Volume 234, number 2, 313–315 FEB 06054 July 1988

The importance of threonine-301 from cytochromes P-450 (laurate (ω-1)-hydroxylase and testosterone 16α-hydroxylase) in substrate binding as demonstrated by site-directed mutagenesis

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Received 8 April 1988

Threonine-301 from rabbit liver cytochromes P-450 (laurate (ω-1)-hydroxylase and testosterone 16α-hydroxylase) has been replaced by histidine via site-directed mutagenesis.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$_{SG1}$, an altered P-450 from a yeast mutant defective in lanosterol 14α-demethylation [4]. We have recently constructed two plasmids for expression in yeast of rabbit liver P-450(ω-1) and P-450(16α) and characterized the P-450s synthesized in yeast transformed with these expression plasmids [5,6]. In this study we constructed mutants of P-450(ω-1) and P-450(16α) by site-directed mutagenesis to examine a possible role of the aforementioned conserved threonine in catalytic activity and report our findings therein.

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Abbreviations: P-450, cytochrome P-450; P450(ω-1), P-450 (laurate (ω-1)-hydroxylase); P-450(16α), P-450 (testosterone 16α-hydroxylase); P-450$_{SG1}$, P-450 from a yeast mutant defective in lanosterol 14α-demethylation; P-450cam, *Pseudomonas putida* P-450

2. MATERIALS AND METHODS

Restriction endonucleases were purchased from Nippon Gene, T_4 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(ω-1) and P-450(16α), 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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00145793/88/$3.50

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(ω-1) and P-450(16α) [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 *Hin*dIII 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 *Hin*dIII site of the expression vector pAAH5. Microsomes prepared from yeast transformed with the expression 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(ω-1:T301H) and P-450(16α: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, α band $\geqq \beta$ band) and similar to that of wild-type P-450(16α) [6]. Since wild-type P-450(ω-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$_{SG1}$ (red shifted Soret band, α 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

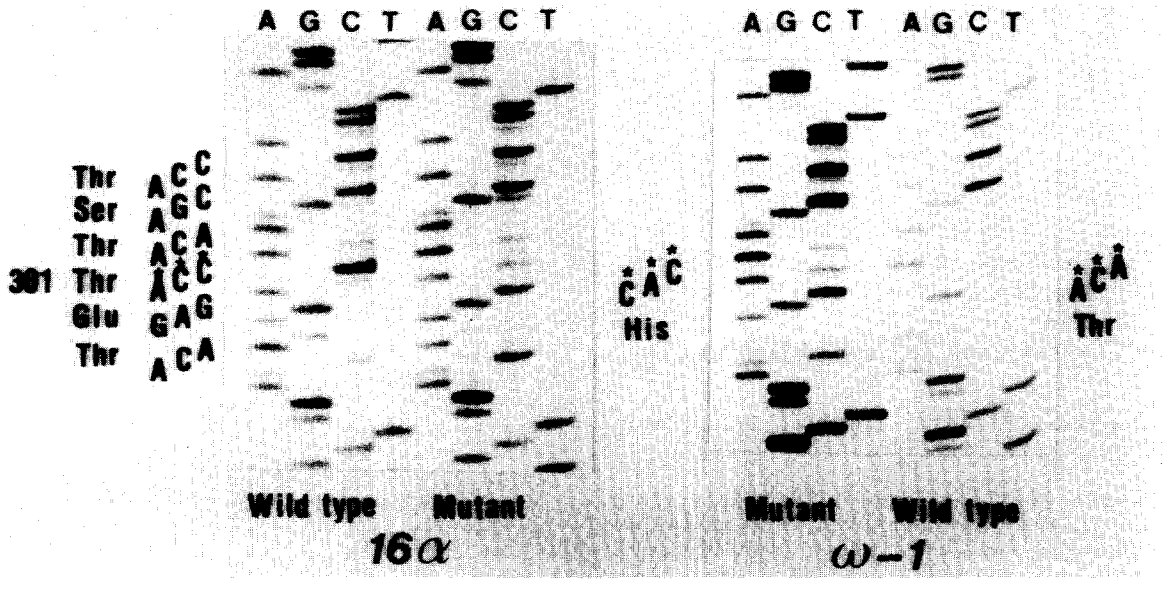

Fig.1. Sequence analysis of P-450 cDNA in the region where desired mutation was expected. The conversion of the codon for threonine-301 (ACA for P-450(ω-1) and ACC for P-450(16α)) to that of histidine (CAC) is marked with asterisks.

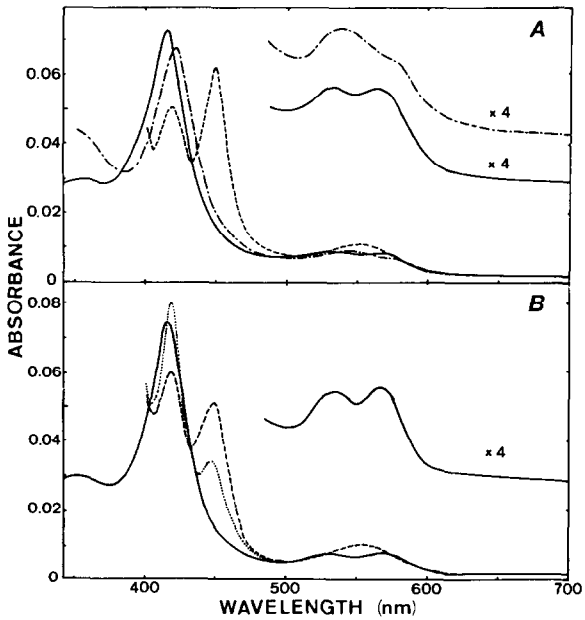

Fig.2. Absolute absorption spectra of mutant P-450s. (A) P-450(ω-1:T301H); (B) P-450(16α:T301H). The partially purified preparation was dissolved in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol and detergents (0.2% Emulgen 913 for A or 0.4% sodium cholate and 0.1% Emulgen 913 for 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 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).

Table 1

Hydroxylase activity of partially purified P-450s

Species	Substrate	Metabolite	Wild-type[a]	T301H mutant[a]
P-450(ω-1)	caprate	9-OH C	8.2	nd
	laurate	11-OH L	7.9	nd
P-450(16α)	testosterone	16α-OH T	3.4	nd
	progesterone	16α-OH P	4.7	nd

[a] 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

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(ω-1), its spectrum is converted to the typical high-spin type (K_d, 7–9 μM) [5]. The addition of the fatty acids (up to 1 mM at least) to ferric P-450(ω-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.

Acknowledgement: We are grateful to Mr J. Miyagawa for determination of fatty acids, steroids, and their hydroxylated products.

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