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MOLECULAR PATHOLOGY OF STEROID 21-HYDROXYLASE DEFICIENCY

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Summary—The molecular pathology of steroid 21-hydroxylase deficiency is attributable to unequal crossover-mediated gene deletion or to large- or small-scale replacement of the functional CYP21B gene sequence by a copy of the analogous CYP21A pseudogene sequence. Because the pathological point mutations originate from the pseudogene which shows only a small number of differences from the functional CYP21B gene sequence, the total number of different pathological point mutations is likely to be small. Mutant *P*450c21 enzymes carrying specific amino acid substitutions seen in patients with 21-hydroxylase deficiency exhibit activities that correlate with the clinical severity of the disease and with biochemical abnormalities such as 17-hydroxyprogesterone levels after ACTH (corticotropin) stimulation.

INTRODUCTION

Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency is a common inherited disorder of cortisol biosynthesis that results in abnormal sexual differentiation and somatic growth [1, 2]. The disease occurs in a wide spectrum of clinical variants, including a severely affected form with a concurrent defect in aldosterone biosynthesis (salt wasting type), a form with apparently normal aldosterone biosynthesis (simple virilizing type) and a mild nonclassical form that may be asymptomatic or may be associated with symptoms of androgen excess developing during childhood or at puberty.

The disorder is inherited as a monogenic autosomal recessive trait closely linked to the HLA major histocompatibility complex on chromosome 6p21.3. In addition to family-based genetic linkage to HLA markers, there are a number of population-based associations between the different forms of 21-hydroxylase deficiency and specific combinations of HLA antigens, or haplotypes. In particular, severe salt wasting disease is strongly associated with the HLA–DR7;Bw47;A3 haplotype, whereas nonclassical disease is associated with the HLA–DR1;B14 haplotype. Based on these associations and on studies of pedigrees containing patients with the different forms of the disease, it appears that these forms are inherited as allelic variants.

The 21-hydroxylase enzyme is a microsomal cytochrome *P*450 termed *P*450XXI or *P*450c21 which catalyses the conversion of 17-hydroxyprogesterone to 11-deoxycortisol in a pathway leading to cortisol synthesis and also the conversion of progesterone to deoxycorticosterone in a pathway leading to aldosterone synthesis. The structural gene encoding *P*450c21 (CYP21B) and a closely related pseudogene (CYP21A) are located in the HLA complex adjacent to, and alternating with, the duplicated C4B and C4A which encode the fourth component of serum complement [3,4]. CYP21B contains 10 exons spanning 3.3 kb. Although CYP21A shows a high degree of sequence relatedness to CYP21B (98% identical in exons and about 96% identical in introns) it is defective in expression as a consequence of the acquisition of a number of deleterious point mutations [5, 6]. The 4 genes span a region of about 60 kb of DNA with the linear order CYP21B-C4B-CYP21A-C4A. Such an organization suggests that sometime in our evolutionary past there was a tandem duplication of a segment of DNA approx. 30 kb long which included an ancestral 21-hydroxylase gene and a C4 gene.

As a consequence of the high degree of sequence relatedness between the neighbouring

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CYP21B-C4B and CYP21A-C4A units unequal pairing between homologous chromosomes (and between sister chromatids) is facilitated in this region [7, 8]. If the homologous chromosome 6s undergo unequal pairing at meiosis then the probability of producing gametes with altered gene organization in the CYP21/C4 gene cluster is enhanced. If such gametes contain a resultant chromosome 6 in which the altered gene organization results in a missing or defective CYP21B gene, they will contribute to 21-hydroxylase deficiency. Analysis of the molecular pathology of the disorder reveals that in all cases so far, 21-hydroxylase deficiency ensues as a result of either partial or complete deletion of the CYP21B gene sequence or partial or complete replacement of the CYP21B gene sequence by the analogous CYP21A pseudogene sequence (gene conversion).

21-HYDROXYLASE DEFICIENCY DUE TO GENE DELETION

Gene deletions which cause 21-hydroxylase deficiency arise as a result of unequal crossover. If two normal homologous chromosome 6s engage in unequal chromosome pairing at meiosis, and then recombination occurs within the area of mispairing, unequal crossover results in the gene organization on both chromosomes being altered [7, 8]. The ensuing crossover products will be a chromosome with 3 CYP21 genes and 3 C4 genes, and a chromosome with 1 CYP21 gene and 1 C4 gene. In the latter case there has effectively been a deletion of about 30 kb eliminating a CYP21-C4 unit. If this deletion results in elimination of the functional CYP21B gene sequence any gametes with the deletion chromosome will no longer have the genetic capacity to encode functional steroid 21-hydroxylase.

Initial attempts to analyse gross alteration in gene organization (e.g. gene deletion) in the CYP21/C4 gene cluster relied on conventional Southern blot hybridization. CYP21- or C4-specific DNA probes are hybridized against genomic DNA samples which have been digested with an appropriately discriminating restriction nuclease. For example, the enzyme Taq I can afford restriction fragment length variants which are usually diagnostic for the individual CYP21A and CYP21B genes or for the C4A and C4B genes. However, this short range restriction mapping method is often open to ambiguity in interpretation because of crossover events which result in hybrid CYP21A/CYP21B genes or which result in transposition of the flanking sequence containing the investigated variable restriction sites. As the deletions which cause 21-hydroxylase deficiency are about 30 kb long, they are not easily analysed by Southern blot hybridization approaches. These problems can be minimized but not eliminated by analysing several digests with enzymes (such as Taq I and Bgl II) that recognize variable restriction sites spaced at intervals in and near the CYP21 genes [9].

The advent of a long-range restriction mapping procedure based on the use of the rare-cutting retriction nuclease Bss HII and pulsed field gel electrophoresis has permitted unambiguous interpretation of gross gene organization in the CYP21/C4 gene cluster [10]. The sizes of CYP21-specific Bss HII fragments which are observed on disease chromosomes always show differences which are integral multiples of the *ca* 30 kb CYP21-C4 unit with the number of units varying from 1 up to 4. Accordingly, large-scale

Table 1. Biochemical and clinical consequences of mutations in the steroid 21-hydroxylase gene

Clinical phenotype	Mutation	% Classic alleles	17-OHP[a]	Symptoms	% Activity
Normal	—	—	200	0	100
Heterozygote	any	—	1000	0	50
Nonclassical	exon 7, Val-281 → Leu	—	5000	0/+	20–50
Simple virilizing	exon 4, Ile-172 → Asn,	6–14[b]	25,000	+ +	1
	intron 2, C → G	50[c]			
Salt wasting	deletion	10–35	25,000	+ + +	0
	intron 2, C → G	50[c]			
	exon 3, −8 bp	3			
	exon 6, codons 235–238	?			
	exon 8, Gln-318 → Ter	4–7			
	exon 8, Arg-356 → Trp	?			

[a]Typical serum values of 17-hydroxyprogesterone measured 60 min after an i.v. bolus of corticotropin, in ng/100 ml.
[b]This mutation is predominantly associated with the simple virilizing phenotype but has been observed in salt wasting patients who are deletion heterozygotes [23].
[c]This mutation occurs in patients with either salt wasting or simple virilizing forms of 21-hydroxylase deficiency.

length polymorphism in the CYP21/C4 gene cluster consistently conforms to a large-scale VNTR (variable number of tandem repeats) model where the basic repeat is the *ca* 30 kb CYP21-C4 unit. Such VNTR polymorphism is exactly what one would predict from the operation of unequal crossover, and direct evidence for the involvement of unequal crossover has recently been obtained following characterization of a *de novo* deletion which contributes to 21-hydroxylase deficiency [11].

Using a combination of short and long range restriction mapping, it has been possible to estimate that the molecular pathology of 21-hydroxylase deficiency is attributable to gene deletion in approx. 30–35% of disease haplotypes in the U.K., Finland and Ireland but in only 12% of Italian disease haplotypes [10, 12, 12a, 12b]. In addition, studies based only on short range mapping have suggested that delections of CYP21B sequences occur on 11–21% of disease haplotypes in the French population [13, 14] and 20% of disease haplotypes in Americans [9]. The high frequency of pathological gene deletion in populations which descend from the northern and western margins of Europe is correlated with a high frequency in these populations of HLA haplotypes which bear the HLA–Bw47 or Bw60 antigens. Of these, the most abundant are the DR6;Bw47;A3 and DR1;B60;A3 haplotypes, which are frequently, but not exclusively associated with CYP21B gene deletions.

Analysis of the residual CYP21 gene on deletion haplotypes associated with disease reveals that in most cases the gene is a hybrid due to unequal crossover occurring within mispaired CYP21A and CYP21B genes. While the 5′ exons of the hybrid gene normally resemble CYP21A, exons nearer to the 3′ end resemble CYP21B. Effectively, the deletion events which give rise to the single CYP21 gene on deletion disease haplotypes result in elimination of a 5′ part of the functional CYP21B gene and a 3′ part of the CYP21A pseudogene, with the crossover points often being located between exons 3–6 and between exons 6–8 [9, 13]. However, deletions which lead to complete elimination of the CYP21B gene sequence as a consequence of extragenic unequal crossover are also known to occur [11].

Deletion haplotypes which are not correlated with 21-hydroxylase deficiency may also be common, notably with HLA–DR3;B8;A1 haplotype in which there is a single C4B gene and a CYP21 gene which resembles CYP21B and is usually, but not always, functional. Such a gene organization is found on nearly 10% of chromosome 6s in the normal U.K. population and almost 1/4 of normal chromosome 6s in Ireland. When such chromosomes engage in meiotic pairing with a homologous chromosome having the conventional CYP21B-C4B-CYP21A-C4A organization, the frequency with which the single CYP21B-C4B unit mispairs with the CYP21A-C4A unit rather than the expected CYP21B-C4B unit may approach 50% of meiosis. Consequently, chromosomes with a single CYP21B-C4B unit may be considered as analogous to premutations in the sense that they facilitate unequal chromosome pairing and consequently enhance the possibility of unequal crossover or gene conversion-like events which can contribute to 21-hydroxylase deficiency [11].

21-HYDROXYLASE DEFICIENCY DUE TO GENE CONVERSION

As mentioned above, approx. 10–35% of disease haplotypes exhibit a CYP21-C4 unit deletion which leads to elimination of part or all of the CYP21B gene sequence. The remaining 65–90% of disease haplotypes show 2 or more CYP21-C4 units. In approx. 5–10% of the nondeletion disease haplotypes there appears to be no recognizable CYP21B gene. Instead, each of the CYP21 genes on such haplotypes appear to resemble CYP21A by exhibiting CYP21A-associated markers and lacking CYP21B-associated markers when investigated by Southern probing [10, 16], PCR-screening [10], oligonucleotide probing [17] and DNA sequencing [18]. In such situations the normal CYP21B gene appears to have been replaced over a large part, if not all, of the gene sequence, by an additional copy of the CYP21A pseudogene, and this process of sequence replacement without deletion has been described as gene conversion as a result of loose analogy with the genetic mechanism of gene conversion which has been studied especially in less complex organisms such as fungi. One extreme example is provided by the occurrence of 8 CYP21 genes in a 21-hydroxylase deficiency patient, each of which appears to be CYP21A-like [10].

The remaining disease haplotypes which show no evidence of gene deletion appear to show at least one CYP21 gene which has CYP21B-like characteristics. However, DNA sequencing of cloned CYP21B mutant genes [19–24] and PCR-

amplified mutant genes [25] or PCR-screening of pathological mutations in selectively amplified mutant CYP21B genes ([26] and Collier S., Tassabehji M. and Strachan T. unpublished) reveals that in all cases the mutant CYP21B genes carry a pathological mutation which is identical to one of the deleterious mutations normally found in the CYP21A pseudogene. The pathological mutations found in sequenced mutant CYP21B genes include the following: a C → G substitution near the 3′ end of intron 2; in 8 bp deletion in exon 3 which introduces a shift in the translational reading frame; a T → A substitution in exon 4 leading to a Ile-172 → Asn substitution; 3 clustered T → A substitutions in codons 235–238 of exon 6, leading to Ile → Asn, Val → Glu and Met → Lys substitutions; a G → T substitution in exon 7, leading to a Val-281 → Leu substitution; and a C → T substitution in exon 8, leading to a Arg-356 → Trp substitution and a C → T nonsense mutation in exon 8. Consequently, again there appears to be a gene conversion-like mechanism whereby a small segment of the CYP21B gene can be replaced by a copy of the analogous segment of the CYP21A pseudogene. If the copied segment of the pseudogene sequence carries a deleterious mutation, the introduction of this mutation into the CYP21B gene will result in defective expression of 21-hydroxylase activity.

The frequencies of the above mutations in patients have been determined by hybridization of DNA samples with allele-specific oligonucleotides. As the pathological point mutations found in mutant CYP21B genes are also found normally in the CYP21A pseudogene, it has been necessary to utilize probing strategies in which the background CYP21A sequences are differentiated from the mutant CYP21B gene sequences. One approach has been to hybridize allele-specific oligonucleotide probes to genomic DNA that has been digested with an enzyme such as Taq I which affords restriction fragments whose lengths are diagnostic for CYP21A or CYP21B [17, 20, 21]. More recently PCR-amplification strategies have been reported in which CYP21B genes are selectively amplified while CYP21A genes are not [14, 25, 26]. The results show that different mutations vary widely in their prevalence. Val-281 → Leu occurs in all or nearly all patients with nonclassic 21-hydroxylase deficiency who carry the HLA haplotype B14;DR1, an association that is presumably due to a founder effect [23]. In certain populations (such as Jews of eastern European origin) this is a very common enzymatic polymorphism with a gene frequency of > 10%. the AG substitution near the 3′ end of intron 2 may be the most frequent nondeletional allele causing classic 21-hydroxylase deficiency; in one study it accounted for 57% of such alleles [26]. The nonsense mutation in codon 318 is seen on 4–7% of classic 21-hydroxylase deficiency alleles ([17, 21, 26] and Collier S., Tassabehji M. and Strachan T., unpublished data), Ile-172 → Asn on about 6–14% of such alleles [17, 20, 26 and Collier S., Tassabehji M. and Strachan T., unpublished data], and the 8 bp deletion in exon 3 on about 3–10% [14, 17, 20, 26]. A 1 bp insertion in exon 7 of the CYP21A gene which, if present in a CYP21B gene would be expected to cause a frameshift of the translational reading frame, has not yet been identified as an independent mutation in any mutant CYP21B gene so far analysed.

The mechanisms underlying the observations of gene conversion, in the CYP21 gene cluster remain to be elucidated. In the case of a chromosome with large-scale replacement of the CYP21B by CYP21A gene sequence, multiple recombination events which may occur at different times in the evolutionary history of the chromosome may be involved [8]. However, when small-scale replacement of the CYP21B by CYP21A gene sequence is observed, the mechanism may involve a nonreciprocal transfer of sequence information. One possibility is mismatch repair following heteroduplex formation: an invading DNA strand from the CYP21A gene pairs with a complementary CYP21B gene strand and DNA repair enzymes recognize a mismatch and correct the acceptor CYP21B gene sequence to make it identical to the invading CYP21A strand over a small region [8]. Analysis of a *de novo* conversion event which contributes to 21-hydroxylase deficiency has indicated that the region of conversion may be only a few hundred bps (Collier S. and Strachan T., unpublished).

BIOCHEMICAL AND CLINICAL CONSEQUENCES OF MUTATIONS

Functional analysis of mutations

Because synthesis of *P*450c21 is restricted to the adrenal gland, the effect of missense mutations on the enzymatic activity of *P*450c21 cannot be directly tested in affected patients. Therefore, a number of attempts have been made to measure the activities of mutant enzymes in cultured cells. Two such studies have

transfected normal and mutated CYP21 genes of *P*450c21 cDNA in plasmid vectors containing a strong promoter to allow expression in COS cells [22, 24]. From these studies it has been inferred that mutant CYP21B genes which contain a C → G substitution near the end of intron 2 are defective in expression as a consequence of aberrant splicing of pre-mRNA. Additionally, the cluster of mutations in codons 235–238 and the Arg-356 → Trp mutation resulted in enzymes with no detectable activity, whereas trace activity was detected in the enzyme carrying the Ile-172 → Asn mutation. However, these studies did not attempt quantitation of activity, possibly because levels of expression were low.

In order to address this problem, several mutant enzymes were synthesized at higher levels in cultured cells using recombinant vaccinia virus [27]. The enzyme with the cluster of substitutions in codons 235–238 again had no activity even when expressed at higher levels. When 17-hydroxyprogesterone was the substrate, the enzyme carrying Ile-172 → Asn had an activity of 0.6% of normal as measured by the first order rate constant, V_{max}/K_m. The Val-281 → Leu mutation resulted in an enzyme with 50% of normal activity when 17-hydroxyprogesterone was the substrate but only 20% of normal activity for progesterone.

Structural effects of mutations

It is not known with certainty how the different missense mutations affect enzymatic activity. The Arg-356 → Trp mutation affects a region that is relatively well conserved in steroid-metabolizing cytochrome *P*450 enzymes, which may be involved in interactions with the substrate [28]. However, defective binding of substrate has not yet been demonstrated. Enzyme carrying either Ile-172 → Asn or Val-281 → Leu are not localized properly in microsomes, suggesting that these mutations both affect the conformation of the enzyme in some manner. It has been speculated [23] that Val-281 → Leu increases the likelihood of forming an α-helix in a region that is not predicted to normally be in a helical conformation. The Ile-172 → Asn mutation involves a region that may normally interact with the membrane of the endoplasmic reticulum [29], and the isoleucine residue at this position is strongly conserved in many different *P*450 enzymes. Mutation of this hydrophobic residue to a polar residue might disrupt such an interaction, weakening the association of the enzyme with the endoplasmic reticulum.

Correlation between genotype and phenotype

Particular mutations may be correlated with the different clinical forms of 21-hydroxylase deficiency (i.e. salt wasting, simple virilizing and nonclassical disease), which behave essentially as allelic variants. Such correlations are best made in persons who are either homozygous for the pathological point mutation or who are effectively hemizygotes in that they have a mutant CYP21B gene on one chromosome 6 while the CYP21B gene on the other chromosome 6 has been deleted.

Nonclassical phenotype. As mentioned, the conservative Val-281 → Leu substitution has been found to be particularly associated with patients with the mild or nonclassical form and results in an enzyme with about 50% of normal activity when 17-hydroxyprogesterone is the substrate but only about 20% of normal activity for progesterone [27]. An individual homozygous for this mutation has nonclassic 21-hydroxylase deficiency with significant biochemical abnormalities and variable symptoms of androgen excess. A heterozygous carrier of a salt wasting mutation might also be expected to have about 50% of normal 21-hydroxylase activity, but such individuals are asymptomatic and have minimal biochemical abnormalities. This suggests that *in vivo* 21-hydroxylase activity in patients with nonclassic 21-hydroxylase deficiency must actually be < 50% of normal. This apparent paradox is resolved by the discovery that progesterone at physiological intra-adrenal concentrations (2–4 μm) [30] acts as a powerful competitive inhibitor of the mutant 21-hydroxylase enzyme for its main substrate, 17-hydroxyprogesterone. Thus, relatively small differences in intra-adrenal progesterone concentration could account for much of the clinical variability that is a hallmark of nonclassical 21-hydroxylase deficiency.

Classical 21-hydroxylase deficiency. An important facet of the clinical variability of 21-hydroxylase deficiency concerns the ability to synthesize adequate amounts of the mineralocorticoid hormone aldosterone. Gene deletions, nonsense mutations (such as the one in codon 318 of exon 8) and frameshift mutations (including the 8 bp deletion in exon 3 and the 1 bp insertion mutation in exon 7) which introduce premature termination codons, completely prevent the synthesis of a functional enzyme and

are associated with salt wasting disease. Homozygotes for these mutations, or individuals who are compound heterozygotes for these mutations consistently show the salt wasting phenotype [10, 18, 21, 22]. Additionally, enzymes with the cluster of mutations Ile-Val-Glu-Met-235–238 → Ans-Glu-Glu-Lys [22] or with the single substitution, Arg-356 → Trp [24] lack any detectable 21-hydroxylase activity and they have also been described in patients with salt wasting disease. However, the splicing mutation at the 3′ end of intron 2 has been found to occur in the homozygous or hemizygous state in individuals who have either the salt wasting or simple virilizing forms of 21-hydroxylase deficiency [22, 26]. Also, while the Ile-172 → Asn mutation is often found in patients with the simple virilizing form of the disease it may also occur in salt wasting patients.

In principle, the simple virilizing phenotype might be produced by a mutation that affected the enzyme's activity for 17-hydroxyprogesterone (a precursor of cortisol) while keeping the activity for progesterone (the precursor of aldosterone) relatively intact. So far, no such mutation has been identified, and consequently, it is more probable that the difference between simple virilizing and salt wasting phenotypes is essentially due to a quantitative difference in overall enzyme activity. Because aldosterone is normally secreted at a rate 100–1000 times lower than that of cortisol, it is apparent that 21-hydroxylase activity would have to decrease to very low levels before it became rate-limiting. Apparently, as little as 0.6% of normal activity as seen in the enzyme carrying the Ile-172 → Asn mutation, allows adequate aldosterone synthesis to prevent significant salt wasting, thus resulting in the simple virilizing phenotype. The finding that some patients homozygous for the intron 2 splicing mutations have the simple virilizing phenotype suggests that not all of the pre-mRNA is aberrantly spliced; a small amount of correctly spliced mRNA must allow synthesis of some functional enzyme, at least in some individuals.

It should be noted, however, that the distinction between the simple virilizing and salt wasting phenotypes is not absolute. One patient with the Ile-172 → Asn mutation has been reported to have an elevated ratio of plasma renin to aldosterone, consistent with mild salt wasting [20] and HLA-identical sib pairs have been reported in which one sib has salt wasting disease whereas the other can synthesize adequate amounts of aldosterone [31]. These findings suggest that additional epigenetic or nongenetic factors can influence the presentation of the salt wasting phenotype. However, profound discordance between HLA-identical sibs in both clinical symptoms and biochemical parameters [32] is more probably attributable to genetic factors including the possibility of germinal mosaicism.

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