Of marsupials and men: “Backdoor” dihydrotestosterone synthesis in male sexual differentiation

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Following development of the fetal bipotential gonad into a testis, male genital differentiation requires testicular androgens. Fetal Leydig cells produce testosterone that is converted to dihydrotestosterone in genital skin, resulting in labio-scrotal fusion. An alternative ‘backdoor’ pathway of dihydrotestosterone synthesis that bypasses testosterone has been described in marsupials, but its relevance to human biology has been uncertain. The classic and backdoor pathways share many enzymes, but a 3α-reductase, AKR1C2, is unique to the backdoor pathway. Human AKR1C2 mutations cause disordered sexual differentiation, lending weight to the idea that both pathways are required for normal human male genital development. These observations indicate that fetal dihydrotestosterone acts both as a hormone and as a paracrine factor, substantially revising the classic paradigm for fetal male sexual development.

1. Role of androgens in fetal male differentiation

Following embryonic sex determination, in which the presence of a Y chromosome drives the differentiation of the primitive, bipotential gonad into a testis, androgens produced by the fetal testis mediate both the stabilization of structures derived from the Wolffian ducts and the closure of the urogenital sinus, resulting in the male phenotype. The indispensable role of androgens is demonstrated by the consequences of human androgen receptor deficiency and androgen-receptor knockout mice, which have female external genitalia despite the presence of testes and abun-
P450 oxidoreductase (POR), HSD17B3 (17α,46,XY males with 5α-reductase deficiency, who typically have testicular androgen deficiency). The resulting DHT induces labio-scrotal fusion, the development of the phallic urethra and the presence of three different enzymes: 5α-reductase, type 1); reductive 3α-HSD activity catalyzed by AKR1C2 and AKR1C4; and oxidative 3β-HSD activity, apparently catalyzed by RoDH (also known as 17α-HSD6). Steroid names include: 17α-Pregnenolone, 17α-hydroxyprogesterone; 17α-DHP, 17α-hydroxydihydroprogesterone (5α-pregnan-3α,17α-ol-20-one); 5α-DHP, 5α-dihydroprogesterone (5α-pregnan-3β,20-dione); 17α-Allopregnanolone, 17α-hydroxyallopregnanolone (5α-pregnan-3β,17α-diol-20-one); androstenediol, androsta-5-ene-3β,17β-diol.

“Classic” Pathway

Alternative/Backdoor Pathway

Fig. 1. The classic and alternative ‘backdoor’ pathways of androgen biosynthesis. The classic pathway proceeds from cholesterol via pregnenolone, 17α-Preg and DHEA to androstenedione or androstenediol and then to testosterone in testicular Leydig cells (shown in blue). Hormonal testosterone from the circulation is then converted to DHT in genital skin. The backdoor pathway proceeds mainly from 17α-Preg to 17α-Preg, 17α-DHP, 17α-Allo, androsterone, androstenediol (3β-Diol) (all shown in green) and thence to DHT (shown in red), all in the testes. The enzymes and proteins shown in the classic pathway are: CYP11A1 (P450ccc, cholesterol side-chain cleavage enzyme), StAR (steroidogenic acute regulatory protein), CYP17A1 (P450c17, 17α-hydroxylase/17,20-lyase), HSD3B2 (3β-HSD2, 3β-hydroxysteroid dehydrogenase, type 2), cytochrome b5, P450 oxidoreductase (POR), HSD17B3 (17α-HSD3, 17β-hydroxysteroid dehydrogenase, type 3), and 5αRed2 (5α-reductase, type 2). The alternative pathway is characterized by the presence of three different enzymes: 5αRed1 (5α-reductase, type 1); reductive 3α-HSD activity catalyzed by AKR1C2 and AKR1C4; and oxidative 3β-HSD activity, apparently catalyzed by RoDH (also known as 17α-HSD6). Steroid names include: 17α-Pregnenolone, 17α-hydroxyprogesterone; 17α-DHP, 17α-hydroxydihydroprogesterone (5α-pregnan-3α,17α-ol-20-one); 5α-DHP, 5α-dihydroprogesterone (5α-pregnan-3β,20-dione); 17α-Allopregnanolone, 17α-hydroxyallopregnanolone (5α-pregnan-3β,17α-diol-20-one); androstenediols, androsta-5-ene-3β,17β-diol.

2. Sexual development and sexual differentiation

Sexual determination and sexual differentiation refer to distinct processes. Sexual determination is the process by which the early bipotential gonad develops into a testis or an ovary, whereas sexual differentiation is the subsequent process by which gonadal hormones determine the anatomic phenotype (Bisson-Lauber, 2010; Grumbach et al., 2003; MacLaughlin and Donahoe, 2004). The human bipotential gonad develops at about 4–6 weeks post conception, testicular development occurs soon thereafter, primarily between 6 and 8 weeks, while ovarian development occurs a bit later, at 10–14 weeks. Once developed, the fetal ovary is hormonally quiescent (Voutilainen and Miller, 1986), and ovarian function is clearly evidenced by the normal female anatomy of newborns with 45,X gonadal dysgenesis (Turner Syndrome). By contrast, the fetal testis is active by 8 weeks, with the Sertoli cells producing Anti Müllerian Hormone (AMH) and the Leydig cells producing androgens, both of which are needed for normal male development. Fetal testicular androgens are required for male sexual differentiation, the process by which the labio-scrotal folds fuse, and the genital tubercle develops into the penis with the urethra traversing its inferior surface. Thus male sexual differentiation is crucially dependent on androgen biosynthesis and action.

3. The “classic” pathway of androgen biosynthesis

The ‘classic’ pathway of androgen biosynthesis is well-known (Fig. 1) (Miller and Auchs, 2011). After cholesterol reaches the inner mitochondrial membrane through the action of the steroidogenic acute regulatory protein (StAR), it is converted to pregnenolone in a three-step process catalyzed by the cholesterol side-chain
cleavage enzyme, mitochondrial cytochrome P450scC, encoded by the CYP11A1 gene. While pregnenolone may be converted to progesterone by 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) in the Leydig cells, the principal pathway (the Δ5 pathway) is that pregnenolone is sequentially converted to 17OH-pregnenolone (17OH-Preg) and thence to DHEA by microsomal cytochrome P450c17, encoded by the CYP17A1 gene (Flück et al., 2003). The Δ5 pathway is preferred because the Km (Michaelis constant) for Δ5 sterols binding to 3βHSD2 is 5.5 μM whereas the Km for binding to P450c17 is 0.8 μM, so that pregnenolone or 17OH-Preg has a 7-fold preference for the Δ5 pathway (Nicolajeff et al., 1999). Further, human P450c17 can convert 17OH-progesterone (17OH-P) to androstenedione with only 2–3% of the efficiency of its conversion of 17OH-Preg to DHEA (Flück et al., 2003; Auchus et al., 1998), so that any 17OH-P that is produced by 3βHSD2 will accumulate and act on 3βHSD2 by end product inhibition to inhibit further conversion of 17OH-Preg to 17OH-P. DHEA may be converted to Δ4-androstenedione by 3βHSD2 and thence converted to testosterone by 17β-hydroxysteroid dehydrogenase, type 3 (17βHSD3), or DHEA may first be reduced to Δ5-androstenediol by 17βHSD3 (Geissler et al., 1994) and then converted to testosterone by 3βHSD2. These two pathways from DHEA to testosterone are quantitatively about equally important. Once produced, testicular testosterone is a classic hormone that is secreted into the circulation and acts at distant targets. One such target is the fetal genital skin, which expresses the enzyme 5α-reductase, type 2 (5αRRed2), which converts testosterone to its substantially more active derivative, dihydrotestosterone (DHT) (Wilson et al., 1994). The synthesis of DHT in fetal genital skin induces labio-scrotal fusion. It appears that activation of the androgen receptor in these cells induces production of extracellular matrix proteins that promote cellular adhesion, but the exact molecular mechanisms of labio-scrotal fusion remain unclear. The essential role of androgen action in the differentiation of the male external genitalia is demonstrated by the female external genital phenotype of 46,XY individuals with non-functional androgen receptors (complete androgen insensitivity syndrome) (French et al., 1990; McPhaul et al., 1993).

Multiple lines of evidence demonstrate the importance of the classic pathway of androgen biosynthesis. In 46,XY individuals, complete absence of the activities of StAR (Bose et al., 1996), P450scC (Tajima et al., 2001; Kim et al., 2008), or P450c17 (Auchus, 2001) results in a female external genital phenotype; the minimal virilization seen in some patients with complete absence of the activities of 3βHSD2 (Moisan et al., 1999; Rheuma et al., 1992) and 17βHSD3 (Geissler et al., 1994; Andersson et al., 1996) has been attributed to the action of the peripheral conversion of DHEA and/or androstenedione to functional androgens. Evidence for the essential role of DHT produced in genital skin comes from the observation that 46,XY individuals with varying defects in 5αRRed2 have a disorder of sexual differentiation called pseudovaginal perineal hypospadia (5α-reductase deficiency); the partial androgenization seen in these individuals at birth is generally ascribed to the action of testicular testosterone. Thus the known pathways of human androgen biosynthesis and the phenotypic consequences of mutations in the androgenic enzymes in this pathway have been consistent; no additional factors were thought to be needed to explain male sexual differentiation. However, as discussed below, the partial virilization seen in patients with 5α-reductase deficiency may also be partially attributable to DHT produced in the fetal testis by a pathway that does not require 5αRRed2.

4. The alternative “backdoor” pathway of androgen synthesis

Marsupials give birth to their young at a stage of development approximately comparable to the end of the first trimester of human pregnancy. The newborn emerges from the birth canal, crawls up the mother’s abdomen and into her pouch and attaches itself to one of her teats, whereupon this ‘pouch young’ can be nourished and finish development in the pouch. This mode of delivery and development permits facile investigation of the early physiology of these animals during their early sexual differentiation (Wilson et al., 2002). In male pouch young of the tammar wallaby (Macropus eugenii), scrotal development is androgen-independent but testicular androgens mediate virilization of the urogenital sinus at 20–45 days postpartum (Renfree et al., 1995). Studies of early testicular steroidogenesis in the tammar wallaby found that this marsupial synthesizes the androgen androstenediol (5α-androstane-3α,17β-diol, referred to herein as 3αDiol) by an alternative pathway that begins with 17OH-P and does not utilize DHEA, androstenedione or testosterone as intermediates, but instead proceeds through 17OH-dihydroprogesterone (5α-pregnan-17α-ol-3,20-dione), 17OH-allopregnanolone (17OH-Allo) (5α-pregnane-3α,17α-ol-3,20-dione) and androsterone to 3αDiol and, apparently, to DHT (Wilson et al., 2003; Auchus; 2004) (Fig. 1).

5. AKR1C2 Mutations in patients: the backdoor pathway is essential for human sex development

The initial characterization of the backdoor pathway focused on the steroids produced, and the enzymes responsible for the steroidal conversions were inferred. A key mode of identifying human steroidogenic pathways has been from studying diseases resulting from mutations in the genes encoding steroidogenic enzymes and other factors (Miller and Auchus, 2011). The steroidal conversions in the backdoor pathway involve a 5α-reductase, 17,20 lyase, a 17β-hydroxysteroid dehydrogenase and both reductive and oxidative 3β-hydroxysteroid dehydrogenases (3βHSD) (Fig. 1). Studies with mice lacking 5α-reductase type 1 (5αRed1) or 5αRed2 indicated that the type 1 enzyme participates in the backdoor pathway (Mahendroo et al., 2004), and studies in vitro shown that human P450c17 readily catalyses 17,20 lyase conversion of 17OH-Allo to androsterone (Gupta et al., 2003), and the 17HSD5 (AKR1C3) and other forms of 17HSD have not been formally excluded. However, the nearly wholly female phenotype in 46,XY patients with complete absence of 17HSD3 suggests that absence of this one enzyme suffices to eliminate essentially all androgen production, suggesting it is the principal 17HSD in both the backdoor pathway as well as in the classic pathway. The enzymes catalyzing the reductive and oxidative 3βHSD activities have been less clear; however, substantial data indicate that 3βHSD activities are catalyzed by members of the AKR1C family of aldo–keto reductases (Couteur et al., 2005; Dufort et al., 2001; Jin and Penning; 2006; Penning et al., 2000, 2004, 1996; Rizner et al., 2003) (Fig. 1). Because many of these enzymatic steps are common to both the classic and backdoor pathways, it has been difficult to determine the relative importance of the backdoor pathway by studying patients with disorders of androgen biosynthesis. The description of 46,XY patients with disordered sexual differentiation due to mutations in AKR1C2 has sustained the hypothesis that both the classic and backdoor pathways are needed for normal human male sexual differentiation (Flück et al., 2011).

5.1. Family A

This family, firstly described in 1972 as having “17,20 desmolase deficiency” (Zachmann et al., 1972) was recently re-investigated. The patients had presented at birth with cryptorchidism and undervirilized external genitalia, and a 46,XY maternal aunt had ambiguous genitalia and no uterus. Studies of urinary steroids
indicated normal production of cortisol and impaired production of androgens (Zachmann et al., 1972). Recent studies showed that the affected individuals lacked mutations in the CYP17A1 gene encoding P450c17, cytochrome P450 oxidoreductase (POR), cytochrome b5, steroidogenic factor-1, and the androgen receptor (Flück et al., 2011). Hormonal data from 1972 appeared to be inconsistent with deficiencies in 17\(\alpha\)-HSD or 5\(\alpha\)-reductase, hence mutations in an alternative pathway were sought. Genes were investigated encoding the AKR1C enzymes that catalyze the 3\(\alpha\)-HSD reactions that are unique to the backdoor pathway, beginning with AKR1C2, an enzyme with reductive 3\(\alpha\)-HSD activity (Jin and Penning, 2006; Rizner et al., 2003).

AKR1C2 mutations were found in all affected individuals (Flück et al., 2011) (Fig. 2A). The 46,XY proband with severe undervirilization and a female sex assignment was a compound heterozygote for the AKR1C2 missense mutations I79V and N300T. A 46,XY heterozygote carrying only I79V was moderately undervirilized, and a heterozygote carrying N300T had normal male external genitalia; both fathered children, although the former required in vitro fertilization. The proband’s 46,XX mother was a compound heterozygote carrying N300T had normal male external genitalia; both fathered children, although the former required in vitro fertilization. The proband’s 46,XX mother was a compound heterozygote carrying N300T had normal male external genitalia; both fathered children, although the former required in vitro fertilization.

A male having one mutation (individual II.1) is normal; a male carrying two mutations on one allele (individual III.4) had impaired fertility, as his reproduction required in vitro fertilization. Two genetic males carrying mutations on both alleles (individuals II.5 and III.2), had severe DSD and were raised as females. An unequal crossover event ablates AKR1C2 activity in another individual (Family B) with 46,XY DSD. The AKR1C1 and AKR1C2 genes, which share about 90% nucleotide sequence identity, apparently mis-paired during meiosis (left). The result is that the affected individual’s Allele 1 lacked an intact AKR1C2 gene and had only the nonfunctional C1/C2 gene from the crossover. Allele 2 in the affected individual had an intact AKR1C1 gene from the father, the hybrid C1/C2 gene and an intact AKR1C2 gene from the mother. However, the maternal AKR1C2 gene carried the missense mutation H222Q (indicated by the light blue spot); this mutation destroyed all measurable 3\(\alpha\)-HSD activity (Flück et al., 2011). N.d. = not done.

Fig. 2. Genetics of identified AKR1C mutations. A. AKR1C mutations correlate with phenotypic severity in the index family A. Females are unaffected, irrespective of AKR1C mutation status, as androgens are not needed for female sexual development or reproduction. The Ile79Val mutation in AKR1C2 and the AKR1C4 splice mutation co-segregate, suggesting they lie on one allele. A male having one mutation (individual II.1) is normal; a male carrying two mutations on one allele (individual III.4) had impaired fertility, as his reproduction required in vitro fertilization. Two genetic males carrying mutations on both alleles (individuals II.5 and III.2), had severe DSD and were raised as females. B. An unequal crossover event ablates AKR1C2 activity in another individual (Family B) with 46,XY DSD. The AKR1C1 and AKR1C2 genes, which share about 90% nucleotide sequence identity, apparently mis-paired during meiosis (left). The result is that the affected individual’s Allele 1 lacked an intact AKR1C2 gene and had only the nonfunctional C1/C2 gene from the crossover. Allele 2 in the affected individual had an intact AKR1C1 gene from the father, the hybrid C1/C2 gene and an intact AKR1C2 gene from the mother. However, the maternal AKR1C2 gene carried the missense mutation H222Q (indicated by the light blue spot); this mutation destroyed all measurable 3\(\alpha\)-HSD activity (Flück et al., 2011).
Similarly, the capacity of these AKR1C2 mutants to oxidize 3-reduced, to 82%, 22%, and 41% of wild-type, respectively (Table 2). The activities of these biosynthetic AKR1C2 proteins were assessed by measuring their ability to reduce 5-epimer. The activities of AKR1C2 mutants I79V, A87P, H90Q, and N300T to convert 5-dihydrotestosterone (DHT) was only modestly reduced (to 28–48%) (Flück et al., 2011).

Thus, the identified AKR1C2 mutations partially impaired 3-oxidalol to androsterone and AKR1C4 can oxidize 3αDiol to DHT (Penning et al., 2000; Lin et al., 1997). Thus AKR1C2 functions almost exclusively as a reductive 3αHSD.

To ascertain the enzymatic consequences of the identified AKR1C2 mutations, each was recreated in an AKR1C2 cDNA expression vector, expressed in bacteria and purified to apparent homogeneity. The activities of these biosynthetic AKR1C2 proteins were assessed by measuring their ability to reduce 5xDHP to Allo and their ability to oxidize 3αDiol to DHT, and the data were expressed as the enzymatic maximal velocity divided by the Michaelis constant (Vmax/Km). The activities of AKR1C2 mutants 179V, H90Q, and N300T to convert 5αDHP to Allo were only modestly reduced, to 82%, 22%, and 41% of wild-type, respectively (Table 2).

Similarly, the capacity of these AKR1C2 mutants to oxidize 3αDiol to DHT was only modestly reduced (to 28–48%) (Flück et al., 2011). Thus, the identified AKR1C2 mutations partially impaired 3αHSD activity, but not to the degree typically seen in recessive disorders of steroidogenesis. These data thus suggested that another factor in addition to AKR1C2 was disordered in the index family. Because the microsatellite data only pointed to the AKR1C locus, the expression of the other genes in this locus was examined more closely by analysis of leukocyte RNA from the affected family members. As shown by reverse transcription followed by polymerase chain reaction (RT-PCR), the mRNAs for four of the five AKR1C enzymes were spliced correctly, but for AKR1C4 there was both correct splicing and incorrect splicing: both the expected product and a shorter product were seen. Sequencing of the AKR1C4 transcripts showed that the incompletely spliced product lacked all of exon 2; this abnormally spliced AKR1C4 mRNA was found in all affected family members, but not in unaffected controls (Flück et al., 2011). The sequence of the AKR1C4 gene in the genomic DNA of affected individuals was normal, except for a G to T mutation in an intron, 106 bases upstream from exon 2. Most intronic mutations that cause aberrant RNA processing are close to intron/exon borders (for example, in the neurofibromatosis 2 gene, 178 of 183 splicing mutations were within 10 bases of an intron/exon border (Baser, 2006), hence finding a mutation 106 bases away would not normally be predicted to alter mRNA splicing. However, assessment of this intronic mutation in vitro by an exon trapping assay showed that it resulted in the loss of exon 2 from the AKR1C4 mRNA. When the encoded truncated protein was assayed in vitro, it had only about 10% of WT activity. Thus the disordered androgen biosynthesis in the index family appeared to arise by ‘digenic inheritance’, wherein partial defects in two genes, AKR1C2 and AKR1C4, combine to yield the phenotype (Fig. 2A and Table 2).

### 5.2. Family B

The central role of AKR1C2 was clarified by finding that an unrelated, 46,XY patient who had female external genitalia and intra-abdominal testes had a complex chromosomal rearrangement in the AKR1C locus that resulted in no functional AKR1C2 enzyme (Flück et al., 2011) (Fig. 2B and Table 2). An unequal crossover between the AKR1C2 gene and the adjacent, 90% identical AKR1C1 gene resulted in the patient having a single C1/C2 hybrid gene on one allele and paternal C1, the C1/C2 hybrid and a maternal C2 on the other allele. However, the intact maternal C2 gene carried the missense mutation H222Q, so that neither allele encoded authentic AKR1C2 (Fig. 2B). When this H222Q mutant was assessed in vitro it lacked any detectable activity to convert 3αDiol to DHT (Flück et al., 2011). Thus this single patient in an unrelated family had a similar but distinct monogenic disorder in which only AKR1C2 was disordered, but in which the AKR1C2 defect was apparently complete. Thus ablation of all AKR1C2 activity appears to be sufficient to interrupt backdoor testicular synthesis of DHT and cause 46,XY DSD.

### 6. Sites of DHT synthesis

These studies have expanded and revised the understanding of the sites of fetal androgen synthesis. The testis appears to be the unique site of expression of 17βHSD3 (Geissler et al., 1994), so that...
the testis is the primary site of testosterone biosynthesis, although the fetal adrenal can produce small amounts of testosterone via 17βHSD5 (Goto et al., 2006). Immunoblotting of human adult and fetal tissues has shown that 5αRed2 is expressed in genital skin but earlier studies did not identify either 5αRed2 or 5αRed1 in fetal testes (Thigpen et al., 1993); however, more sensitive stud-
cies using RT-PCR found abundant mRNA for 5αRed1 but not for 5αRed2 in fetal testes, and abundant 5αRed2 but not 5αRed1 in adult testes (Flück et al., 2011). Similarly, RT-PCR showed that AKR1C2 is abundantly expressed in the fetal testis but minimally expressed in the adult testis (Flück et al., 2011) (Fig. 3A). Thus AKR1C2 appears to participate in the backdoor pathway to DHT in the human fetal testis, but not in the adult testis, and its mutation appears to cause incomplete male genital development. RoDH (17βHSD6) was also detected in the fetal testes, suggesting it may catalyze the oxidative 3αHSD reaction needed to complete the backdoor pathway to DHT, but this identification remains uncertain. Adult adrenals expressed abundant AKR1C2 (Fig. 3B), so that the backdoor pathway to DHT production may be important in situations where intra-adrenal 17OHP concentrations are high, such as in 21-hydroxylase deficiency. AKR1C2 is not expressed in the fetal ovary, consistent with the fact that androgens are not crucial in female sex development, whereas its mRNA is readily detectable in the adult gonad (Fig. 3C).

7. Modeling structural studies

The AKR1C enzyme family has high amino acid sequence conservation, and the same catalytically important residues are found in the same positions (Asp50, Tyr55, Lys84 and His117) in all family members (Fig. 4A). For catalytic activity, Tyr55 acts as an acid, Lys84 decreases the pKₐ of the tyrosine, and Asp50 forms a salt-bridge to the lysine. The substrate-binding pocket consists of 5 loops; loop A (117–143), B (217–238), C (299–323), β1-α1 (23–32) and β2-α2 (51–57); allowing binding of structurally diverse substrates through an “induced-fit” mechanism (Couture et al., 2005). Two of the amino acids that were mutated in our patients, Ile79 and Asn300, are at the beginning of loops that make up the steroid binding pocket (Fig. 4B); amino acid changes at these locations may disturb the Lys84, Trp86 and Leu308 residues further down the loops that interact with the substrates at the catalytic site. The His90 residue that was found to be mutated is in close proximity (5.2 Å) to His117 and in contact with Ile116 and Phe118, which are part of catalytic center. In our analysis, the His117-NADPH contact was broken in the His90Gln variant, potentially from the additional interactions of Gln90 with side chains of neighboring residues. His117 is essential for the activity in all AKR family members, as it interacts with a central acidic ion to act as a bridge between steroid substrate and NADPH. A change in interaction between NADPH and His117 will likely affect co-factor binding and electron transfer. Comparison of the structures of rat AKR1C9 and human AKR1C2 shows that changing His222 to Ser222 pushes the side-chain of neighboring Trp227, resulting in testosterone binding deeper than in human AKR1C2, where testosterone binds closer to surface. The substitution of His for Gln (His222Gln), as found in one of our patients, does not shift the side-chain of Trp227, so that the steroid binding pocket remains unchanged. However, flipping of the side-chain of Gln222 pushed it into the NADPH binding tunnel (Fig. 4B), potentially blocking NADPH entry. In human AKR1C3 and AKR1C4, His222 is naturally replaced with Gln222, but this change is accommodated by other amino acid replacements (as compared to AKR1C2) that may alter the co-factor binding patterns. A major factor in AKR1C2 reactions is the release of cofactors following catalysis; minor changes in charge caused by Asn300Thr may influence how a particular steroid or co-factor could exit the catalytic site. An enzyme with slower release of product or co-factor will impair binding of the next molecule of substrate or the co-factors. The three-dimensional model of AKR1C2 and C4 with mutations is depicted in Fig. 4.

Fig. 4. AKR1C2/4 Structure and Mutations. A The AKR enzyme family has high amino acid sequence conservation, and the same catalytically important residues are found in the same positions (Asp50, Tyr55, Lys84 and His117) in all family members. For catalytic activity, Tyr55 acts as an acid, Lys84 decreases the pKa of the tyrosine, and Asp50 forms a salt-bridge to the lysine. The substrate-binding pocket consists of 5 loops; loop A (117–143), B (217–238), C (299–323), β1-α1 (23–32) and β2-α2 (51–57); allowing binding of structurally diverse substrates through an “induced-fit” mechanism (Couture et al., 2005). Molecular dynamic simulation analysis of the AKR1C4 structure revealed movements of short flexible fragments comprising AA 9–14 and 50–59, opening and closing access to the catalytic center. A model of AKR1C4 lacking the 56 amino acids (Val29-Lys84) encoded by exon 2 results in a stable structure that may still bind NADPH, but which lacks several catalytically essential residues, rendering the truncated mutant inactive. The active site tyrosine (Tyr55) and Asp50, Leu54 and Lys84 are missing from the truncated AKR1C4 mutant. Leu54 is potentially involved in determining substrate specificity in AKR1C4, and Asp50 and Lys84 together balance the pKa of the active site similarly to their role in AKR1C2, and are necessary for transferring electrons from NADPH to the steroid substrate. B. Ile79 and Asn300 (mutated in some patients) are near the steroid-binding pocket; mutations may disturb the interaction of the steroid with the catalytic site. His90 is in close proximity (5.2 Å) to His117, which acts as a bridge between the steroid substrate and NADPH. The His117-NADPH contact was broken in the His90Gln variant, which is involved in binding the steroid and the co-factors. The His222Gln mutation results in the side-chain of Gln222 to be pushed into the NADPH binding tunnel, potentially altering interaction with NADPH. The Asn300Thr mutation is adjacent to Asp301 (whose mutation is important for steroid binding by providing base-acid potential with Tyr55), and may influence how a particular steroid or co-factor could enter or exit the catalytic site due to a change in the charge/ hydrophobicity of the steroid-binding tunnel.

Molecular dynamic simulation analysis of the AKR1C4 structure revealed movements of short flexible fragments comprising AA 9–14 and 50–59, opening and closing access to the catalytic center. A
has been that fetal testicular testosterone is a circulating hormone known that this model is incomplete (Wilson et al., 2002). The alternative/backdoor pathway (Fig. 1) and that this DHT production seems to be required indicating that the fetal testis produces DHT intratesticularly through the alternative pathway, which will be formed through the activity of 5α-reductase type 2. We now have data indicating that the fetal testis produces DHT intratesticularly through the alternative/backdoor pathway (Fig. 1) and that this DHT production seems to be required for the normal differentiation of the male external genitalia. Thus we add a novel genetic cause, AKR1C2 deficiency, to the list of 46,XY disorders of sexual development (DSD) associated with androgen biosynthesis.

model of AKR1C4 lacking the 56 amino acids (Val29-Lys84) encoded by exon 2 results in a stable mature form that may still bind NADPH, but which lacks several catalytically essential residues, rendering the truncated mutant inactive. The active site tyrosine (Tyr55) and Asp50, Leu54 and Lys84 are missing from the truncated AKR1C4 mutant. Leu54 is potentially involved in determining substrate specificity in AKR1C4, and Asp50 and Lys84 together balance the pKa of the active site similarly to their role in AKR1C2 (Fig. 4A), and are necessary for transferring electrons from NADPH to the steroid substrate.

8. Conclusions

The conventional model of male external genital development has been that fetal testicular testosterone is a circulating hormone that is converted to DHT by 5αRed2 in genital skin, which then acts as a paracrine factor to stimulate fusion of the labio-scrotal folds. However, it has long been known that this model is incomplete (Wilson et al., 2002). The finding that human AKR1C2 mutations cause disorders of sexual differentiation hints to the fact that DHT produced in the fetal testis via the backdoor pathway seems to act as a hormone that collab- rate with DHT produced in genital skin by 5αRed2 to induce labioscrotal fusion. Thus both the classic and backdoor pathways participate in human fetal androgen synthesis, and DHT acts in male genital development as both a paracrine factor and as a hormone; both pathways appear to be needed for male differentiation of the urogenital sinus and external genitalia.

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