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MOLECULAR BIOLOGY OF 11β-HYDROXYLASE AND 11β-HYDROXYSTEROID DEHYDROGENASE ENZYMES

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Summary—There are two steroid 11β-hydroxylase isozymes encoded by the *CYP11B1* and *CYP11B2* genes on human chromosome 8q. The first is expressed at high levels in the normal adrenal gland, has 11β-hydroxylase activity and is regulated by ACTH. Mutations in the corresponding gene cause congenital adrenal hyperplasia due to 11β-hydroxylase deficiency; thus, this isozyme is required for cortisol biosynthesis. The second isozyme is expressed at low levels in the normal adrenal gland but at higher levels in aldosterone-secreting tumors, and has 11β-hydroxylase, 18-hydroxylase and 18-oxidase activities. The corresponding gene is regulated by angiotensin II, and mutations in this gene are found in persons who are unable to synthesize aldosterone due to corticosterone methyloxidase II deficiency. Thus, this isozyme is required for aldosterone biosynthesis.

Cortisol and aldosterone are both effective ligands of the "mineralocorticoid" receptor *in vitro*, but only aldosterone is a potent mineralocorticoid *in vivo*. This apparent specificity occurs because 11β-hydroxysteroid dehydrogenase in the kidney converts cortisol to cortisone, which is not a ligand for the receptor. This enzyme is a "short-chain" dehydrogenase which is encoded by a single gene on human chromosome 1. It is possible that mutations in this gene cause a form of childhood hypertension called apparent mineralocorticoid excess, in which the mineralocorticoid receptor is not protected from high concentrations of cortisol.

OUTLINE

1. 11β-Hydroxylase Isozymes
   1.1. Zonal distribution of 11β-hydroxylase activity
   1.2. Steroid 11β-hydroxylase deficiency
   1.3. Corticosterone methyloxidase II deficiency
   1.4. Dexamethasone- (or glucocorticoid) suppressible hyperaldosteronism (DSH)
   1.5. Types of mutations observed in the *CYP11B* genes
2. 11β-Hydroxysteroid Dehydrogenase

Glucocorticoids and aldosterone, the primary mineralocorticoid, are hydroxylated at the 11β position. Thus, regulation of 11β-hydroxylation may be a key mechanism by which the body controls levels of active glucocorticoids and mineralocorticoids. Such regulation may occur by control of synthesis of 11β-hydroxylated steroids and, under some circumstances, by control of their removal from the pool of active hormones by dehydrogenation to 11-oxo steroids. This article reviews the molecular biology of the enzymes that mediate these reactions.

1. 11β-HYDROXYLASE ISOZYMES

1.1. Zonal distribution of 11β-hydroxylase activity

Cortisol is synthesized from cholesterol in the zona fasciculata under the control of ACTH [1]. Synthesis of cortisol requires five enzymatic steps (Fig. 1): cleavage of the cholesterol side-chain to yield pregnenolone, 3β-dehydrogenation to progesterone, and successive hydroxylations at the 17α, 21 and 11β positions by 3 distinct cytochrome *P*450 enzymes. A "17-deoxy" pathway is also active in the zona fasciculata, in which 17α-hydroxylation does not occur and the final product is normally corticosterone (in fact, rodents do not express 17α-hydroxylase in the adrenal gland, and corticosterone is their predominant glucocorticoid).

The same 17-deoxy pathway is active in the zona glomerulosa, which contains no 17α-hydroxylase activity. However, corticosterone is

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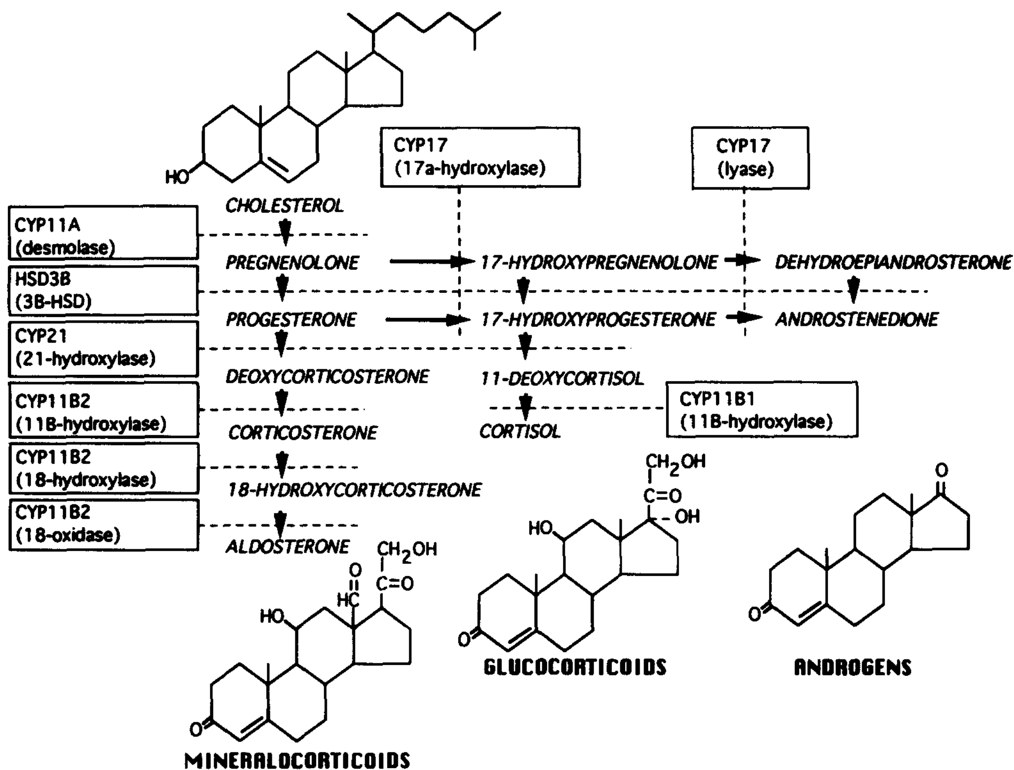

Fig. 1. Adrenal steroidogenesis. Gene products mediating each biosynthetic step are boxed with the corresponding enzymatic activity listed in parentheses; note that one enzyme may have more than one activity. The planar structures of cholesterol, aldosterone, cortisol and androstenedione are shown.

not the final product in the zona glomerulosa; instead, corticosterone is successively hydroxylated and oxidized at the 18 position to yield aldosterone (the 18-hydroxylase and 18-oxidase activities are also respectively termed corticosterone methyloxidase I and II). Because the zona fasciculata is much larger than and is regulated differently from the zona glomerulosa, it is apparent that 18-oxidase activity must remain absent from the zona fasciculata or aldosterone synthesis would be inappropriately regulated. In principle, different mechanisms by which 18-oxidase activity might be suppressed are possible depending on the identity of the 18-oxidase enzyme.

Biochemical studies of animal adrenal glands have yielded contradictory answers regarding the identity of the 18-oxidase enzyme. Mitochondria isolated from bovine or porcine zona fasciculata cannot synthesize aldosterone, whereas mitochondria from the zona glomerulosa can, consistent with observations in intact animals. However, the purified 11β-hydroxylase enzyme (CYP11B1, *P*450XIB1, *P*450c11) is biochemically identical in the zonae fasciculata and glomerulosa and has both 18-hydroxylase and 18-oxidase activities [2, 3]. Biochemical [4] and genetic [5] studies suggest the existence of two closely related 11β-hydroxylase isozymes in cattle with similar activities and identical distributions in the zonae fasciculata and glomerulosa. The significance of these isozymes is unclear, and it remains possible that they are allelic variants. In any case, some factor must biochemically suppress 18-hydroxylase and 18-oxidase activities of bovine or porcine CYP11B1 in the zona fasciculata without significantly affecting 11β-hydroxylase activity.

In contrast, CYP11B1 purified from rat zona fasciculata does not have 18-oxidase activity. If rats are deprived of dietary sodium and fed excess potassium (stresses that increase aldosterone secretion), an additional isozyme with 18-oxidase activity can be purified from the zona glomerulosa. This isozyme has a distinct amino-terminal sequence and is undetectable in rats fed a normal diet [6, 7]. Cloned cDNA corresponding to this enzyme has recently been characterized [8, 9]. Thus, appropriate zonation of aldosterone biosynthesis in the rat involves a distinct enzyme that is apparently regulated on the transcriptional level [10]. Genes encoded two distinct enzymes have also been characterized in the mouse [11].

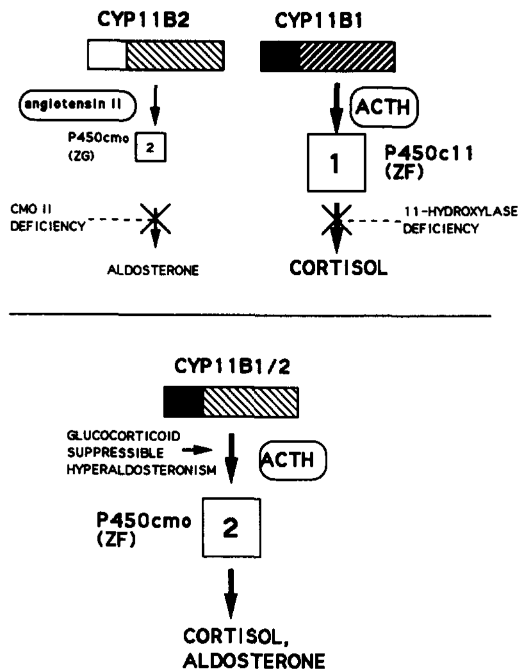

Fig. 2. Functions of the *CYP11B1* and *CYP11B2* genes. The genes are shown schematically with the promoter of each gene being in a contrasting pattern. *CYP11B2* is normally transcribed at low levels and is required for aldosterone synthesis, whereas *CYP11B1* is normally transcribed at high levels and is required for cortisol synthesis. In glucocorticoid-suppressible hyperaldosteronism, an intergenic recombination juxtaposes the promoter of *CYP11B1* with coding sequences of *CYP11B2*, leading to abnormal regulation of aldosterone biosynthesis.

Recent work has demonstrated that humans also have distinct isozymes that are responsible for cortisol and aldosterone biosynthesis (also see a related review in this volume [12]). Humans carry two genes [13] on chromosome 8q21-q22 [14, 15] that encode 11β-hydroxylase isozymes with predicted amino acid sequences that are 93% identical. One gene, *CYP11B1*, is expressed at high levels in normal adrenal glands [13], and transcription of this gene is appropriately regulated by cAMP (the second messenger for ACTH) [16]. Transcripts of the other gene, *CYP11B2*, cannot be detected by hybridization to Northern blots of normal adrenal RNA [13], but such transcripts have been detected by hybridization to RNA from an aldosterone-secreting tumor [17].

Low levels of *CYP11B2* transcripts have been detected in normal adrenal RNA using a more sensitive assay wherein RNA was reverse-transcribed and then amplified using the polymerase chain reaction (RT-PCR) [18]. When an RNA sample of an aldosterone-secreting adrenal tumor was examined in this manner, it contained a concentration of *CYP11B1* transcripts slightly lower than that of the normal adrenal, but *CYP11B2* transcripts were increased 5-fold over the normal gland.

To determine if levels of *CYP11B2* transcripts were appropriately regulated, the zona glomerulosa was dissected out of human adrenal surgical specimens and cultured in the presence of angiotensin II or corticotropin (ACTH) before preparing RNA. Angiotensin II markedly increased levels of both *CYP11B1* and *CYP11B2* transcripts. ACTH increased *CYP11B1* mRNA levels more effectively than angiotensin II, but it had no effect on *CYP11B2* transcription [18].

The enzymes encoded by *CYP11B1* and *CYP11B2* have been studied by expressing the corresponding cDNAs in cultured cells [17, 18] and after actual purification from aldosterone secreting tumors [19]. CYP11B2 (also termed *P*450XIB2, *P*450cmo, *P*450c18 or *P*450aldo), 11β-hydroxylates 11-deoxycorticosterone to corticosterone and 11-deoxycortisol to cortisol. It 18-hydroxylates corticosterone and cortisol, and further oxidizes 18-hydroxycorticosterone to aldosterone. In contrast, human CYP11B1 has a strong 11β-hydroxylase activity but 18-hydroxylates only about one-tenth as well as CYP11B2. CYP11B1 does not synthesize detectable amounts of aldosterone from 18-hydroxycorticosterone.

These data suggest that CYP11B1 synthesizes cortisol in the zona fasciculata whereas CYP11B2 synthesizes aldosterone in the zona glomerulosa. This hypothesis has been confirmed by studying individuals with defective cortisol or aldosterone synthesis due to respective deficiencies in 11β-hydroxylase and corticosterone methyloxidase II (CMO II) activities (Fig. 2).

1.2. Steroid 11β-hydroxylase deficiency

The inherited inability to synthesize cortisol is termed congenital adrenal hyperplasia. Whereas the most common cause of this disorder is 21-hydroxylase deficiency (failure to convert 17-hydroxyprogesterone to 11-deoxycortisol, accounting for more than 90% of cases), classic 11β-hydroxylase deficiency comprises 5–8% of cases, occuring in about 1/100,000 births in the general Caucasian population [20]. A large number of cases have been reported in Israel among Jewish immigrants from Morocco, a relatively inbred population. The incidence in this group is currently estimated to be 1/5000–1/7000 births, with a gene frequency of 1.2–1.4% [21].

Patients with this disorder are unable to convert 11-deoxycortisol to cortisol. Elevated levels of ACTH cause steroid precursors to accumulate proximal to the blocked step. Many of these precursors are shunted into the pathway for androgen biosynthesis as occurs in 21-hydroxylase deficiency. Thus, female patients with this disorder are born with masculinized external genitalia and affected individuals of both sexes undergo rapid somatic growth with premature epiphyseal closure, resulting in short adult stature.

A parallel defect usually exists in the synthesis of 17-deoxy steroids, so that deoxycorticosterone is not converted to corticosterone and instead accumulates. Because deoxycorticosterone and some of its metabolites have mineralocorticoid activity, elevated levels may cause hypertension and hypokalemia. About two thirds of untreated patients become hypertensive, sometimes early in life [22]. This clinical feature distinguishes 11β-hydroxylase deficiency from 21-hydroxylase deficiency, in which poor aldosterone synthesis causes renal salt wasting in the majority of patients.

In the only published analysis of a mutation causing this disorder [23], six families carrying an allele for 11β-hydroxylase deficiency were studied; all were Jews originating from Morocco. Eleven of 12 mutant alleles carried the same mutation, a single base change in exon 8 of *CYP11B1*. Codon 448, CGC, encoding arginine, was changed to CAC, histidine (R448H).

The sulfhydryl of Cys-450 in CYP11B1 is presumed to constitute the fifth ligand to the iron atom of the heme prosthetic group. This residue is completely conserved in all cytochrome *P*450 enzymes and the surrounding "heme-binding peptide" is also highly conserved. In particular, Arg-448 is conserved in all eukaryotic *P*450 enzymes examined thus far (see references in [24]) suggesting that substitutions at this position are poorly tolerated. Thus, it is reasonable to speculate that the R448H mutation interferes with binding or functioning of the heme functional group. In unpublished studies, we found that this mutation indeed abolished normal enzymatic activity.

Although patients in 5/6 families in this study were presumably genotypically identical, there were significant differences in signs and symptoms of androgen and mineralocorticoid excess, even within families. For example, all affected females were born virilized, but only 5/7 males had an abnormally large penis in infancy. Only 8/11 patients were hypertensive when untreated. Thus, as is the case with 21-hydroxylase deficiency, other epigenetic or nongenetic factors probably influence the clinical phenotype of the disorder.

1.3. Corticosterone methyloxidase II deficiency

CMO II deficiency is an inherited defect of aldosterone biosynthesis [25]. 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 [26].

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* [27].

In Iranian Jews with CMO II deficiency, no mutations were found in *CYP11B1*, whereas two missense mutations were identified in *CYP11B2* [28]. The first of these, in exon 3, codon 181, is CGG (arginine)→TGG (tryptophan) (R181W), a substitution of an amino acid with a large non-polar group for a basic amino acid, whereas the second in exon 7, codon 386, is GTG (valine)→GCG (alanine) (V386A), a more conservative substitution of one amino acid with a non-polar side chain for another. All individuals affected with CMO II deficiency were homozygous for both of these mutations, whereas no unaffected individuals carried both mutations; individuals homozygous for either one of the mutations alone were asymptomatic.

When normal and mutant CYP11B2 were expressed in cultured cells, the R181W mutant had normal 11β-hydroxylase activity, decreased 18-hydroxylase activity and undetectable 18-oxidase activity. The V386A mutant had slightly decreased activity as compared with the normal enzyme. No differences could be demonstrated between the enzymes carrying R181W alone and in combination with V386A, but the studies on patients suggest that the double mutant enzyme

must have even more severely compromised 18-oxidase activity than the enzyme carrying R181W alone.

It is likely that the results presented here for both 11β-hydroxylase and CMO II deficiencies reflect founder effects. There was relatively little intermarriage in many relatively small Jewish communities prior to emigration to Israel, and so genetic heterogeneity at certain loci may be limited.

1.4. Dexamethasone- (or glucocorticoid) suppressible hyperaldosteronism (DSH)

This form of hypertension is inherited as an autosomal dominant disorder [29]. It is distinguished from primary aldosteronism by suppression of moderate aldosterone hypersecretion within 48 h of dexamethasone administration [30]. Accompanying effects of dexamethasone administration in DSH include elevation of suppressed plasma renin activity to the normal range and restoration of the normal aldosterone responsiveness to changes in posture and dietary sodium manipulation. In contrast, infusion of ACTH in a glucocorticoid treated patient causes recrudescence of the hypertension. A pathognomic biochemical abnormality in this disorder is a high urinary concentration of a 17α-hydroxylated analog of aldosterone, 18-oxocortisol [31]. The inappropriate regulation of aldosterone synthesis by ACTH and the resence of a 17α-hydroxylation analog of aldosterone (which cannot be produced in the zona glomerulosa due to the lack of 17α-hydroxylase activity in that zone) suggest that aldosterone is being synthesized in the zona fasciculata or a functional equivalent. This implies that *CYP11B2* must be inappropriately regulated in a manner similar to the normal regulation of *CYP11B1*. This might occur if an intergenic recombination juxtaposed the promoter of *CYP11B1* with coding sequences of *CYP11B2*, in which case the hybrid gene would be expressed in the zona fasciculata and transcription would be stimulated mainly by corticotropin. A single copy of such an abnormally regulated gene should be sufficient to cause the disease, consistent with the known mode of inheritance as an autosomal dominant disorder.

Indeed, we [31a] and others [32] have recently found unequal crossovers in such patients that cause exactly the predicted rearrangement, although the enzymatic activity of the encoded hybrid protein (the amino terminal region is that of CYP11B1) has not yet been determined.

1.5. Types of mutations observed in the CYP11B genes

Steroid 21-hydroxylase deficiency, the most common cause of congenital adrenal hyperplasia, is due to mutations in the *CYP21* gene encoding the enzyme *CYP21* (also termed *P*450c21 or *P*450XXI). *CYP21* and a 98% identical pseudogene, *CYP21P*, are closely linked on chromosome 6p21.3 in the major histocompatibility complex. All reported mutations causing 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 (approx. 20% of alleles) or apparent gene conversions in which deleterious mutations normally present in *CYP21P* are transferred to *CYP21* (reviewed in [33]).

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 should be the result of mutations that are not gene conversions. Indeed, we have characterized three *CYP11B1* mutations causing 11β-hydroxylase deficiency in addition to R448H, and all are *de novo* point mutations or small insertions and not large deletions or gene conversions (unpublished observations). Similarly the R181W mutation in *CYP11B2* associated with CMO II deficiency is also a simple point mutation (like R448H, it is a mutation of CpG to TpG, the most common type of point mutation in higher eukaryotes). 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.

The relative positions of the human *CYP11B1* and *CYP11B2* genes has not been determined with certainty. *CYP11B2* is on the left (if the genes are pictured as being transcribed left to right) in the mouse [11], and studies of crossovers in families with CMO II deficiency and dexamethasone-suppressible

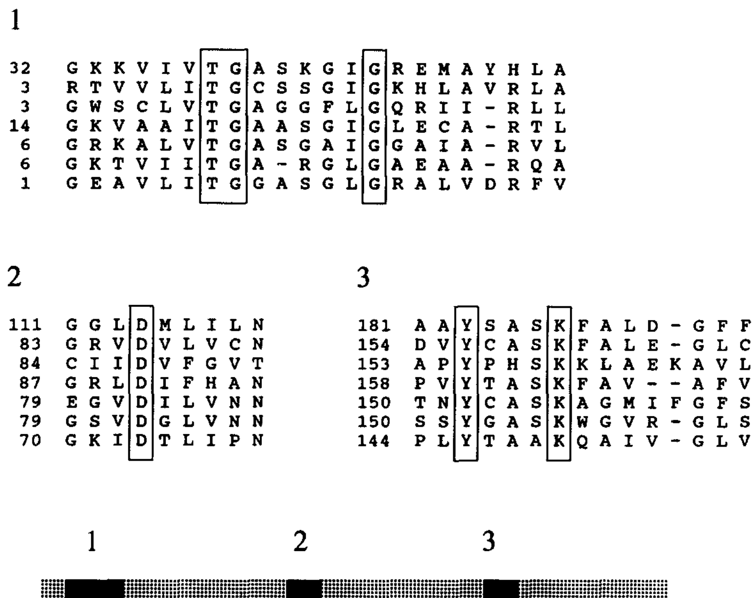

Fig. 3. Conserved regions in 11β-hydroxysteroid dehydrogenase and related enzymes (condensed from Ref. [42]). Sequences displayed for each of three regions are from (in descending order): human 11-HSD, human 17β-hydroxysteroid dehydrogenase, human placental 3β-hydroxysteroid dehydrogenase, ribitol dehydrogenase from *Klebsiella aerogenes*, *nodG* protein from *R. meliloti*, 3α, 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* and dihydrodiol dehydrogenase from *Pseudomonas sp.* Amino acid residues are listed in single letter codes and absolutely conserved residues are boxed. The relative positions of these regions are indicated by the dark boxes within the shaded box, with the amino terminus to the left (human 11-HSD is 292 residues long).

hyperaldosteronism suggest that the same arrangement exists in humans. As mentioned, unequal crossovers resulting in a *CYP11B1*/*CYP11B2* duplication are associated with the latter disorder. The reciprocal deleted chromosome should carry a single *CYP11B* gene with a 5′ end corresponding to *CYP11B2* and a 3′ end corresponding to *CYP11B1*. Like *CYP11B2*, such a gene should be expressed at low levels, and probably only in the zona glomerulosa, but it should have an enzymatic activity similar to the normal product of *CYP11B1*. Such a chromosome might be expected to function as a "wild card" allele that, depending on the allele in *trans*, would be expressed as either 11β-hydroxylase deficiency due to its low level of expression, or CMO II deficiency due to its lack of 18-oxidase activity. As yet, such chromosomes have not been detected.

2. 11β-HYDROXYSTEROID DEHYDROGENASE

In humans, 11β-hydroxysteroid dehydrogenase (11-HSD) catalyzes the interconversion of cortisol and its inactive metabolite cortisone in the presence of the cofactor NADP+/NADPH. Because the Type I (mineralocorticoid) receptor has identical affinities for cortisol and aldosterone *in vitro* [34], it appears that the dehydrogenase activity of 11-HSD is necessary to protect the renal Type I receptor from normally higher serum concentrations of cortisol, allowing aldosterone to regulate sodium homeostasis [35, 36]. This is an unusual mechanism for achieving receptor specificity (reviewed in [37]).

In the absence of 11-HSD activity, cortisol, which is present physiologically at 100–1000 times the concentration of aldosterone, has free access to the Type I receptor, and is able to act as a mineralocorticoid. This may occur as an inborn metabolic defect termed apparent mineralocorticoid excess [38], or due to pharmacologic inhibition of the enzyme, as occurs in licorice intoxication [39]. Individuals with these conditions develop severe hypertension and hypokalemic metabolic alkalosis. Apparent mineralocorticoid excess has been shown to occur in siblings whose parents metabolize cortisol normally, consistent with inheritance as an autosomal recessive disorder [40].

Rat cDNA [41] and human cDNA and genomic [42] clones encoding the enzyme have been isolated. The rat enzyme contains 287 amino acid residues and has a M_w of 34 kD. Expression of rat cDNA clones has demonstrated that the enzyme in fact catalyzes both

dehydrogenation (corticosterone to 11-dehydrocorticosterone) and the reverse reduction reaction with similar kinetic constants at pH 7 [43].

The human gene is present in a single copy on chromosome 1 and consists of six exons spaced over at least 9 kb.

A search of sequence databases [42] revealed that the predicted sequence of 11-HSD was related to several other prokaryotic and eukaryotic enzymes including human estradiol 17β-hydroxysteroid dehydrogenase [44, 45]. Examination of these alignments revealed a total of nine residues that were conserved in all proteins. These residues are likely to be structurally or functionally important. Although it could not be aligned with 11-HSD using computer algorithms, human 3β-hydroxysteroid dehydrogenase retains six of these nine residues in a similar arrangement [46] (Fig. 3). Three of these residues are in an area near the amino terminus which is similar to known nucleotide cofactor binding sites of other enzymes, including yeast alcohol dehydrogenase [47]. If the three absolutely conserved residues distal to the cofactor binding site (Asp-114, Tyr-183 and Lys-187) participate in the catalytic function of the enzyme, they should be near the pyridine ring of NADP+ and/or the 11α position of the steroid. X-ray crystallographic analysis of a related enzyme, 3α,20β-hydroxysteroid dehydrogenase of *S. hydrogenans* [48] has confirmed that the conserved region near the amino terminus does form part of the nucleotide cofactor binding site. The conserved tyrosine residue (Tyr-152 in 3α,20β-HSD) is indeed located near the pyridine ring of the cofactor in a cleft that is presumed to be the steroid binding site. The conserved lysine is directly behind the tyrosine (i.e. on the opposite side of the tyrosine ring from the cofactor). There is demonstrable bridging of electrons between the phenolic hydroxyl of tyrosine and the ε-amino group of lysine, suggesting an interaction between these groups. These findings support the idea that the conserved tyrosine and lysine participate in the catalytic function of the enzyme by facilitating the transfer of a hydride radical from the steroid to the cofactor. In contrast, the conserved aspartate (Asp-82 in 3α,20β-HSD) is not located near the cofactor or the steroid, and its functional significance is difficult to assess from these studies.

Preliminary studies in which residues in 11-HSD were modified by *in vitro* mutagenesis and expression of the cDNA in cultured cells demonstrated that changing the tyrosine and lysine residues abolished enzymatic activity [48a]. Mutating the analogous tyrosine residue in a related enzyme, 15-hydroxyprostaglandin dehydrogenase, to alanine also destroys activity [49].

By aligning the amino acid sequence of 11-HSD with those of related enzymes, it is apparent that 11-HSD has an amino terminal extension of approx. 30 residues which is not present in any related protein. The sequences encoding this extension are entirely contained on the first exon of the *HSD11* gene, and the first residue of the second exon is a methionine in a good context for initiation of translation. Thus, initiation of transcription in the first intron, producing an mRNA that does not contain the first exon, should yield a truncated protein that might be enzymatically active. We have used RT-PCR to determine whether any such alternative transcripts exist, and have found them at a relatively high level exclusively in the kidney (unpublished observations). Transcripts of different sizes have also been observed in Northern blots of kidney RNA [50]. The functional significance of these transcripts is not yet clear.

Because both dehydrogenase and reductase activities apparently reside in the same enzyme, it will be of obvious interest to search for mutations in the HSD11 gene associated with AME (in which 11-reductase activity is apparently spared) and correlate their effects on enzymatic function with clinical phenotype.

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