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Molecular Endocrinology of Hydroxysteroid Dehydrogenases*

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I. Introduction

HYDROXYSTEROID dehydrogenases (HSDs) play pivotal roles in the biosynthesis and inactivation of all steroid hormones. In steroidogenic tissues they catalyze the final steps in androgen, estrogen, and progesterone biosynthesis. In peripheral tissues, including steroid hormone target tissues, they convert potent steroid hormones into inactive metabolites and regulate the amount of hormone that can bind to members of the nuclear receptor superfamily, ultimately regulating gene expression. Target cells may depend on these reactions to control specificity of response to steroid hormones.

Because HSDs catalyze bidirectional reactions, it has been difficult to understand how they can be involved in both the synthesis and inactivation of steroid hormones. cDNA cloning indicates that each HSD exists in multiple isoforms, which show tissue specificity in expression; this, coupled with the properties of each isoform (reductase or dehydrogenase), can determine the role of the enzyme in steroid hormone action. Advances in cDNA isolation have led to the concept that HSDs belong to at least two distinct protein phylogenies: the short-chain dehydrogenase/reductase family (SDR; formerly short-chain alcohol dehydrogenase) whose members include the 3β-HSD/ketosteroid isomerase (3β-HSD/KSI), 11β-HSD, and 17β-HSD (1, 2), and the aldo-keto reductase (AKR) superfamily (3–6) whose members include 3α-HSD and 20α-HSD. Three-dimensional structures now exist for mammalian HSDs that belong to each of the two families and serve as templates for structure-function studies on HSDs within each protein family. These represent the first available structures for mammalian enzymes involved in steroidogenesis and steroid metabolism.

Because of their central role in steroid hormone action, HSDs as a group of enzymes are considered therapeutic targets. Several reviews (7, 8) have been written on the design

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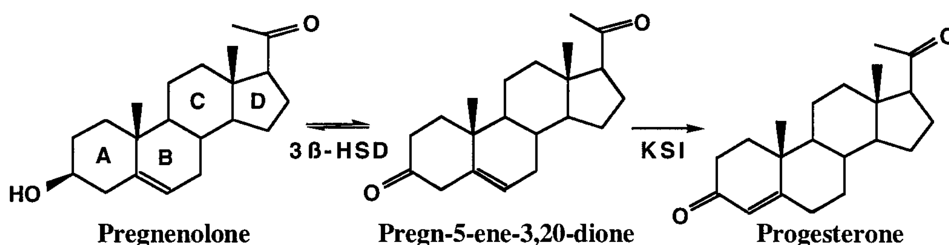
of HSD inhibitors, and that material will not be reviewed here. It is sufficient to say that specificity in drug design may depend on the successful targeting of a tissue-specific isoform.

II. 3β-Hydroxysteroid Dehydrogenase/Ketosteroid Isomerase (3β-HSD/KSI)

A. Physiological and pharmacological significance

The dual functional 3β-HSD/KSI catalyzes the formation of Δ^4-3-ketosteroids. The enzyme catalyzes two reactions, the dehydrogenation of 3β-equatorial hydroxysteroids and the subsequent isomerization of the Δ^5-3-ketosteroid products to yield the α,β-unsaturated ketones. The majority of steroid hormones, with the exception of estrogens and the 5α-reduced androgens, contain this functional group. Since these latter hormones are themselves derived from Δ^4-3-ketosteroids, 3β-HSD/KSI is required for all steroid hormone biosynthesis. It should be noted that, in the placenta and corpus luteum, 3β-HSD/KSI is responsible for the final steps in progesterone biosynthesis necessary for the maintenance of pregnancy (Fig. 1). In the adrenal cortex the 3β-HSD/KSI plays a pivotal role in the formation of glucocorticoids and mineralocorticoids.

Development of selective inhibitors of tissue-specific isoforms of HSDs is a desirable goal. This is nowhere more apparent than in the 3β-HSD/KSIs. Inhibitors of the placental and ovarian 3β-HSD/KSI would prevent the biosynthesis of progesterone, and such agents would be useful as antiprogestational agents for the termination of pregnancy and could act as abortifacients and postcoital contraceptives. However, if the adrenal 3β-HSD/KSI were inhibited, chronic administration of such agents would lead to adrenal hyper-



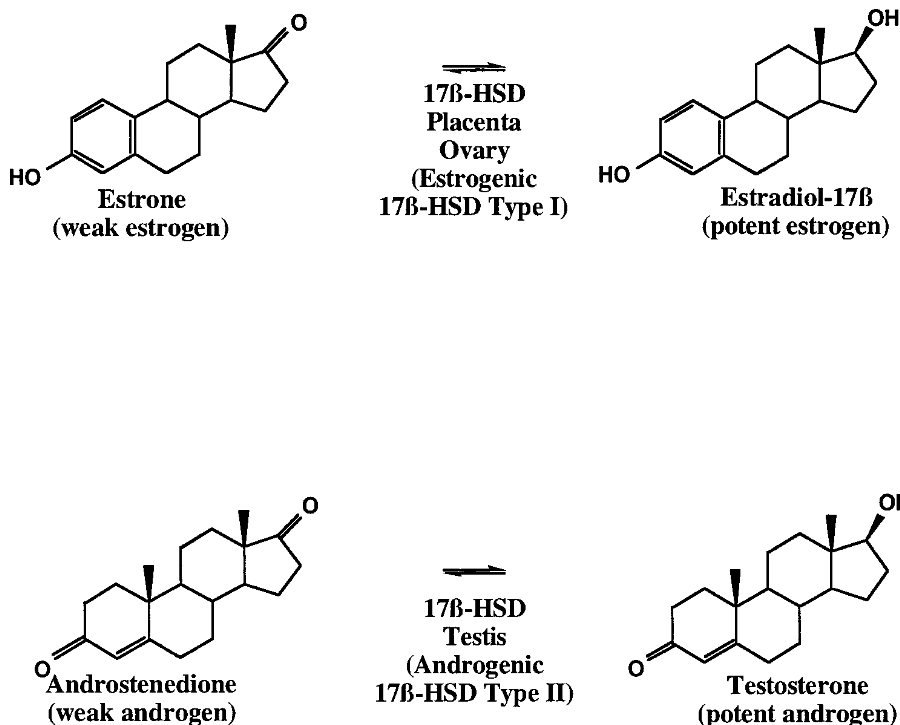


FIG. 1. Reactions catalyzed by HSDs in steroidogenic tissues. Reactions (*top*) are those catalyzed by the bifunctional 3β-HSD/KSI found in the adrenal, placenta, ovary, testis, and skin. Reactions (*bottom*) are those catalyzed by the estrogenic and androgenic 17β-HSDs.

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plasia. cDNA cloning indicates that because the human isoforms present in the adrenal and placenta are distinct (9–11), further chronic use of antiprogestational agents may not be required for an antiprogestational effect. This suggests that the development of antiprogestins targeting 3β-HSD/KSI isoforms is an achievable goal. One effective compound, epostane (12), has withstood a clinical trial in Europe (13). In fact, epostane was found to lower circulating progesterone levels markedly and thereby terminate early and midstage pregnancy.

B. Cloning and expression of the 3β-HSD/KSI cDNAs

The purification of the dual functional 3β-HSD/KSI from human placenta microsomes (14) led to the development of a polyclonal antibody, which enabled the screening of a human placental cDNA λgt-11 expression library (9). This was a remarkable achievement because numerous investigators had failed to solubilize and purify the 3β-HSD/KSI activities from either adrenal or placental microsomes. Because the cDNA for human placental 3β-HSD/KSI was the first to be isolated, it was termed type I 3β-HSD/KSI. The cDNA for the type I enzyme encodes a protein of 372 amino acids with a calculated molecular weight of 42,216. The type I enzyme was transiently expressed in mammalian cells and catalyzed the conversion of dehydroepiandrosterone and pregnenolone to produce androstenedione and progesterone, respectively (10, 11). In addition, when transfected cell homogenates were supplemented with NAD^+, they efficiently oxidized 5α-androstane-3β,17β-diol to 5α-dihydrotestosterone (5α-DHT), and upon addition of NADH the homogenates reduced 5α-DHT back to the diol. These data ended an ongoing and lengthy debate by establishing unequivocally that a single polypeptide chain could catalyze both the dehydrogenase and isomerase reactions. The availability of this cDNA clone led to the isolation of a cDNA for human adrenal or type II 3β-HSD/KSI (11). Human type I and type II 3β-HSD/KSI share in excess of 90% sequence identity. Transient expression of these two isoforms revealed that the catalytic efficiency (V_{max}/K_m) of type I is 5.9-, 4.5-, and 2.8-fold higher than that of the type II 3β-HSD using pregnenolone, dehydroepiandrosterone, and dihydrotestosterone (reverse direction) as substrates, respectively (11). The higher Michaelis-Menten constant (K_m) values observed for type II 3β-HSD, which is expressed mainly in steroidogenic tissues, could be related to the higher levels of endogenous substrates present in these tissues (15).

Subsequently, four subtypes of rat 3β-HSD/KSI have been cloned (type I through type IV); because numbers were assigned in order of discovery, the rat isoforms do not correspond to the human isoforms by number (16–18). Rat type I and type II 3β-HSD/KSI share 93.8% sequence homology, yet the type I enzyme has a relative specific activity that is 64- and 46-fold higher than the type II enzyme for pregnenolone and dehydroepiandrosterone, respectively. The lower activity of the type II enzyme is due to a change in four amino acids in a membrane-spanning region predicted to exist by hydropathy plots (16). Construction of chimeric 3β-HSDs, in which the membrane-spanning domain was reinserted into the type II enzyme, restored the specific activity of the protein (16). Rat type III 3β-HSD/KSI appears to be found exclusively in the liver where it appears to function predominantly as a liver-specific 3-ketoreductase to inactivate steroid hormones (17). Rat type IV 3β-HSD/KSI is a novel form found in the skin but, remarkably, is the predominant form found in rat placenta. Ribonuclease (RNase) protection studies have provided a thorough analysis of the tissue distribution of these isoforms (18). Such studies have shown that rat type IV is equivalent to human type I 3β-HSD/KSI (placental form), and rat types I and II are related to human type II 3β-HSD/KSI (adrenal form). Since the isolation of the human and rat clones, cDNA clones encoding for the murine adrenal (19), bovine ovary (20), and macaque ovary (21) have been isolated. Initial pair-wise comparison of these sequences indicate that they share in excess of 80–90% homology with each other but little or no homology with other HSDs (Table 1).

C. Structure, regulation, and tissue-specific expression of the 3β-HSD/KSI genes

Most *in vivo* regulation studies on the 3β-HSD isoforms have been performed in the rat and may be relevant to their human orthologs. Interest in the regulation of the adrenal 3β-HSD/KSI exists because of its pivotal role in adrenal steroidogenesis and the knowledge that deficiencies in this

TABLE 1. Percent amino acid sequence identities among the major forms of 3βHSD/KSI

	Mouse			Rat				Bovine	Macaque	Human	
	II[a]	III	IV	I	II	III	IV	I	I	I	II
Mouse I	85	83	77	88	86	77	86	73	72	72	71
Mouse II	—	91	75	84	84	78	88	75	73	72	72
Mouse III	—	—	73	82	82	75	89	74	71	71	70
Mouse IV	—	—	—	78	77	83	77	67	68	68	68
Rat I	—	—	—	—	94	80	91	74	73	72	72
Rat II	—	—	—	—	—	80	88	74	72	72	71
Rat III	—	—	—	—	—	—	79	69	68	68	67
Rat IV	—	—	—	—	—	—	—	76	75	73	73
Bovine I	—	—	—	—	—	—	—	—	79	79	78
Macaque I	—	—	—	—	—	—	—	—	—	94	96
Human I	—	—	—	—	—	—	—	—	—	—	94

[a] As mouse II is a partial cDNA, missing amino acids 1–109, comparisons with mouse II include only amino acids 110–373. For comparison of all other forms, amino acids 1–373 were included. [Reproduced with permission from T. R. Clarke *et al.*: *Mol Endocrinol* 7:1569–1578, 1993. © The Endocrine Society.]

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enzyme can result in congenital adrenal hyperplasia. Studies on the rat adrenal 3β-HSD/KSI revealed that its mRNA was up-regulated by ACTH in intact male rats and down-regulated by corticosterone. Hypophysectomy reduced mRNA levels markedly, and these could be elevated by the administration of ACTH (22). These data indicate that the gene is regulated by the adrenal-pituitary axis in a manner consistent with controlling circulating glucocorticoid levels. The stimulating effect of ACTH on 3β-HSD/KSI expression has also been observed in bovine adrenocortical cells (23). The ACTH receptor is a member of the G protein-coupled receptor family and acts through the protein G_s to activate adenylyl cyclase and increase intracellular cAMP. The protein kinase A- dependent signaling mechanism that results in altered transcription of the 3β-HSD/KSI gene, however, has not been fully elucidated.

Interest in the regulation of the ovarian 3β-HSD/KSI exists because it catalyzes the final step in progesterone biosynthesis. Down-regulation of this enzyme may contribute to declining progesterone levels, which is a sign of luteolysis. In rat ovary, 3β-HSD/KSI mRNA and enzyme activity are decreased by ovine PRL and up-regulated by human CG (hCG) (24, 25). These responses are physiologically relevant because PRL stimulates luteolysis whereas hCG is luteotrophic. Changes in mRNA were confirmed by *in situ* hybridization studies using luteinized porcine granulosa cells. The inhibitory effect of PRL on 3β-HSD expression and activity is correlated with a progressive decrease in serum progesterone concentration while serum pregnenolone levels are elevated. These findings indicate that inhibition of 3β-HSD/KSI gene expression in the corpus luteum occurs early in the luteolytic process induced by PRL and could well play a role in PRL-induced luteolysis.

In luteinized porcine granulosa cells, gonadotropins, as well as agents that increase intracellular cAMP accumulation (cholera toxin, forskolin, $(Bu)_2$cAMP), increased 3β-HSD mRNA levels (25). In contrast, activation of the protein kinase C pathway induced cAMP accumulation but led to a marked inhibition of the stimulatory effect of hCG, LH, forskolin, and cholera toxin on 3β-HSD mRNA levels (24). The cross-talk that occurs between these two signaling pathways to regulate 3β-HSD/KSI expression in granulosa cells remains to be elucidated.

In the rat testis, 3β-HSD/KSI is required for androgen biosynthesis. Not surprisingly, hCG, acting as a mimic of LH, caused a 3.0-, 19.7-, and 11.5-fold increase in steady state levels of testicular 3β-HSD mRNA, immunoreactive 3β-HSD/KSI protein, and enzyme activity, respectively, in hypophysectomized male rats (15).

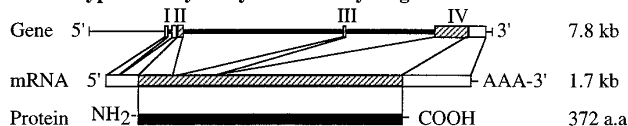
In rat placenta, the temporal modulation of type I, II, and IV 3β-HSD/KSI has been measured during the second half of gestation. It will be recalled that rat type IV 3β-HSD/KSI is most similar to human placental type I 3β-HSD/KSI. It was found that type I and II 3β-HSD mRNA levels are modulated in a closely parallel manner, peaking sharply on day 15 but falling abruptly thereafter. On the other hand, type IV 3β-HSD mRNA levels remained elevated and were stable from days 10–17 of gestation, suggesting that this form of the enzyme is responsible for the substantial increase in 3β-HSD and hence progesterone production that occurs in midpregnancy (15).

Collectively, the studies on the regulation of rat tissue-specific isoforms of 3β-HSD/KSI indicate that expression is under the control of trophic hormones and that alterations in expression occurs in response to the need to change circulating steroid hormone levels. The ability of the appropriate trophic hormone to increase 3β-HSD/KSI mRNA levels and protein and enzyme activities on demand in the adrenal, ovary, and testis points to common mechanisms of regulation that involve cAMP.

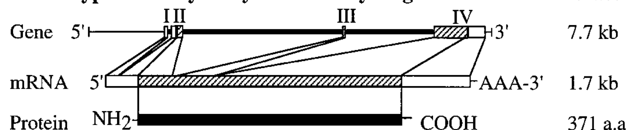
To gain insight into the regulation of the human 3β-HSD/KSI genes, type I and type II genes have been cloned (26, 27). Each gene is 7.8 kb in length and consists of four exons and three introns. Both genes are localized to chromosome 1p13.1 (Fig. 2). Both genes encode for transcripts that are 1.7 kb in length. Because the type I gene is expressed at high levels in syncyctial trophoblasts and the type II gene is expressed at high levels in the adrenal cortex, gene regulation studies were performed in human choriocarcinoma (JEG-3) cells and human adrenocortical tumor cell lines H295 cells, respectively.

In human JEG-3 cells, the 1.7-kb transcript is up-regulated by a cAMP-dependent mechanism and by phorbol esters. The phorbol ester response is not mediated by a diacylglycerol-dependent kinase (28). The increases in steady state levels observed in 3β-HSD/KSI mRNA after treatment with

Human Type I 3ß-Hydroxysteroid Dehydrogenase/Δ^5-Δ^4 Isomerase



Human Type II 3ß-Hydroxysteroid Dehydrogenase/Δ^5-Δ^4 Isomerase



Human Type I 17ß-Hydroxysteroid Dehydrogenase Gene

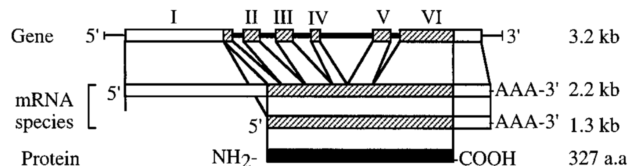


FIG. 2. Organization of the human type I 3β-HSD/KSI, type II 3β-HSD/KSI, and Type I 17β-HSD genes. Noncoding exons are *open boxes*, coding exons are *stippled boxes*, and *narrow solid lines* are introns. [Reprinted from F. Labrie *et al.*: *Baillieres Clin Endocrinol Metab* 8:451–474, 1994 (15) by permission of the publisher, W. B. Saunders Company Limited, London.]

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cAMP or phorbol esters can be observed in the presence of cycloheximide and occur independently of new protein synthesis.

In human adrenal cells, activators of the protein kinase A-signaling pathway [*e.g.* forskolin, $(Bu)_2cAMP$] enhance the production of dehydroepiandrosterone and androstenedione with a concomitant increase in steady state levels of type II 3β-HSD and 17α-hydroxylase (CYP17). In contrast, phorbol ester treatment dramatically elevated 3β-HSD mRNA but attenuated 17α-hydroxylase mRNA. The 5′-flanking region of the 3β-HSD type II gene was found to contain a consensus sequence for the orphan nuclear receptor steroidogenic factor 1 (SF-1). The functionality of the SF-1 site was tested in promoter-chloramphenicol acetyl transferase constructs in which the SF-1 site was either present or deleted. It was found that the phorbol ester response had an absolute requirement for the SF-1 element, and that the response was further enhanced if the cDNA encoding for SF-1 was cotransfected (29).

In an attempt to identify *cis*-regulating elements that may control tissue-specific expression of the type I and type II 3β-HSD/KSI genes in the placenta and adrenal, respectively, it was found that a strong positive regulatory element exists in the first intron of the type II gene. This 3β 1-A element was able to drive transcription of a reporter gene in both placenta cells (JEG-3) and adrenal cells (SW13) and was found to bind four proteins including SP-1 (30). Therefore, this intronic element appears to be important for gene transcription but is not responsible for the tissue-specific expression of the type II gene (30).

D. 3β-HSD deficiencies

Classic 3β-HSD/KSI deficiency can cause the salt-wasting and non-salt-wasting forms of congenital adrenal hyperplasia due to cortisol deficiency with or without aldosterone deficiency, respectively. It is estimated that defects in this enzyme are responsible for 10% of the patients presenting with congenital adrenal hyperplasia and that the remainder have defects in either the steroid 21-hydroxylase (CYP21) or 17α-hydroxylase/20-lyase genes (CYP17). In its most severe form, 3β-HSD deficiency results in the blockade of steroidogenesis in both the adrenal and gonads, and as a result there is an elevated steroid-5-ene to steroid-4-ene ratio. This manifests itself in males as pseudohermaphroditism and in females as virilization at birth. Since the human type II 3β-HSD gene is the predominant form in these tissues, it is not surprising that the defects occur in this gene product. Simard and colleagues (31–33) analyzed 12 male and four female patients who had defects in this gene. It was found that there were 14 unique point mutations in the type II 3β-HSD gene. In the most severe form of the disease, which is salt-wasting, the mutations detected were as follows: W171Stop (nonsense), which may coexist with an insertion of a single C between codon 186 and 187, which leads to a truncated protein at 202; deletion of K273 (frameshift) and point mutations Q142K; Y253N; G15D; L108W; and P86L (missense) (34–36). In the more moderate form of the disease, the non-salt-wasting disease, the following point mutations (missense) were detected: A245P; G129R; N100S; Y254D; L173R; and A82T (31, 37, 38). Several of these mutations were introduced into the type II 3β-HSD/KSI cDNA, and the resultant mutant proteins were expressed in COS-1 cells. Kinetic analysis of the mutants revealed that of the point mutations present in the salt-wasting form, the G15D and the G15A mutants showed a 4-fold decrease in catalytic efficiency for the conversion of pregnenolone to progesterone, and the L108W and P186L mutants resulted in a decrease in catalytic efficiency of 40-fold (Table 2). The reduction in catalytic efficiencies observed with the G15D and G15A mutants has been partially explained by the increase in K_m for NAD^+. This glycine residue is predicted to form part of a Gly-XXX-Gly-X motif found in the Rossmann fold for cofactor binding (39)(see *Sections VII* and *VIII*). Interestingly, the N100S mutant found in the non-salt-wasting form of the disease resulted in a 30-fold decrease in catalytic efficiency. The similar decreases in catalytic efficiency observed with mutations found in the salt and non-salt wasting forms of the disease indicate that they alone cannot explain the genetic lesions responsible for the differences in the two diseases.

III. 17β-Hydroxysteroid Dehydrogenases

A. Physiological and pharmacological significance

Like the 3β-HSD/KSIs, the 17β-HSDs play essential roles in steroidogenesis. 17β-HSD isozymes catalyze the final steps in androgen and estrogen biosynthesis. In the Leydig

TABLE 2. Kinetic parameters of native type II 3β-HSD and mutant proteins found in patients with the salt-wasting form of classic 3α-HSD deficiency (G15D, G15A, N100S, L108W, and P186L) and mutant proteins found in patients with the non-salt-wasting form of classic 3β-HSD deficiency (N100S)

Protein	K_m (μM) PREG	Relative V_{max} (%) PREG	Relative V_{max} PREG/K_m PREG	K_m (μM) NAD	Relative V_{max} (%) NAD	Relative V_{max}NAD/K_m NAD
Type II 3β-HSD	0.72	100	139	22	100	4.54
G15D	3.2	120	37.5	113	121	1.07
G15A	3.4	134	39.4	148	116	0.78
Type II 3β-HSD	1.7	100	58.8	24	100	4.2
L108W	12.0	16.1	1.3	678	17.4	0.025
P186L	18.0	20.7	1.2	920	17.4	0.019
Type II 3β-HSD	3.5	100	28.6	20	100	5
N100S	25	19.3	0.8	650	30	0.05

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cells of the testis, 17β-HSD converts androstenedione (a weak androgen) into testosterone (a potent androgen) (40). By contrast, 17β-HSD in the placenta and ovary will catalyze the final step in estrogen biosynthesis by converting estrone (a weak estrogen) into 17β-estradiol (a potent estrogen) (41, 42) (Fig. 1). When these actions occur in androgen or estrogen target tissues, 17β-HSD subtypes are in a unique position to control ligand occupancy of the respective steroid hormone receptor and could work as molecular switches. Such actions of 17β-HSD may control the growth of hormonally dependent cancers of the prostate and mammary gland (15, 43, 44). There is thus a need to develop isozyme-specific 17β-HSD inhibitors, and this area has been reviewed recently (8).

B. Cloning and expression of the 17β-HSD cDNAs

Different isozymes have been designated for 17β-HSD (types I-IV) and are named according to the chronological order in which their cDNAs were cloned. The type I 17β-HSD is the soluble form originally purified from human placenta by Jarabak *et al*. (41, 42); it converts estrone to 17β-estradiol using NADPH as cofactor and is also known as estrogenic 17β-HSD. This is the principal activity of this enzyme since it displays a 100-fold higher affinity for C18 than for C19 steroids. It functions as a homodimer and displays modest 20α-HSD activity. Its availability led to the development of a polyclonal antibody that was used to screen a bacteriophage expression library. The cDNA clone for human type I 17β-HSD was isolated by three groups (45–47) and encodes a protein of 327 amino acids. It contains no obvious membrane-spanning domains. When the recombinant enzyme was transiently expressed in cultured mammalian cells, it almost exclusively catalyzed the reduction of estrone to 17β-estradiol as predicted by the earlier biochemical studies (48). The enzyme has also been overexpressed in *Baculovirus*-infected Sf9 insect cells, and the resultant recombinant protein retained the properties of the purified enzyme (49, 50).

Type II 17β-HSD is a microsomal form and uses NAD^+ as cofactor. It catalyzes the oxidation of testosterone and 17β-estradiol to form androstenedione and estrone, respectively. Its principal function is to inactivate circulating androgens and estrogens. It is located in the liver, small intestine, secretory endometrium, and placenta, which would be consistent with its role in steroid hormone inactivation (40). The cDNA for type II 17β-HSD was isolated by expression cloning of a cDNA library from human prostate mRNA using cytomegalovirus promoter constructs (51). The cDNA encodes a protein of 387 amino acids, which contained an amino-terminal signal anchor motif and a carboxy-terminal endoplasmic reticulum retention motif that would locate the enzyme in the membrane. The presence of a hydrophobic amino acid terminus in type II 17β-HSD distinguishes the enzyme from the type I 17β-HSD, which has a hydrophilic amino acid terminus. When the cDNA for type II 17β-HSD was expressed in human embryonal kidney 293 cells, it also displayed modest 20α-HSD activity and converted 20α-hydroxyprogestrone to progesterone (51), a reaction that produces an active progestin.

Type III 17β-HSD, also known as androgenic 17β-HSD, is found in the microsomes of the testis where it reduces androstenedione to testosterone (40). It prefers NADPH as cofactor. Using an expression-cloning strategy, the cDNA for human testis type III 17β-HSD was obtained (52). The cDNA encodes a protein of 310 amino acids. Analysis of the amino acid sequence for the type III enzyme shows a hydropathy profile that does not support the presence of any membrane-spanning regions, but the short hydrophobic N- and C-termini may associate the protein with the endoplasmic reticulum. Pairwise comparisons between the three 17β-HSD isozymes show that the amino acid identities between the type I, type II, and type III proteins are approximately 23% (51, 52), indicating that these enzymes are members of the same family (53).

Recently, a type IV 17β-HSD has been found in human, mouse, and pig. It is similar to the type II isoform in that it is NAD^+ dependent and is principally involved in the oxidation and therefore inactivation of estrogens and androgens. In porcine gonads, immunofluorescence detected the type IV 17β-HSD in granulosa cells as well as the Leydig cells and Sertoli cells in the testis. The cDNA encodes an 80-kDa protein featuring domains not present in other 17β-HSDs (54). The 80-kDa protein is cleaved at the N terminus to yield a 32-kDa fragment with 17β-HSD activity. Both the 80-kDa and the N-terminal 32-kDa fragment have 17β-HSD activity (55). The central domain (324–596 amino acids) is 40% identical to the C-terminal domain of the fatty acid hydratase/dehydrogenase of yeast and catalyzes β-oxidation of fatty acids. The C-terminal domain is 39% identical with sterol carrier protein. Type IV 17β-HSD is found in a number of human cells including those from the breast (MCF-7 and T47D) and liver (HepG2) (56). The peroxisomal location of this isozyme suggests that the protein may have other as yet undefined roles in lipid and steroid metabolism (57).

C. Structure, regulation, and tissue-specific expression of the 17β-HSD genes

Two major mRNA species for type I 17β-HSD, 2.2 kb and 1.3 kb in length, have been identified in human placenta. S1 nuclease analysis indicates that in placenta the major transcript is 1.3 kb in length and starts nine nucleotides upstream from the start codon. The 2.2-kb transcript is a minor species and contains approximately 971 nucleotides upstream from the same in frame start codon (58). Type I 17β-HSD mRNA is distributed in placenta, liver, ovary, endometrium, prostate, testis, and adipose tissue. Immunochemical analyses have confirmed the presence of the type I enzyme in the syncytiotrophoblast of human placenta (59), the granulosa cells of human ovary (60), the epithelial cells of human breast (43), and the endometrium (61). In studies on estrogen-sensitive human breast cancer cell lines (ZR-75–1, MCF-7, T-47D, and R-27), type I 17β-HSD was elevated and favored formation of 17β-estradiol. In estrogen receptor-negative cell lines, the activity and expression were considerably lower (44, 62) and estrone formation was favored. Data such as these indicate that type I 17β-HSD may be an important component in estrogen-dependent growth of mammary tissues and a target for drug development.

The regulation of the type I 17β-HSD 1.3- kb transcript has been measured in JEG-3 cells. The mRNA for 17β-HSD was

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elevated with an analog of cAMP and a protein kinase C agonist, phorbol-12-myristrate-13-acetate. The effects of cAMP were mimicked with forskolin and isobutyl methyl xanthine (a phosphodiesterase inhibitor) but were not abolished by a cAMP-dependent protein kinase inhibitor. By contrast, the effects of protein kinase C were abolished with a diacylglycerol kinase inhibitor. This indicates that type I 17β-HSD is regulated by a nonclassic cAMP-dependent mechanism that remains to be elucidated (28, 63).

The expression pattern of the type II 17β-HSD mRNA in human tissues supports its role as a steroid hormone inactivator. The 1.5-kb transcript is most highly expressed in liver, followed by the placenta, small intestine, and endometrium. The glandular epithelium of the endometrium almost exclusively expressed the type II 17β-HSD, and it is positively regulated by progestins to inactivate estrogens (64).

The 1.3-kb mRNA encoding type III 17β-HSD has been detected only in the testis, which is consistent with its role in the formation of testicular androgens (52).

To understand the basis of the tissue-specific and regulated 17β-HSD gene expression, the human type I, II, and III 17β-HSD genes have been cloned (52, 53, 58, 65). The type I gene consists of seven exons and five introns that span 6.2 kb and encode a transcript of 2.2 kb with a long 5′-untranslated region (UTR) (Fig. 2). A highly homologous pseudogene (previously designated 17β-HSD I) has also been cloned, but there is evidence that the pseudogene is not expressed (46, 63, 66). The pseudogene contains eight exons and five introns. The two genes appear in tandem and together span more than 21 kb. These genes were mapped to the long arm of chromosome 17 and more specifically to the region q11-q12 or q12–21 (46, 63, 66). It is now known that the gene for human type I 17β-HSD is closely linked to the susceptibility gene for hereditary breast and ovarian cancer, the *BRCA1* gene on chromosome 17q21 (46). Both 17β-HSD genes contain a promoter region with a TATA box as well as GC and inverse CAAT boxes. The 5′-flanking regions contained consensus sequences for *cis*-acting elements that may function as regulators of 17β-HSD gene expression. These sequences included estrogen, progesterone, and glucocorticoid response elements and a cAMP response element (67). No functional studies on the 17β-HSD type I gene promoter have been reported.

The type II 17β-HSD gene is located on chromosome 16q24. The gene was isolated from a human leukocyte genomic DNA library (65). The type II gene contains seven exons and spans 40 kb. This gene gives rise to two alternatively spliced mRNAs, but only the major transcript (1.45 kb) is functional and encodes for the 387-amino acid protein. The cap site is between 179 and 167 nucleotides upstream from the ATG start codon (65).

In contrast, the type III 17β-HSD gene (androgenic 17β-HSD) is found on chromosome 9q22. This gene contains 11 exons and is in excess of 60 kb in length (52, 53). Although the lack of chromosomal synteny between the type I-III 17β-HSDs and the low degree of sequence homology suggested that they arose by convergent evolution, they appear to be SDR family members (53).

D. 17β-HSD deficiency

Defects in 17β-HSD were originally described by Saez and colleagues (68), who reported familial male pseudohermaphroditism due to a deficiency in testicular 17-ketoreductase. The characteristic phenotype is a 46,XY individual with testes and male Wolffian duct-derived urogenital structures (*e.g.* epididymus, vas deferens, and seminal vesicles), but with external female genitalia. These individuals are usually classified as females at birth but are genetic males and develop a male phallus at puberty. It is recognized that the 17β-HSD deficiency, which results in pseudohermaphroditism, results from autosomal recessive mutations in the type III 17β-HSD gene (androgenic form) (69–71). This defect underscores the importance of the type III 17β-HSD in androgen formation. Examination of 14 male pseudohermaphrodites showed that a number of unique mutations occurred within the type III 17β-HSD gene. These mutations included 10 point mutations (missense): S65L, R80Q, Q176P, V205E, A203V, F208I, E215D, S232L, M235V, and P282L (53, 72); three splice-junction abnormalities; and one small deletion Δ777 that results in a frame shift and slightly truncated protein. Mutations were also found in introns 3 and 8, which disrupted the splice-acceptor sites. The point mutations in the open-reading frame were introduced into the cDNA for type III 17β-HSD, and their effect on enzyme activity was measured after transient transfection into human embryonal kidney 293 cells. It was found that eight of the nine missense mutant enzymes were devoid of catalytic activity, establishing that these mutations were the cause of the deficiency. The exception was the R80Q mutation, which had one fifth the specific activity of the wild type enzyme, and the decrease in activity was found to result from a reduced affinity for NADPH. The residual activity observed was consistent with the low formation of testosterone observed in the affected patient.

IV. 11β-Hydroxysteroid Dehydrogenases

A. Physiological and pharmacological significance

The concept that HSDs can function as molecular switches and regulate occupancy of steroid hormone receptors is a theme that can be developed for many of the HSDs. This concept is seen vividly with 11β-HSD. A paradox that existed for some time was that both gluco- and mineralocorticoids have the same affinity for the mineralocorticoid receptor in the kidney, yet glucocorticoids are present at much higher concentrations than aldosterone; thus, one would predict that the outcome would be hypertension due to mineralocorticoid excess. This, in fact, is prevented in kidney cells because 11β-HSD converts cortisol (an active hormone) into cortisone (an inactive hormone) and thereby prevents glucocorticoids from binding to the type I mineralocorticoid receptor and exerting their mineralocorticoid activity. In this example steroid hormone specificity is not receptor-driven but is HSD-dependent (73–75) (Fig. 3).

Apparent mineralocorticoid excess (AME) is associated with a deficiency in the kidney-specific type II 11β-HSD (76). AME is characterized by hypertension, excessive salt retention, and hypokalemia. The disease can be mimicked by the

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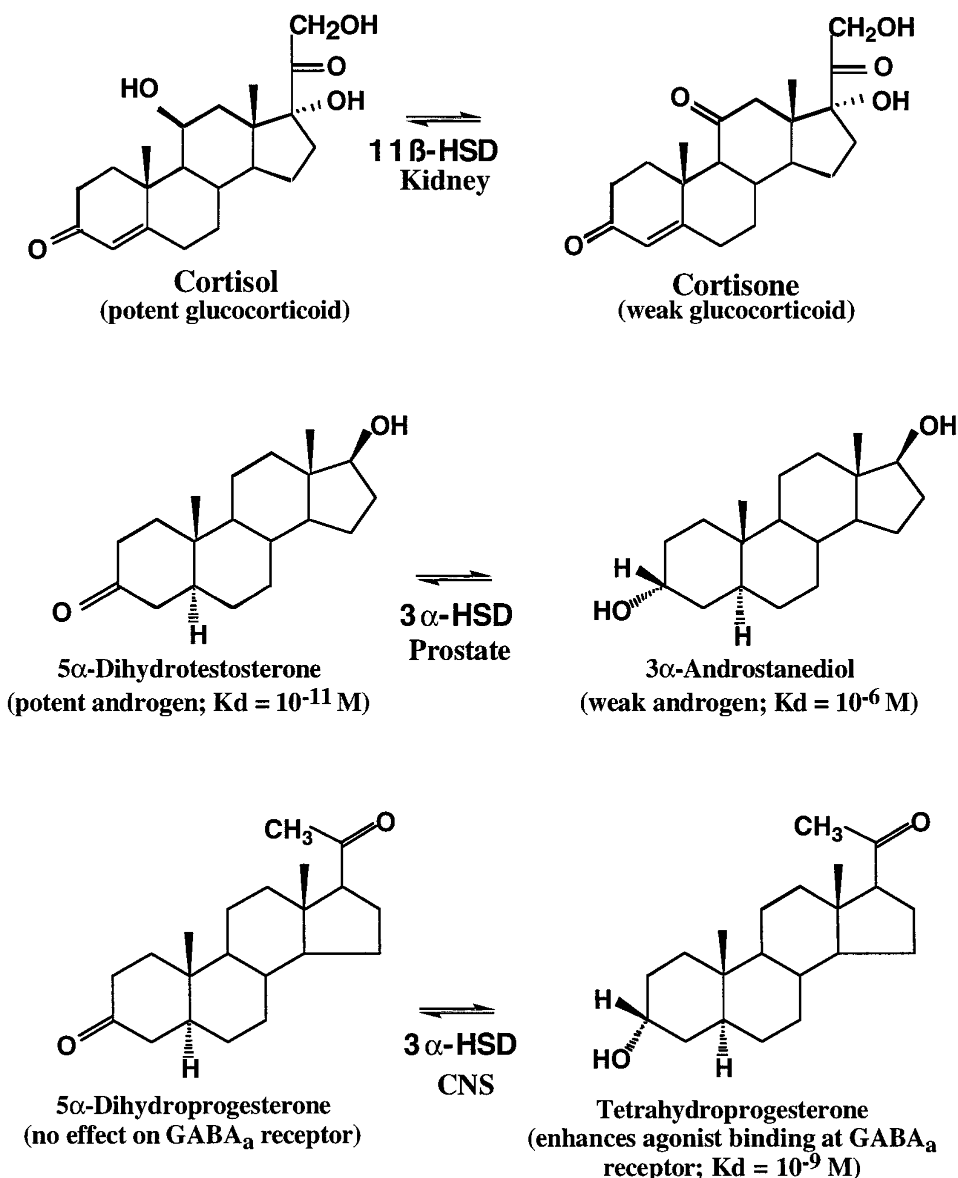

FIG. 3. Reactions catalyzed by HSDs in steroid target tissues. In these reactions, 3α-HSD and 11β-HSD act as molecular switches by interconverting potent steroid hormones with their cognate inactive metabolites. K_d values are for steroid receptors.

ingestion of licorice, the active ingredient being glycyrrhetinic acid, which is the aglycone of glycyrrhizic acid and a potent inhibitor of 11β-HSD (77, 78).

B. Cloning and expression of the 11β-HSD cDNAs

Rat liver 11β-HSD was one of the first mammalian HSDs to be cloned (79) and was expressed using a vaccina virus infection protocol (80). The cDNA contained a 861-bp open reading frame that encodes a protein of 287 amino acids. Sequence comparison indicated that there was low but significant identity to ribitol dehydrogenase, the product of the *Nod G* gene, and *Drosophila* alcohol dehydrogenase, which are recognized as being members of the SDR family (79). It is now known that two isoforms of 11β-HSD exist and both belong to this superfamily.

The type I 11β-HSD refers to the form that was originally purified and cloned from rat liver. It is a glycoprotein and the molecular mass of the mature protein is 34 kDa. It catalyzes oxidation and reduction using NADP(H) as cofactor, and it is expressed in a variety of rat and human tissues including liver, lung, testis, colon, and kidney. A remarkable feature of this enzyme is that, when it is purified from rat liver, it functioned only as a dehydrogenase. However, the recombinant enzyme, expressed using vaccina virus, exhibited both oxidation and reduction of steroids (80). These observations led to the speculation that the level of glycosylation determined directionality of enzyme catalysis (80). When tunicamycin, an inhibitor of glycosylation, was added to the vaccina virus expression system, enzyme activity was reduced by more than 50%. This represents one of the first examples in which the function of a mammalian HSD may be regulated by posttranslational modification. It was originally proposed that the type I 11β-HSD was responsible for conferring mineralocorticoid specificity; however, immunocytochemical studies within the rat kidney indicate that it is localized in the proximal tubular elements and not colocal-

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ized with the type I mineralocortioid receptor in the distal tubular elements (81). Studies on the hepatic form of 11β-HSD in the squirrel monkey also indicate that the type 1 11β-HSD does not protect against AME. This enzyme shares 75% sequence identity with the rat type 1 11β-HSD but is only expressed in the liver and fibroblasts. Since squirrel monkeys have highly elevated cortisol levels but do not display Cushing's disease or AME, an enzyme other than the hepatic 11β-HSD must protect the mineralocorticoid receptor from cortisol occupancy (82).

In contrast, a type II 11β-HSD was found in the kidney and placenta microsomes of sheep and humans. Clones encoding the kidney isozyme have been isolated from sheep and human kidney cDNA libraries (83, 84). The human type II 11β-HSD is 41 kDa in length and consists of 405 amino acids. It is NAD^+ specific and it has a lower K_m (10–100 nM) for steroid substrate than the type I enzyme (75). It catalyzes only 11β-dehydrogenation, *i.e.* it inactivates cortisol. The type II 11β-HSD and the type II 17β-HSD share 35% sequence identity and suggest that both these dehydrogenases belong to the SDR superfamily.

The pattern of diformazan disposition (a dye that produces a blue color after the reduction of cortisol by cofactor in the presence of enzyme) colocalized the type II 11β-HSD activity with the mineralocortioid receptor (85). It is now clear that it is the type II enzyme that is important in regulating occupancy of the mineralocorticoid receptor (75, 76, 85).

C. Structure, regulation, and tissue-specific expression of the 11β-HSD genes

At least four different mRNA species hybridize with the type I 11β-HSD cDNA in rat kidney (86), indicating that one or more alternative forms of 11β-HSD exist that may be products of either alternative splicing of the pre-mRNA or the use of a different cap site. The multiple RNA transcripts for type I 11β-HSD can be generated by either differential promoter usage by the type I 11β-HSD gene (86) or by differential polyadenylation. Isoforms have been termed type IA and type IB 11β-HSD. Type IB is exclusively expressed in the kidney medulla and cortex, and interest originally existed in this form because this enzyme had the potential to colocalize with the mineralocorticoid receptor (87). The type IB 11β-HSD mRNA encodes for a protein in which the first 26 amino acids from the N terminus are missing (87). This truncated enzyme is encoded by a transcript that originates from intron-1 of the type I gene. This truncation results in the loss of a hydrophobic polypeptide chain that may be important in intracellular transport of the enzyme. Expression of the type IB 11β-HSD cDNA failed to yield active enzyme using either cortisol or corticosterone as substrates and suggests that the truncated region is essential for enzyme activity. This truncation stops just before the $NADP^+$ binding site, which is predicted to be located at the N terminus.

Using the cDNA for type I 11β-HSD and polyclonal antibody, it was found that this enzyme was expressed in tissues that were not mineralocorticoid target tissues (*e.g.* liver, lung, skin, and testis) (88). In addition, no type I 11β-HSD was found in several mineralocorticoid target segments in the kidney (*e.g.* cortical collecting ducts and collecting tubules) (85, 88, 89). This profile of tissue-specific expression further confirms that type I 11β-HSD does not protect against mineralocorticoid activity of the glucocorticoids.

The human type I 11β-HSD gene has been cloned and is located on chromosome 1 and contains six exons that span a total length of 9 kb (90) (Fig. 4). The human type II 11β-HSD gene consists of five exons and four introns and is 6.2 kb in length and, by contrast to the type I gene, is located on chromosome 16q22 (91). The 5′-flanking regions of these genes have not been analyzed for the presence of *cis*-acting elements, and functional studies on these promoters have not been described.

D. 11β-HSD deficiency

AME is an inborn error in metabolism that effects occupancy of the type I mineralocorticoid receptor in the kidney tubule. The defect is characterized by increased half-lives for cortisol, retention of sodium (despite low mineralocorticoid

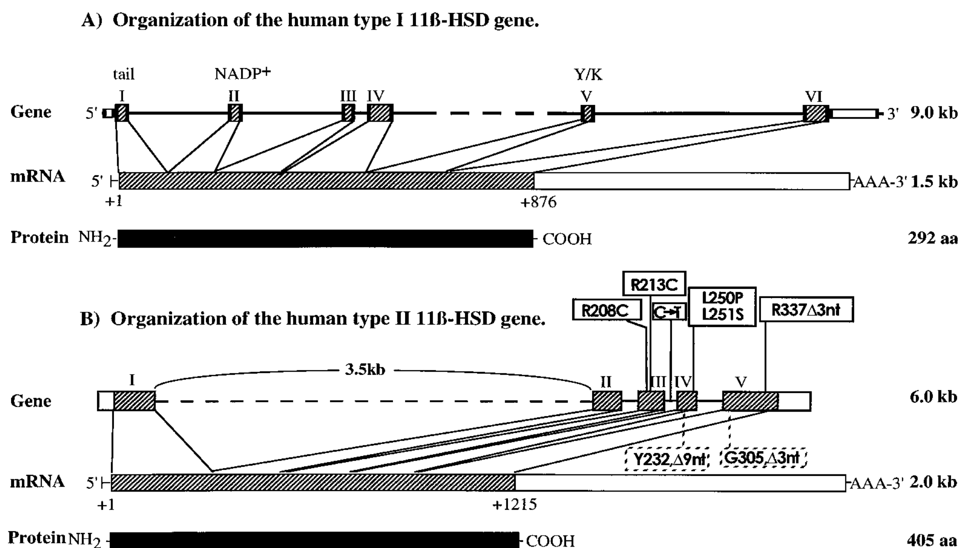

FIG. 4. Organization of the human type I and type II 11β-HSD genes. A, Organization of the type I 11β-HSD gene is shown. Noncoding exons are *open boxes*, coding exons are *stippled boxes*, and *narrow solid lines* are introns. Exons encoding the N-terminal tail and the conserved cofactor-binding ($NADP^+$) and catalytic (Y/K) domains are indicated. [Derived from Ref. 92.] B, Organization of the type II 11β-HSD gene. Noncoding exons are *open boxes*, coding exons are *stippled boxes*, and *narrow solid lines* are introns. Mutations detected in patients with AME are shown. *Dotted lines* around mutations denote heterozygosity and *solid lines* denote homozygosity. [Derived from Ref. 76.]

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levels), and hypokalemia. It is treatable with the aldosterone receptor antagonist, spironolactone, and a low salt diet. Mutations in the gene for type I 11β-HSD were suspected, but early analysis revealed that none existed (92). The molecular basis for the deficiency has been elucidated after the isolation of the gene for type II 11β-HSD, which is the human kidney-specific isoform (76). The gene encoding this enzyme has been analyzed in 11 AME patients. Mutations were found on both alleles in nine of the 11 patients, and these mutations were found to adversely affect enzyme activity. The mutations detected included R208C, R213C, R337H, deletion mutants of Y232 and Y338, and the point mutations L250P and L251S. In an independent study a missense mutation of R337C was observed in three siblings (93, 94). Several of these mutations were generated in the type II 11β-HSD cDNA, and the mutant enzymes were expressed in mammalian cells. The majority of mutant enzymes had less than 10% of the enzyme activity of the wild type recombinant protein, whereas the R337C mutant displayed a 10-fold increase in the K_m for cortisol (95). One additional mutation in the gene was observed 14 bp downstream from the splice site in intron 4. It was found that this mutation adversely affected the splicing of the pre-mRNA and resulted in the elimination of exon 4 from the expressed protein. The low activity of many of the point or deletion mutants in 11β-HSD can be rationalized on the basis of the crystal structure for *Streptomyces hydrogenans* 3α,20β-HSD; both these enzymes are members of the short-chain dehydrogenase/reductase family and are inhibited by carbenoxolone (a derivative of glycyrrhetinic acid, the active ingredient of licorice) (see *Sections VII* and *VIII*). Deletion of Y232 is anticipated to result in inactive enzyme since this residue is part of a Tyr-X-X-X-Lys catalytic motif that is conserved (see *Sections VII* and *VIII*). Similarly, R208 and R213 are predicted to make up portions of the steroid-binding pocket, and changes in K_m for steroid substrate are anticipated.

V. 3α-Hydroxysteroid Dehydrogenases

A. Physiological and pharmacological significance

Mammalian 3α-HSDs work in concert with the 5α- and 5β-reductases to reduce 3-ketosteroids to produce the 5α,3α- and 5β,3α-tetrahydrosteroids. Although these reactions are responsible for the metabolism of the majority of steroid hormones, they are not without consequence. In the prostate, 3α-HSD works as a molecular switch and regulates occupancy of the androgen receptor. In this tissue the enzyme interconverts 5α-DHT [dissociation constant (K_d) for the androgen receptor of 10^{-11} M] to 3α-androstanediol [K_d for the androgen receptor of 10^{-6} M], thereby reducing the affinity of the steroid ligand for the androgen receptor by 5 orders of magnitude (96–99) (Fig. 3). In the dog prostate, evidence has mounted that the enzyme works predominantly as a dehydrogenase and works in concert with 5α-reductase to maintain high levels of 5α-DHT, which is required for both the normal and abnormal growth of the prostate (100, 101). Inhibitors of prostatic 3α-HSD would block the interconversion of 5α-DHT to 3α-androstanediol and may be useful in preventing the build-up of 5α-DHT. Such inhibitors could be useful in combination with Finasteride for the treatment of benign prostatic hyperplasia and prostatic cancer.

In the brain, 3α-HSD converts 5α-reduced steroids (*e.g.* 5α-dihydroprogesterone) to yield tetrahydrosteroids (*e.g.* 5α,3α-tetrahydroprogesterone), which are potent allosteric effectors of the γ-aminobutyric acid $(GABA)_a$ receptor (Fig. 3). These steroids do not bind to the $GABA_a$ receptor by themselves but, in the presence of GABA, nanomolar concentrations of these steroids increase the affinity of the receptor for this neurotransmitter. As a consequence, these neurosteroids increase GABA-dependent chloride conductance and have anxiolytic and anesthetic properties (102–104). The 3α-hydroxysteroid, alphaxalone, was developed as an anesthetic based on this mechanism of action (105). Thus, 3α-HSD regulates occupancy of steroid hormone receptors, whether they are members of the nuclear receptor superfamily, such as the androgen receptor, or a membrane-bound chloride-ion gated channel such as the $GABA_a$ receptor.

B. Cloning and expression of the 3α-HSD cDNAs

The first mammalian 3α-HSD to be purified was from rat liver cytosol. This enzyme was obtained in milligram amounts (106) and led to the development of a polyclonal antibody (107). Immunoscreening of a λgt-11 expression library led to the isolation of a full-length cDNA clone that encodes a protein of 322 amino acids (3). The fidelity of the clone was confirmed by its overexpression in *E. coli* (108). The same cDNA was cloned and expressed independently by several groups (109–111). The cDNA for rat liver 3α-HSD was found to share no sequence identity with other HSDs that had been cloned at that time, including 3β-HSD/KSI (9–11, 16), 11β-HSD (79), and 17β-HSD (45–47). Instead, a search of GenBank revealed that it shared high sequence identity with aldose-reductases from rat and bovine lens (112, 113), human placental aldose reductase (114, 115), prostaglandin $F_{2\alpha}$ synthase (116), and ρ-crystallin from frog lens (117). These proteins all share in excess of 58% sequence identity and are known members of the AKR superfamily (see *Section VII*).

After this initial work a number of potential human 3α-HSD cDNAs were cloned, including human liver DD1, DD2, and DD4, where DD refers to the associated dihydrodiol dehydrogenase activity. These enzymes display greater than 70% sequence similarity with rat liver 3α-HSD. Expression of these clones reveals that DD1 acts predominantly as a 20α-HSD rather than a 3α-HSD and differs from DD2 in only seven amino acids (118, 119). Recombinant DD2 corresponds to the human bile acid-binding protein (119, 120). In contrast, DD4 appears to be identical to chlordecone reductase (119). Chlordecone reductase is now known to be human type I 3α-HSD (121). Neither DD2 nor DD4 functions as the human ortholog of the rat liver 3α-HSD. DD2, like the rat liver enzyme, binds bile acids with high affinity but differs in that it is not an efficient 3α-HSD. In contrast, DD4 is an efficient 3α-HSD but does not bind bile acids well. Recently, a type II 3α-HSD has been cloned from human liver (121). Recombinant type I and type II 3α-HSDs differ in their K_m values for 5α-DHT. The type I enzyme has a K_m of 1.2 μM, and the type

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II enzyme has a K_m of 19.2 μM. Type I and type II 3α-HSDs display high sequence identity. The availability of these clones has also led to the cloning and expression of two different 3α-HSDs from human prostate libraries (122, 123). The prostatic 3α-HSD cDNA isolated by Lin *et al.* (122) was originally expressed as a his-tag protein in *E. coli* and was devoid of 3α-HSD activity, even though it displayed high sequence identity with the rat and human liver isoforms. Subsequently, expression without the his-tag has led to the overexpression of the human prostatic 3α-HSD activity in *E. coli*. Kinetic characterization of the recombinant enzyme reveals that it favors 5α-DHT formation (H.-K. Lin and T. M. Penning, unpublished results). The prostatic 3α-HSD cDNA clone isolated by Dufort *et al.* (123) was transiently expressed in human embryonal kidney 293 cells and also had demonstrable 3α-HSD activity and weak 20α-HSD activity. This clone has 86% sequence identity with human liver type II 3α-HSD. Which of the two cDNAs represents the major human prostatic 3α-HSD is unknown. No attempt has been made to determine which prostate cells (epithelial *vs.* stromal cells) express 3α-HSD, and no RNase protection studies have been performed to determine their relative abundance across tissues.

C. Structure, regulation, and tissue-specific expression of the 3α-HSD genes

Using rat liver 3α-HSD cDNA as a probe, expression of a 2.7-kb transcript was found to be limited to peripheral tissues such as the liver, lung, and small intestine (124). When poly(A)$^+$ RNA was used, expression was also detected in the testis, mammary gland, and ovary. In the ovary two smaller transcripts, 1.2 and 1.6 kb, were observed. 3α-HSD is a member of the AKR superfamily and, because members share high-sequence identity, probes based on open-reading frames may result in cross-hybridization and complicate data interpretation concerning tissue-specific expression.

In rat liver, 3α-HSD displays high constitutive expression and represents 0.5–1.0% of the soluble protein. It also displays sexual dimorphic expression; mRNA, enzyme protein, and enzyme activity are all elevated in adult female rat liver, and expression appears to be under the control of estrogens. Maximal expression is seen after 21 days in males but will decline unless estrogens are administered (125). This sexual dimorphism has been observed primarily in rat liver for other enzymes involved in steroid hormone metabolism. Feminization of steroid hormone metabolism involves elevation of microsomal 5α-reductase and decreased 6β- and 16α-hydroxylase levels and has been shown to be governed by gonadal regulation of GH secretion (126–128). In these studies estrogens stimulated the constant secretion of GH, and androgens stimulated the pulsatile release of GH (128). The mechanism by which constant exposure to GH controls the coordinate expression of select steroid-metabolizing enzymes is unknown. In hypophysectomized female and male rats, levels of 3α-HSD mRNA, enzyme protein, and enzyme activity were decreased 8-fold. However, the administration of either GH or estrogens alone failed to elevate levels to those seen in intact animals. The data would suggest that GH is insufficient by itself to cause increased expression of the 3α-HSD gene. One explanation for this phenomenon is that in rat liver the estrogen receptor is regulated by GH (129), and the liver may need priming by GH before it will respond to estrogens.

The genes for rat 3α-HSD (130), human type I 3α-HSD (chlordecone reductase/DD4) (121), and human type II 3α-HSD (121) have been cloned and show similar organization (Fig. 5). They contain at least nine exon-intron boundaries that are highly conserved within members of the AKR superfamily (131, 132). The rat 3α-HSD gene is more than 47 kb long, whereas the type I and type II human 3α-HSD genes are 20 and 16 kb in length, respectively (121). The human type I 3α-HSD is located on chromosome 10 at p14 (133). No

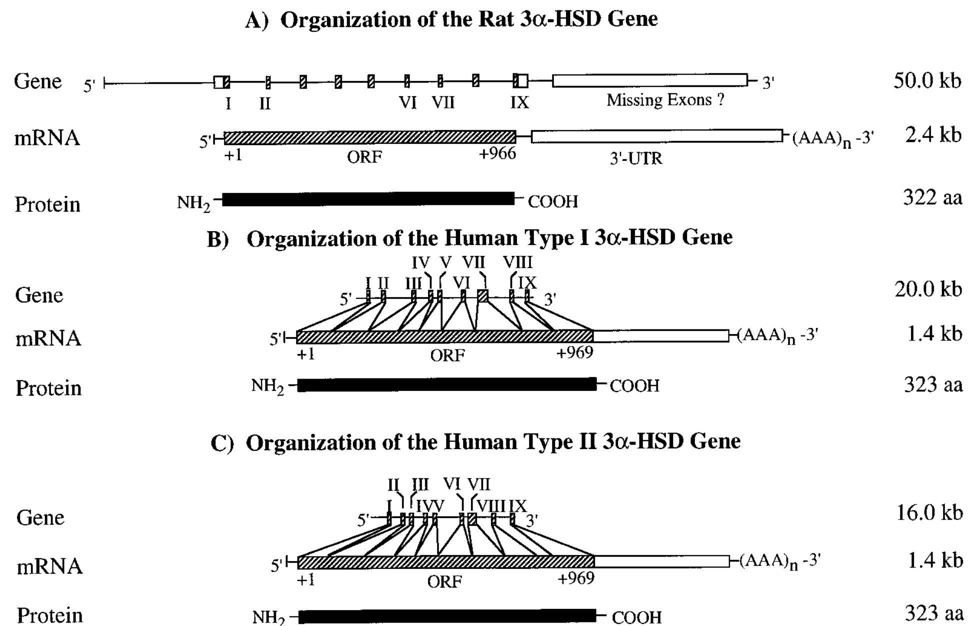

FIG. 5. Organization of the rat and human 3α-HSD genes. Noncoding exons are *open boxes*, coding exons are *stippled boxes*, and *narrow solid lines* are introns. A label indicates that the exon has been completely sequenced and located. ORF, Open reading frame; UTR, untranslated region. [The structure of the rat 3α-HSD gene has been modified from T. M. Penning: *J Endocrinol* 150: S175–S187, 1996, and the structures of the human type I and type II genes have been derived from Ref. 133.]

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location of the human type II 3α-HSD gene has been reported.

To understand what controls high constitutive expression and regulated expression in rat liver, detailed functional studies have been performed on the rat 3α-HSD gene (130). The bulk of the 2.7-kb transcript encodes for a long 3′-UTR which may be an important determinant in mRNA stability (134, 135). Estimates of the t½ of rat liver 3α-HSD mRNA are of the order of 12 h. Exons encoding for the long 3′-UTR present in the mRNA have not been isolated (Fig. 5). Primer extension analysis has located the transcription start site at −54 or −55 bp upstream (+1). Upstream from this site is a TATTA box that presumably binds RNA polymerase in forming the basal transcriptional complex. A genomic clone containing 9.5 kb of the 5′-flanking region of the gene has been isolated, and 2.5 kb immediately upstream from the start site have been sequenced. Analysis of this region for transcription factor consensus sequences shows the presence of a large number of imperfect steroid hormone response elements, including estrogen response elements, progesterone response elements, and glucocorticoid response elements. Interspersed between these elements are several AP-1, AP-2, and Oct sites. It has been suggested that these elements may comprise a steroid response unit.

Nested deletions of the 5′-flanking region of the gene fused to the reporter gene chloramphenicol acetyltransferase (CAT) have been generated and cotransfected into human hepatoma (HepG2) cells to identify the *cis*-elements involved in regulating constitutive expression (130). With respect to the transcription start site (+1), it was found that −199 to +55 bp contained the basal promoter, and gave a level of CAT expression similar to that observed in a pSV40-CAT positive control. Between −499 and −199 bp, a proximal enhancer was identified that elevated CAT activity 1.8- to 2.5-fold; between −755 and −498 bp, a silencer was observed that prevented CAT expression. Further upstream a powerful distal enhancer was located between −4.0 and −2.0 kb, which elevated CAT expression 40-fold and is thought to control high liver-specific expression. An examination of the silencer or negative response element (NRE) indicated that it contained two unique Oct sites, one on either strand. Band shift and supershift assays using a radiolabeled NRE and anti-Oct monoclonal antibodies provided evidence for the binding of Oct transcription factors to the NRE and suggest that Oct factors may be repressors of the 3α-HSD gene (130).

The function of the steroid response unit on the 5′-flanking region of the 3α-HSD gene has also been examined (136). It was found that in isolated rat hepatocytes, dexamethasone was able to increase steady state 3α-HSD mRNA levels, in a time- and concentration-dependent manner, and that this effect was blocked by antiglucocorticoids such as RU486. Nuclear-run off assays established that the effect of dexamethasone was at the level of new gene transcription (136). It was Tomkins (137, 138) who first implicated hepatic 3α-HSD as playing a major role in glucocorticoid metabolism. These findings suggest that glucocorticoids may increase their own metabolism by inducing 3α-HSD gene expression. This may comprise a positive feedback loop to eliminate these steroids.

D. 3α-HSD deficiencies

Although, no 3α-HSD deficiencies have been described, a clue may exist in glaucoma patients. These patients are sensitive to increases in intraocular pressure induced by the administration of corticosteroids (139). Studies on the metabolism of glucocorticoids in peripheral blood monocytes obtained from glaucoma patients indicate that 5β-reductase activity is elevated, and 3α-HSD activity is attenuated (139). This would cause a build up of 5β-dihydrocorticoids, which are known to increase intraocular pressure (140). Whether this is a mere correlation or a casual relationship, resulting from a genetic defect, requires further work.

VI. 20α-Hydroxysteroid Dehydrogenases

A. Physiological and pharmacological significance

One of the least studied HSDs is 20α-HSD; this enzyme was originally characterized and purified from rat ovary (141–143). In the ovary it plays an important role in converting progesterone (a potent progestin) into 20α-hydroxyprogesterone (a weak progestin) (Fig. 6). In steroid target tissues, it would regulate the amount of progestin that can bind to the progesterone receptor, and in this regard may be important in the placenta and endometrium. Early studies on the luteolytic effects of prostaglandin $F_{2\alpha}$ on rat ovary established that 20α-HSD activity is induced 150-fold. Because of the ability of this enzyme to metabolize progesterone to its inactive metabolite, 20α-hydroxyprogesterone, the underlying induction mechanism may contribute to luteolysis (144). 20α-Hydroxyprogesterone is unable to sustain pregnancy and, in the rat, the increase in luteal 20α-HSD activity contributes to the lower circulating progestin levels associated with the termination of pregnancy. The possibility exists that inhibitors of the human isoform may help maintain pregnancy before the luteal-placental shift in steroidogenesis.

It is now clear that there are several distinct proteins that catalyze 20α-HSD activity. In the ovary 20α-HSD catalyzes the inactivation of progesterone as described. But this should be distinguished from the enzyme found in other steroidogenic tissues (testis and adrenal) where its principal substrates may be either 17α-hydroxypregnenolone or 17α-hydroxyprogesterone. By converting the C20 ketone to an alcohol, the 17,20-lyase would be deprived of substrate, and this would prevent the conversion of C21 to C19 steroids. In the human placenta, 20α-HSD activities are catalyzed by type I or type II 17β-HSD.

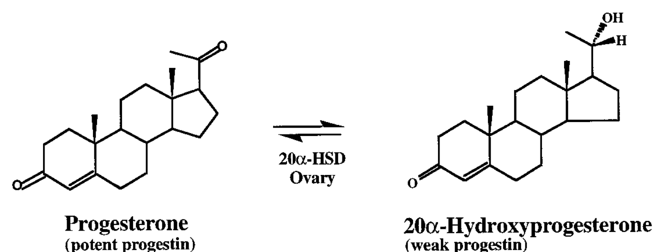

FIG. 6. Reaction catalyzed by ovarian 20α-HSD.

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B. Cloning and expression of the 20α-HSD cDNAs

cDNAs for rat and rabbit ovarian 20α-HSD have been cloned (5, 6). These enzymes display high-sequence identity with the mammalian 3α-HSDs and are members of the AKR superfamily. Recently, bovine testicular 20α-HSD has been cloned and has been found to be identical to aldose reductase (145). The testicular enzyme will only catalyze the reduction of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone and will not utilize progesterone as a substrate. The mRNA for this enzyme is also found in the adrenal cortex. Both type I and type II 17β-HSD also display 20α-HSD activity (51, 146). It is proposed that the type I enzyme (which is estrogenic and a reductase) inactivates progesterone whereas the type II enzyme (which inactivates estrogens and acts as a dehydrogenase) converts 20α-hydroxyprogesterone to progesterone. This switching mechanism may contribute to the estrogen- and progestin-dependent regulation of endometrial growth.

C. Structure, regulation, and tissue-specific expression of the 20α-HSD genes

In rat granulosa cells, GnRH or FSH alone increases 20α-HSD activity whereas PRL decreases the activity of the enzyme (147). The availability of the cDNA for rat ovarian 20α-HSD, which detects a 1.2-kb transcript in corpora lutea, has led to an extension of these studies. mRNA levels were markedly reduced by PRL treatment and appear to be under tight control by PRL (148). Tissue distribution studies indicate that the expression of the 1.2-kb transcript is specific for the rat corpus luteum, it being almost nondetectable in the uterus, kidney, lung, and heart. Its tissue-specific expression and tight control by gonadotrophs confirm the potential importance of this enzyme in luteolysis.

The structure of genes encoding ovarian 20α-HSD has not been reported. By contrast, the gene structures of several AKRs have been described (131, 132). These have a genomic organization similar to that described for rat 3α-HSD and contain the nine highly conserved exon-intron boundaries. A similar genomic organization is anticipated for 20α-HSDs that are AKRs.

VII. HSDs Belong to Two Protein Families

A. HSDs that belong to the SDR superfamily

Analysis of the cDNAs for 3β-HSD/KSI, 11β-HSD, and 17β-HSD isoforms indicates that they belong to the SDR family (Fig. 7) (149–152). SDR family members are nonmetalloenzymes that have monomers of 25–28 kDa. The monomer molecular masses of the type I-IV 17β-HSDs and the type II 11β-HSD are somewhat larger. These proteins often function as multimers, and they share approximately 25% sequence identity. Despite this relatively low homology, family members share identical protein folds (1, 2). Part of the protein fold includes an arrangement of α-helix and β-strands $(\beta\text{-}\alpha\text{-}\beta\text{-}\alpha\text{-}\beta)_2$ to produce a Rossmann fold for cofactor binding. Binding of the cofactor, NAD(P)(H) (all nicotinamide adenine dinucleotides) is in the N-terminal part of the molecules, where a common Gly-XXX-Gly-X-Gly motif occurs. In addition, they all share a conserved consensus sequence Tyr-X-X-X-Lys, which has been implicated by site-directed mutagenesis to play a role in catalysis (Table 2) (153–155). Originally, 3β-HSD/KSI was not considered part of this family but these enzymes contain both the Gly-XXX-Gly-X-Gly and the Tyr-X-X-X-Lys motifs. Other SDR family members include porcine testicular 20β-HSD (156), 3α,20β-HSD from *Streptomyces hydrogenans* (157), 15-hydroxyprostaglandin dehydrogenase (158), *Drosophilia* alcohol dehydrogenase (154), ribitol dehydrogenase from *Klebsiella aerogenes* (159), carbonyl reductase (160), the ACT111 gene product of *Streptomyces coelicolor* (161), and AP27, an adipocyte protein of unknown function (162). There are currently 57 members in the protein family, and two crystal structures exist for HSDs that belong to this family; these are for 3α,20β-HSD from *Streptomyces hydrogenans* and the human placenta type-1 17β-HSD (163–166).

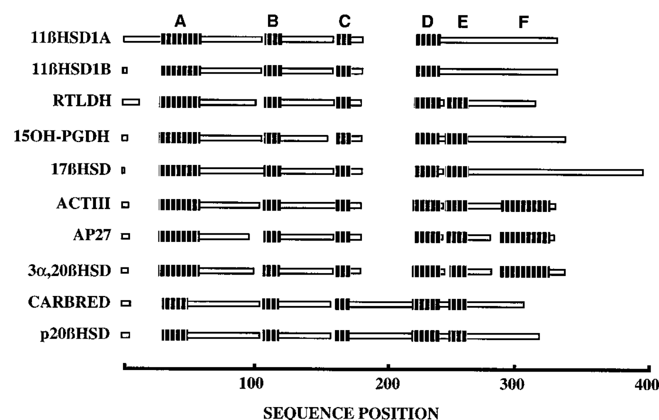

FIG. 7. A schematic of representative members of the SDR family. Conserved domains (A-F) are indicated by *stippled boxes*. Domain A represents the cofactor-binding region, and domain D represents the active site of the enzyme. The functions of the other conserved domains are unknown. Abbreviations are as follows: 11β-HSD1A, 11β-hydroxysteroid dehydrogenase from liver and kidney; 11β-HSD1B, the truncated 11β-hydroxysteroid dehydrogenase protein as predicted from the cDNA cloned from the kidney; RTLDH, ribitol dehydrogenase; 15OH-PGDH, 15-hydroxyprostaglandin dehydrogenase; 17β-HSD, type 1 17β-hydroxysteroid dehydrogenase; ACTIII, gene product of *Streptomyces coelicolor*; AP27, an adipocyte protein of unknown function; 3α,20β-HSD, 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*; CARBRED, carbonyl reductase; p20β-HSD, pig 20β-hydroxysteroid dehydrogenase. [Reprinted by permission of the publisher from Z. S. Krowzowski *et al.*: *Steroids* 59:116–120, 1994 (75). © Elsevier Science, Inc.]

B. HSDs that belong to the AKR superfamily

Analysis of the cDNAs for rat and human 3α-HSDs, as well as the cDNAs for rat ovarian 20α-HSD (6, 148), rabbit ovarian 20α-HSD (5), and bovine testicular 20α-HSD (145), indicates that they are all members of the AKR superfamily. Other HSDs assigned to this superfamily include a murine liver 17β-HSD (A-face specific) (4) and a human type V 17β-HSD. As more HSDs are cloned and sequenced, membership in this superfamily is anticipated to grow.

There are now more than 40 proteins that belong to the AKR superfamily. The 5β-reductases that are steroid double-bond reductases, which precede the action of the 3α- and 3β-HSDs in steroid hormone metabolism, also belong to this superfamily (167, 168). A cluster analysis indicates that the AKR superfamily breaks into a number of discrete subdivi-

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sions based on substrate specificity; these subdivisions include the aldose reductases, the aldehyde reductases, and the HSDs (Fig. 8). Common features of AKR family members are that they are all monomers of 34 kDa in size. They will utilize NAD(P)(H) as cofactor. They do not contain a Rossmann fold for binding cofactor; instead they bind the pyrophosphate bridge of the dinucleotide via a "salt-linked" safety-belt. Invariant at their active site is a catalytic tetrad consisting of Asp 50, Tyr 55, Lys 84, and His 117 (numbering according to the 3α-HSD sequence). Site-directed mutagenesis supports a role for a Tyr/Lys pair to perform catalysis, in a manner similar to that described for the SDR family members (see *Section VIII. G*). A comparison of the properties of HSDs in the SDR and AKR superfamilies is given in Fig. 9.

VIII. Structure/Function of HSDs

A. Kinetic mechanism of HSDs

HSDs catalyze the oxidoreduction of steroids using NAD(P)(H) as cofactor. These bisubstrate reactions can follow either a random kinetic mechanism in which either steroid or cofactor can bind first to form a binary complex, or these reactions can follow a sequential ordered bi-bi mechanism in which pyridine nucleotide binds first and leaves last (Fig. 10). Complete kinetic mechanisms have been solved for many HSDs. Kinetic mechanisms for SDR family members reveal that 3α,20β-HSD from *Streptomyces* has an ordered kinetic mechanism (169, 170) whereas human placenta type I 17β-HSD has a random mechanism (171). In contrast, an ordered kinetic mechanism appears to be a hallmark feature of members of the AKR superfamily (172–174). Examination of the individual rate constants for reactions catalyzed by AKRs indicate that the binding and release of cofactor is the rate-limiting step in complex formation. This rate is slower than the rate of diffusion, suggesting that a conformational change must occur before cofactor binds to yield a productive E·NAD(P)(H) complex capable of binding second ligand. Transient fluorescence changes occur in aldose reductase after binding NADPH and indicate that a further transition takes place before the second ligand can be accommodated.

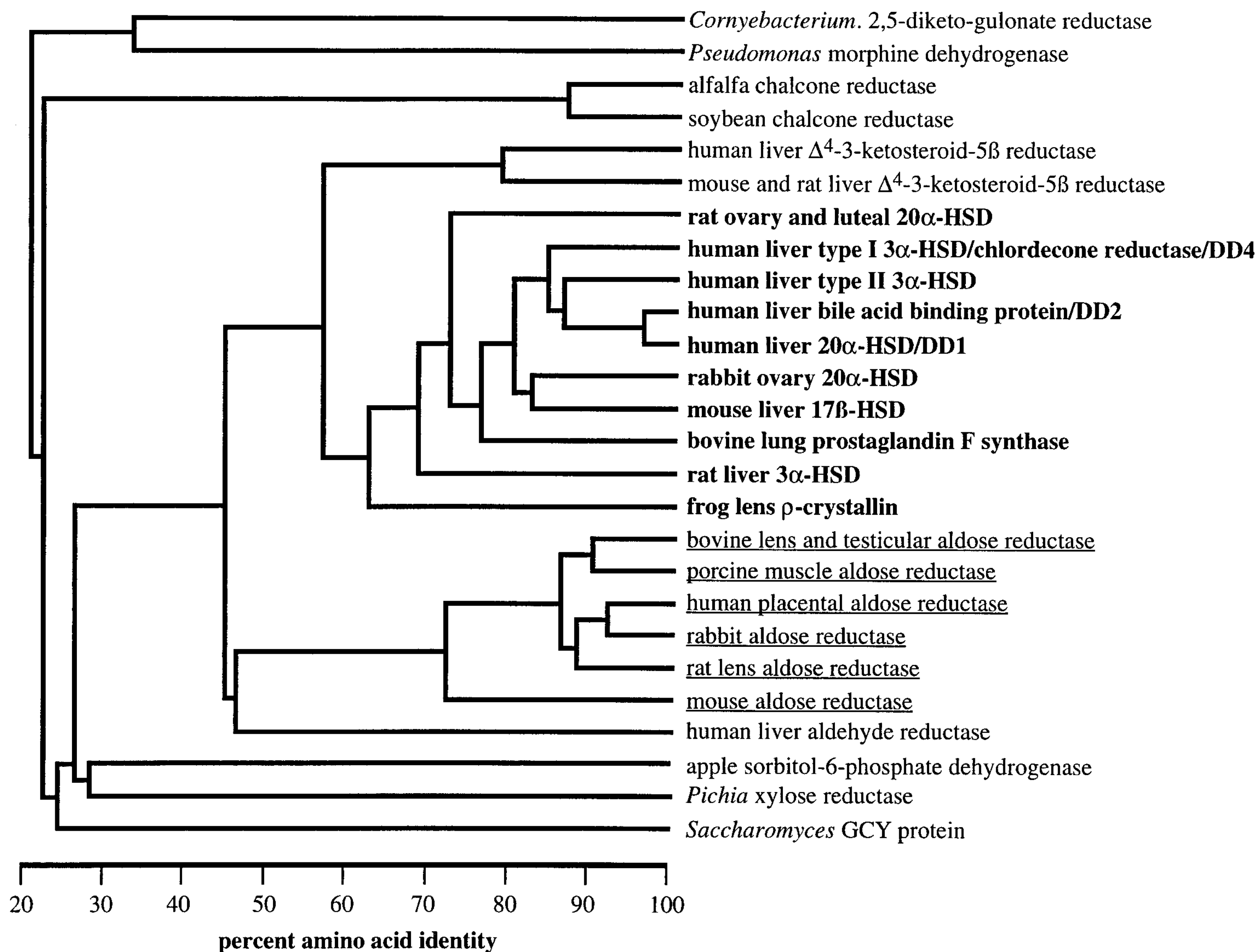

FIG. 8. Cluster analysis of the AKR superfamily. The HSDs that are members of the AKR superfamily are in *boldface type*. [Reprinted by permission of the publisher from T. M. Penning *et al.*: Mammalian 3 alpha-hydroxysteroid dehydrogenases. *Steroids* 61:508–523. © 1996 by Elsevier Science, Inc.]

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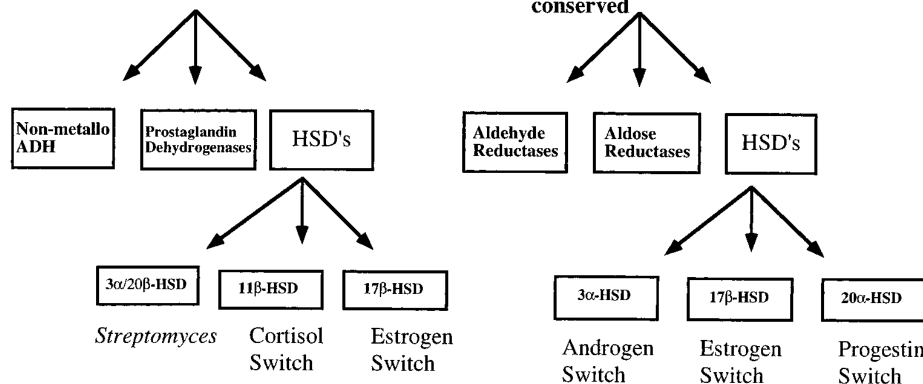

FIG. 9. Comparison of the SDR and AKR superfamilies.

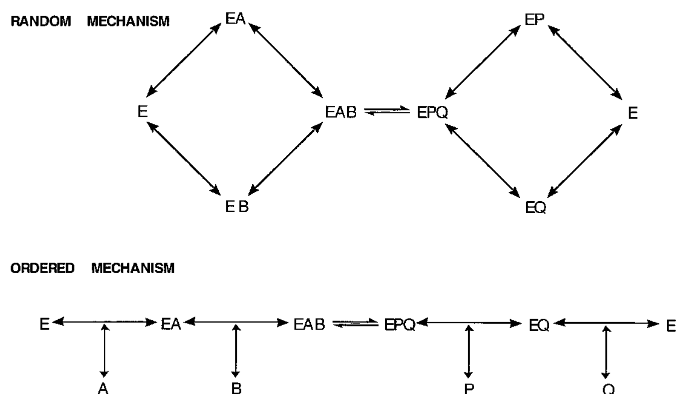

FIG. 10. Kinetic mechanisms for HSDs. *Top* panel shows order of ligand binding in a random kinetic mechanism; *bottom* panel shows order of ligand binding in an ordered kinetic mechanism. E, Enzyme; A, $NAD(P)^+$; B, steroid substrate; P, steroid product; and Q, NAD(P)H. Standard Cleland nomenclature is used. [Reproduced with permission from T. M. Penning and J. W. Ricigliano: *J Enzyme Inhib* 5:165–198, 1991 (7). © Harwood Academic Publishers.]

In this transition, E·NADPH isomerizes to an *E·NADPH complex (activated binary complex) (175–177). In contrast, the turnover of the ternary complex occurs readily.

B. Catalytic mechanism of HSDs

HSDs are nonmetalloenzymes and thus, unlike horse liver or yeast alcohol dehydrogenase, do not contain zinc at the active site. In the reduction direction the catalytic mechanism requires direct hydride transfer from the C4 position of the nicotinamide ring to the acceptor carbonyl of the steroid substrate (178, 179), which becomes protonated to form the steroid alcohol. The reaction may be concerted, in which case the hydride transfer and protonation steps occur simultaneously (Fig. 11). Protonation of the carbonyl group could precede hydride transfer, and thus the formation of a full or partial carbonium ion could occur (180). In this mechanism an electrophilic carbon (carbonium ion or carbocation) is stabilized by adjacent carbons in the steroid ring or side chain so that a Markovnikov addition (hydride transfer to the most stable carbonium ion) could occur. Alternatively, hydride transfer could precede protonation and an oxyanion intermediate would result. Since there is no metal ion to polarize the acceptor carbonyl, a general acid is invoked to facilitate the reaction. In the oxidation direction the same amino acid may function as a general base. The stereochemistry of hydride transfer has been elucidated for a number of HSDs using stereospecifically labeled 4-pro(*R*)- or 4-pro(*S*)-NAD(P)H (172, 181–184). As an example, the reaction catalyzed by 3α-HSD is shown in Fig. 12 in which the 4-pro(*R*)-hydrogen is transferred from the A-face of the cofactor to the β-face of the steroid to form a 3α-axial alcohol.

C. x-Ray crystal structures of HSDs in the SDR superfamily

The first x-ray structure for an HSD in the SDR family was determined for 3α,20β-HSD from *Streptomyces hydrogenans* (163–165). Although this bacterial enzyme shares only 15–18% sequence identity with other mammalian HSDs in this family, the protein-fold is maintained. The *Streptomyces* enzyme crystallizes as a tetramer, and each monomer of the tetramer shares an identical protein fold. This fold consists of seven-stranded parallel β-sheets surrounded by six α-helices, with three helices existing either side of the β-sheets. This arrangement provides a Rossmann fold for binding cofactor (Fig. 13). The structures of the *Streptomyces* 3α,20β-HSD with either NAD^+ or the inhibitor carbenoxolone bound have been solved (164, 165). Because type I and type II 11β-HSD are also inhibited by licorice derivatives, this structure has been used to rationalize the basis for inhibitor binding. In 3α,20β-HSD, NAD^+ lies across the Rossmann fold in an extended *syn*-conformation. This orients the B-face of the cofactor into the active site cleft so that 4-pro-(*S*)-hydride transfer can occur. Residues from each of the monomers contribute to the steroid hormone-binding site and may explain why this enzyme functions as a tetramer. The structure also provides a rationale for how the enzyme can transform steroid alcohols at both the 3β- and 20α-positions. This is achieved by binding either the A and B rings or the C and

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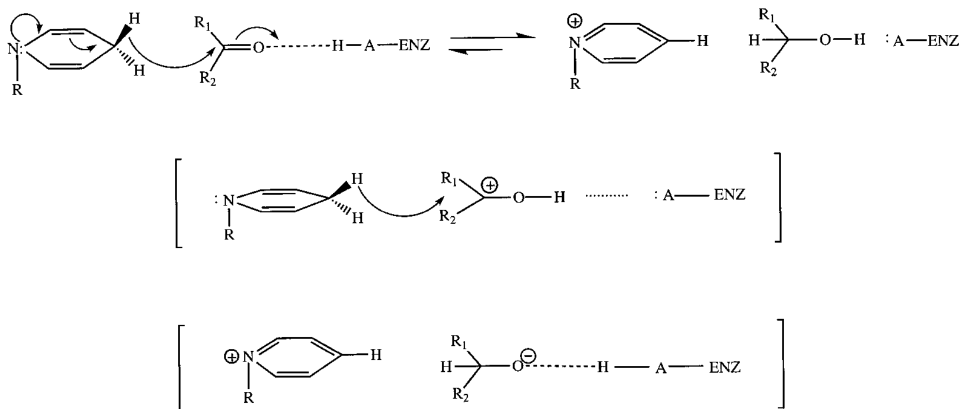

FIG. 11. Catalytic mechanisms for HSDs. Enz, Enzyme; A-H, general acid at the enzyme active site; R_1 and R_2, components of either the steroid nucleus or steroid side chain. The *top* reaction shows a concerted mechanism in which hydride transfer and protonation occur simultaneously. In *brackets* two alternative mechanisms are proposed. *Middle* shows hydride transfer as the second step and occurs after the formation of a formal carbonium ion. *Bottom* shows hydride transfer as the first step with the formation of an oxyanion intermediate.

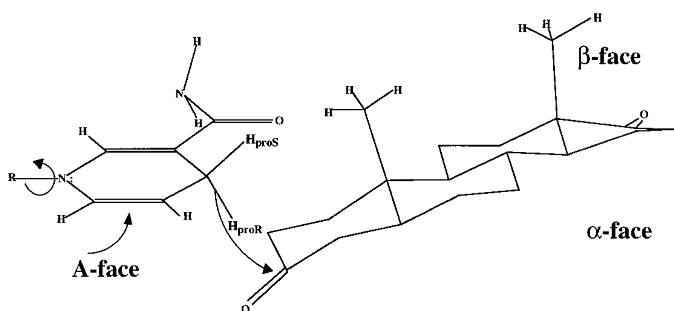

FIG. 12. Stereochemistry of hydride transfer for 3α-HSD. The 4-pro-(*R*)-hydrogen is transferred from the A-face of the cofactor to the β-face of the steroid to produce a 3α-axial alcohol. [Reprinted by permission of the publisher from T. M. Penning *et al.*: Mammalian 3 alpha-hydroxysteroid dehydrogenases. *Steroids* 61:508–523. © 1996 by Elsevier Science, Inc.]

D rings near the catalytic residues, *i.e.* steroid ligands can bind backwards. The structure of the E•carbenoxolone complex revealed that the hemisuccinate at the C3 position of the steroid ligand was within hydrogen bond distance of the conserved Tyr 152, which is part of the catalytic motif Tyr-X-X-X-Lys. The positions of these ligands provide points of reference for modeling cofactor and substrate into other HSDs that belong to the SDR superfamily.

The structure of the human placental or estrogenic apo-17β-HSD (type I 17β-HSD) has also been solved at 2.2 Å resolution by a combination of isomorphous-heavy atom derivatives and molecular replacement using the *Streptomyces* 3α,20β-HSD as a search model (166). Inserted into the protein fold were three additional helices and a helix-turn-helix motif. Although, $NADP^+$ was included in the crystallization conditions, no cofactor was observable within the electron density. Therefore, $NADP^+$ was modeled into the structure using the coordinates for the 3α,20β-HSD structure. This model predicts that the B-face of the co-factor will present itself to an apolar cleft presumed to bind estrone. In this manner the stereochemistry of 4-pro(*S*)-hydrogen transfer would be maintained as is the case for the 3α,20β-HSD structure.

Knowing that estrone is reduced to 17β-estradiol via hydride transfer to the α-face of the steroid, the crystal structure of estrone was modeled into the apolar cleft of the 17β-HSD structure (166). It was found that the 17-ketone was in close proximity with the C4 position of the nicotinamide ring, and that the phenolic hydroxyl group on the A-ring of the steroid formed a hydrogen bond with the imidazole nitrogen of His 221. Other amino acids in the cleft included His and Cys residues that were previously identified by bromoacetoxysteroid affinity-labeling studies to be involved in steroid binding (185, 186). The active site also contained Tyr 155 and Lys 159 near the C17 ketone, and these residues are believed to be catalytic. In the model, the β-face of the steroid orients itself along one side of the cleft, which contains an apolar surface comprising Tyr 218, Leu 262, and Leu 263. Interestingly, Tyr 218 lies immediately over the aromatic A ring of the steroid substrate and may provide some binding energy to attract phenolic steroids. The α-face of the steroid is exposed to Met 193 and Phe 226, and at the bottom of the cleft, His 221 and Ser 222 interact with the 3′-hydroxyl group of the steroid. A model of the transition state has been proposed, in which Tyr 155, Lys 159, and a water molecule form a hydrogen bond network juxtaposition to the C17-hydroxyl/ketone group transformed in catalysis (166) (Fig. 14A). Several but not all of these interactions have been supported by recently reported structures of the E•estradiol binary complex and the E•$NADP^+$•estradiol ternary complex (187, 188).

D. Site-directed mutagenesis on HSDs that are SDR members

The conservation of the catalytic motif (Tyr-X-X-X-Lys) found in HSDs that are SDRs is shown in Table 3. When site-directed mutagenesis was conducted on the related 15-hydroxyprostaglandin dehydrogenase, the Tyr in this motif was shown to be essential for catalysis (153). When both the Tyr and Lys in this motif were mutated in type I 11β-HSD, enzyme activity was also abolished (155). The first evidence that the function of the Lys was to lower the pK_a of the adjacent Tyr, so that it could function as the general acid, came from the work on *Drosophilia* alcohol dehydrogenase (154). In these studies, Lys was mutated to His with some retention of enzyme activity indicating the need for a residue that could function as a base or its conjugate acid. Site-directed mutagenesis studies on type I 17β-HSD have continued to confirm the general importance of the conserved Tyr. Thus when Tyr 155 was mutated to Ala it was found that the *Baculovirus*-expressed protein displayed only residual enzyme activity (49). In the same study, the H221A mutant had a dramatic effect on kinetic parameters: the K_m for es-

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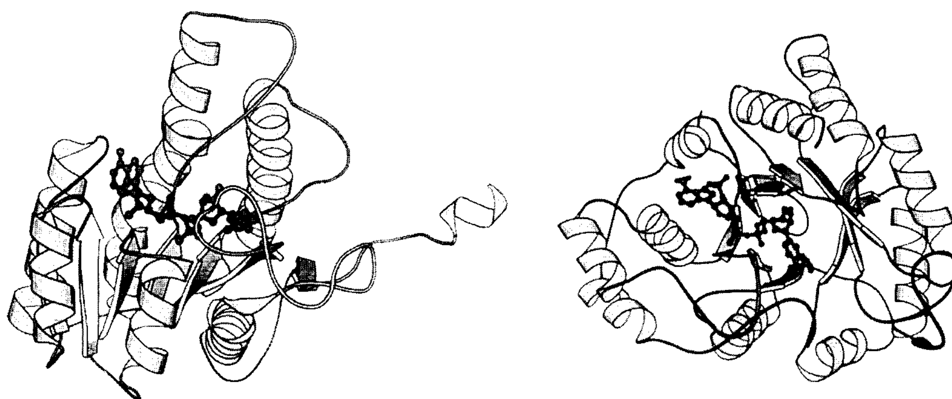

FIG. 13. Comparison of the protein folds present in HSDs. Protein fold characteristic of the SDR family (*right*) in which seven-stranded parallel β-sheets are surrounded on either side by three α-helices. Protein fold characteristic of the aldo-keto reductase superfamily (*left*) in which there is an alternating arrangement of α-helix and β-sheet to form an $(\alpha/\beta)_8$-barrel. Cofactors are shown as ball and stick representations.

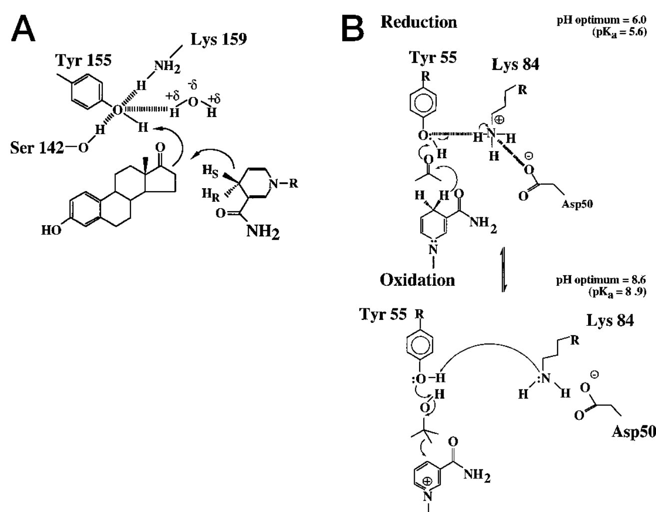

FIG. 14. Proposed mechanism for the reduction of estrone to 17β-estradiol catalyzed by human placental 17β-HSD (an SDR) deduced from the crystal structure (A). *Curved arrows* show the direction of electron flow in which a hydride ion is transferred to the C17 ketone group. Hydrogen bonds are proposed between the tyrosine hydroxyl group and a water molecule. The δ^+ and δ^- signs show the charge distribution on the water molecule. The hydroxyl group on Ser 142 is shown hydrogen bonded to the hydroxyl group on tyrosine. Another role for Ser 142 would be to form a hydrogen bond with the C17 ketone of the ketosteroid and play a facilatory role in polarizing the carbonyl. [Reproduced with permission from D. Ghosh *et al.*: *Structure* 3:503–513, 1995 (166). © Current Biology.] Proposed catalytic mechanism for 3α-HSD (an AKR) deduced from the crystal structure (B). [Reproduced with permission from J. E. Pawlowski and T. M. Penning: *J Biol Chem* 269:13502–13510, 1994 (108). © American Society for Biochemistry and Molecular Biology.]

tradiol was increased 3-fold and the V_{max} was decreased by 10-fold, leading to a 30-fold reduction in catalytic efficiency. These data are consistent with the location of His 221 at the active site.

E. x-Ray crystal structures of HSDs in the AKR superfamily

The x-ray crystal structure of rat liver 3α-HSD has been determined, and this represents the first structure of a mammalian HSD that belongs to the AKR superfamily. Based on the high amino acid sequence identity that exists between HSD members of the AKR superfamily, the general features of this structure may apply to all HSDs within the family, including the liver, brain, and prostatic 3α-HSDs. The structures of the apoenzyme (native 3α-HSD) and the binary complex $E{\cdot}NADP^+$ have been reported (189, 190). The apoenzyme structure was solved by molecular replacement using the coordinates for human placental aldose reductase, which shares 58% sequence identity with rat liver 3α-HSD (191). The binary complex structure was solved using the apoenzyme structure as the search molecule. Like aldose reductase, rat liver 3α-HSD adopts a triose-phosphate isomerase barrel motif. In this motif, there is alternating α-helix and β-strand arrangement that occurs eight times $(\alpha/\beta)_8$, from which the β-strands form the staves of a barrel in the core of the structure.

The structure of the apoenzyme solved to 3.2 Å resolution (189) is characterized by two large loops at the back of the barrel with disordered electron density that were built into the structure using the coordinates for aldose reductase (Fig. 13). Using the coordinates for NADPH from the aldose reductase·NADPH binary complex structure, the cofactor was modeled into the apoenzyme. In close proximity to the C4 position of the nicotinamide ring, four hydrophilic residues were found to exist (Asp 50, Tyr 55, Lys 84, and His 117), which may comprise a catalytic tetrad. These residues are invariant in HSDs that belong to the AKR superfamily. On the basis of this structure, a catalytic mechanism was proposed in which Tyr 55 was implicated as the general acid, and its effective pK_a was lowered by hydrogen bonding with Lys 84, which in turn was salt-linked to Asp 50 (Fig. 14B). Based on this model, the apolar cleft would consist of a side of hydrophobic residues (Leu 54, Trp 86, Phe 128, Phe 129) and an opposing flexible loop (loop-B) on which Trp 227 resides.

The structure of the binary complex was recently solved to 2.7 Å resolution with good geometry and provides further evidence in support of the structural model (190). The position of $NADP^+$ in the structure closely resembles that modeled for NADPH. The nicotinamide ring is in close enough proximity with the phenolic ring of Tyr 216 and may permit sharing of the pi-electrons between the two aromatic rings. In this orientation the position of the carboxamide side chain of the nicotinamide ring is dictated by hydrogen bonds with Ser 166, Asn 167, and Gln 190 (Fig. 15). The residues around the adenine ring are indicative of the preference for NADPH (K_d = 190 nM) over NADH (K_d = 165 μM) (172, 192). Two amino acid residues, Arg 276 and Ser 271, provide a counterion for the negatively charged phosphate and a hydrogen bond, respectively, to stabilize the additional phosphate in

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TABLE 3. Alignment of the Tyr-X-X-(Ser)-Lys consensus sequence of the short chain dehydrogenases/reductases (SDRs) in HSDs

3α-HSD	203 Leu	Asp	**Tyr**	Cys	Lys	**Ser**	**Lys**	Asp	Ile	Ile	Leu	Val	Ser	Tyr	217 Cys
11β-HSD	176 Ala	Ser	**Tyr**	Ser	Ala	**Ser**	**Lys**	Phe	Ala	Leu	Asp	Gly	Phe	Phe	190 Ser
17β-HSD	153 Asp	Val	**Tyr**	Cys	Ala	**Ser**	**Lys**	Phe	Ala	Leu	Glu	Gly	Leu	Cys	167 Glu
3β-HSD/KSI	268 Leu	Asn	**Tyr**	Thr	Leu	**Ser**	**Lys**	Glu	Phe	Gly	Leu	Arg	Leu	Asp	282 Ser
3α-HSD	151 Leu	Ala	**Tyr**	Ala	Gly	**Ser**	**Lys**	Tyr	Ala	Val	Thr	Cys	Leu	Ala	165 Arg
3β-HSD	149 Ala	Gly	**Tyr**	Ser	Ala	**Ser**	**Lys**	Ala	Ala	Val	Ser	Ala	Leu	Thr	163 Arg
3α20β-HSD	150 Ser	Ser	**Tyr**	Gly	Ala	**Ser**	**Lys**	Trp	Gly	Val	Arg	Gly	Leu	Ser	164 Lys
7α-HSD	157 Ile	Ala	**Tyr**	Gly	Thr	**Ser**	**Lys**	Ala	Ala	Ile	Asn	Tyr	Leu	Thr	171 Lys
15-OH PGDH	148 Pro	Val	**Tyr**	Cys	Ala	**Ser**	**Lys**	His	Gly	Ile	Val	Gly	Phe	Thr	162 Arg
DADH	150 Pro	Val	**Tyr**	Ser	Gly	Thr	**Lys**	Ala	Ala	Val	Val	Asn	Phe	Thr	164 Ser
cis-**DD**	43 Val	Leu	**Tyr**	Thr	Ala	Gly	**Lys**	His	Ala	Val	Ile	Gly	Leu	Ile	57 Lys

Abbreviations are as follows: 3α-HSD, rat liver 3α-hydroxysteroid dehydrogenase, which is not a SDR; 11β-HSD, rat liver 11β-hydroxysteroid dehydrogenase; 17β-HSD, human placental or type 1 17β-hydroxysteroid dehydrogenase; 3β-HSD/KSI, human placenta; 3β-hydroxysteroid dehydrogenase/ketosteroid isomerase; 3α-HSD, from *Pseudomonas* sp.; 3β-HSD, from *Pseudomonas testosteroni*; 3α,20β-HSD, from *Streptomyces hydrogenans*; 7α-HSD, from *Eubacterium*; 15-OH PGDH, 15-hydroxyprostaglandin dehydrogenase from human placenta; DADH, *Drosophila* alcohol dehydrogenase; *cis*-DD, *cis*-benzene-dihydrodiol dehydrogenase from *Pseudomonas putida*. [Reproduced from T. M. Penning: *J Endocrinol* 150: S175–S187, 1996, by permission of the Journal of Endocrinology, Ltd].

2′-AMP. Of the 18 amino acid residues that are involved in cofactor binding to aldose reductase, 11 are positionally conserved in the structure of the binary complex of 3α-HSD and interact with the cofactor as shown in Fig. 15. A novel feature of this binary complex is that a water molecule is observed within hydrogen bond distance of the C4 position of the nicotinamide ring. In other aldose reductase structures, this water molecule is in the same position as the oxygen atom of inhibitors, *e.g.* cacodylate, citrate, glucose-6-phosphate, and may provide a good reference point for the C3 position of 3-ketosteroid substrates. Based on the proximity of the water molecule to Tyr 55 and the nicotinamide ring, Tyr 55 would appear to be the most likely candidate for the general acid.

Interestingly, Trp 227, which is located on loop B of the structure, is inserted into the apolar cleft of the other protein molecule in the asymmetric unit of the binary complex. This implies that Trp 227 prefers to be in association with the apolar cleft rather than in solution and suggests that it may mimic the binding of steroid ligand (190). Considering the stereochemistry of hydride transfer, the position of the water molecule, and the location of the apolar cleft, it is proposed that the α-face of the steroid must orient itself toward that side of the cleft which contains Trp 86 and that loop-B including Trp 227 interacts with either the β-face or the edge of the steroid. A crystal structure for the ternary complex E·NAD(P)(H)·Steroid has been recently determined and supports these conclusions.

F. Site-directed mutagenesis on HSDs that are AKR members

The x-ray crystal structures of rat liver 3α-HSD have been used to guide and interpret site-directed mutagenesis studies. These studies have relevance to HSDs that belong to the AKR superfamily. The structure implicates Asp 50, Tyr 55, Lys 84, and His 117 in catalysis. Furthermore, the primary structure contains the conserved sequence Tyr-X-X-X-Lys, which is the catalytic motif in SDR family members (Table 3). In 3α-HSD the corresponding Tyr/Lys pair is Tyr 205 and Lys 209. PCR site-directed mutagenesis was performed on these candidate catalytic residues. The following mutant enzymes were expressed in *E. coli* and purified to homogeneity in milligram amounts: Y55S, Y55F, K84M, K84R, D50N, D50E, H117A, and Y205F (108, 193).

The Y205F mutant was found to be catalytically indistinguishable from wild type r3α-HSD. This implies that Y205 is not the general acid. This represents the first time that the Tyr in the conserved Tyr-X-X-X-Lys consensus sequence has been mutated with retention of enzyme activity. Since Tyr 205 is located on an α-helix on the periphery of the structure, these data suggest that this motif does not correspond structurally to the same sequence in the SDRs. The Y55S, Y55F, K84M, K84R, D50N, and D50E mutants displayed a dramatic loss of enzyme activity. Each of these mutants, however, retained the ability to bind first ligand, namely NADPH, without a dramatic change in K_d. Thus the K_d for NADPH fluctuated only 2-fold. The Y55F and Y55S mutants were unable to catalyze steroid oxidoreduction, and this was also true of the K84R and K84M mutants. In contrast, the D50N, D50E, and H117A mutants had catalytic efficiencies of 1/30 th, 1/30 th, and 1/500 th of wild type r3α-HSD, respectively, indicating that although these residues are essential for maintaining a high *kcat* they are not the general acid or base. Knowing that testosterone is a competitive inhibitor, the ability of the mutants to form a ternary complex of E·NADH·testosterone was measured by equilibrium dialy-

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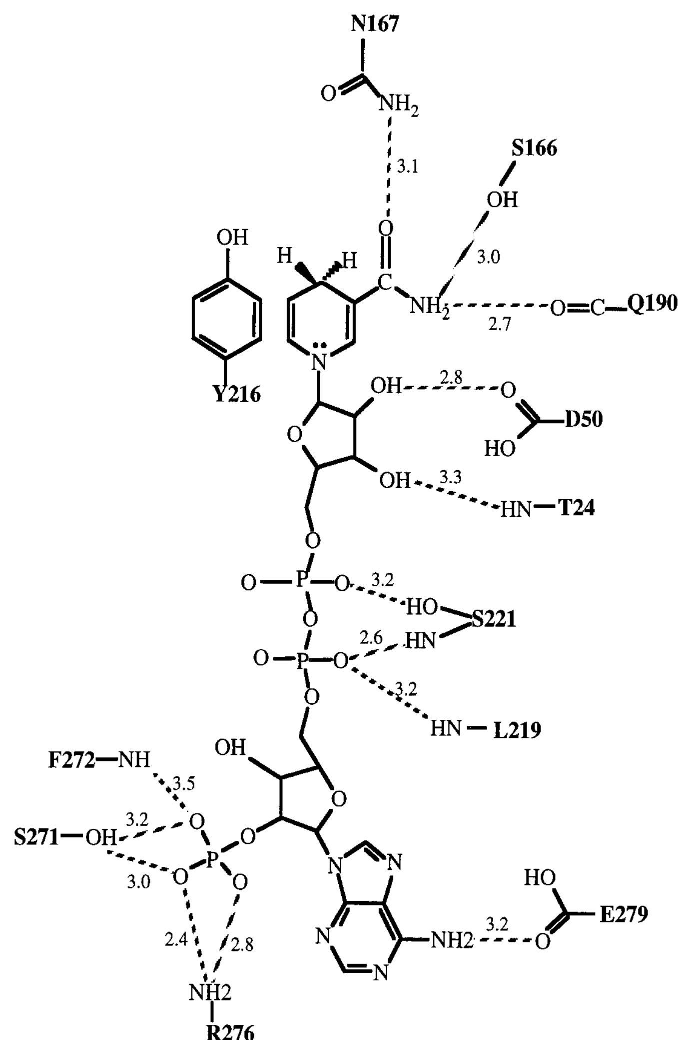

FIG. 15. NADPH recognition by 3α-HSD. Amino acid residues that make individual contact with the cofactor are labeled. These contacts are either from the amino acid side chains or the amide group (NH) of the peptide bond. *Numbers* refer to distances in angstroms. [Reprinted with permission of the publisher from T. M. Penning *et al.*: Mammalian 3 alpha-hydroxysteroid dehydrogenases. *Steroids* 61: 508–523. © 1996 by Elsevier Science, Inc.]

sis. It was found that although the Y55F and Y55S mutants displayed a 30-fold increase in the K_d for testosterone, this reduced affinity for steroid hormone was insufficient to explain the complete loss of enzyme activity. In contrast, the Lys 84 and His 117 mutants were unable to bind steroid hormones at the limit of detection (50 μM). Since the Y55 mutants are still capable of forming ternary complexes, but cannot perform catalysis, these data would support the mechanism described in which Tyr 55 is the general acid and its effective pK_a may be lowered by Lys 84. However, these data do not rule out a role for either Lys 84 or the water molecule to act as the general acid.

G. Convergent evolution to a common reaction mechanism

As described earlier, cDNA cloning indicates that HSDs belong to two main protein superfamilies, the SDRs and the AKRs. Despite the large differences in three-dimensional structures between the two protein families, it is remarkable that site-directed mutagenesis suggests that both families may have evolved similar catalytic mechanisms to perform their functions involving the use of a Tyr/Lys pair. To investigate this further, the nicotinamide rings in 3α,20β-HSD (a SDR) and 3α-HSD (an AKR) were superimposed from their respective crystal structures. It was found that the catalytic tyrosines and lysines fall into the same three-dimensional space, providing strong evidence of conservation of catalytic mechanism across both protein families (Fig. 16) (190).

H. Engineering alternate substrate specificity

An eventual goal is to develop a model of how steroid hormones bind to each HSD and use this model to predict what governs the exquisite positional and stereoselectivity of these enzymes. The best test of a model would be to engineer in a predicted activity using site-directed mutagenesis. This is not merely an exercise in protein engineering. Only when these protein structures can be manipulated in a predictable fashion can it be claimed that we understand the structural basis of steroid hormone recognition. With this knowledge we will have access to the rational design of HSD inhibitors, which may have therapeutic value. Perhaps the best approach will be to build by homology the steroid-binding clefts found in the structures of related HSDs. This could be achieved using the three-dimensional structures of placental 17β-HSD and rat liver 3α-HSD as predictors of the steroid-binding pockets of mammalian HSDs that are either SDRs or AKRs, respectively.

Based on the high sequence identity that exists in the AKR superfamily, homology modeling may be more straightforward with members of this family. We have compared the residues that reside in the steroid-binding pockets of HSDs that belong to the AKR superfamily (Table 4). When residues common to rat liver 3α-HSD and human type I and type II 3α-HSDs are eliminated, several differences appear in the apolar cleft and the C terminus, which may be determinants of steroid specificity.

IX. Conclusions

HSDs belong to two protein families, the SDR and the AKR families. Good structural templates exist for members of the SDR and AKR families and can be used to predict the three-dimensional structures of other HSDs that are assigned to these families. These templates can be used to interpret site-directed mutagenesis data. The value of this approach is to determine the details of catalytic mechanism, to elucidate the structural determinants that are responsible for the exquisite regio- and stereospecificity of the mammalian HSDs, and to rationalize why point mutations result in HSD deficiencies. Site-directed mutagenesis will ultimately lead to an increased understanding of ligand recognition by these enzymes and will offer clues to the design of structure-based inhibitors that will have therapeutic value.

The regulation of many HSDs are controlled by trophic hormones so that steroid hormones can be synthesized on demand. Although the signaling mechanisms involve cAMP-dependent protein kinases and protein kinase C, the

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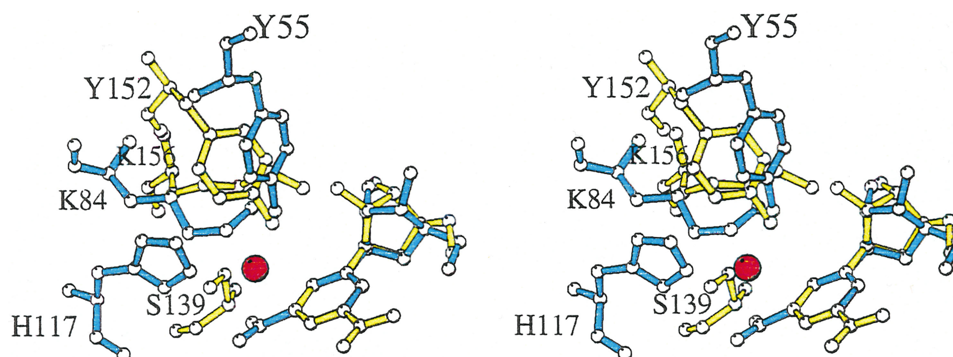

FIG. 16. Convergent evolution to a common reaction mechanism for HSD catalysis. Comparison of SDR and AKR enzymes in which the protein folds differ but the active site residues superimpose. Stereoscopic figures of active site residues in 3α,20β-HSD, a member of the SDR superfamily (*yellow*), superimposed with the active site residues of 3α-HSD, a member of the AKR superfamily (*blue*). All nonhydrogen atoms in Ser 139, Tyr 152, Lys 156, and the nicotinamide ribose in the 3α,20β-HSD·NAD^+ binary complex and all nonhydrogen atoms in Tyr 55, Lys 84, His 117, and the nicotinamide ribose in the 3α-HSD·$NADP^+$ binary complex are shown. The water molecule in 3α-HSD that may mimic the position of the carbonyl oxygen in a 3-ketosteroid substrate is represented by its oxygen atom in *red*. The superimposition was based on the nicotinamide ring position, excluding the carboxamide substituent at the C3 position. The reason for excluding this substituent was that 3α,20β-HSD transfers the *pro-S* hydrogen, while 3α-HSD transfers the *pro-R* hydrogen, so that although the nicotinamide rings lie in the same plane, they are flipped 180° relative to one another and the carboxamide groups do not superimpose. [Reprinted with permission from M. J. Bennett *et al.*: *Biochemistry* 35:10702–10711, 1996 (189). © American Chemical Society.]

TABLE 4. Residues in the steroid-binding pockets of HSDs that belong to the AKR superfamily

Residue[a]	50	52	54	55	84	86	117	118	120	128	129	137	139	227	306	308	310
Protein																	
Rat liver 3α-HSD	D	A	L	Y	K	W	H	F	M	F	F	L	F	W	N	A	Y
Human type I 3α-HSD	D	A	L	Y	K	W	H	F	M	P	L	V	F	W	V	M	F
Human type II 3α-HSD	D	A	L	Y	K	W	H	S	M	L	S	V	F	W	F	S	S
Mouse liver 17β-HSD	D	A	M	Y	K	W	H	F	M	Y	L	L	Y	W	I	G	S
Rat ovary 20α-HSD	D	S	L	Y	K	W	H	F	V	L	L	L	L	C	F	A	M
Rabbit ovary 20α-HSD	D	A	F	Y	K	W	H	F	T	I	I	A	F	W	V	A	F

Residues 306, 308, and 310 are at the C terminus.

[a] Residue numbering is relative to rat liver 3α-HSD. [Reproduced from T.M. Penning: "Hydroxysteroid dehydrogenases: new drug targets of the aldo-keto reductase superfamily" In: Enzymology and Molecular Biology of Carbonyl Metabolism 6, Advances in Experimental Medicine and Biology, vol 414: 475–490, Plenum Press, New York, 1977 (1997).]

exact mechanisms by which this leads to altered gene transcription have not been elucidated for each enzyme. Understanding how these genes are regulated will provide a basis for the molecular mechanisms by which steroidogenesis, steroid hormone metabolism, and occupancy of steroid hormone receptors are ultimately controlled at the level of HSDs. The molecular biological approach has enabled the isolation of the genes for the HSDs that are important both clinically and endocrinologically. Identification of genetic lesions in individual genes offers hope for diagnosis and treatment of HSD deficiencies. The molecular biological approach will also pave the way to examine the contribution of individual HSD subtypes to steroid-hormone action by allowing the generation of transgenic mice in which individual HSDs have been targeted for inactivation.

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