Mutations of P450c21 (Steroid 21-Hydroxylase) at Cys⁴²⁸, Val²⁸¹, and Ser²⁶⁸ Result in Complete, Partial, or No Loss of Enzymatic Activity, Respectively

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Abstract

Steroid 21-hydroxylase deficiency is the major cause of congenital adrenal hyperplasia (CAH), a common genetic disease. To define the relationship between gene mutations and enzyme deficiency, we generated missense mutations of the 21-hydroxylase cDNA at three different sites and characterized the mutant proteins after expressing them in cultured mammalian and yeast cells. Among them, Ser²⁶⁸ and Val²⁸¹ have been found to be mutated in CAH patients, whereas Cys⁴²⁸ has been implicated as the heme ligand. Our results show mutations at these sites result in complete, partial, or no loss of the enzymatic activity. All the Cys⁴²⁸ mutants had neither enzymatic activity nor P450 absorption, thus supporting the notion that Cys⁴²⁸ is the heme ligand. All the 268-mutants exhibited the same activity as normal 21-hydroxylase, demonstrating that the clinically observed Ser²⁶⁸ → Thr change represents a polymorphism rather than the cause of the enzyme deficiency. The 281-mutants had normal K_m but greatly reduced V_{max} values that also paralleled the reduction in the heme content, in the order Val²⁸¹ (normal, 100%) > Ile²⁸¹ (50%) > Leu²⁸¹ (20%) > Thr²⁸¹ (10%). Our findings suggest that the methyl group at the β -carbon of Val²⁸¹ is required for heme incorporation and consequently enzymatic activity. (*J. Clin. Invest.* 1991. 88:519–523.) Key words: protein expression • congenital adrenal hyperplasia • steroidogenesis • cytochrome P450 • heme binding

Introduction

P450c21 (steroid 21-hydroxylase) is a member of the heme-containing cytochrome P450 superfamily that act as a terminal monooxygenase in electron transport systems (1, 2). It accepts electrons from NADPH via NADPH-reductase and catalyzes the conversion of progesterone to deoxycorticosterone and 17-hydroxyprogesterone to 11-deoxycortisol during the synthesis of mineralocorticoids and glucocorticoids (3). 21-Hydroxylase deficiency causes decreased synthesis of cortisol which in turn triggers overstimulation of the adrenal glands by adrenocorticotropin and ultimately results in the symptoms of adrenal hyperplasia and virilization (4).

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A 21-hydroxylase deficiency accounts for > 90% of the cases of congenital adrenal hyperplasia (CAH)¹ which have variable clinical presentations ranging from the mild, nonclassical form to the severe, salt-wasting form (4). The wide range of clinical presentation is due to the variety of mutations associated with the CYP21B gene which encodes P450c21 (5, 6). These mutations include gene deletions (7, 8), splicing errors (9), large gene conversions (10, 11), and apparent point mutations (12–15). Gene deletions result in the salt-wasting form of CAH (16), whereas point mutations have been found in a spectrum of CAH cases, including the salt-wasting, simple-virilizing, and nonclassical forms (12–15). The severity of the disease depends on the functional importance of the mutation.

To study the changes in the structure and function of 21-hydroxylase caused by missense mutations, we have expressed mutant proteins in both cultured mammalian cells and yeast. Three sites in 21-hydroxylase were chosen for functional analysis: Ser²⁶⁸, Val²⁸¹, and Cys⁴²⁸.

Among the three sites, a Ser²⁶⁸ → Thr change was found in patients suffering from CAH (17) and Val²⁸¹ → Leu mutation was previously identified in a patient with nonclassical CAH characterized by partial enzyme deficiency (15). Cys⁴²⁸ is the invariant Cys among all cytochromes P450 and has been predicted to be the heme ligand (18, 19). We mutated Ser²⁶⁸ to Thr, Cys, and Met to see if these changes altered the function of 21-hydroxylase. We changed Val²⁸¹ to Leu, Ile, and Thr in order to investigate the effect of these amino acid substitutions on the structure and enzymatic activity of 21-hydroxylase. Val, Leu, and Ile share similar properties, therefore substituting one with another should not drastically disturb the structure of the protein. Cys⁴²⁸ has been changed to Thr, Met, and Ser in our study to explore the importance of Cys⁴²⁸.

We have assayed the enzymatic activities and heme-binding properties of the mutant proteins and conclude that Ser²⁶⁸ is a polymorphic site whose mutation does not cause CAH, Val²⁸¹ is involved in heme interaction, and Cys⁴²⁸ is the heme ligand.

Methods

Site-directed mutagenesis. Oligonucleotides were synthesized to contain the mutated sequence in the middle and 10 nucleotides of homologous sequences flanking each side. The sequence of the oligos are as follows: GGCGCAGCCGACCATGGAAGAG for Thr²⁶⁸, GGGTGGCGCAGCCGATGATGGAAGAGGGCTC for Met²⁶⁸, GGCGCAGCCGTGCATGGAAGA for Cys²⁶⁸, TGCCCGCTGAGCCTGGGCGA for Ser⁴²⁸, GCGGTGCCCCGCGTGATGCTGGGCGAGCCGCT for Met⁴²⁸, GCGGTGCCCCGCGTGACACTGGGCGAGCCGCT for Thr⁴²⁸, GGAAGGGCACCTGCACATGGC for

1. Abbreviations used in this paper: CAH, congenital adrenal hyperplasia; phc21, 21-hydroxylase cDNA; RSV- β -gal, β -galactosidase gene.

Leu²⁸¹, TCCTGGAAGGGCACATTACATGGCTGCAGT for Ile²⁸¹, and TCCTGGAAGGGCACACGCACATGGCTGCAGT for Thr²⁸¹. Site-directed mutagenesis of 21-hydroxylase cDNA was performed in an M13 vector as described (20) and all the mutant cDNAs were sequenced in order to confirm that the point of mutation was as expected and not elsewhere. The mutant cDNA was then subcloned into two plasmid-expression vectors.

Cells and plasmids. The calcium-phosphate precipitation method was used to transfect COS-1 cells with 21-hydroxylase cDNA (phc21) and a β -galactosidase gene (RSV- β -gal) which was used as an internal control to normalize the transfection efficiency (21). 2 d after transfection, cells were incubated with 1 μ M [¹⁴C]-17-hydroxyprogesterone for 1–24 h. Steroids in the media were extracted and the cells were harvested for protein immunoblotting assays (21).

All of the plasmids for mammalian expression were derived from phc21 which contained the SV40 early promoter, splice sites, and polyadenylation sites (20). The cDNAs were first digested completely with Xho I, then partially with Bam HI; and the resulting 2-kb cDNA fragment with 180 bp of the 5'-untranslated region was inserted into the yeast vector pYE8 at the Eco RI site after filling the ends with Klenow Fragment. pYE8 vector contains the yeast glyceraldehyde-3-phosphate dehydrogenase promoter and the 2 μ origin of replication (22). Transformation of plasmids into *S. cerevisiae* strain 20B-12 has been described previously (21, 23).

Activity assay and heme-content measurement. Yeast microsomes were collected after zymolyase digestion, sonication, and centrifugation at 3,000 g, 11,000 g, and 206,000 g. The high-speed pellet was resuspended in a potassium-phosphate buffer (pH 7.4) and stored at –70°C until further testing. The CO-difference spectra were obtained by bubbling 3 mg/ml reduced microsomal protein with CO gas before recording absorbance from 350 to 550 nm by a model 557 spectrophotometer (Hitachi Ltd., Tokyo). The heme content of the P450 proteins was calculated from the absorbance at 450 nm.

The kinetic properties of 21-hydroxylase were measured by first incubating microsomes with 0.15 μ M of substrate at different time intervals to determine the linear range of the reaction. The microsomes were then incubated with 0.15, 0.3, 0.45, 0.6, 0.8, 1.0, 1.5, and 2.0 μ M [¹⁴C]-17-hydroxyprogesterone at the time points when the reaction was linear. The substrate and the product were separated by thin layer chromatography. The velocities of the reaction were calculated after counting the radioactivity of the substrate and the product. The K_m and V_{max} values were deduced from double reciprocal plots using Lineweaver-Burk transformation of the initial reaction rate versus the substrate concentration.

Results

Both wild-type and mutant proteins are expressed. We generated missense mutations at codons 268, 281, and 428 to characterize the properties of the mutant proteins. As shown in Fig. 1, all of the mutant proteins were synthesized in COS-1 cells and reacted with P450c21 antiserum as did the wild-type protein. The control cells that contained no 21-hydroxylase cDNA did not produce any detectable P450c21. The amount of mutant protein production was about the same as the wild type, indicating that the missense mutations did not alter the transcriptional or the translational capability of the gene.

The Met⁴²⁸ mutant protein migrated more slowly compared to the rest of the P450c21 (Fig. 1 C, lane 4). We have sequenced the entire coding region of the mutant plasmid and found no errors. Several independent mutants obtained by independent transformations all exhibited this same property (data not shown). Therefore we are certain that this aberrant mobility is due to the intrinsic property of the protein and not a cloning artifact. Whether this is due to an inadvertent post-

Figure 1. Production of mutant P450c21 in COS-1 cells detected by Western Blot. After COS-1 cells in 6-cm dishes were transfected with plasmids which are marked at the top of each lane, 1/4 of the cell extract was loaded onto 10% SDS polyacrylamide gel, transferred to nitrocellulose, and reacted with anti-P450c21 antisera. (A) 268 mutant cDNAs transfected in duplicate. (B) 281 mutants. (C) 428 mutants. WT represents wild type cDNA used in transfection. In lane 1 of A and C, cells received no DNA.

translational modification in COS-1 cells or another reason is not known. Similarly, a single amino acid substitution that altered the electrophoretic behavior of the protein NADPH-cytochrome P-450 oxidoreductase has been reported (24). The seemingly slower migration of Thr-281 in Fig. 1 B, lane 4, is a gel artifact, as many other gels show that Thr²⁸¹ exhibits no difference in mobility from other P450c21.

All 268-mutants retained full activity. The enzymatic activity of P450c21 mutants with substitutions at amino acid 268 was compared with that of the wild-type 21-hydroxylase. Like the wild-type enzyme, all the 268-mutants converted ~ 50% of the substrate (17-hydroxyprogesterone) into product (deoxycortisol) in 1 h (Fig. 2 A). Thr²⁶⁸-, Cys²⁶⁸-, and Met²⁶⁸-mutants had on average 103±15, 93±7, and 107±21% of the wild type 21-hydroxylase activity. Therefore, the mutations of Ser²⁶⁸ did not significantly change the enzymatic activity.

Val²⁸¹ mutants retained partial enzymatic activity. The enzymatic activities of the 281-mutants when compared with that of the normal 21-hydroxylase were greatly reduced (Fig. 2 B). In 1 h, 52% of the substrate was converted into product by the wild-type 21-hydroxylase, whereas there was only 10, 32, and 8% conversion by the Leu²⁸¹-, Ile²⁸¹-, and Thr²⁸¹-mutants, respectively. When the same experiments were carried out with a shorter reaction time (30 min), the activities of the mutants were 7–20% of the wild type for the Leu²⁸¹-mutant, 60–70% for the Ile²⁸¹-mutant, and 10–15% for the Thr²⁸¹-mutant.

Figure 2. 21-Hydroxylase activities from normal and mutant P450c21 expressed in COS-1. After COS-1 cells were transfected with 5 μ g each of the P450c21 cDNA and RSV- β -gal plasmids, 1 μ M [14 C]-17-hydroxyprogesterone was added to the media and incubated for 1 h in A and B, and for 24 h in C. Product was separated from substrate by thin layer chromatography followed by autoradiography. The activity was then normalized for transfection efficiency using β -galactosidase activity as an internal control. S refers to the position of substrate; P refers to product. The 268-mutant activities were tested in duplicate.

Cys⁴²⁸ mutants have no enzymatic activity. None of the Cys⁴²⁸-mutants produced by COS-1 cells exhibited any 21-hydroxylase activity. Even after a prolonged reaction time, when the wild-type 21-hydroxylase utilized 95% of the substrate, none of the 428-mutants had any activity (Fig. 2 C). The complete inactivation of the Cys⁴²⁸-mutants is distinct from other classes of the mutations.

Kinetic comparison of mutant P450c21. To gain further insight into the mechanism of complete loss or partial retention of enzymatic activities in the mutants, kinetic properties of the wild-type and mutant enzymes were studied using proteins expressed in yeast. The fast growth rate of yeast enables production of enough proteins for such kind of measurement. Like the mammalian expression, both the wild-type and mutant proteins were expressed in equal quantities in yeast (data not shown).

Table I. Comparison of Normal and Mutant 21-Hydroxylase Activities in Yeast

Protein type	Enzyme activity	K_m	V_{max}	Heme content
	%	μ M	nmol/h/mg microsomal protein	nmol/mg microsomal protein
Val ²⁸¹ (WT)	100	0.27	40	0.076
Leu ²⁸¹	18 $\pm$ 9	0.4	2.5	0.006
Ile ²⁸¹	45 $\pm$ 5	0.29	26	0.028
Thr ²⁸¹	10 $\pm$ 8	0.48	2.1	ND
Ser ⁴²⁸	0	ND	ND	ND

ND, not detected. For enzymatic activity assay, 100 μ g yeast microsomal protein was incubated with 1 μ M [14 C]-17-hydroxyprogesterone at 37°C for 5 min for the wild type or 30 min for the mutant enzymes. The product was separated from substrate by thin layer chromatography and the amount of substrate conversion was quantified by scintillation counting. Enzyme activity is shown as mean $\pm$ SD ($n = 3$). K_m , V_{max} , and heme content were calculated as described in Methods.

All the 428-mutants expressed in yeast lacked enzymatic activity just like the 428-mutants in COS-1 cells.

The 281-mutants expressed in yeast retained partial activity as observed in mammalian cells (Table I). The K_m values for 17-hydroxyprogesterone of Leu²⁸¹, Ile²⁸¹, and Thr²⁸¹-mutants were 0.4, 0.29, and 0.48 μ M, respectively, which was similar to the normal K_m value of 0.27 μ M for wild-type 21-hydroxylase.

Figure 3. CO-difference spectra of wild type and mutant P450c21. Microsomes (3 mg/ml) in potassium phosphate buffer were reduced with sodium dithionite, bubbled with CO gas, and the difference spectra were recorded. (YE8) Vector control; (YE-S428) Ser-428 mutant. YE-T281, L281, and I281 are Thr, Leu, and Ile-281 mutants; YE21 is the wild type P450c21.

			*	*		
450c21human	260	MLQGVAQPSMEEGSGQLLEGHVHMAAVDLLIGGTETTANTLSWAVVFLLLHHP	315			
450c21bovine	259	MLQGVGRQRVEEGPGQLLEGHVHMSVVDLFIGGTETTASTLSWAVAFLLHHP	314			
450c21mouse	255	MLQGVKQRDQKDEEQLHEGHVHMSVVDLFIGGTETTATTLWAVAFLLHHP	310			
450c21porcine	259	MLQEAGRQRVEEGQGQLLEGHVHMSVVDLFIGGTETTANTLSWAVVYLLHHP	314			
450cam	224	ANGQV-----NGRPITSDEAKRM-CGLLVGGGLDTVVFFLSFSMEFLAKS	372			
					Helix I	

		*	
450c21human	420	AFGCGARVCLGEP LARLELFVVLTRLLQAF TLLP	453
450c21bovine	419	AFGCGARVCLGES LARLELFVLLRLLQAF TLLP	452
450c21mouse	412	SFGCGARVCLGEP LARLELFVVLARLLQAF TLLP	445
450c21porcine	419	AFGCGARVCLGEP LARLELFVVLVQLLQAF TLLP	452
450cam	349	TFGHGSHLC LGOHLARREIIVTLKEWLTRIPDFS	382

Helix I.

Figure 4. Alignment of P450c21 and P450cam. The sequences of four P450c21s and P450cam were aligned at the helix I and helix L regions. The number in front of each sequence refers to amino acid position of each P450. Asterisks refer to amino acid positions 268, 281, and 428.

The $V_{\max}$ values, however, decreased 20-fold for the Leu²⁸¹- and Thr²⁸¹-mutants. The Ile²⁸¹-mutant, which retained ~ 40–50% wild-type activity, also possessed ~ 65% of the wild-type $V_{\max}$ value.

Heme-content of the mutant P450c21. The reason for the change of the catalytic rates in the mutants was explored by studying the structure of the active site of P450c21: the heme-binding pocket. As shown in Fig. 3, the wild-type P450c21 exhibited a characteristic peak at 450 nm upon binding to carbon monoxide, whereas microsomes from yeast transformed with the original vector plasmid showed no absorption at 450 nm but had a small peak at 420 nm. The Ser⁴²⁸-mutant also exhibited no absorption at 450 nm, indicating that Ser⁴²⁸ no longer bound heme.

The Ile²⁸¹- and Leu²⁸¹-mutants, on the other hand, had decreased but detectable absorption at 450 nm. The 450 nm peak exhibited by the Thr²⁸¹-mutant was too weak to be detected. The heme content of the proteins calculated from the peak height showed that both the Leu²⁸¹- and Ile²⁸¹-mutants bound less heme than the wild-type protein, whereas no heme was detected in the Thr²⁸¹- and Ser⁴²⁸-mutants (Table I).

Discussion

We have generated single amino acid substitutions in 21-hydroxylase at three sites to correlate the clinical phenotypes with the biochemical properties of the mutant enzymes and to characterize the structure-function relationship of P45021. We have provided biochemical evidence that 21-hydroxylase with Ser²⁶⁸ replaced by Thr, Cys, and Met has normal enzymatic activity. Ser²⁶⁸ is not a conserved amino acid when the sequence surrounding it is aligned with that of P450c21 from other species (Fig. 4 A). Based on these observations, we conclude that Ser²⁶⁸ does not participate directly in the catalysis of P450c21. The recent report on the association of the Ser²⁶⁸ → Thr change with an Nco I polymorphism in normal individuals is compatible with our result (25).

Val²⁸¹ → Leu mutation was identified in a patient suffering from nonclassical CAH, which is often characterized by a mild enzyme disorder (15). We demonstrate that a Val²⁸¹ → Leu

substitution results in a mutant protein with 10–20% the wild-type activity. A recent study using the vaccinia virus expression system reports 50% of the wild-type activity towards 17-hydroxyprogesterone for the Leu²⁸¹-mutant (26). Compared with this report, we have characterized the 281-mutation in more detail by changing Val²⁸¹ to Ile and Thr as well and by analyzing the heme-binding property of the mutant proteins.

The loss of enzymatic function in 281-mutants can be accounted for by the reduction of hemoprotein content as shown in the CO-difference spectra. The heme content of the protein paralleled the enzymatic activity with the Ile²⁸¹-mutant possessing the highest amount of heme binding and also the highest residual activity. Therefore, the dynamics of heme binding affected the enzymatic function of the 281-mutants.

When sequences of 34 cytochromes P450 at the position of Val²⁸¹ were compared (19), Leu is the most common amino acid (13/34), with Ile next (11/34) and Val the third (7/34). Although it is not conserved amongst different subfamilies of P450s, this residue is mostly hydrophobic and conserved within certain subfamilies (27). Among steroidogenic P450s, Val is present at the highest frequency (5/6) with Ile next (1/6). But Val is the only amino acid at this site in P450c21 from four different mammalian species (Fig. 4 A). Therefore, only similar types of P450 such as steroidogenic P450s share similar structures surrounding Val²⁸¹.

When the sequence of P450c21 is aligned with that of P450cam (19), whose crystal structure has been solved, Val²⁸¹ is located at a position corresponding to the edge of helix I, although homology breaks past the core of helix I (Fig. 4 A). In P450cam, the heme moiety is sandwiched between helix I and helix L (28). A substitution of Val²⁸¹ at the edge of helix I may perturb the heme-binding property of the protein, resulting in a partial enzyme deficiency.

The three 281-mutants showed different enzymatic activities and hemoprotein contents, Ile²⁸¹ being the highest, Leu²⁸¹ the second, and Thr²⁸¹ the third. Ile, Leu, and Val are hydrophobic amino acids, whereas Thr has a polar group. Like the wild type residue Val, Ile has a methyl group at its β -carbon. Because Ile has nearly normal activity and the highest heme content among the mutants, this methyl group may be impor-

tant in the interaction with the heme moiety. Both Leu²⁶⁸- and Thr²⁸¹-mutants bind heme less well probably because the methyl group in Leu is located at the γ -carbon and Thr has a polar hydroxyl group.

Cys⁴²⁸ is considered to be the heme ligand as predicted from its homology with the heme-binding site near helix L in P450cam (18). Amino acid alignment studies also indicate that this position is the only invariant Cys in all known P450s (19, and Fig. 4 B). Shimizu et al. have mutated this Cys and its surrounding residues in P-450d and also conclude that this Cys is necessary for enzymatic activity (29). Our data show that changing Cys⁴²⁸ of P450c21 to three other amino acids results in complete loss of heme-binding and enzymatic activity. This, as well as its invariant location near helix L, suggests that Cys⁴²⁸ is the heme ligand in P450c21.

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