Naturally Occurring Mutants of Human Steroid 21-Hydroxylase (P450c21) Pinpoint Residues Important for Enzyme Activity and Stability*

(Received for publication, July 21, 1997, and in revised form, December 29, 1997)

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Three mutants (deletion of E196, G291S, and R483P) of steroid 21-hydroxylase (P450c21) from patients with inherited congenital adrenal hyperplasia had reduced activity toward progesterone and 17-hydroxyprogesterone after transient expression in cultured mammalian cells. In addition, both the E196 deletion and the R483P mutant had shorter half-lives than the wild-type enzyme, whereas the half-life of the G291S mutant was comparable with that of the normal protein. These results directly link the clinical situation with the three mutations and suggest that G291 is important for the catalytic activity of P450c21.

Cytochrome P450s are a superfamily of enzymes essential for the oxidative metabolism of different endogenous compounds and xenobiotics (1). For many members of the family, enzyme activity varies between individuals due to polymorphisms in the coding parts of the genes. Four cytochrome P450 enzymes are involved in the synthesis of cortisol in the adrenal cortex (2). Impaired production of cortisol due to reduced function of any of these enzymes causes increased secretion of adrenocorticotropic hormone (ACTH) from the pituitary and congenital adrenal hyperplasia (CAH) (3). Of particular interest is the cytochrome P450 steroid 21-hydroxylase (P450c21) since more than 90% of all cases of CAH are due to mutations in the gene encoding the enzyme. P450c21 participates in the synthesis of both glucocorticoids and mineralocorticoids. In addition to lack of cortisol and aldosterone, P450c21 deficiency leads to overproduction of adrenal androgens. The disorder has a wide spectrum of manifestations (4), ranging from a life-threatening neonatal condition in both sexes with ambiguous genitalia in girls, to inopercious symptoms of hyperandrogenism in adult women.

P450c21 is a protein of 494 amino acids (5, 6). The gene locus has a complicated structure, with an active gene (CYP21) and a highly homologous inactive pseudogene (CYP21P) (5–8). Misalignment followed by recombination events between the two homologous genes during meiosis with transfer of sequences from CYP21P to CYP21 account for around 95% of the mutations of P450c21 that lead to CAH (9). Rarely, mutations also arise independently of the pseudogene. We have characterized three such rare missense mutations, which were found in patients with CAH. To investigate the molecular mechanisms behind the impaired enzyme function displayed by the patients, the mutations were reconstructed by in vitro site-directed mutagenesis, and normal and mutant enzymes were transiently expressed in COS-1 cells. Enzyme activities toward the two natural substrates, 17-hydroxyprogesterone (17-OHP) and progesterone, were determined. In addition, to assess whether the impaired function resulted from reduced stability of the mutant proteins, their half-lives were determined by pulse-chase experiments followed by immunoprecipitation.

EXPERIMENTAL PROCEDURES

Patients—The CYP21 mutations were found in five patients with CAH. The siblings numbers B39 and B40, as well as patient number B103, have been reported previously (10, 11), whereas the younger brother of B103, patient E. I., as well as patient number 1293 have not been described. Genotyping was performed by allele-specific polymerase chain reaction (12) and direct sequencing of CYP21 (10). Numbering of nucleotides and amino acids follows the reference sequence of White et al. (5). Subjects numbers B39 and B40 were compound heterozygotes, with the unique R483P mutation on one allele and the well-known I172N mutation (13) on the other. They had a moderate form of the disease, with early clitoral enlargement in the female sibling but without salt-wasting. Subject number B103 and his younger brother E. I. had inherited the unique G291S mutation on their paternal allele, whereas the maternal allele was deleted. They had the most severe, salt-wasting form of CAH. Patient number 1293 was a compound heterozygote for the I172N mutation and a novel sequence aberration, where nucleotides 1158–1160 (AGG) in exon 5 of CYP21 were deleted, causing loss of a glutamic acid residue at position 196 in P450c21, delE196 (Fig. 1). This female patient was born in Norway in 1968 and was affected with a moderate form of CAH with some signs of prenatal virilization but without salt-wasting.

Construction of Plasmids, Site-directed Mutagenesis, in Vitro Expression, and Assay of Enzyme Activity—pGEM constructs as well as pCMV4 expression vectors were constructed to contain the cDNAs encoding the wild-type and the three mutant enzymes, essentially as described previously (14). The plasmids thus generated were named pGEM-CYP21P (encoding the wild-type enzyme), pGEM-CYP21(delE196), pGEM-CYP21G291S, pGEM-CYP21R483P, and pCMV4-CYP21P, pCMV4-CYP21(delE196), pCMV4-CYP21G291S, and pCMV4-CYP21R483P. All constructs were sequenced to verify the correct incorporation of the mutations and to exclude additional aberrations. Enzymatic activities of the mutants and the wild-type enzyme were determined in intact COS-1 cells after transient expression, using 17-OHP and pro-
Effects of delE196, G291S, and R483P Mutations on P450c21

RESULTS

Enzyme Activity of Mutants—Table I shows that all mutants had a fraction of the activity of the wild-type enzyme. The delE196 mutant retained 6% activity toward 17-OHP and 23% activity toward progesterone. The G291S mutant displayed 0.8% of normal activity in both cases, whereas the R483P mutant displayed 1% of normal activity toward 17-OHP and 2.2% activity toward progesterone. The activity of the wild-type enzyme was defined as 100%.

In Vitro Translation—To investigate whether the three sequence alterations of CYP21 had any effect on the translation of the gene, an in vitro-coupled transcription/translation assay was employed. None of the mutations affected the rate of translation in this in vitro system (data not shown).

Stability of Mutant Versus Wild-type Protein—Fig. 2 shows the degradation profiles of immunoprecipitated wild-type and mutant P450c21s for chase periods of up to 4 h. Fig. 3 shows the same results after quantification of the immunoreactive bands with a phosphoimager. The degradation of each enzyme species was comparable.

Fig. 1. Nucleotide sequence in the vicinity of the delE196 mutation. Part of the sequence of exon 5 of CYP21 is shown, including the novel mutation found in patient number 1293. Nucleotides 1158–1160 (AGG) are deleted, resulting in a protein lacking residue glutamic acid 196. The identification of the other two mutations described in this paper (G291S and R483P) has been reported previously (10, 11).

Fig. 2. Degradation profiles of wild-type and mutant P450c21s transiently expressed in COS-1 cells. Cells were transfected with pCMV4-CYP21 (wild-type protein) and pCMV4-CYP21(delE196) (A) and with pCMV4-CYP21(G291S) and pCMV4-CYP21(R483P) (B), pulse-labeled, and chased for, from right to left, 0, 1, 2, 3, and 4 h. Cells were then lysed, and their supernatants were treated with rabbit P450c21 antiserum. Immunoprecipitated material was analyzed by SDS-PAGE (10% gels). The arrow indicates the band corresponding to P450c21.

Fig. 3. Quantification of degradation profiles of wild-type and mutant P450c21s transiently expressed in COS-1 cells. Results are expressed as percentage of remaining protein, with the amount at time 0 (just before chase) defined as 100%. •, wild-type P450c21; △, delE196 mutant; ●, G291S mutant; □, R483P mutant. Values for 0, 1, 2, 3, and 4 h represent the average of two independent pulse-chase experiments.
between the normal and mutant enzymes (data not shown). Fig. 3 indicates that the wild-type enzyme was the most stable, whereas all mutants had decreased stability. The R483P was most rapidly degraded and was barely detectable at the end of the 4-h chase. Half-lives were calculated as 1 h, 10 min (wild-type P450c21), 30 min (delE196), 55 min (G291S), and 20 min (R483P).

**DISCUSSION**

Defective function of P450c21 is the major cause of CAH. To examine whether this was the case for three novel mutant P450c21s from patients with CAH, we studied the enzymatic activities and half-lives of the mutants. All three mutations impaired enzyme activity toward the two natural substrates of the enzyme when assayed after transient transfection in cultured cells. The delE196 mutant was partially active toward both substrates. P450c21 G291S resulted in a protein with less than 1% of normal activity, whereas the R483P mutant retained 1–2% of the activity of the native enzyme. This is in agreement with the clinical phenotypes of the patients. Those carrying delE196 and R483P had moderate forms of CAH and were compound heterozygotes with the I172N mutation on the other chromosome. The latter mutation is generally associated with the moderate, simple virilizing phenotype (9, 17, 18). The brothers carrying G291S had deleted their 21-hydroxylase gene from both chromosomes and had the most severe, salt-wasting form of the disease.

To examine whether the reduced enzymatic activity of the mutants was a consequence of reduced stability rather than decreased turnover of substrate, the half-lives of the wild-type and the three mutant enzymes were compared by pulse-chase experiments. The half-life of the wild-type P450c21 (1 h, 10 min) was longer than those of all mutants. However, these differences in stability were more pronounced for the mutants. The delE196 and R483P mutants were most rapidly degraded (half-lives 30 and 20 min, respectively). These data suggest that the phenotypes resulting from the delE196 and R483P mutations can be explained in view of little if any available P450c21. In contrast, the half-life of G291S (55 min) and wild-type P450c21 (1 h, 10 min) were comparable, it seems that G291 plays an essential role in the maintenance of enzyme activity. Generally, little is known about structure-function relationships of mammalian cytochrome P450 enzymes. One reason is that mammalian P450s contain hydrophobic segments and are therefore difficult to crystallize. However, three-dimensional structures of four nonmembrane-associated bacterial cytochrome P450s (P450cam (19), P450BM-3 (20), P450terp (21), and P450eryF (22)) have been determined. A correlation of important residues among different classes of cytochrome P450s has been made by Nelson and Strobel (23) after alignment of 34 different P450s, including P450c21 and P450cam (24). According to their model, G291 would be hydrogen-bonded with T295 to form together an oxygen-binding pocket. Another model obtained from the structural information of P450BM-3 (20) implies that residues 291–297 of human P450c21 are involved in proton transfer from water to heme. Thus, G291 is intricately involved in catalysis and the three-dimensional structures of two extremely different bacterial P450s support the hypothesis that G291 is an essential residue for catalysis.

In conclusion, enzymatic activity and stability analyses of three naturally occurring mutants of P450c21 from patients with CAH provided a potential explanation for the clinical symptoms and identified residues important for the in vitro stability and catalytic activity of the enzyme.

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