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Genetics

Mutations in the human *CYP11B2* (aldosterone synthase) gene causing corticosterone methyloxidase II deficiency

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ABSTRACT **Corticosterone methyloxidase II (CMO-II) deficiency is an autosomal recessive disorder of aldosterone biosynthesis, characterized by an elevated ratio of 18-hydroxycorticosterone to aldosterone in serum. It is genetically linked to the *CYP11B1* and *CYP11B2* genes that, respectively, encode two cytochrome P450 isozymes, P450XIB1 and P450XIB2. Whereas P450XIB1 only catalyzes hydroxylation at position 11β of 11-deoxycorticosterone and 11-deoxycortisol, P450XIB2 catalyzes the synthesis of aldosterone from deoxycorticosterone, a process that successively requires hydroxylation at positions 11β and 18 and oxidation at position 18. To determine the molecular genetic basis of CMO-II deficiency, seven kindreds of Iranian-Jewish origin were studied in which members suffered from CMO-II deficiency. No mutations were found in the *CYP11B1* genes, but two candidate mutations, R181W and V386A, were found in the *CYP11B2* genes. When these mutations were individually introduced into CYP11B2 cDNA and expressed in cultured cells, R181W reduced 18-hydroxylase and abolished 18-oxidase activities but left 11β-hydroxylase activity intact, whereas V386A caused a small but consistent reduction in the production of 18-hydroxycorticosterone. All individuals affected with CMO-II deficiency were homozygous for both mutations, whereas eight asymptomatic subjects were homozygous for R181W alone and three were homozygous for V386A alone. These findings confirm that P450XIB2 is the major enzyme mediating oxidation at position 18 in the adrenal and suggest that a small amount of residual activity undetectable in *in vitro* assays is sufficient to synthesize normal amounts of aldosterone.**

Aldosterone, the principal mineralocorticoid hormone in humans, is synthesized from deoxycorticosterone in mitochondria of the zona glomerulosa of the adrenal cortex. Conversion of deoxycorticosterone to aldosterone requires hydroxylation at position 11β to form corticosterone, hydroxylation at position 18 to form 18-hydroxycorticosterone, and finally oxidation at position 18. The enzymes required for these three steps have recently been characterized. Humans (1–3) have distinct cytochrome P450 isozymes that catalyze hydroxylation at position 11β (conversion of 11-deoxycortisol to cortisol) in the zona fasciculata (this isozyme is termed P450c11, P450XIB1, or CYP11B1) and all three reactions (i.e., conversion of deoxycorticosterone to aldosterone) in the zona glomerulosa (an isozyme termed P450cmo, P450aldo, P450XIB2, or CYP11B2). Similar enzymes have been identified in the rat (4, 5), but a distinct P450cmo isozyme has not as yet been identified in cattle (6).

The human P450c11 and P450cmo enzymes are predicted to be 93% identical in amino acid sequence. They are, respectively, encoded by the *CYP11B1* and *CYP11B2* genes (7), which are both located on chromosome 8q22 (8). Whereas both enzymes mediate hydroxylation at position 11β of 11-deoxycorticosterone *in vitro*, P450c11 catalyzes hydroxylation at position 18 at <10% of the rate at which P450cmo mediates this reaction (1–3). Further conversion of 18-hydroxycorticosterone to aldosterone by P450c11, if it occurs, is below the level of detection by experiments reported to date.

Corticosterone methyloxidase II (CMO-II) deficiency is an inherited defect of aldosterone biosynthesis (9, 10). Patients with this disorder are subject to potentially fatal electrolyte abnormalities as neonates and a variable degree of hyponatremia and hyperkalemia combined with poor growth in childhood, but they may have no symptoms as adults. Asymptomatic adults with this disorder have been ascertained in the course of family studies because affected individuals invariably have an elevated ratio of 18-hydroxycorticosterone to aldosterone, which has been presumed to reflect a block in the final step of the biosynthetic pathway (11).

CMO-II deficiency is apparently rare in the general population, but it has been found at an increased frequency among Jews of Iranian origin. In this population, the disease is inherited as an autosomal recessive trait and is genetically linked to a unique *Msp* I polymorphism in *CYP11B1* (12).

To investigate the genetic basis of this disease, we have characterized the *CYP11B1* and *CYP11B2* genes in members of Iranian-Jewish families in which CMO-II deficiency was segregating. Whereas no candidate mutations were found in *CYP11B1*, two missense mutations were identified in *CYP11B2*. All individuals affected with CMO-II deficiency were homozygous for both of these mutations, whereas no unaffected individuals carried both mutations, confirming that the *CYP11B2* gene product is required for normal aldosterone biosynthesis.

MATERIALS AND METHODS

Subjects. DNA samples were prepared from 37 members of seven Jewish families of Iranian origin, five of which were consanguineous. Twelve subjects had CMO-II deficiency. Disease status was defined by an elevated ratio of 18-hydroxycorticosterone to aldosterone, as has been reported elsewhere (12–15). The presence of 17α-hydroxylase deficiency, which also leads to an elevated ratio of 18-hydroxycorticosterone to aldosterone, was excluded by the presence of normal blood and urine levels of 17α-hydroxylated steroids (16).

Amplification and Sequencing of Exons. At the nucleotide level, *CYP11B1* and *CYP11B2* are 95% identical in exons and 90% identical in introns. Exons from each gene were amplified in several segments by polymerase chain reactions (PCRs) using oligonucleotides corresponding to intronic sequences where the two genes differed (17). Reactions were performed for 35 cycles at 94°C for 1 min, 60°C or 65°C for 1

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Abbreviation: CMO-II, corticosterone methyloxidase II.
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min, and 72°C for 2 min with an additional 5 sec each cycle. The specificity of the reactions was confirmed by control reactions on cloned DNA from each gene and by comparison of resulting sequence data. Amplified fragments from each gene were purified by agarose gel electrophoresis, recovered on an NA45 sheet (Schleicher & Schuell), and subcloned into pCR1000 (Invitrogen, San Diego). Exons and exon–intron boundaries were sequenced by the dideoxynucleotide chain-termination method using DNA denatured in the presence of dimethyl sulfoxide (18). Sequence changes were confirmed in independent subclones and then checked in other family members by direct sequencing of PCR-amplified DNA that had been denatured in the presence of dimethyl sulfoxide (18, 19) and by hybridizing PCR-amplified DNA with allele-specific oligonucleotides.

Mutagenesis and Subcloning of Constructs. Candidate mutations were introduced into the normal sequence of CYP11B2 cDNA by a PCR using oligonucleotides containing the desired change (20). Briefly, CYP11B2 cDNA (3) was amplified with *Thermococcus litoralis* DNA polymerase ($Vent_R$ polymerase; New England Biolabs) in two overlapping segments, each using one oligonucleotide corresponding to either the 5′ or 3′ end of the cDNA coding sequence and one containing the desired mutant sequence. The 5′ and 3′ amplified segments were then gel-purified, combined in a single reaction mixture, denatured, annealed, and extended with $Vent_R$ DNA polymerase to produce a full-length molecule, which was then amplified using the primers from the 5′ and 3′ ends of the coding sequence. Restriction sites were included in these flanking primers to facilitate cloning into the expression vector pCMV4 (21). The complete sequence of each resulting recombinant was then checked to ensure that no unwanted mutations had been introduced by the PCR.

An expression plasmid containing cDNA for human adrenodoxin, pCMV4-Adx, was constructed by amplifying the insert of hAdx-7 (22) by a PCR using the $Vent_R$ polymerase and primers specific for the 5′ and 3′ ends of the insert (AAAGGTACCATGGCTGCCGCTGGGGGC and CACACTAGTTCAGGATGGCTTG; underlined bases are *Kpn* I and *Spe* I sites). The insert was digested with *Kpn* I and *Spe* I and ligated to pCMV4 DNA that had been digested with *Kpn* I and *Xba* I. The resulting construct was sequenced to verify the absence of PCR-induced mutations.

An expression plasmid containing human adrenodoxin reductase cDNA, pCMV4-AR, was produced by ligating the insert of λhtAR-1 (23) (this clone and hAdx-7 were gifts of W. L. Miller) into the *Bgl* II site of pCMV4 after rendering both DNA fragments blunt ended with the Klenow fragment of DNA polymerase I.

Transient Transfection Assays. Plasmid DNA was prepared by the rapid boiling method, treated with StrataClean resin (Stratagene), and ethanol-precipitated. DNA was transfected into COS-1 cells using cationic liposomes (24) (TransfectAce; GIBCO/BRL). The following five constructs were used: pC11-B1, containing the normal sequence for *CYP11B1* (identical to pCMV4-B1 in ref. 3), pCMO-B2, containing the normal sequence for *CYP11B2* (identical to pCMV4-B2 in ref. 3); pCMO-R181W, containing a mutation in exon 3; pCMO-V386A, containing a mutation in exon 7; pCMO-X3X7, containing the mutations in both exon 3 and exon 7. Expression plasmids containing cDNA coding for human adrenodoxin and adrenodoxin reductase, pCMV4-Adx and pCMV4-AR, respectively, were also transfected into the cells because previous studies (25) suggested that COS cells do not express sufficient levels of adrenodoxin to permit full activity of mitochondrial cytochrome P450 enzymes. Approximately 10^6 cells at 80% confluence in 35-mm culture wells were exposed for 24 h to 10 μg of TransfectAce, 1–2 μg of pCMV4-Adx, and 1–2 μg of pCMV4-AR, with no additional DNA, with 1–2 μg of pC11-B1, or with 1–2 μg of one of the pCMO constructs in 1 ml of Opti-MEM reduced serum medium (GIBCO/BRL). Cells were allowed to recover for 24 h in Dulbecco's modified Eagle's medium with 5% (vol/vol) newborn calf serum before addition of 1 nmol of [^{14}C]deoxycorticosterone. Medium was removed 24 h later, extracted with 3 ml of methylene chloride, dried under nitrogen gas, resuspended in 20 μl of methylene chloride, and loaded on TLC plates. Plates were developed in methylene chloride/methanol/H_2O, 300:20:1 (vol/vol). Specific products were identified by the use of appropriate labeled and unlabeled steroids as standards.

In parallel experiments, biosynthesis of corticosterone and aldosterone was quantitated by radioimmunoassay. Transfected cells were treated identically to those used for TLC assays, except that 1 nmol of nonradioactive deoxycorticosterone was used. After extraction with methylene chloride, steroids were resolved by Celite column chromatography and the concentrations of deoxycorticosterone, corticosterone, and aldosterone were determined by RIA as described (26, 27).

RNA Preparation and Analysis. RNA was prepared from transfected cells by the guanidinium isothiocyanate method (28). The RNA was prepared from the same transfected cells utilized for the enzyme assays. Total RNA was subjected to electrophoresis at 100 V for 2.75 h in 0.74 M formaldehyde/1.2% agarose gels, transferred to nylon membranes (Magnagraph; Micron Separation, Westborough, MA), fixed by drying at 60°C, and hybridized with a PCR product spanning exon 3 of *CYP11B2* that had been labeled with ^{32}P by random priming. Final stringent washes were carried out at 60°C in 0.03 M NaCl/0.003 M sodium citrate/0.1% SDS.

RESULTS

Identification of Mutations and Segregation Analysis. Initial sequence data were obtained from two patients with CMO-II deficiency and compared to published data (1, 7, 29) and to sequences of additional CYP11B1 and CYP11B2 cDNA clones isolated in our laboratory from individuals without CMO-II deficiency (unpublished observations). No sequence polymorphisms resulting in changes to the coding sequence were found in the *CYP11B1* genes of the CMO-II-deficiency patients. Two candidate missense mutations were found in *CYP11B2* genes that were not present in any other data sources (Fig. 1). The first of these, in exon 3 at codon 181, is CGG(Arg) → TGG(Trp) (R181W), the substitution of an amino acid with a large nonpolar group for a basic amino acid, whereas the second, in exon 7 at codon 386, is GTG(Val) → GCG(Ala) (V386A), the more conservative substitution of an amino acid with a nonpolar side chain for one with a similar nonpolar side chain containing two additional methyl groups.

To clarify the role of these mutations in the disease, we sequenced exons from all other family members to determine the segregation pattern of the mutations. The results in Fig. 2 show that homozygosity for both mutations is completely correlated with the presence of CMO-II deficiency; all affected individuals are doubly homozygous whereas there are no doubly homozygous unaffected individuals. The individual mutations are themselves less-well related to disease status. Eight apparently unaffected individuals are homozygous for the R181W mutation alone, and three such individuals are homozygous only for the V386A mutation. The frequency of the R181W polymorphic variant is evidently very high in these families, as only two "normal" homozygotes were found. Possibly the frequency of this allele is very high among the entire population of Iranian-Jewish descent, but we have not yet had the opportunity to study Iranian-Jewish families that do not carry CMO-II deficiency. In a sample of 40 unrelated individuals from an ethnically heterogenous New York population, none was found to carry

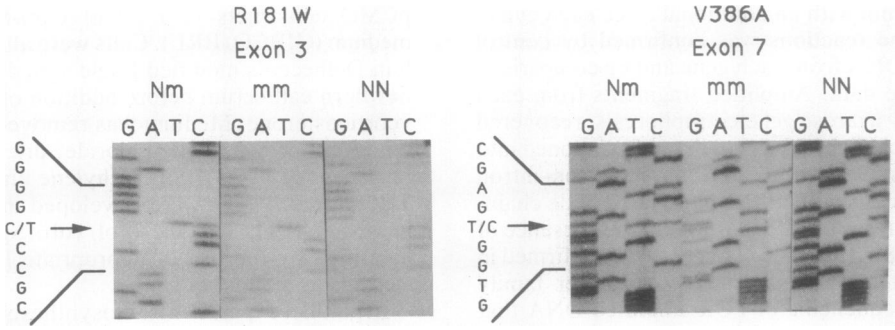

FIG. 1. Mutations detected in *CYP11B2* genes. Segments of *CYP11B2* genes were amplified from DNA from individuals with or without CMO-II deficiency, gel-purified, and sequenced directly. Photographs of sequencing gels for parts of exons 3 and 7 are shown with the corresponding sequence to the left. Each mutant nucleotide is marked with an arrow. For exons 3 and 7, a sample from a subject who is heterozygous for that mutation (Nm), homozygous for the mutation (mm), or homozygous for the normal sequence (NN) is shown.

R181W (unpublished observations). Population studies of this mutation may be facilitated by the fact that it alters a *Sma* I restriction site in the coding sequence.

Activity of Mutant Enzymes. Cells transfected with an expression plasmid (pCMO-B2) encoding P450cmo are able to synthesize corticosterone (25 pmol, detected by radioimmunoassay), 18-hydroxycorticosterone, and aldosterone (2.5 pmol), which are clearly visible on autoradiography or scanning of TLC plates (Fig. 3). Expression of the corresponding CYP11B1 construct (pC11-B1) leads to the synthesis of corticosterone (280 pmol), a minimal amount of 18-hydroxycorticosterone, and no aldosterone by radioimmunoassay. Similar results have previously been obtained (1, 3). The enzyme expressed from pCMO-V386A has a very similar profile of activities to that from pCMO-B2, synthesizing corticosterone (46 pmol), 18-hydroxycorticosterone, and aldosterone (4.6 pmol). In repeated transfections with these constructs a small but consistent reduction in the production of 18-hydroxycorticosterone was observed when the V386A mutation was present, as judged from the autoradiograms. In contrast, the enzyme expressed from pCMO-R181W resembles the activity profile observed in transfections with pCMV-B1, making corticosterone (178 pmol), a reduced amount of 18-hydroxycorticosterone, and no detectable aldosterone. Transfections with the cDNA construct contain-

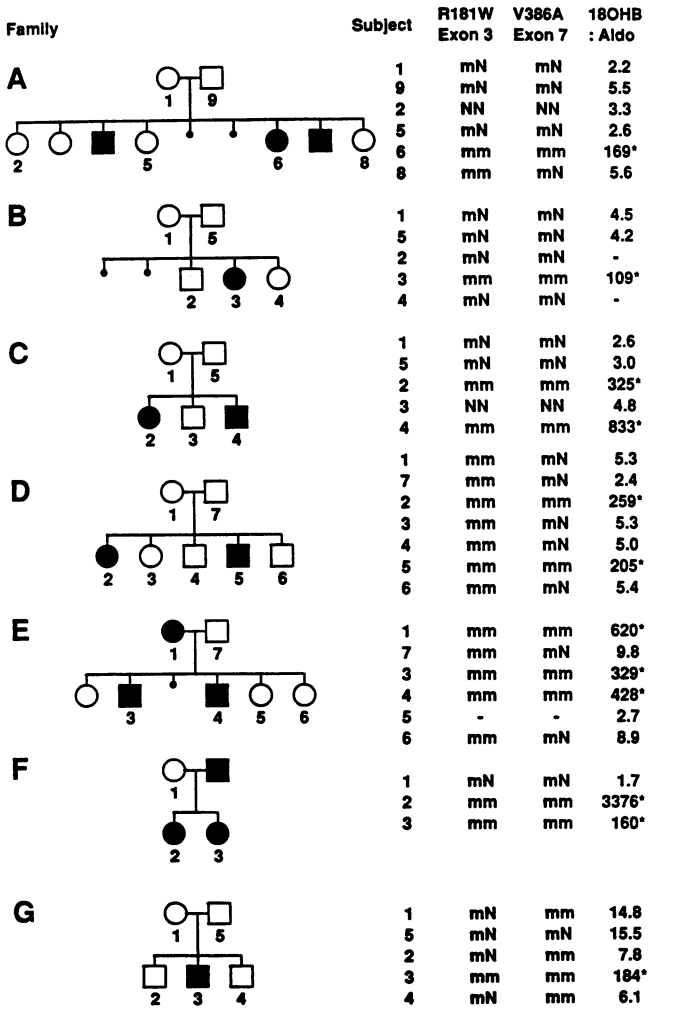

FIG. 2. Segregation of *CYP11B2* mutations in families carrying CMO-II deficiency. Pedigrees of the seven kindreds studied (*A–G*) are shown with affected individuals represented by solid symbols. All subjects studied are numbered within each nuclear family as in ref. 12 where appropriate. Results of sequencing are shown at right with Nm, mm, and NN as defined in Fig. 1. 18OHB:Aldo, ratio of 18-hydroxycorticosterone to aldosterone.

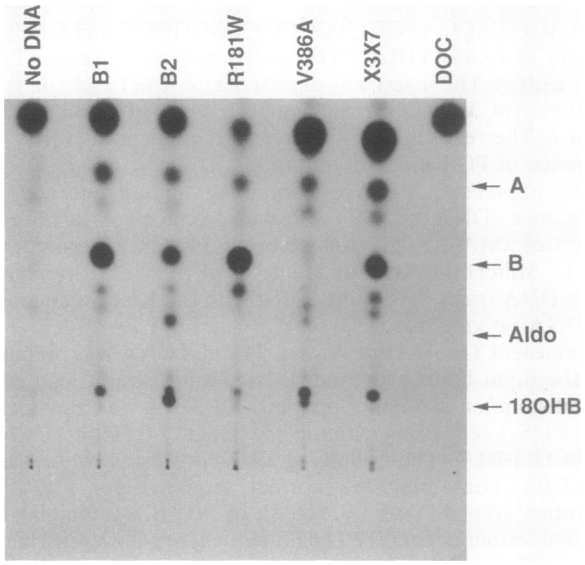

FIG. 3. Enzymatic activity of cells transfected with normal and mutant cDNA constructs. COS-1 cells were transfected with 2 μg of pCMV4-Adx, 2 μg of pCMV4-AR and 2 μg of pC11-B1 (*CYP11B1*), 2 μg of pCMO-B2 (*CYP11B2*), 2 μg of pCMO-R181W (R181W), 2 μg of pCMO-V386A (V386A), 2 μg of pCMO-X3X7 (X3X7), or no additional DNA (No DNA). The transfected cells were incubated with 11-[^{14}C]deoxycorticosterone and the resulting metabolites were identified by TLC and autoradiography. Positions of steroids are marked on the autoradiogram as follows: DOC, 11-deoxycorticosterone; A, 11-dehydrocorticosterone [which is produced from corticosterone by 11β-hydroxysteroid dehydrogenase activity intrinsic to COS-1 cells (3)]; B, corticosterone; 18OHB, 18-hydroxycorticosterone; Aldo, aldosterone.

ing both mutations (pCMO-X3X7) yielded results similar to those performed with pCMO-R181W, with 98 pmol of corticosterone and no aldosterone synthesized. Similar results were obtained in a second identical experiment and in a parallel experiment in which an expression plasmid encoding bovine adrenodoxin (25) (a gift from Michael Waterman) was used instead of the human adrenodoxin and adrenodoxin reductase constructs (data not shown).

Transfection of all five constructs was confirmed by the detection of transcripts on Northern blots (Fig. 4) and by the expected 49-kDa protein observed on immunoprecipitation of lysed transfected cells (data not shown). The RNA level produced by transfection of pC11-B1 was higher than those from the pCMO constructs, but the reason for this was not investigated in detail. There were no differences in RNA levels between the various pCMO constructs.

DISCUSSION

Genetic Origins of the R181W and V386A Mutations. The most common genetic disorder of aldosterone biosynthesis is steroid 21-hydroxylase deficiency (failure to convert progesterone to deoxycorticosterone) (30). This disorder, which also affects cortisol biosynthesis, is due to mutations in the *CYP21* gene encoding the enzyme P450c21 (P450XXI). *CYP21* and *CYP21P*, a 98% identical pseudogene, are closely linked on chromosome 6p21.3 in the major histocompatibility complex. All reported mutations causing a 21-hydroxylase deficiency are apparently the result of recombinations between *CYP21* and *CYP21P*. These are either deletions of *CYP21* due to unequal meiotic crossing-over or apparent gene conversions in which deleterious mutations normally present in *CYP21P* are transferred to *CYP21*.

Like *CYP21* and *CYP21P*, *CYP11B1* and *CYP11B2* are closely linked homologs, but *CYP11B1* and *CYP11B2* both encode active enzymes. Thus, gene conversions that transfer polymorphic sequences between *CYP11B1* and *CYP11B2* might not be expected to have major adverse effects on enzymatic activity, in which case genetic deficiencies of the encoded enzymes (P450c11 and P450cmo) should be the result of mutations that are not gene conversions. Indeed, *CYP11B1* mutations, characterized thus far, that cause 11β-hydroxylase deficiency (failure to convert 11-deoxycortisol to cortisol) are *de novo* point mutations and not deletions or gene conversions (ref. 17 and unpublished observations), and the R181W mutation in *CYP11B2* that was identified in the present study is also a simple point mutation [R181W is a mutation of CpG to TpG, the most common type of point mutation in higher eukaryotes (31)]. In contrast, V386A is normally present in *CYP11B1* and thus its presence in the mutant *CYP11B2* genes of CMO-II deficiency patients may be the result of an ancestral gene conversion, although an independent mutation is also possible. As predicted, V386A itself has a minimal effect on enzymatic activity.

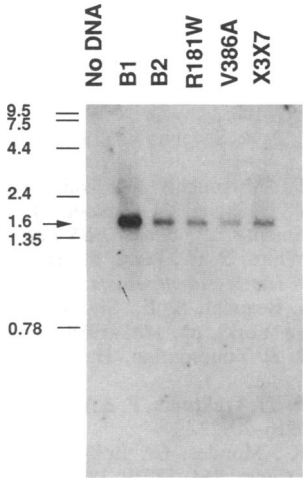

FIG. 4. Levels of CYP11B1 or CYP11B2 mRNA in cells transfected with normal and mutant cDNA constructs. Lane headings are as in Fig. 3 and each of the experimental lanes was loaded with ≈4 μg of total RNA. Transcripts from the transfected constructs were detected by probing with a PCR product spanning exon 3 and are estimated to represent 0.3% of the cellular mRNA. High molecular weight RNA size standards (GIBCO/BRL) were visualized by staining with ethidium bromide.

It is surprising that all four possible phases of the alleles for codons 181 and 386 are present in the study sample (as seen in Fig. 2, subjects may carry both, either, or neither of the two mutations). The most parsimonious explanation for this observation is that there has been a crossover between exons 3 and 7 in a previous generation. As the presence of V386A is in complete linkage disequilibrium with a *CYP11B1* polymorphism (12), we would predict that *CYP11B1* is adjacent to the 3′ end of *CYP11B2*. This hypothesis could be confirmed by isolating genomic clones linking *CYP11B1* and *CYP11B2*.

Roles of the R181W and V386A Mutations in the Pathogenesis of CMO-II Deficiency. When expressed *in vitro*, the enzyme carrying R181W does not synthesize detectable amounts of aldosterone, suggesting that its ability to produce this compound is at most a few percent of normal. Nevertheless, simple homozygosity for R181W is insufficient to cause CMO-II deficiency. This suggests that this mutant enzyme in fact possesses a small amount of residual activity that is sufficient to synthesize normal amounts of aldosterone. Possibly the mutant enzyme is expressed *in vivo* at markedly elevated levels to compensate for its poor activity. A similar phenomenon occurs in congenital adrenal hyperplasia due to 21-hydroxylase deficiency, in which homozygosity for mutations that abolish enzymatic activity (such as gene deletions) lead to inability to synthesize aldosterone whereas mutations that reduce enzymatic activity to about 1% of normal permit adequate synthesis of aldosterone *in vivo* even though cortisol synthesis is severely compromised (32).

Alternatively, it is possible that the enzyme carrying R181W has more 18-oxidase activity in zona glomerulosa cells than is displayed in COS cells; variations in the 18-oxidase activity of bovine and porcine P450c11 in different cell types have been reported (6).

In the patients studied, CMO-II deficiency requires the homozygous presence of both the R181W and V386A mutations, suggesting that the enzyme carrying both mutations is less active than the enzyme carrying R181W alone. In the *in vitro* assays, the only difference in enzymatic activity between P450cmo(R181W) and the double mutant was a small difference in efficiency of production of 18-hydroxycorticosterone. However, it remains possible that V386A further decreases the already undetectable 18-oxidase activity of P450cmo(R181W) even though it has little effect on enzymatic activity when present by itself; a high-level expression system such as vaccinia virus will be necessary to determine whether this is the case. Alternatively, there may be an additional undetected mutation in an intron or the 3′ flanking region that affects transcription, mRNA processing, or stability and is in complete genetic linkage disequilibrium with the allele carrying both R181W and V386A.

Although the ability of the R181W and doubly mutant enzymes to synthesize 18-hydroxycorticosterone *in vitro* is impaired, levels of this compound are elevated in patients with CMO-II deficiency. This may reflect the blocked 18-oxidase activity of the doubly mutant enzyme. Additionally, *CYP11B1*, which is normally expressed in the adrenal cortex at much (perhaps 100 times) higher levels than *CYP11B2*, is capable of producing 18-hydroxycorticosterone. Levels of

CYP11B1 transcripts in zona glomerulosa cells are increased by angiotensin II, a primary stimulus of aldosterone biosynthesis (3). Poor synthesis of aldosterone in patients with CMO-II deficiency should stimulate production of angiotensin II, augmenting expression of *CYP11B1* and thus increasing levels of corticosterone and 18-hydroxycorticosterone despite the relatively poor ability of P450c11 to synthesize the latter steroid.

It is necessary to explain the fact that patients with CMO-II deficiency who reach adulthood become asymptomatic and are able to synthesize adequate amounts of aldosterone at the expense of elevated levels of aldosterone precursors. Perhaps P450cmo carrying both mutations is still capable of producing trace amounts of aldosterone, but it is also possible that P450c11, which is normally expressed at much higher levels than P450cmo, has in addition to its low 18-hydroxylase activity a trace amount of 18-oxidase activity, which can synthesize adequate amounts of aldosterone if the enzyme is expressed at increased levels and presented with elevated levels of corticosterone and 18-hydroxycorticosterone. A very low (<1% that of wild-type P450cmo) level of 18-oxidase activity in P450c11 has not been ruled out (1–3), to our knowledge. Alternatively, a completely unrelated enzyme might be involved. The conversion of 18-hydroxycorticosterone to aldosterone need not even be mediated by an oxidase (i.e., it need not involve molecular oxygen, as would be the case for any reaction mediated by a cytochrome P450) but might instead be a dehydrogenation (a direct transfer of a hydride to an acceptor such as $NADP^+$). The conversion of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase is an example of the latter class of reaction (33).

The existence of ostensibly asymptomatic individuals with significantly compromised function of P450cmo suggests that the apparent rarity of CMO-II deficiency may reflect problems of ascertainment. Mild defects of 21-hydroxylase activity, similarly, are approximately 100 times as frequent as severe defects (30). It is possible that a mild CMO-II deficiency is in fact relatively common. Although asymptomatic as adults, affected children might be unusually susceptible to the stress of sodium deprivation (from diarrheal illness, for example) and might require increased dietary sodium to grow normally.

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