Natural Mutagenesis Study of the Human Steroid 5α-Reductase 2 Isozyme†

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ABSTRACT: The enzyme steroid 5α-reductase utilizes NADPH to reduce the double bonds of a variety of steroid substrates with generalized 3-oxo, $\Delta^{4,5}$ structures. One substrate for this membrane-bound enzyme is testosterone, whose reduction to dihydrotestosterone is required for the embryonic differentiation of the external male genitalia and prostate. There are two 5α-reductase isozymes, designated types 1 and 2, which have different biochemical and pharmacological properties. Inherited deficiencies of 5α-reductase type 2 result in a form of male pseudohermaphroditism in which the external genitalia fail to develop normally. Here, nine additional mutations in the 5α-reductase 2 gene in subjects with 5α-reductase deficiency are described. The biochemical consequences of these mutations, as well as 13 previously identified missense mutations, were characterized by recreating the mutations in an expressible cDNA and transfecting into mammalian cells. Twelve of the 22 mutations inactivated the enzyme. The remaining 10 mutations impaired enzyme function by affecting either substrate or cofactor binding. Almost all mutations decreased the half-life of the protein, and all but one of the impaired enzymes had an altered pH optimum. The mutations provide insight into functional domains in the protein as well as an unusual acidic pH optimum characteristic of the 5α-reductase type 2 isozyme.

The steroid 5α-reductases are a small family of isozymes that participate in the catabolism and anabolism of steroid hormones (Russell & Wilson, 1994). By reducing $\Delta^{4,5}$ bonds in the A ring of steroid hormone substrates, the enzymes render 3-oxo groups susceptible to reduction by 3α- and 3β-hydroxysteroid dehydrogenases, which results in inactivation of the steroid (Bondy, 1981). The action of 5α-reductase can also enhance steroid hormone activity. For example, the reduction of testosterone to dihydrotestosterone produces an androgen that is 50-fold more potent than testosterone (Wilson et al., 1993).

Two 5α-reductase isozymes, designated types 1 and 2, have been identified by cDNA cloning in the human and rat (Russell & Wilson, 1994). The isozymes are approximately 250 amino acids in length, share 50% sequence identity, and are both integral membrane proteins of the endoplasmic reticulum. Despite these similarities, the isozymes have quite different kinetic, biochemical, and pharmacological properties. The type 1 isozyme has a low affinity for steroid substrates and a basic pH optimum and is sensitive to certain benzoquinolinone inhibitors (Jenkins et al., 1992; Thigpen et al., 1993a, Hirsch et al., 1993). The type 2 isozyme has a higher affinity for steroid substrates, an acidic pH optimum, and a unique sensitivity profile to certain 4-azasteroid inhibitors (Andersson et al., 1991; Thigpen et al., 1993a).

Of the two 5α-reductase isozymes, the physiological role of the type 2 protein is better understood due to the occurrence of natural mutations in the gene (symbol *SRD5A2*) encoding the type 2 isozyme (Wilson et al., 1993). This isozyme is responsible for the embryonic synthesis of dihydrotestosterone that is required for the development of the external genitalia and prostate. Males homozygous for mutations in the type 2 gene fail to develop these reproductive structures (Wilson et al., 1993). To date, 20 different mutations in the *SRD5A2* gene have been characterized at the molecular level in individuals with 5α-reductase deficiency (Andersson et al., 1991; Thigpen et al., 1992a,b). Three of these mutations (G34R, G196S, and R246W) affected the ability of the type 2 isozyme to interact with substrate and cofactor (Thigpen et al., 1992a,b).

Despite the central role played by 5α-reductases in steroid hormone metabolism, little is known about functional domains in these enzymes. Although they share sequence identity with several viral proteins, they do not share obvious homologies with other NADPH-utilizing proteins or enzymes that act on steroid substrates (Russell & Wilson, 1994). Here, we took two approaches to gain insight into structure–function relationships in 5α-reductase. In the first, we screened for additional mutations in the *SRD5A2* gene in subjects with 5α-reductase 2 deficiency. In the second, the biochemical consequences of 22 of these naturally occurring mutations for enzyme activity and stability were determined.

EXPERIMENTAL PROCEDURES

Materials. DNA modifying enzymes were purchased from New England Biolabs. Rabbit polyclonal antisera directed against amino acid residues 227–251 of the human type 2 isozyme were generated as described (Thigpen et al., 1993b). Trans^{35}S-label *in vivo* radiolabeling mix was from ICN. [^{14}C]-Testosterone was from DuPont–New England Nuclear.

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Detergents and NADPH were purchased from Sigma Chemical Co. Protein A–Sepharose was from Pharmacia LKB Biotechnologies Inc. K.CAT^r, a program for the analysis of kinetic data, was from BioMetallics Inc. (Princeton, NJ). Protein concentration was determined with reagents from Bio-Rad.

Subjects. The diagnosis of 5α-reductase deficiency was made based on physical examination, analysis of plasma testosterone and dihydrotestosterone levels, pedigree analysis, and in some cases by measurement of 5α-reductase enzyme activity in biopsy samples. The individuals in whom additional mutations in the *SRD5A2* gene were detected are as follows:

(*1*) *5R2-Paris-2* is case 2 of Mauvais-Jarvis et al. (1981) and is homozygous for the R103* mutation in exon 2.

(*2*) *5R2-Paris-3* is a 28-year-old 46, XY phenotypic female of Algerian descent studied at the Hospital Necker in Paris. Her basal plasma testosterone was 730 ng/dL and plasma dihydrotestosterone was 26 ng/dL. An enzyme activity of 4.5 ± 2.2 fmol of dihydrotestosterone (μg of DNA)$^{-1}$ h^{-1} was measured in cultured genital skin fibroblasts. This patient underwent orchiectomy and lives as a female. She is homozygous for the P59R mutation in exon 1.

(*3*) *5R2-Paris-6* is a 31-year-old 46, XY phenotypic female of Caucasian descent studied at the Hospital Necker in Paris. Her plasma testosterone was 760 ng/dL and plasma dihydrotestosterone was 27 ng/dL. These values increased to 2340 and 34 ng/dL upon stimulation with human chorionic gonadotrophin. An enzyme activity of 1.7 ± 2.9 fmol of dihydrotestosterone (μg of DNA)$^{-1}$ h^{-1} was measured in cultured genital skin fibroblasts. This patient lives as a female and is homozygous for the Y235F mutation in exon 5.

(*4*) *5R2-New Guinea-2* is described in Warne et al. (1988) and is a Class 2B heterozygote (Wilson et al., 1993) for the R145W mutation in exon 2.

(*5*) *5R2-Chicago-4* is a 46,XY individual of Mexican–American descent who was raised as a female, underwent a reversal of gender role behavior, and now lives as a man. He was diagnosed by Dr. R. Sharifi of the University of Illinois, Chicago. No enzyme activity could be detected in cultured genital skin fibroblasts from this subject. This individual was homozygous for the P212R mutation in exon 4.

(*6*) *5R2-New York-5* is subject B of Saenger et al. (1978) and is a compound heterozygote with the P181L mutation of exon 3 and the H230P mutation of exon 4.

(*7*) *5R2-Sao Paulo-6* is described in Mendonca et al. (1987) and is a compound heterozygote harboring the Q126R mutation of exon 2 and the N193S mutation of exon 4.

(*8*) *5R2-Los Angeles-3* is reviewed in Wilson et al. (1993) and is homozygous for the Y91D mutation of exon 1.

Mutation Detection and Site-Directed-Mutagenesis. Mutations in the *SRD5A2* gene were detected by single-stranded DNA conformation analysis (SSCP) as described (Thigpen et al., 1992a), except that the P181L mutation in exon 3 of subject 5R2-New York-5 was detected on a 0.5× mutation detection enhancement gel (J. T. Baker, Phillipsburg, NJ), and that the oligonucleotide pair 5′-dTTCGCGGTGC-CCGCGGGGATCCTCG-3′ and 5′-dCGGGAGCAGG-GCAGTGCGCTGCACT-3′ was used to amplify the 3′-end of exon 1 and the 5′-end of intron 1. Oligonucleotide-directed mutagenesis of the 5α-reductase type 2 cDNA was performed as previously described using mutagenic oligonucleotides 21 bases long (Thigpen & Russell, 1992).

Expression Analysis. Normal and mutant cDNAs were transfected into human embryonic 293 cells (American Type Culture Collection, CRL 1573, Rockville, MD) as described (Andersson et al., 1991). *In vivo* and *in vitro* enzyme activity assays, pH optimum determination, and analysis of apparent kinetic constants were carried out as described (Thigpen & Russell, 1992).

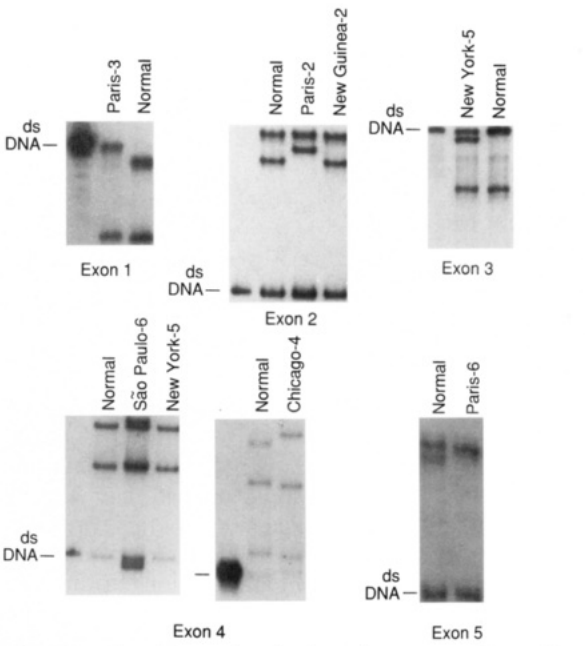

FIGURE 1: Detection of mutations in 5α-reductase type 2 gene by SSCP analysis. Individual exons of the *SRD5A2* gene were amplified from genomic DNA isolated from the indicated subject and electrophoresed on neutral polyacrylamide gels as described in Experimental Procedures. The position to which the nondenatured double-stranded DNA migrated in each gel is shown on the left of the autoradiograms. Bands not marked as double-stranded DNA represent single-stranded exon DNA. The mutations Y91D (not shown), R145W (exon 2), and H230P (exon 4) did not alter the mobility of the single-stranded DNAs and were detected by DNA sequencing.

Immunoprecipitation. Human embryonic kidney 293 cells were plated in 100-mm dishes at 9 × 10^5 cells/dish. Cells were transfected with either normal or mutant cDNAs on day 0 by a calcium phosphate procedure. On day 2, cells were washed in 37 °C pulse medium [cysteine/methionine-deficient Dulbecco's modified Eagle's medium (DMEM), pH 7.0] and then radiolabeled in 5 mL of medium supplemented with 300 μCi of Trans^{35}S-label for 2 h at 37 °C. Cells were washed twice in 37 °C chase medium [DMEM supplemented with 10% (v/v) fetal calf serum, 30 μM cysteine, and methionine], and incubated in chase medium at 37 °C. After incubation for 0–66 h, cells were lysed and immunoprecipitation was performed as described (Thigpen et al., 1993).

RESULTS

Initial genetic analyses of 5α-reductase deficient subjects revealed 20 different mutations in the *SRD5A2* gene (Andersson et al., 1991; Thigpen et al., 1992a,b). The mutations were present in 19 different ethnic backgrounds, suggesting a high degree of genetic heterogeneity in this disorder. A systematic review of the literature revealed a number of additional cases of 5α-reductase deficiency that had not been analyzed at the molecular level (Wilson et al., 1993). To determine if different mutations were present in the *SRD5A2* gene in these unstudied individuals, SSCP and DNA sequence analysis was performed on DNA fragments amplified from genomic DNA. As shown in Figure 1, six previously unreported mutations (P59R, R103*, P181L, N193S, P212R, and Y235F) were detected by SSCP analysis. Three additional

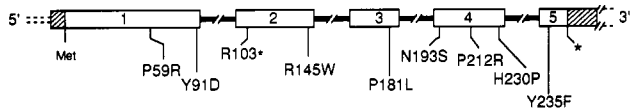

FIGURE 2: Location of mutations in the *SRD5A2* gene described in this study. A schematic of the *SRD5A2* gene is shown in the center of the figure composed of five exons (open blocks) connected by four introns (broken solid lines). The initiator methioninie is indicated in exon 1 by the abbreviation Met, and the termination codon in exon 5 is indicated by an asterisk. Untranslated regions in the gene are delineated by hatched blocks. Amino acids are indicated by the single-letter code. The nomenclature of the mutations is as follows: the first amino acid is the normal residue, the number refers to the position in the protein, and the second amino acid represents the residue found in the mutant protein.

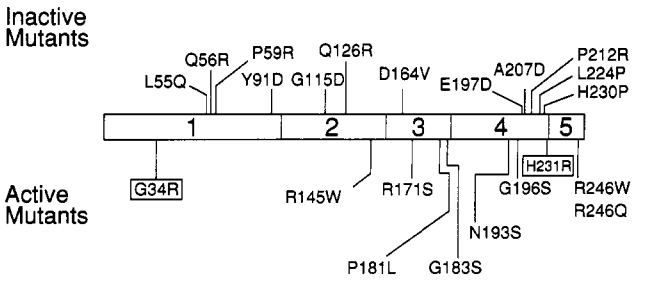

FIGURE 3: Locations of mutations that inactivate or impair 5α-reductase type 2 enzyme activity. A schematic of the protein coding region is shown in the center with the portions encoded by individual exons demarcated by blocks and numbered. Mutations that inactivate the enzyme are shown above the schematic. Those that impair enzyme activity are indicated below the schematic. Boxed mutations decrease the affinity of the protein for substrate. Non-boxed mutations below the protein schematic decrease the affinity of the enzyme for NADPH cofactor. See legend to Figure 2 for mutation nomenclature.

mutations (Y91D, R145W, and H230P) could not be detected by SSCP analysis (Figure 1 and data not shown), but were found after DNA sequence analysis of individual exons. The locations of the mutations identified in the current study are summarized in Figure 2.

To determine the effects of these missense mutations and others previously identified on enzyme activity, the appropriate base-pair substitutions were introduced into an expressible cDNA encoding the 5α-reductase type 2 isozyme. After DNA sequence analysis, the mutated cDNAs were individually transfected into cultured human embryonic kidney 293 cells and the ability of the transfected cells to convert [^{14}C]-testosterone into [^{14}C]dihydrotestosterone was determined (Table 1). As summarized in Figure 3, 12 mutations inactivated the enzyme and 10 mutations severely impaired enzyme activity.

Mutations that impaired enzyme activity were studied further to determine their biochemical effects. For each mutant enzyme, an apparent K_m for substrate (testosterone) and cofactor (NADPH) was determined. The results from a representative series of assays are shown in Figure 4. The calculated Michaelis constants (determined at V_{max}) for the individual mutations are summarized in Table 1. Two of the mutations (G34R and H231R) primarily affected the ability of the enzyme to bind testosterone, whereas the remaining eight mutations (R145W, R171S, P181L, G183S, N193S, G196S, R246W, and R246Q) decreased the affinity of the enzyme for NADPH.

We next determined the pH optima of the mutant proteins with residual enzyme activity. With one exception (R145W), the substitution mutations altered the pH optimum of the 5α-reductase type 2 isozyme. As shown in Figure 4C, the normal enzyme has a narrow acidic pH optimum centered around pH 5.0, whereas the mutant enzymes had more basic pH optima. The mutations could be divided into two classes, including (1) those that shifted the optimum approximately 0.5 pH unit and maintained the symmetric shape of the normal pH curve and (2) those that shifted the optimum more than 0.5 unit and substantially broadened the pH curve (Figure 5).

The absence of enzyme activity in cells transfected with the mutant cDNAs could be due to rapid turnover of the resulting abnormal protein or to inactivity of the enzyme. To distinguish

Table 1

mutation	activity in transfected whole cells (% conversion)[a]	pH optimum	V_{max} (nmol of DHT min^{-1} mg^{-1})[b]	K_m (testosterone) (μM)	K_m (NADPH) (μM)	protein half-life (h)
normal	55	5.0	2.0–5.0[c]	0.5–1.0	10–20	20–30
G34R[d]	1.2	5.2–6.0	0.4–0.6	10–12	8–15	20–30
L55Q	<0.2					<1
Q56R	<0.2					15–20
P59R	<0.2					5–10
Y91D	<0.2					1–2
G115D	<0.2					15–20
Q126R	<0.2					1–2
R145W	0.75	5.0	0.03–0.05	0.9–1.2	483, 584	10–15
D164V	<0.2					20–25
R171S	22.0	5.6–6.0	0.8–1.5	1.2–2.3	320, 340	15–20
P181L	0.65	6.0–6.5	0.004–0.007	1.9, 2.2	211, 526	2–4
G183S	12.0	5.1–5.3	0.3, 0.4	0.8, 1.8	144, 316	10–20
N193S	5.0	5.5–6.5	0.3, 0.4	1.8, 1.2	184, 325	5–10
G196S[d]	5.7	5.1–5.2	0.06	0.5–1.0	150–180	1–2
E197D	<0.2					1–2
A207D	<0.2					2–4
P212R	<0.2					1–2
L224P	<0.2					4–5
H230P	<0.2					1–2
H231R	8.0	5.4–6.5	0.5–1.0	13, 14	18–25	20–30
R246Q	1.5	5.3–5.5	0.002, 0.002	1.4, 0.9	201, 401	1–2
R246W[d]	2.7	5.3–5.5	0.04–0.08	0.5–1.0	600–650	1–2

[a] Percent conversion measured in transfected whole cells in the presence of 2 μM [^{14}C]testosterone after 30 min at 37 °C. [b] Maximum velocity determined at optimum pH and expressed as nanomoles of dihydrotestosterone (DHT) formed per minute per milligram of cell lysate protein. [c] A range is given when three or more assays were performed. Individual experimental values are shown when two or less assays were done. [d] Partially characterized in previous studies (see text). These mutations were restudied here and are included for completeness and to report protein half lives.

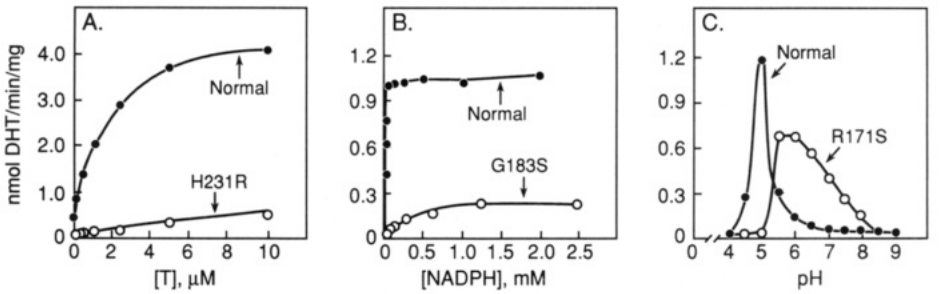

FIGURE 4: Biochemical characterization of normal and mutant 5α-reductase type 2 enzymes. Normal or mutant cDNAs were transfected into cultured 293 cells on day 0 and cell lysates were prepared on day 2. Aliquots of protein (5–150 μg) were then assayed for their ability to convert [^{14}C]testosterone to [^{14}C]dihydrotestosterone under the indicated conditions and as described in Experimental Procedures. (A) The concentration of NADPH was held constant at 10 mM and the concentration of substrate was varied as indicated. (B) The concentration of [^{14}C]testosterone was held constant at 4 μM and the level of NADPH was varied as indicated. (C) The concentration of [^{14}C]testosterone and NADPH were held constant at 5 μM and 10 mM, respectively, and the pH of the reaction buffer was varied as indicated. (●) Normal cDNA, (○) mutant cDNA. See legend to Figure 2 for mutation nomenclature.

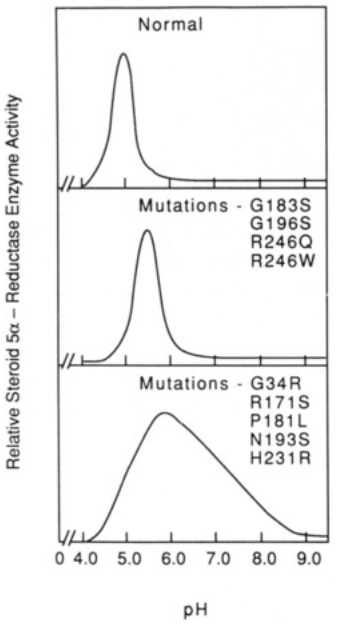

FIGURE 5: pH profiles of normal and mutant 5α-reductase type 2 enzymes. Relative 5α-reductase activity is indicated on the ordinate and pH is indicated on the abscissa. Mutations can be divided into two classes based on the shape of the activity curve.

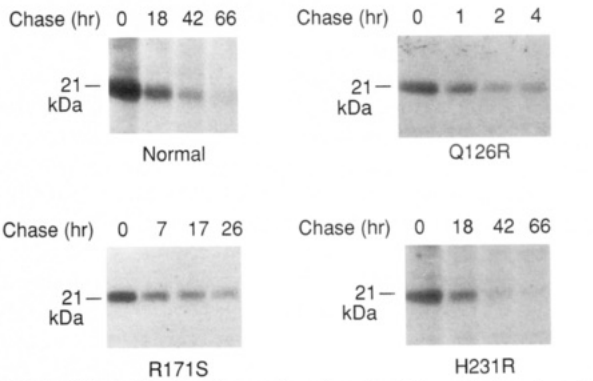

FIGURE 6: Biosynthesis of normal and mutant 5α-reductase type 2 enzymes. The indicated cDNA was transfected into cultured 293 cells on day 0 of the experiment. On day 2, cells were pulse-labeled with 300 μCi of [^{35}S]TransLabel for 2 h and then chased for the indicated periods of time in the presence of fresh medium containing excess unlabeled amino acids. Cells were harvested and lysates were subjected to immunoprecipitation as specified in Experimental Procedures. Exposure times varied from 48 to 120 h. The calculated half-lives of the 5α-reductase enzymes for the experiments shown were normal, 28 h; Q126R, 2h; R171S, 15 h; and H231R, 22 h.

these possibilities and to determine the effects of the mutations on protein turnover, the half-lives of the individual mutant enzymes were measured in pulse–chase studies in transfected cells. As shown in Figure 6 and summarized in Table 1, each of the mutant cDNAs produced an immunoprecipitable enzyme. The normal 5α-reductase type 2 protein had a half-life of approximately 20–30 h in the transfected 293 cells. The mutant proteins could be divided into three classes, including (1) those with very short half-lives of approximately 1–2 h (e.g., Q126R), (2) those with intermediate half-lives ranging from 7 to 10 h (e.g., R171S), and (3) those with essentially normal half-lives (e.g., H231R). There was no readily apparent correlation between protein half-life and enzyme activity, suggesting that most mutations directly inactivated the enzyme.

DISCUSSION

We describe the identification of nine additional mutations in the *SRD5A2* gene in subjects with 5α-reductase deficiency. The biochemical consequences of several of these mutations plus 15 previously described missense mutations were determined in cultured cells. Twelve of the mutations inactivated the enzyme, and 10 severely impaired enzyme function. Of mutations in the latter class, eight affected the ability of the enzyme to utilize NADPH cofactor and mapped in the conserved carboxyl-terminal half of the molecule, whereas two mutations affected substrate binding and mapped at both ends of the protein. Nine mutations that decreased enzyme activity also shifted the pH optimum to a more alkaline value. Twenty of the 22 missense mutations decreased the half-life of the protein.

A total of 29 mutations have now been described in the *SRD5A2* gene in subjects with 5α-reductase deficiency (Andersson et al., 1991; Thigpen et al., 1992a,b). At the molecular level, the mutations include two deletions, two nonsense mutations, one splicing defect, and 24 missense mutations. These lesions are evenly distributed among the five exons of the gene and show both founder gene effects, in which a single mutation is prevalent among a particular ethnic group, and mutational hot spots, in which the same mutation appears in multiple ethnic groups (Wilson et al., 1993). A wide spectrum of phenotypes is associated with 5α-reductase deficiency; however, a comparison of these phenotypes with the biochemical consequences of the various mutations determined here did not reveal a correlation between impairment of the enzyme and the phenotype of a given subject.

In a previous study, no mutations in the *SRD5A2* gene were detected in subjects 5R2-Los Angeles-3 and 5R2-London-3, and only one mutation was found in four other affected individuals (Thigpen et al., 1992a). Since the clinical diagnosis

of 5α-reductase deficiency was unambiguous in these subjects and since the disorder is inherited in an autosomal recessive manner, it was hypothesized that other mutations were present in regions of the gene not screened in the study. In particular, a portion of exon 1 was not examined because this region of the gene had not been cloned. Here, the missing segment was isolated both by us and by another group (Labrie et al., 1992), and analysis of this region revealed a mutation (Y91D) in subject 5R2-Los Angeles-3. However, no additional mutations were detected in this region of exon 1 in any of the other subjects. It remains likely that other mutations are present in the introns or the extreme 5′- or 3′-ends of the 5α-reductase 2 gene in the above subjects.

The 24 missense mutations provide insight into structure–function relationships in the enzyme. Since two different base-pair changes cause the same substitution of a glycine for arginine at position 34 (G34R) (Thigpen et al., 1992a), there are 23 different mutations at the amino acid level. Here, we have analyzed all but one (Y235F) of these mutations to determine their biochemical consequences for the protein. All of the mutated amino acids represent residues that are conserved between the rat and human type 1 and type 2 isozymes of 5α-reductase [see Normington and Russell (1992) for an amino acid alignment] and would thus be predicted to be important for enzyme function. In agreement with this observation, 12 of the mutations completely inactivated the enzyme (Figure 3). The mechanism of this inactivation was not determined, but it does not appear to be related to failure to synthesize protein since all mutated cDNAs produced an immunodetectable protein in transfected cells. In addition, these 12 inactivating mutations were distributed throughout the isozyme.

Ten mutations gave rise to enzymes with diminished activity. Two of these, G34R and H231R, altered testosterone binding as deduced from Lineweaver–Burk plots. Glycine 34 and histidine 231 are located at different ends of the 254 amino acid protein, suggesting that the substrate binding domain of the 5α-reductase type 2 isozyme is composed of nonlinear determinants. Amino acids involved in finasteride binding, an inhibitor that is competitive with substrate, were mapped to residues 26–29 of the type 1 isozyme of 5α-reductase (Thigpen & Russell, 1992). The comparable amino acids in the type 2 isozyme are located between residues 21 and 24 and are thus close to the G34R mutation. If these amino acids in the type 2 isozyme also interact with finasteride, then one portion of the substrate binding domain may map between residues 21 and 34 at the amino terminus of the molecule. The mechanism by which the G34R mutation disrupts substrate binding was not determined; however, it seems likely that the effect is related to the substitution of the bulky and charged arginine for the smaller glycine residue. It remains to be determined how and if the amino-terminal segment interacts with histidine 231 to form the substrate binding domain.

Histidine 231 is located in a stretch of three histidines (residues 230–232) in the type 2 isozyme. Both the rat and human type 1 and type 2 isozymes have two or three histidines at this location and mutations in two of these (residues 230 and 231) inactivate or impair, respectively, the type 2 isozyme. This data and the conservation among isozymes and species imply that these histidine residues are catalytically important. A logical role for one or more of these residues would be in metal binding; however, 5α-reductase does not appear to require metal cations for activity as high concentrations of chelators do not inhibit the enzyme (Wilson, 1975). Alternatively, one or more of these histidines may actively participate in catalysis.

Eight different amino acids that contribute to NADPH cofactor binding reside in the carboxyl-terminal half of the type 2 isozyme (Figure 3). The mechanism by which mutation of these residues alters the affinity of the enzyme for NADPH has not been determined. However, these mutant proteins maintained a near normal affinity for testosterone substrate (Table 1); thus, it is unlikely that the mutations result in a global denaturation of the protein. The 5α-reductase enzymes do not have consensus NADPH selectivity residues or adenine nucleotide binding sequences identified in other more well-studied reductase enzymes (Perham et al., 1991). It is therefore conceivable that these eight residues define a novel class of NADPH binding sites. Mutations in these eight residues also decrease the half-life of the protein as determined by immunoprecipitation experiments (Table 1). Similar conclusions regarding enzyme stability and NADPH binding were reached by analyzing mutant 5α-reductase enzymes in fibroblasts biopsied from subjects with 5α-reductase deficiency (Leshin et al., 1978). Taken together, these findings suggest that NADPH binding may stabilize the enzyme within the cell and that variations in the intracellular levels of cofactor could regulate turnover of 5α-reductase.

Nine of the 10 mutations that decrease but do not eliminate enzyme activity also alter the pH optimum of the type 2 isozyme (Figure 5). The normal type 2 isozyme has an unusually narrow and acidic pH optimum in cell lysates but is thought to function at a neutral pH within the cell (Thigpen et al., 1993). The symmetric shape of the pH curve suggests that a small number of amino acid residues, presumably in or around the substrate or cofactor binding sites, give rise to this apparent *in vitro* phenomenon. However, the finding that mutations mapping throughout the enzyme alter the pH optimum argues that this suggestion may be an oversimplification.

The type 2 isozyme has a roughly 50-fold higher affinity for testosterone at neutral pH versus acidic pH (Faller et al., 1993; Thiigpen et al., 1993). This difference is another indication that the enzyme functions at a neutral pH within the cell. In the current studies, measurement of apparent substrate K_ms was carried out at the optimum pH for each mutant enzyme (Table 1). We also attempted to determine this value at neutral pH; however, the very low levels of mutant enzyme activity precluded an accurate determination of the apparent K_m at this pH.

In summary, the current studies identify amino acid residues that participate in substrate and cofactor binding in the type 2 isozyme of 5α-reductase. These apparently crucial amino acids will serve as guides in future mutagenesis studies to further dissect structure–function relationships in this membrane-bound reductase enzyme.

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REFERENCES

Andersson, S., Berman, D. M., Jenkins, E. P., & Russell, D. W. (1991) *Nature 354*, 159–161.

Bondy, P. K. (1981) in *Williams Textbook of Endocrinology* (Wilson, J. D., & Foster, D. W., Eds.) pp 816–890, W. B. Saunders, Philadelphia, PA.

Faller, B., Farley, D., & Nick, H. (1993) *Biochemistry 32*, 5705–5710.

Hirsch, K. S., Jones, C. D., Audia, J. E., Andersson, S., McQuaid, L. A., Stamm, N. B., Neubauer, B. L., Pennington, P., Toomey, R. E., & Russell, D. W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5277–5281.

Jenkins, E. P., Andersson, S., Imperato-McGinley, J., Wilson, J. D., & Russell, D. W. (1992) *J. Clin. Invest. 89*, 293–300.

Labrie, F., Sugimoto, Y., Luu-The, V., Simard, J., Lachance, Y., Bachvarov, D., Leblanc, G., Durocher, F., & Paquet, N. (1992) *Endocrinology 131*, 1571–1573.

Leshin, M., Griffin, J. E., & Wilson, J. D. (1972) *J. Clin. Invest. 247*, 685–691.

Mauvis-Jarvis, P., Kuttenn, F., Mowszowicz, I., & Wright, F. (1981) *Clin. Endocrinol. 14*, 459–469.

Mendonca, B. B., Batista, M. C., Arnhold, I. J. P., Nicolau, W., Madureira, G., Lando, V. S., Kohek, M. B. F., Carvalho, D. G., & Bloise, W. (1987) *Rev. Hosp. Clin. Fac. Med. Sao Paulo 42*, 66–68.

Normington, K., & Russell, D. W. (1992) *J. Biol. Chem. 267*, 19548–19554.

Perham, R. N., Scrutton, N. S., & Berry, A. (1991) *BioEssays 13*, 515–525.

Russell, D. W., & Wilson, J. D. (1994) *Annu. Rev. Biochem. 63*, 25–61.

Saenger, P., Goldman, A. S., Levine, L. S., Korth-Schutz Muecke, E. C., Katsumata, M., Doberne, Y., & New, M. I. (1978) *J. Clin. Endocrinol. Metab. 46*, 627–634.

Thigpen, A. E., & Russell, D. W. (1992) *J. Biol. Chem. 267*, 8577–8583.

Thigpen, A. E., Davis, D. L., Milatovich, A., Mendonca, B. B., Imperato-McGinley, J., Griffin, J. E., Francke, U., Wilson, J. D., & Russell, D. W. (1992a) *J. Clin. Invest. 90*, 799–809.

Thigpen, A. E., Davis, D. L., Gautier, T., Imperato-McGinley, J., & Russell, D. W. (1992b) *N. Engl. J. Med. 327*, 1216–1219.

Thigpen, A. E., Cala, K. M., & Russell, D. W. (1993) *J. Biol. Chem. 268*, 17404–17412.

Warne, G. L., Macaulay, J. O., Reed, G., Montalto, J. E., Yong, A. B. W., Whorwood, C. B., Pitt, J., & Barnett, J. S. (1988) Program of the 8th International Congress of Endocrinology, Abstract 16-22-287.

Wilson, J. D. (1975b) *Handb. Physiol. 5*, 491–508.

Wilson, J. D., Griffin, J. E., & Russell, D. W. (1993) *Endocrine Rev. 14*, 577–593.