22 [ϱ^+] cells were transformed with the expression plasmid pAC25 and the vector pAAH5. Total cellular protein fractions prepared from the recombinant yeast strains were analyzed by Western immunoblotting using anti-rat $P450_{LMT25}$ IG (Fig. 2). The AH22/pAC25 strain (lane 3) contained a protein band reacting with anti-$P450_{LMT25}$ IG at a slightly upper position as compared with that of the authentic mature $P450_{LMT25}$ (lane 1). The control AH22/pAAH5 strain (lane 2) did not contain the corresponding band. The apparent molecular weight of the recombinant $P450_{LMT25}$ protein was smaller than that estimated from the cDNA encoding the precursor of $P450_{LMT25}$. So, it appeared that the mitochondrial signal peptide of the rat $P450_{LMT25}$ precursor was processed in the yeast, but may be differently as compared with in the rat liver.

Fig. 3 shows the reduced CO-difference spectra of the whole cells of both AH22/pAAH5 (control) and AH22/pAC25 strains. A typical P450 peak was found in the AH22/pAC25 strain, but not in the control strain, indicating that the $P450_{LMT25}$ protein produced in the yeast contained a protoheme in the molecule. The content of the $P450_{LMT25}$ hemoprotein was estimated to be about 5×10^4 molecules per cell on the basis of the reduced CO-difference spectra. Both mitochondrial and microsomal fractions prepared from the recombinant yeast cells showed a Soret peak at around 450 nm (data not shown). The ratio of the $P450_{LMT25}$ hemopro-

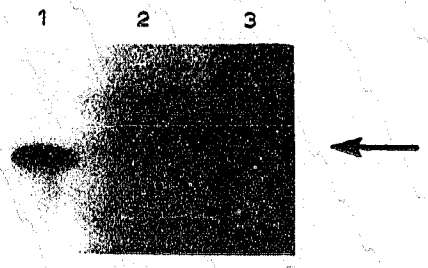

Fig. 2. Western immunoblotting of the recombinant yeast strain producing $P450_{LMT25}$ protein. SDS-solubilized spheroplasts prepared from 5×10^7 transformed yeast cells were analyzed by gel transfer immunoassay with anti-rat $P450_{LMT25}$ IG. (Lane 1) The purified mature $P450_{LMT25}$; (lane 2) the control AH22/pAAH5 strain; (lane 3) the AH22/pAC25 strain. The arrow indicates the position where the recombinant $P450_{LMT25}$ protein migrates.

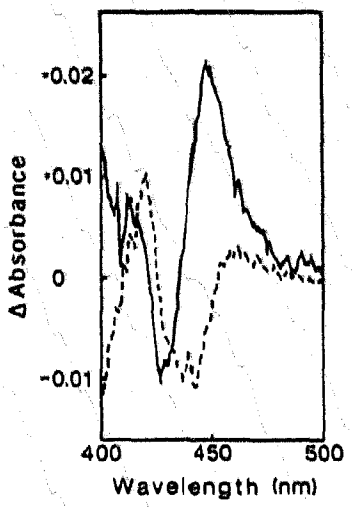

Fig. 3. Reduced CO-difference spectra of the recombinant yeast strains. Reduced CO-difference spectra of the control AH22/pAAH5 (···) and AH22/pAC25 (—) strains were measured in 0.1 M potassium phosphate buffer (pH 7.0) at a concentration of 10^9 cells/ml.

tein in the mitochondrial and microsomal fractions was roughly estimated to be about 1:1, although a large amount of unlysed spheroplasts were in the cell debris fraction.

The mitochondrial fraction prepared from the recombinant yeast cells was assayed for $P450_{LMT2S}$-dependent monooxygenase activities in an in vitro reconstituted system containing bovine adrenodoxin and NADPH-adrenodoxin reductase. The AH22/pAC25 mitochondrial fraction converted 1α-(OH)-D_3 into 1α,25-dihydroxyvitamin D_3 (1α,25-(OH)-D_3), while the control AH22/pAAH5 mitochondria did not. The turnover number of 25-hydroxylation toward 1α-(OH)-D_3 in the AH22/pAC25 mitochondrial fraction was calculated as 0.14 mol product/min · mol P450 (Table I). Also, the AH22/pAC25 mitochondrial fraction showed 27-hydroxylation activity toward THC to yield 5β-cholestane-3α,7α,12α,27-tetrol (TeHC), while the control AH22/pAAH5 mitochondria did not. The turnover number was 20 mol product/min · mol P450 (Table I). These results indicated that the $P450_{LMT2S}$ hemoprotein produced in the yeast mitochondria catalyzed both 25-hydroxylation toward 1α-(OH)-D_3 and 27-hydroxylation toward THC, although the turnover number for THC 27-hydroxylation was much higher than that for 1α-(OH)-D_3 25-hydroxylation.

4. DISCUSSION

The amino acid sequence deduced from the nucleotide sequence of the cDNA for rat liver mitochondrial $P450_{LMT2S}$ revealed that the enzyme consists of 501 amino acid residues preceded by 32 amino acid signal sequence [6]. We constructed the yeast expression plasmid for the precursor protein of rat $P450_{LMT2S}$ including the signal sequence. The $P450_{LMT2S}$ protein produced in the yeast contained a protoheme and located in both mitochondrial and microsomal fractions. However, there was a possibility of contamination of $P450_{LMT2S}$ into the microsomes from the mitochondrial $P450_{LMT2S}$ during fractionation. The mitochondrial fraction prepared from the recombinant yeast cells exhibited both 25-hydroxylase activity toward 1α-(OH)-D_3 (0.14 mol/min · mol P450) and 27-hydroxylase activity toward THC (20 mol/min · mol P450) in the in vitro reconstituted system. These turnover numbers of the $P450_{LMT2S}$ produced in the yeast mitochondria were less than those of $P450_{LMT2S}$ purified from rat liver mitochondria (1.4 mol 1α,25-$(OH)_2$-D_3/min · mol P450 and 36.0 mol TeHC/min · mol P450) [5], particularly in the assay using 1α-(OH)-D_3 as substrate. However, we must be careful for comparison of these turnover numbers obtained under different assay conditions.

We have reported that *S. cerevisiae* was suited for the functional expression of microsomal P450 monooxygenase enzymes and their modified ones [8]. The present study also showed that the yeast was suitable for expression of the mitochondrial $P450_{LMT2S}$. Several mitochondrial P450 species such as $P450_{SCC}$ [13], $P450_{11\beta}$ [14], $P450_{LMT2S}$ [7], P450cc24 [15] and sterol 26 (or 27)-hydroxylase [16] were expressed in nonsteroidogenic COS cells. However, the corresponding P450 hemoproteins were not detected by the reduced CO-difference spectrum. This may be in part due to low expression levels of the heterologous genes in the COS cells. In addition, on the in vivo assays for

Table I

Monooxygenase activities of the mitochondrial fraction prepared from the recombinant yeast cells expressing $P450_{LMT2S}$ in an in vitro reconstituted system

Mitochondrial fraction prepared from the recombinant yeast strain	Monooxygenase activity[a] (mol/min · mol P450)	
	25-Hydroxylase activity toward 1α-(OH)-D_3	27-Hydroxylase activity toward THC
AH22/pAAH5	N.D.	N.D.
AH22/pAC25	0.14	20

[a]The monooxygenase activities were measured in a reconstituted system containing the mitochondrial fraction prepared from the indicated recombinant yeast strain. The reaction mixture (0.5 ml) contained 4 pmol mitochondrial $P450_{LMT2S}$/63 μg protein, 0.2 unit/ml bovine NADPH-adrenodoxin reductase, 8 μM bovine adrenodoxin, a substrate (200 μM 1α-(OH)-D_3 or 28 μM [^{3}H]THC), 100 mM Tris-HCl buffer, pH 7.8, and 0.5 mM EDTA. The reaction was started by addition of NADPH to a final concentration of 100 mM. After incubation at 37°C for 10 min, the reaction mixture was analyzed by HPLC for 1α-(OH)-D_3 25-hydroxylation and by TLC for THC 27-hydroxylation

$P450_{LMT2S}$-dependent monooxygenase activities, the exogenously added labeled substrates were probably diluted with the endogenous substrates present in the serum medium. So, it is reasonable to presume that the monooxygenase activities calculated from the radioactivity were possibly underestimated. On the other hand, the recombinant AH22/pAC25 yeast cells produced a fairly large amount of $P450_{LMT2S}$ and contained no endogenous substrates for the enzyme. Therefore, the $P450_{LMT2S}$-dependent activities were easily measured under low background conditions.

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