

# The importance of threonine-301 from cytochromes P-450 (laurate ( $\omega$ -1)-hydroxylase and testosterone 16 $\alpha$ -hydroxylase) in substrate binding as demonstrated by site-directed mutagenesis

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Threonine-301 from rabbit liver cytochromes P-450 (laurate ( $\omega$ -1)-hydroxylase and testosterone 16 $\alpha$ -hydroxylase) has been replaced by histidine via site-directed mutagenesis. In the oxidized state the mutant P-450s exhibited typical low-spin type absorption spectra of P-450 and their reduced CO complexes showed a Soret peak at 450 nm. However, no spectral change was induced on addition of substrates for their wild-type counterparts. The mutant P-450s were also completely devoid of the hydroxylase activity. These findings suggest that threonine-301, which is highly conserved in P-450s and located at the distal heme surface, plays an important role in substrate binding.

Cytochrome P-450; Laurate; Testosterone; Hydroxylation; Site-directed mutagenesis

## 1. INTRODUCTION

Cytochromes P-450 constitute a large family of heme-thiolate proteins involved in oxidative metabolism of a wide variety of xeno- and endobiotic compounds. In the ferric low-spin state the heme iron of P-450 is hexacoordinated and a water molecule or hydroxide anion occupies the axial 6th coordination position *trans* to the thiolate 5th ligand [1]. X-ray crystallographic studies have shown that threonine-252 of P-450cam from *Pseudomonas putida* is located at the distal heme surface [1] and this threonine residue is highly conserved in P-450s [1–3]. On the other hand, the 6th coordination position is thought to be occupied by a histidine imidazole in P-450<sub>SG1</sub>, an altered P-450 from a yeast mutant defective in lanosterol 14 $\alpha$ -

demethylation [4]. We have recently constructed two plasmids for expression in yeast of rabbit liver P-450( $\omega$ -1) and P-450(16 $\alpha$ ) and characterized the P-450s synthesized in yeast transformed with these expression plasmids [5,6]. In this study we constructed mutants of P-450( $\omega$ -1) and P-450(16 $\alpha$ ) by site-directed mutagenesis to examine a possible role of the aforementioned conserved threonine in catalytic activity and report our findings therein.

## 2. MATERIALS AND METHODS

Restriction endonucleases were purchased from Nippon Gene, T<sub>4</sub> DNA ligase (DNA ligation kits) from Takara Shuzo, and M13 cloning, sequencing, and in vitro mutagenesis kits from Amersham International. The mutagenic oligonucleotide primer, 5'-CTGGAACAGAGCACACAAGCACCA-3', and the cDNA sequencing primer (for screening of the mutants), 5'-CCCTCGGGACTTTATTG-3', were prepared by the phosphoramidite method [7] using an automated DNA synthesizer (Applied Biosystems model 381A). pAH3P2 and pAHF3, expression plasmids for P-450( $\omega$ -1) and P-450(16 $\alpha$ ), respectively, were described in [5,6]. The yeast expression vector pAAH5 [8] was supplied by Dr A. Ito of Kyushu University, and *Saccharomyces cerevisiae* strain AH22 by Dr Y. Oshima of Osaka University.

Site-directed mutagenesis was performed by the method of Nakamaye and Eckstein [9] according to the instruction pro-

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*Abbreviations:* P-450, cytochrome P-450; P450( $\omega$ -1), P-450 (laurate ( $\omega$ -1)-hydroxylase); P-450(16 $\alpha$ ), P-450 (testosterone 16 $\alpha$ -hydroxylase); P-450<sub>SG1</sub>, P-450 from a yeast mutant defective in lanosterol 14 $\alpha$ -demethylation; P-450cam, *Pseudomonas putida* P-450

vided by the kit supplier. Expression plasmids for the mutated P-450s were constructed from the mutated P-450 cDNAs and pAAH5 as described [5]. Transformation and cultivation of yeast, preparation of yeast microsomes and purification of P-450s were carried out by the methods described previously [5,6]. Reconstituted hydroxylase activities were measured as described [5,6,10]. Nucleotide sequences were determined by the dideoxy termination method [11].

### 3. RESULTS AND DISCUSSION

Sequence alignment of P-450s indicates that threonine-252 of P-450cam corresponds to threonine-301 of P-450( $\omega$ -1) and P-450(16 $\alpha$ ) [2,3], and is located in a threonine-serine cluster that is invariably found in rabbit liver P-450s [2]. To replace this threonine by histidine, the inserts of pAH3P2 and pAHF3 (1.78 and 1.64 kbp *Hind*III fragments, respectively) were cloned into M13mp11 and subjected to site-directed mutagenesis (see section 2). The desired mutants were selected by sequencing of the single-stranded DNA insert prepared from progeny phages (fig.1). Complete sequencing of the inserts also confirmed that unwanted mutations had not occurred in the selected mutants. Each of the mutated P-450 cDNAs thus obtained was inserted into the *Hind*III site of the expression vector pAAH5. Microsomes prepared from yeast transformed with the expres-

sion plasmids having the mutated cDNAs contained spectrophotometrically detectable P-450, although its content was lower than in microsomes from yeast carrying pAH3P2 or pAHF3 (not shown). The mutated P-450s, P450( $\omega$ -1:T301H) and P-450(16 $\alpha$ :T301H), were solubilized from the corresponding microsomes and purified partially as described [5,6] with a yield of 3–5 nmol from 3 l of culture.

As shown in fig.2, both mutant P-450s in the oxidized state show absorption spectra that are typical of a low-spin type of P-450 (Soret band at 416–417 nm,  $\alpha$  band  $\geq \beta$  band) and similar to that of wild-type P-450(16 $\alpha$ ) [6]. Since wild-type P-450( $\omega$ -1) exhibits a mixed-spin type spectrum in the oxidized state [5], it seems that replacement of threonine-301 by histidine in this P-450 has caused a change in the heme environment. At any rate, the spectra of the ferric mutant P-450s are clearly different from that of ferric P-450<sub>SG1</sub> (red shifted Soret band,  $\alpha$  band  $< \beta$  band) [4], which is essentially identical with those of 1-methylimidazole complexes of the ferric mutant P-450s (fig.2A). These results indicate that the mutant P-450s have not a histidine imidazole as the 6th ligand of the ferric low-spin heme. Spectra of the 1-methylimidazole complexes of the mutant and wild-type P-450s are superimposable on each other.  $K_d$

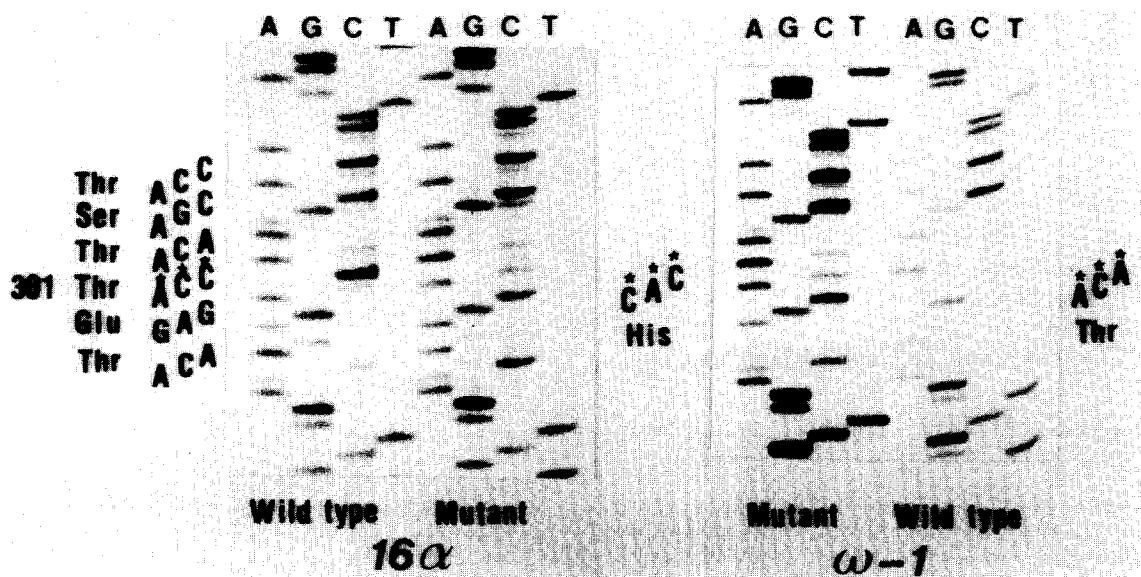


Fig.1. Sequence analysis of P-450 cDNA in the region where desired mutation was expected. The conversion of the codon for threonine-301 (ACA for P-450( $\omega$ -1) and ACC for P-450(16 $\alpha$ )) to that of histidine (CAC) is marked with asterisks.

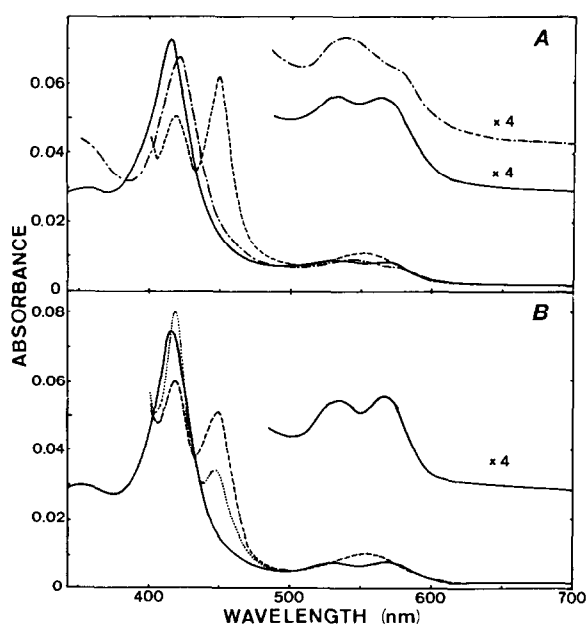


Fig.2. Absolute absorption spectra of mutant P-450s. (A) P-450( $\omega$ -1:T301H); (B) P-450(16 $\alpha$ :T301H). The partially purified preparation was dissolved in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol and detergents (0.2% Emulgen 913 for A or 0.4% sodium cholate and 0.1% Emulgen 913 for B). (—) Oxidized, addition of 1 mM sodium laurate (A) or 0.5 mM testosterone (B) caused no change in this spectrum; (---) and (...) reduced CO complex recorded 2 and 8 min after the addition of sodium dithionite, respectively; (- - - -) 1-methylimidazole (final concentration, 25 mM) was added to the oxidized form (A).

values of the mutant P-450s for 1-methylimidazole were determined to be approximately the same as those of the wild-type P-450s. The reduced CO complexes of the mutant P-450s show a Soret peak at 450 nm, as is the case for their wild-type

Table 1

Hydroxylase activity of partially purified P-450s

Species	Substrate	Metabolite	Wild-type <sup>a</sup>	T301H mutant <sup>a</sup>
P-450( $\omega$ -1)	caprate	9-OH C	8.2	nd
	laurate	11-OH L	7.9	nd
P-450(16 $\alpha$ )	testosterone	16 $\alpha$ -OH T	3.4	nd
	progesterone	16 $\alpha$ -OH P	4.7	nd

<sup>a</sup> Results expressed as nmol/min per nmol P-450

The activities were measured by reconstituting the hydroxylase system as described previously [5,6,10]. nd, not detectable

counterparts [5,6], together with a peak at 420 nm due to the denatured form. These findings support the view that in the mutant P-450s the native axial ligands of the heme iron are still maintained, although their reduced CO complexes were very unstable and converted rapidly to their denatured forms (fig.2B).

Despite the evidence for preservation of native axial ligands, the mutant P-450s could not catalyze the hydroxylation of substrates that can be metabolized by the wild-type P-450s (table 1). When laurate or caprate binds to ferric P-450( $\omega$ -1), its spectrum is converted to the typical high-spin type ( $K_d$ , 7–9  $\mu$ M) [5]. The addition of the fatty acids (up to 1 mM at least) to ferric P-450( $\omega$ -1:T301H), however, induced no spectral change, indicating that the mutant P-450 is defective in substrate binding. The results described above suggest strongly that threonine-301 plays an important role in substrate binding. The possibility still remains that the histidine residue in the place of the threonine residue affects the function of the threonine-serine cluster in which the conserved threonine is located. Investigations are in progress to examine this possibility by replacing threonine-301 with other amino acids.

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