PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 69 (1999) 211–225

The Journal of Steroid Biochemistry & Molecular Biology

Molecular determinants of steroid recognition and catalysis in aldo-keto reductases. Lessons from 3α-hydroxysteroid dehydrogenase ☆

T.M. Penning*

Department of Pharmacology, University of Pennsylvania School of Medicine, 3620 Hamilton Walk, Philadelphia, PA 19104-6084, USA

Abstract

Hydroxysteroid Dehydrogenases (HSDs) regulate the occupancy of steroid hormone receptors by converting active steroid hormones into their cognate inactive metabolites. HSDs belong to either the Short-chain Dehydrogenase/Reductases (SDRs) or the Aldo-Keto Reductases (AKRs). The AKRs include virtually all mammalian 3α-HSDs, Type 5 17β-HSD, ovarian 20α-HSDs as well as the steroid 5β-reductases. Selective inhibitors of 3α-HSD isoforms could control occupancy of the androgen and $GABA_A$ receptors, while broader based AKR inhibitors targeting 3α-HSD, 20α-HSD and prostaglandin $F_{2\alpha}$ synthase could maintain pregnancy. We have determined three X-ray crystal structures of rat liver 3α-HSD, a representative AKR. These structures are of the apoenzyme (E), the binary-complex ($E.NADP^+$), and the ternary complex ($E.NADP^+$.testosterone). These structures are being used with site-directed mutagenesis to define the molecular determinants of steroid recognition and catalysis as a first step in rational inhibitor design. A conserved catalytic tetrad (*Tyr*55, *Lys*84, *His*117 and *Asp*50) participates in a 'proton-relay' in which *Tyr*55 acts as general acid/base catalyst. Its bifunctionality relies on contributions from *His*117 and *Lys*84 which alter the pK_b and pK_a, respectively of this residue. Point mutation of the tetrad results in different enzymatic activities. H117E mutants display 5β-reductase activity while Y55F and Y55S mutants retain quinone reductase activity. Our results suggest that different transition states are involved in these reaction mechanisms. The ternary complex structure shows that the mature steroid binding pocket is comprised of ten residues recruited from five loops, and that there is significant movement of a C-terminal loop on binding ligand. Mutagenesis of pocket tryptophans shows that steroid substrates and classes of nonsteroidal inhibitors exhibit different binding modes which may reflect ligand-induced loop movement. Exploitation of these findings using steroidal and nonsteroidal mechanism based inactivators may lead to selective and broad based AKR inhibitors.

1. Introduction

Hydroxysteroid Dehydrogenases (HSDs) are drug targets since tissue specific isoforms interconvert potent steroid hormones with their cognate inactive metabolites. In so doing they regulate the occupancy and activation of steroid hormone receptors. Examples include: the kidney Type 2 11β-HSD which regulates occupancy of the mineralocorticoid receptor by converting cortisol (a potent glucocorticoid with mineralocorticoid activity) to cortisone (a weak gluco- and mineralo-corticoid) [2–4]; estrogenic Type 1 17β-HSD which regulates occupancy of the estrogen receptor in breast tumors by converting estrone (a weak estrogen) into 17β-estradiol (a potent estrogen) [5–7]; and androgenic Type 3 17β-HSD as well as the labile Type 5 17β-HSD which may regulate the occupancy of the androgen receptor by converting 4-androstene-3,17-dione into testosterone [8,9]. Inhibitors of these enzymes would be useful for the treatment of hypotension and estrogen and androgen dependent tumors, respectively.

☆ Proceedings of the Xth International Congress on Hormonal Steroids, Quebec City, Quebec, 17–21 June 1998.

* Tel.: +1-215-898-9445; fax: +1-215-573-2236.
E-mail address: penning@pharm.med.upenn.edu (T.M. Penning)

0960-0760/99/$ - see front matter
PII: S0960-0760(99)00038-2

Other examples include the mammalian 3α-HSDs which can regulate the occupancy of the androgen receptor by interconverting the potent androgen, 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone, 5α-DHT) ($K_d = 10^{-11}$ M for the androgen receptor) with the weak androgen 5α-androstane-3α,17β-diol (3α-androstanediol) ($K_d = 10^{-6}$ M for the androgen receptor) [10–12]. These enzymes can thus regulate the normal and abnormal growth of the prostate [13,14]. Characterization of homogeneous recombinant human Type 2 3α(17β)-HSD suggests that it may function to eliminate 5α-DHT from the prostate and protect the androgen receptor from androgen excess [1]. In contrast, androgenic 3α-HSDs exist which can oxidize 3α-androstanediol to 5α-DHT and increase the prostate androgen pool, candidate enzymes include the Type 3 3α-HSD and human retinol dehydrogenase and are targets for androgen ablative therapy of prostate disease [15].[1] In the CNS, GABAergic 3α-HSD converts 5α-pregnane-3,20-dione (5α-dihydroprogesterone) to 3α-hydroxy-5α-pregnane-20-one (5α-tetrahydroprogesterone) which is an allosteric effector of the $GABA_A$ receptor. These neurosteroids increase chloride conductance and have anxiolytic and anesthetic effects [16–19]. Inhibitors of 3α-HSD in the CNS would be useful in attenuating the sedative effects of neurosteroids. From studies on Type 1 5α-reductase knock-out mice, uterine 3α-HSD generates 3α-androstanediol which acts as a parturition hormone and inhibition of this anti-gestational activity may help maintain pregnancy [20].

Additionally, mammalian 20α-HSDs convert the potent progestin, progesterone into the weak progestin 20α-hydroxy-pregn-4-ene-3,20-dione (20α-hydroxyprogesterone) [21,22] and inhibitors of this enzyme may be useful in preventing miscarriage.

These HSD drug targets belong to two different gene superfamilies. The Type 2 11β-HSD, Type 1 and Type 3 17β-HSDs belong to the short-chain dehydrogenase/reductases (SDRs) [23–25]. These enzymes are often multimeric, with monomers of at least 25 kDa. They are NAD(P)H specific, they contain a Rossmann fold and a conserved catalytic *Tyr*-X-X-X-*Lys* motif. Importantly, crystal structures exist for Type 1 17β-HSD complexed with estrogens [26,27]. By contrast, the mammalian 3α-HSD and 20α-HSDs belong predominantly to the AKR gene superfamily [28–33]. They are soluble, monomeric proteins of 34 kDa, they are NAD(P)H specific, they contain a conserved catalytic motif of *Tyr*55, *Lys*84, *His*117 and *Asp*50 and crystal structures exist for rat Type 1 3α-HSD. It is important to realize that the SDRs and the AKRs contain many enzymes other than HSDs, and therefore it is essential to develop a structure-based approach to drug design to gain the desired selectivity. This article will focus on the molecular determinants of catalysis and steroid recognition in 3α-HSDs and how this relates to inhibitor design in the AKR gene superfamily.

2. The AKR gene superfamily

Cluster analysis of cDNA sequences for mammalian HSDs indicates that many belong to the AKR1C family of the AKR gene superfamily [34], Fig. 1. HSDs that belong to this family include virtually all mammalian 3α-HSDs [28,31,35,36], rat and rabbit ovarian, and bovine testicular 20α-HSD [32,33,37], and murine liver 17β-HSD (Type A stereospecificity) [38]. As other HSDs are cloned and sequenced, more are likely to be assigned to this superfamily. The AKR1C family also includes the steroid double-bond reductases (5β-reductases) which precede the action of 3α-HSD in steroid hormone metabolism [39,40]. The 5β-reductases cloned so far are those found in rat and human liver and play an important role in bile acid biosynthesis. Since 5β-reduced steroids are produced elsewhere it is likely that other 5β-reductase isoforms will be cloned which are involved in steroid hormone inactivation, and that these may become drug targets for the treatment of certain hormone insufficiencies. We predict that AKRs will ultimately be identified which regulate the occupancy of diverse nuclear receptors (e.g., vitamin D receptor, retinoic acid receptors, etc), and that the appropriate AKRs await cloning and characterization.

2.1. Drug targets in the AKR gene superfamily

The AKR gene superfamily contains diverse drug targets. Thus the aldose reductases (AKR1B) convert glucose to the hyperosmotic sugar sorbitol and are targets to eradicate the complications arising from diabetes, e.g., diabetic retinopathy, neuropathy and nephropathy [41]. The aldehyde reductases (AKR1A)

[1] The properties of these human 3α-HSD isoforms are based on the characterization of the homogeneous recombinant isoforms expressed in *E. coli*. Type 2 3α-HSD also has 17β-HSD activity, it will reduce 17β-hydroxy-5α-androstan-3-one (5α-DHT) to 5α-androstane-3α,17β-diol (3α-androstanediol), and oxidize 3α-androstanediol to androsterone and 5α-androstane-3,17-dione. It can thus eliminate 5α-DHT from the prostate androgen pool [1]. It is identical to Type 5 17β-HSD described by Van-Luu The (see these proceedings). When expressed in human kidney embryonic 293 cells the enzyme displays higher 17β-HSD than 3α-HSD activity and can convert 4-androstene-3,17-dione to testosterone. This higher 17β-HSD activity is labile and its relevance to testosterone formation in the prostate in vivo remains to be determined. The recombinant Type 3 3α-HSD will reduce 5α-DHT to 3α-androstanediol but it will also catalyze the reverse reaction and could replenish the 5α-DHT pool from circulating 3α-androstanediol [Lin & Penning, unpublished data]. It is also likely that other human 3α-HSD isoforms exist.

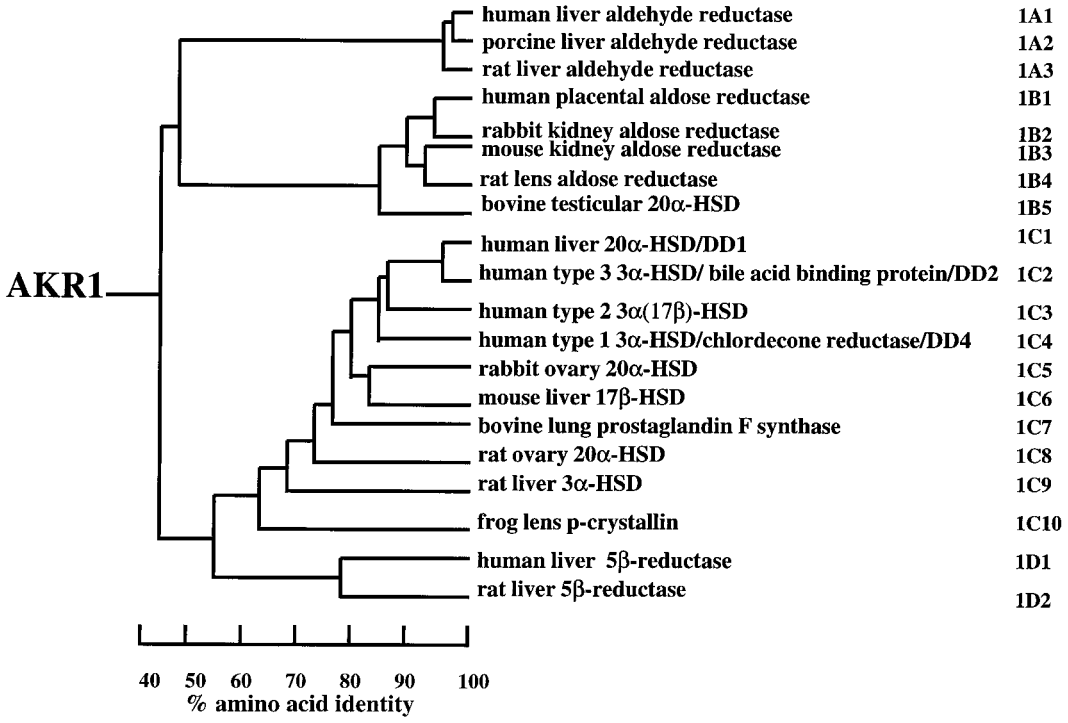

Fig. 1. Cluster analysis of the AKR1C family shows the relationship between steroid transforming aldo-keto reductases. The dendrogram is based on the pairwise-sequence alignments performed by the GCG program PILEUP and indicates the percent amino acid sequence identity among the proteins.

are involved in the metabolism of monoamine neurotransmitters and have been targeted for anti-depressant activity [42]. HSDs (AKR1Cs) which are potential drug targets include: androgenic 3α-HSD for the treatment of BPH and PCA (e.g., Type 3 3α-HSD, AKR1C2); anti-androgenic 3α-HSD for the treatment of androgen insufficiency (e.g., Type 2 3α-HSD, AKR1C3) and GABAergic 3α-HSD to attenuate the sedative properties of neurosteroids (e.g., CNS 3α-HSD). Inhibition of each of these enzymes would require the development of 3α-HSD isoform specific inhibitors. By contrast broad based AKR inhibitors targeting uterine 3α-HSD, ovarian 20α-HSD and prostaglandin F synthase may be desirable to maintain pregnancy.

2.2. Rat liver 3α-HSD as a representative AKR

Each of the steroid transforming AKRs have cDNA clones that share greater than 80% sequence identity yet they maintain regiopositional- and stereo-specificity. Thus knowledge of one structure will be pertinent to the rest and can be used to elucidate the molecular determinants of catalysis and steroid recognition.

Rat liver 3α-HSD is the most thoroughly characterized HSD of the AKR superfamily and serves as a good template for structure-based drug design for all steroid transforming AKRs (e.g., 3α-HSD, 17β-HSD, 20α-HSD and 5β-reductase). It has been purified to homogeneity [43] and has a monomer mol. wt. 37029 kDa. Milligram quantities of the recombinant enzyme can be obtained by overexpressing the cDNA in *E.coli* [28,44]. Its complete kinetic mechanism is known [45]. The enzyme follows a sequential ordered bi-bi reaction in which pyridine nucleotide cofactor binds first and leaves last. Thus the binding of cofactor is an obligatory event for the binding of steroid hormone. The catalytic mechanism involves direct hydride transfer from the 4-pro(*R*)-position of NAD(P)(H) to the C3 ketone of the steroid which is protonated by a general acid [45]. In the reverse direction a general base must deprotonate the steroid alcohol substrate to facilitate hydride transfer to the cofactor. The identity of the general acid and base is now known (see below). Crystal structures of the apoenzyme (E), the binary complex (E.NADPH), and the ternary complex ($E.NADP^+$.testosterone) exist as the first step in a structure-based drug-design approach for AKRs [46–48]. Importantly, the ternary complex structure was obtained with recombinant enzyme suggesting that structures of mutant enzymes and their complexes will be forthcoming.

The X-ray crystal structure of the apoenzyme was solved by molecular replacement at a resolution of 3.1 Å using the structure of human placental aldose re-

ductase which shares 48% sequence identity with the rat liver enzyme [46,49], Fig. 2. This was the first X-ray crystal structure of an HSD that is an AKR superfamily member. The apoenzyme adopts an $(\alpha/\beta)_8$-barrel in which there is an alternating arrangement of α-helix and β-strands. The β-strands coalesce in the middle of the structure forming the staves of the barrel. At the back of the structure three large loops are apparent, the A-loop, the B-loop and the C-terminal loop, Fig. 2A and B. Of these the B-loop could not be seen in the electron density and was modeled into the structure using the coordinates for placenta aldose reductase. Later, the structure of the binary complex was solved at a resolution of 2.7 Å [47]. In this structure the cofactor lies perpendicular to the barrel axis with the nicotinamide ring facing the core of the barrel and the adenine ring lying at the periphery. The structure shows why the binary complex is unsatisfactory to understand steroid recognition at the atomic level. Loop structures A, B and C are flexible and may change position once steroid is bound. For these reasons we recently solved the X-ray crystal structure of E.NADP$^+$.testosterone to 2.5 Å resolution [48]. This is the first structure of a protein with the male sex hormone testosterone bound. In this structure the steroid lies perpendicular to the cofactor, the A-ring of the steroid and the nicotinamide ring of the cofactor converge in the core of the barrel. The mature steroid binding pocket consists of 5-loops and loop-B and the C-terminal loop undergo significant loop movement.

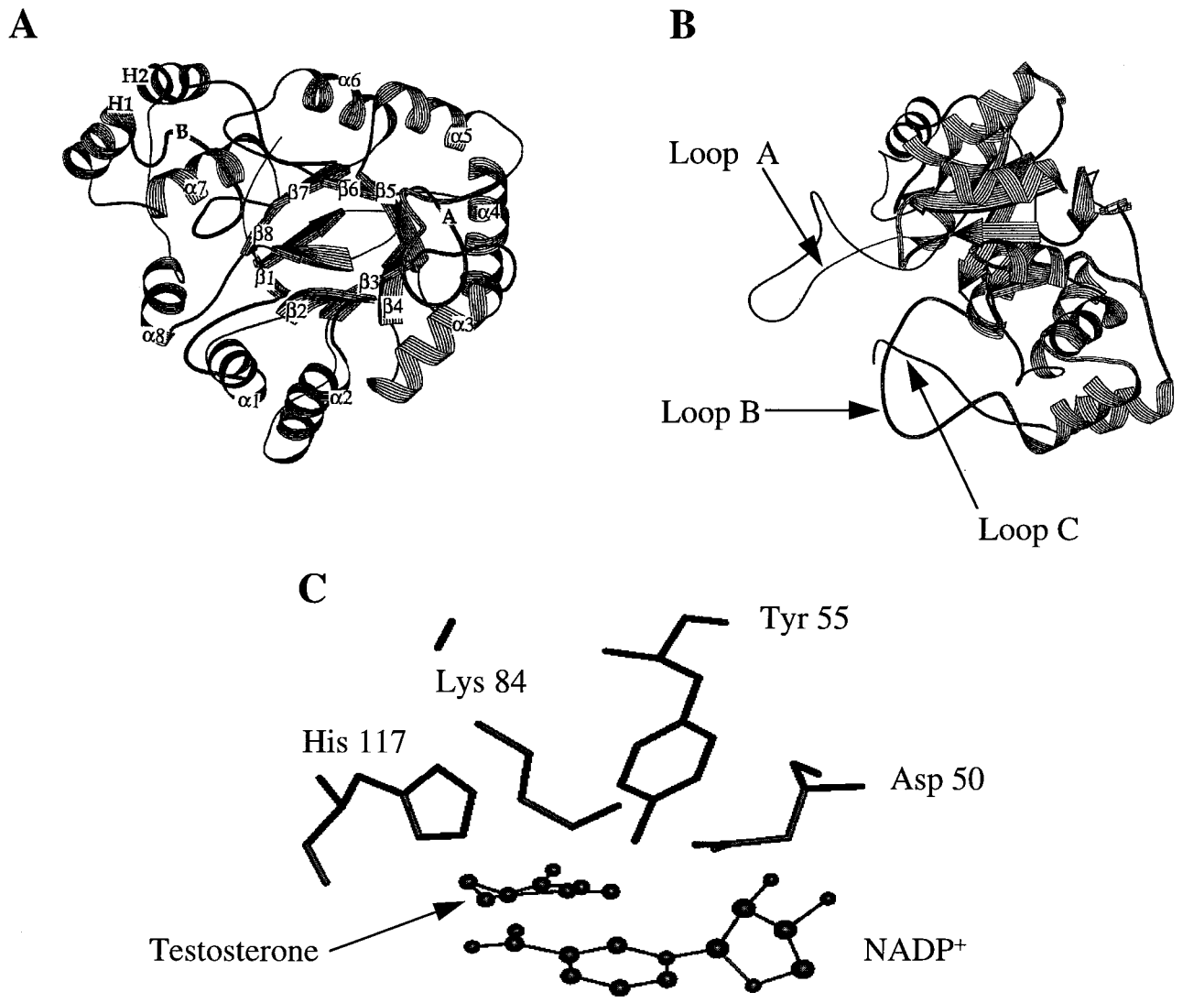

Fig. 2. Barrel structure, loops and the catalytic tetrad in 3α-HSD. (A) Shows the $(\alpha/\beta)_8$ barrel structure of the apoenzyme; (B) shows (A) rotated 90° around the vertical plane to display loops A, B and C at the back of the barrel; and (C) shows the position of the catalytic tetrad with respect to the C4 position of the cofactor and the C3 ketone of testosterone based on the structure of the ternary complex E.NADP$^+$.testosterone [48].

The structure emphasizes the danger of trying to understand the structural basis of steroid recognition without a steroid-enzyme complex. The ternary complex structure reveals the relationship between: the 4-pro(*R*)-hydrogen of the nicotinamide ring, the C3 ketone of testosterone (a competitive inhibitor) and the putative catalytic tetrad, Fig. 2C. This tetrad of *Tyr*55, *Lys*84, *His*117 and *Asp*50 is conserved throughout the AKR superfamily and is a clear target for site-directed mutagenesis to establish the chemical mechanism.

3. Molecular determinants of catalysis

Mutational analysis of the catalytic tetrad has revealed the chemical mechanism for HSDs. In addition point mutations of the tetrad resulted in new enzymatic activities (introduction of 5β-reductase activity and retention of quinone reductase activity). These engineered activities point to different transition states for the individual reactions and have implications for transition state analog design. The work on the quinone reductase mutants will be discussed elsewhere [50].

3.1. Site-directed mutagenesis reveals the chemical mechanism mechanism for HSDs

Seven active site mutants (Y55F, Y55S, H117A, D50N, D50E, K84R, K84M) were overexpressed and purified to homogeneity from *E. coli* [44,51]. All mutants formed the binary complex E.NADPH unimpeded. The Y55F and Y55S mutants displayed only a 10-fold decrease in steroid affinity yet they were the most severely catalytically impaired. By monitoring the conversion of [^{14}C]-5α-DHT to 3α-androstanediol the contribution of each residue to rate enhancement k_{cat}/k_{noncat} was assessed. All mutants catalyzed this reaction but the major contributor to rate enhancement was *Tyr*55, where k_{cat}/k_{noncat} was reduced by 10^4. These data support a role for *Tyr*55 as the general acid. The ability to detect enzyme activity in all the mutants permitted the generation of detailed k_{cat} vs pH-rate profiles; an experiment rarely performed on site-directed mutants of HSDs. It was predicted that the mutant which abolished the titratable group with the largest effect on the pH-independent value of k_{cat} would be the candidate general acid.

The enzymatic reduction of 5α-androstane-3,17-dione and 5α-DHT by wild type enzyme required a group that must be protonated (a general acid) for maximal activity and has a pK_b = 7.3, Fig. 3. Conversely the enzymatic oxidation of androsterone or 3α-androstanediol required a group that must be deprotonated for maximal activity (a general base) and has a pK_a = 7.3, Fig. 3. The same group may function as both the general acid and base. For 5α-DHT reduction, the titratable group was eliminated in both the Y55F and H117A mutants but was retained in the K84 M mutant. The largest effect on the pH-independent value of k_{cat} was seen with Y55F. For 3α-androstanediol oxidation the titratable group was lost both with the Y55F and the K84 M mutant but was retained in the H117A mutant. Only the Y55F mutant abolished the titratable group in both directions indicating that it functions as the general acid-base. This bifunctionality requires participation from other

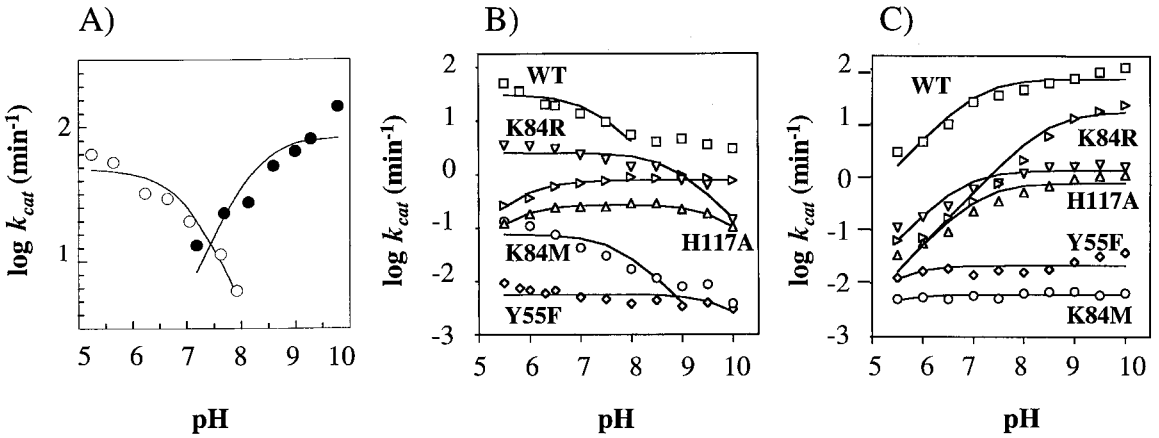

Fig. 3. (A) log k_{cat} vs pH for steroid oxidoreduction catalyzed by the wild type and mutant forms of 3α-HSD: 5α-androstane-3,17-dione reduction (○) and androsterone oxidation (●) catalyzed by wild-type enzyme. (B) log k_{cat}(app) vs pH profiles for [^{14}C]-5α-DHT reduction catalyzed by wild type and tetrad mutants of 3α-HSD: wild-type (□), K84R (▽), D50N (▷), H117A (△), K84M (○), and Y55F (◇). (C) log k_{cat}(app) vs pH profiles for [^{3}H]-3α-androstanediol oxidation catalyzed by wild type and tetrad mutants of 3α-HSD: wild-type (□), D50N (▷), K84R (▽), H117A (△), Y55F (◇), K84 M (○). Lines indicate the best fit to the equations described [59]. Reproduced with permission from the American Chemical Society.

groups in the tetrad. In the reduction direction *His*117 facilitates proton donation by *Tyr*55 and in the oxidation direction *Lys*84 facilitates proton removal by *Tyr*55, Fig. 4. Thus *Tyr*55 relies on different partners in the tetrad to act as a general acid-base catalyst. In the reduction direction 4-pro(*R*) hydride transfer is facilitated by a proton relay to a delocalized positive charge on the imidazole ring of *His*117 and explains the pK_b observed. In the oxidation direction the proton relay is initiated by *Lys*84 which forms a phenolate anion on *Tyr*55. This base extracts the proton from the steroid alcohol to facilitate hydride transfer and explains the pK_a observed. This 'push-pull' mechanism may be applicable to all AKRs which retain this conserved catalytic tetrad.

3.2. Engineering 5β-reductase activity into 3α-HSD

A test of how well the catalytic mechanism of AKRs is understood is whether it is possible to engineer new enzyme activities into 3α-HSD. 5β-reductase precedes 3α-HSD in steroid hormone metabolism and is also an AKR. Alignment of tetrad residues in AKRs indicated that *His*117 had been replaced with *Glu*117 in 5β-re-

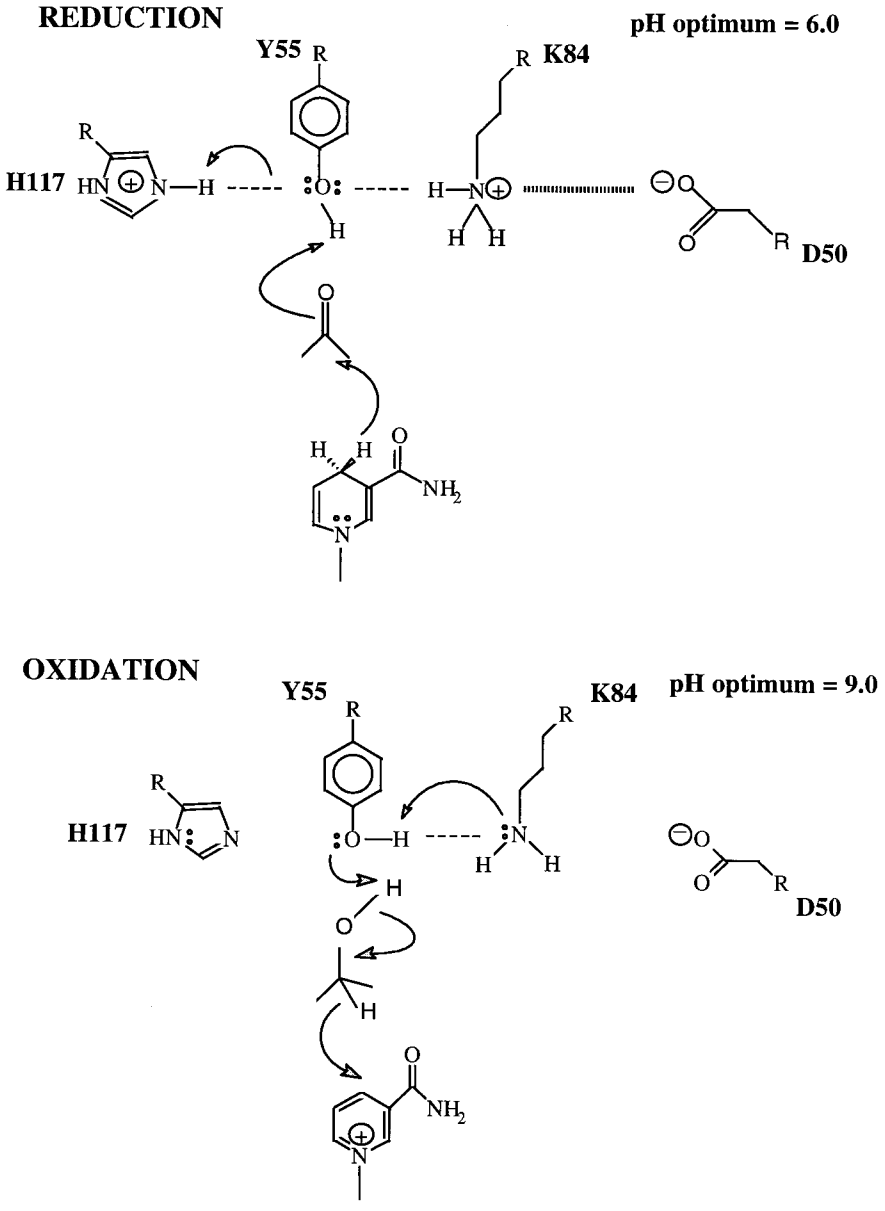

Fig. 4. Catalytic mechanism for HSDs. In the reduction direction *Tyr*55 acts as a general acid by donating its proton to the acceptor carbonyl of the steroid. This is facilitated by protonation of the tyrosyl hydroxyl group by the imidazole ring of *His*117 to yield the observed pK_b. In the oxidation direction *Tyr*55 acts as a general base by using its phenolate anion to abstract a proton from the steroid alcohol. This is facilitated by deprotonation of the tyrosyl hydroxyl group by the ε-amino group of *Lys*84, to yield the observed pK_a.

ductase. The H117E mutant of 3α-HSD was constructed to see if it was possible to introduce 5β-reductase activity into 3α-HSD [52]. Homogeneous H117E mutants were examined for their ability to reduce [^{14}C]-testosterone to 17β-hydroxy-5β-androstan-3-one (5β-dihydrotestosterone) and reduce [^{14}C]-progesterone to 5β-pregnane-3,20-dione (5β-dihydroprogesterone) using NADPH as cofactor. In each case 5β-reductase activity was observed. The enzyme also retained 3α-HSD activity since the 5β-dihydrosteroid products were converted to the corresponding 5β,3α-tetrahydrosteroids, Fig. 5. Importantly, calculation of k_{cat} showed that the H117E mutant had turnover numbers similar to those described for homogeneous rat liver 5β-reductase [53].

k_{cat} vs pH profiles were informative for the H117E mutant. The wild-type enzyme showed the presence of a titratable group for 5β-DHT (3-ketone reduction) with a pK_b = 7.0 which has been assigned to *Tyr*55. The H117A mutant as before showed the loss of this titratable group. In the H117E mutant the titratable group is restored and the titration curves observed for

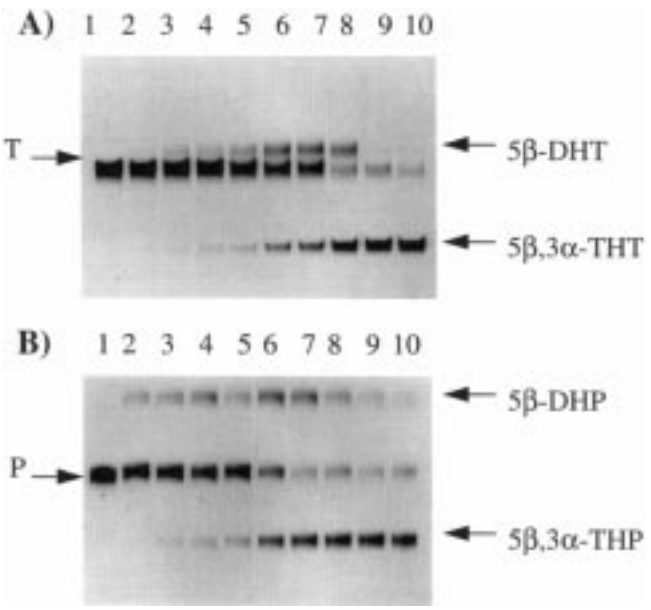

Fig. 5. Steroid double-bond reduction catalyzed by the H117E mutant of 3α-HSD. (A), [^{14}C]-testosterone (T) (50 μM) and 2.3 mM NADPH were incubated in 100 mM potassium phosphate pH 7.0 with 10 μg H117E for 0 min (lane 1), 2 min (lane 2), 5 min (lane 3), 10 min (lane 4), 20 min (lane 5), 40 min (lane 6), 60 min (lane 7), 120 min (lane 8), 180 min (lane 9) and 240 min (lane 10). The formation of 5β-dihydrotestosterone (5β-DHT) and 5β-androstane-3α,17β-diol (5β,3α-THT) are clearly visible. (B), [^{14}C]-progesterone (P) (50 μM) and 2.3 mM NADPH were incubated in 100 mM potassium phosphate pH 7.0 with 10 μg H117E for 0 min (lane 1), 2 min (lane 2), 5 min (lane 3), 10 min (lane 4), 20 min (lane 5), 40 min (lane 6), 60 min (lane 7), 120 min (lane 8), 180 min (lane 9) and 240 min (lane 10). The formation of 5β-dihydroprogesterone (5β-DHP) and 3α-hydroxy-5β-pregnan-20-one (5β,3α-THT) are clearly visible.

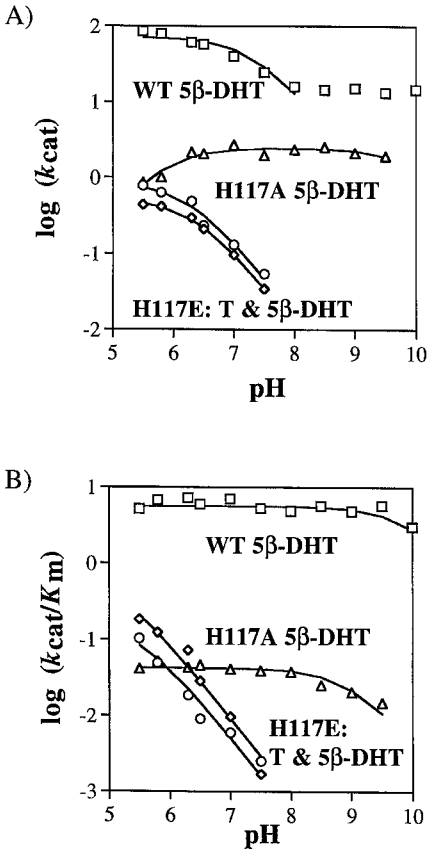

Fig. 6. pH dependence of 5β-reductase and 3α-HSD activities catalyzed by wild-type 3α-HSD and the H117E and H117A mutants. (A) log k_{cat} vs pH and (B) log (k_{cat}/K_m) vs pH profiles for 5β-DHT reduction catalyzed by wild-type 3α-HSD (□) and the H117A (△) and H117E (○) mutants and for testosterone reduction catalyzed by the H117E (◇) mutant.

5β-DHT (3-ketone) and testosterone (Δ^{-4}-ene) reduction are now superimposable, suggesting that the same acid is required for both reactions, Fig. 6. However, the pK_b of *Tyr*55 has been acid-shifted to a pK_b = 6.3 suggesting that the acidity of this residue has been increased by a fully protonated *Glu*117. These results are consistent with acid catalyzed enolization of the enone. In the proposed mechanism the *Glu*117 lowers the pK_b of *Tyr*55. This increased acidity promotes enolization of the Δ^{-4}-3-ketone and facilitates hydride transfer to a partial positive charge at C5, Fig. 7.

3.3. Implications for transition state analog design

What do these studies mean for AKR drug design? They provide information about the transition state and hence the design of transition state analogs, Fig. 8. For HSDs, hydride transfer and proton donation could be concerted suggesting that a bi-substrate transition state analog would be preferred. If hydride ion transfer occurs first a tetrahedral oxyanion transition state would be formed. If protonation occurs first a carbocation transition state analog would be produced.

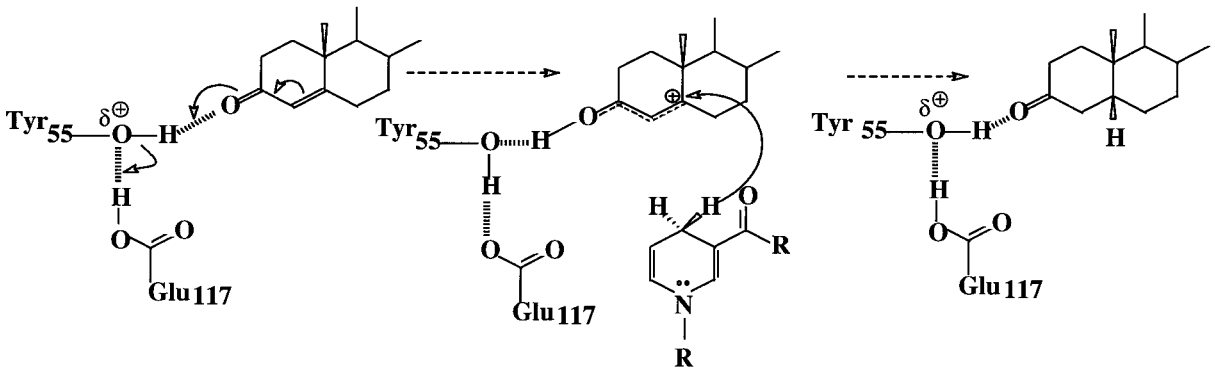

Fig. 7. Proposed mechanism for double-bond reduction in the 3α-HSD H117E mutant and native 5β-reductase, in which a protonated *Glu*117 facilitates the reaction catalyzed by *Tyr*55, the general acid.

The mechanism proposed by site-directed mutagenesis supports the formation of an oxyanion intermediate that is stabilized by the delocalized positive charge of the imidazole ring of H117. Thus candidate transition state analogs would include steroid boronic acid derivatives that have sp^3 character, or steroid carboxylates that could mimic the oxyanion and bind to the oxyanion hole. For 5β-reductases, hydride transfer and proton donation could be concerted and a bi-substrate analog would be desirable. If hydride transfer occurs first then a transition state analog based on an enolate anion would be preferred. If protonation occurs first then preference would exist for a carbocation intermediate. The mechanism proposed by site-directed mutagenesis would support the formation of *Tyr*-OH_2^+ suggesting that protonation occurs first forming a partial carbocation. Candidate transition state analogs that could inhibit 5β-reductase might include steroid heterocycles, not unlike finasteride, that can form a partial positive charge in the A-ring. Such compounds should be based on A/B-*cis*-ring fused or bent steroids.

4. Molecular determinants of steroid recognition

4.1. Lessons from a ternary complex structure

To develop potent reversible HSD inhibitors, compounds must form multiple contacts with steroid binding pocket residues to increase the binding energy.

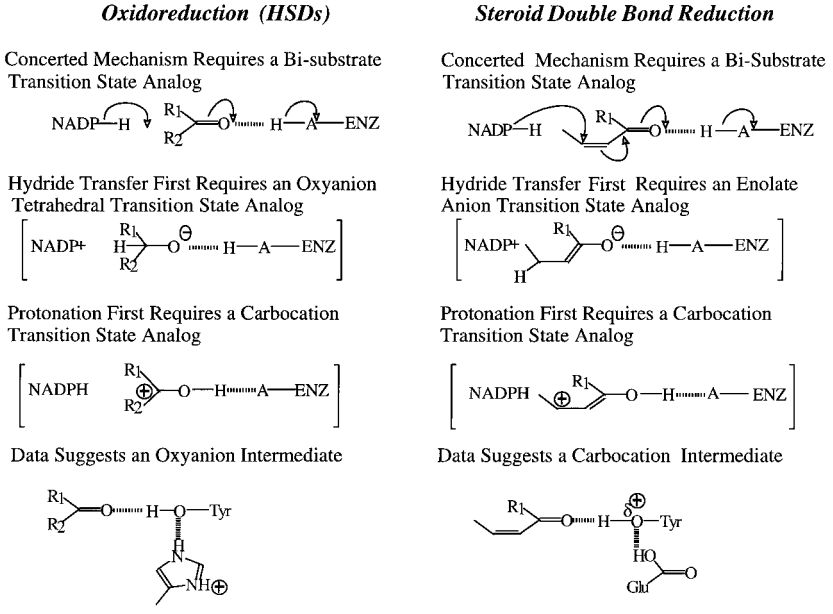

Fig. 8. Implications for transition state analog design based on site-directed mutagenesis of the catalytic tetrad in 3α-HSD. For C3 ketone reduction the reaction proceeds via an oxyanion intermediate with hydride transfer occurring first. In contrast steroid double bond reduction occurs via a partial carbonium ion with proton donation occurring first. In the former case transition state analogs should be designed based on a steroid oxyanion intermediate and in the latter case the transition state analog should be designed on steroids that contain a partial positive charge in the A-ring.

Table 1
Alignment of the ten loop residues that comprise the steroid binding pockets of HSDs in the AKR gene superfamily[a]

Loop Position	N-terminus	β2-α2-loop		Loop-A			Loop-B		C-terminus	
	24	54	55	117	118	129	226	227	306	310
Rat liver 3α-HSD	T	L	Y	H	F	F	T	W	N	Y
Human Type 1 3α-HSD	Y	L	Y	H	F	L	L	W	V	F
Human Type 2 3α-HSD (3α,17β-HSD)	Y	L	Y	H	S	S	R	W	F	S
Human Type 3 3α-HSD (3α,20α-HSD)	Y	V	Y	H	F	I	P	W	L	I
Rat ovary 20α-HSD	Y	L	Y	H	F	L	Y	C	F	M
Rabbit ovary 20α-HSD	Y	F	Y	H	F	I	E	W	V	F
Murine liver 17β-HSD	Y	M	Y	H	F	L	Q	W	I	S

[a] Bovine testis 20α-HSD is excluded because it is identical to aldose reductase and does not reduce progesterone. Although *Trp*86 is not a loop residue, it is in the core of the pocket and close to *His*117.

Therefore it is necessary to understand the structural basis for steroid recognition and the basis by which HSDs can display positional and stereospecificity for different steroid hormone substrates. The 3α-HSD ternary-complex structure showed that two-loops move significantly when testosterone is bound [48]. Thus the B-loop collapses to fold onto the steroid and the C-terminal loop rotates to fold over the A-ring of the steroid. These loops are at the back of the $(\alpha/\beta)_8$-barrel structure. In all 10-residues make up the mature steroid-binding site and are recruited from 5-loops, Table 1 and Fig. 9A. Of the 322 amino acids in the protein, *Tyr*310 on the C-terminal loop stacks against the A-ring of the steroid. As a result of the loop movement 95% of the surface of testosterone is solvent inaccessible. Based on this structure the equivalent ten residues

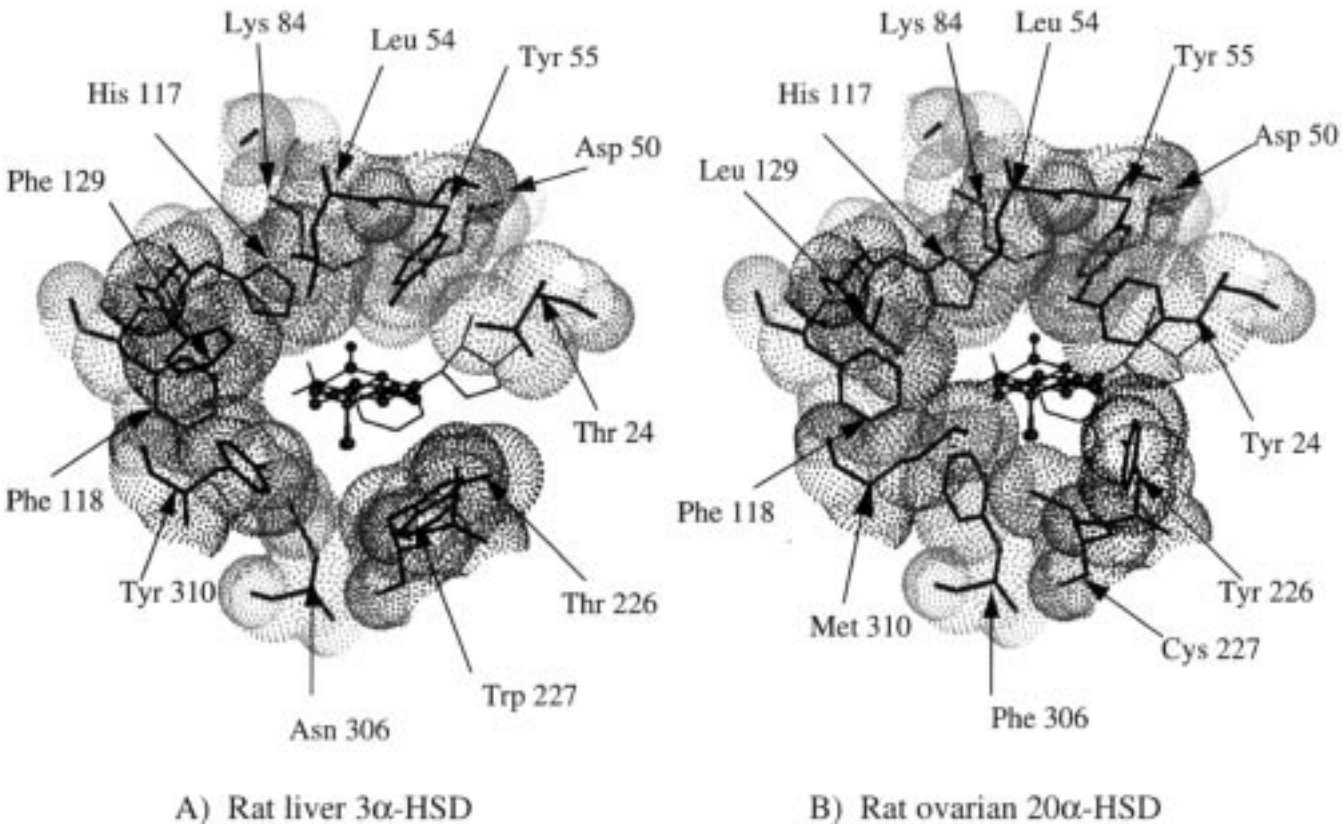

Fig. 9. Steroid binding pockets in AKRs. (A) The mature steroid binding pocket of 3α-HSD. The view shown is taken from the E.$NADP^+$.testosterone ternary complex [48]. (B) The predicted steroid binding pocket of rat ovarian 20α-HSD. The amino acid residues in the binding pocket of 3α-HSD were mutated to those found in 20α-HSD. Testosterone was modeled into the site and significant clashing is observed with the following residues *Tyr*226, *Cys*227, *Trp*227, *Phe*306 and *Met*310.

in other HSDs that are AKRs were aligned to elucidate the structural basis of steroid specificity, Table 1.

Using the rat 3α-HSD ternary complex as a template molecular modeling has been performed to predict the three-dimensional structures of other AKR steroid binding sites. When this is conducted for rat ovarian 20α-HSD the pocket no longer accommodates testosterone. The models predict that several mutations would be required to change 3α-HSD to 20α-HSD. Additionally, inspection of the amino acid alignments (Table 1) shows that once the catalytic residues and the conserved and semi-conserved residues are eliminated only a few residues are left to define steroid specificity. Those that remain are on loop-B and the C-terminal loop. Preliminary point-mutagenesis in this pocket shows that it is difficult to alter the positional preference of a given HSD by making single or multiple substitutions. Greater success can be achieved with loop chimeras suggesting that different residues may be recruited from the loops to define the pocket for a given steroid ligand, i.e., there is substrate induced-fit [Ma and Penning, unpublished observations].

4.2. Evidence for different ligand binding modes within the same pocket

Mutagenesis of two tryptophans within the steroid binding pocket, *Trp*86 in the core of the pocket and *Trp*227 on loop-B, supports the concept that there are ligand dependent binding modes [54]. Thus W86Y mutants show low catalytic efficiency (k_{cat}/K_m) for small ring substrates, e.g., *p*-nitroacetophenone while W227Y mutants show high catalytic efficiency with small ring substrates. This situation is reversed as the substrate ring size increases, Fig. 10. These data indicate that different binding modes exist within the same pocket for small ring nonsteroidal and steroidal based substrates.

The existence of different binding modes could be exploited to develop nonsteroidal reversible inhibitors for these HSDs. It is known that nonsteroidal anti-inflammatory drugs (NSAIDs) are potent inhibitors of 3α-HSD, ($K_i = 0.8$ μM for indomethacin) [43]. Additionally ponalrestat and zolpolrestat are exceedingly potent inhibitors of aldose reductase, a related AKR, yielding K_i values between 3–10 nM [55,56]. Yet ponalrestat and zolpolrestat only inhibit 3α-HSD with micromolar affinity. Thus aldose reductase inhibitors display a 1000-fold selectivity for their target AKR. Interestingly, both the NSAIDs and aldose reductase inhibitors have an identical pharmacophore, a carboxylic acid that could bind to the oxyanion hole, linked to a heterocycle with a bridge to an additional aromatic ring system, Fig. 11. When these compounds were evaluated as inhibitors for the W86Y and W227Y mutants different outcomes were observed. Neither mutant affected the binding of the aldose reductase inhibitors whereas the W86Y mutant severely impeded the binding of NSAIDs. Further, the W227Y mutant had no effect on binding NSAIDs yet severely affects the binding and turnover of steroid substrates. This suggests that aldose reductase inhibitors, NSAIDs and steroid substrates all have different binding modes within the same apolar pocket [54]. These different binding modes could be exploited for the development

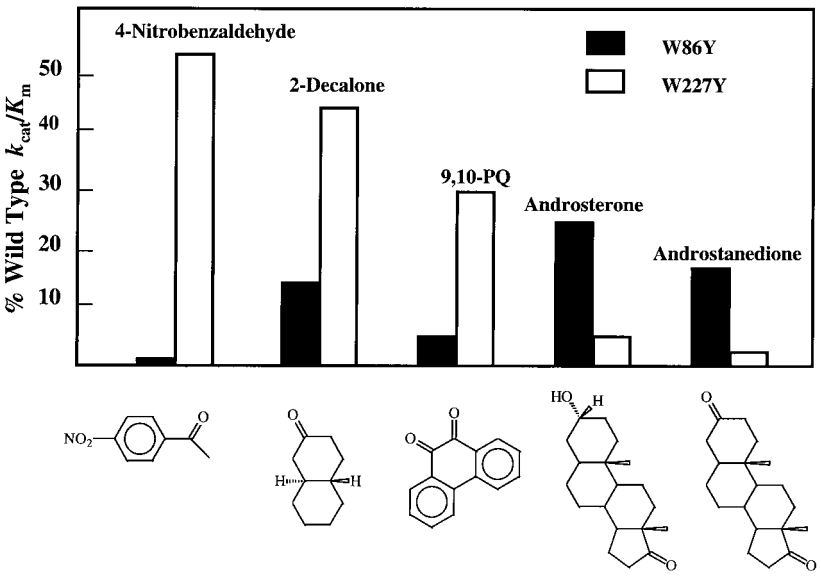

Fig. 10. Percent log k_{cat}/K_m (catalytic efficiency) for wild type and the W86Y and W227Y mutants for the turnover of one to four ring substrates demonstrates different ligand binding modes within the same AKR pocket.

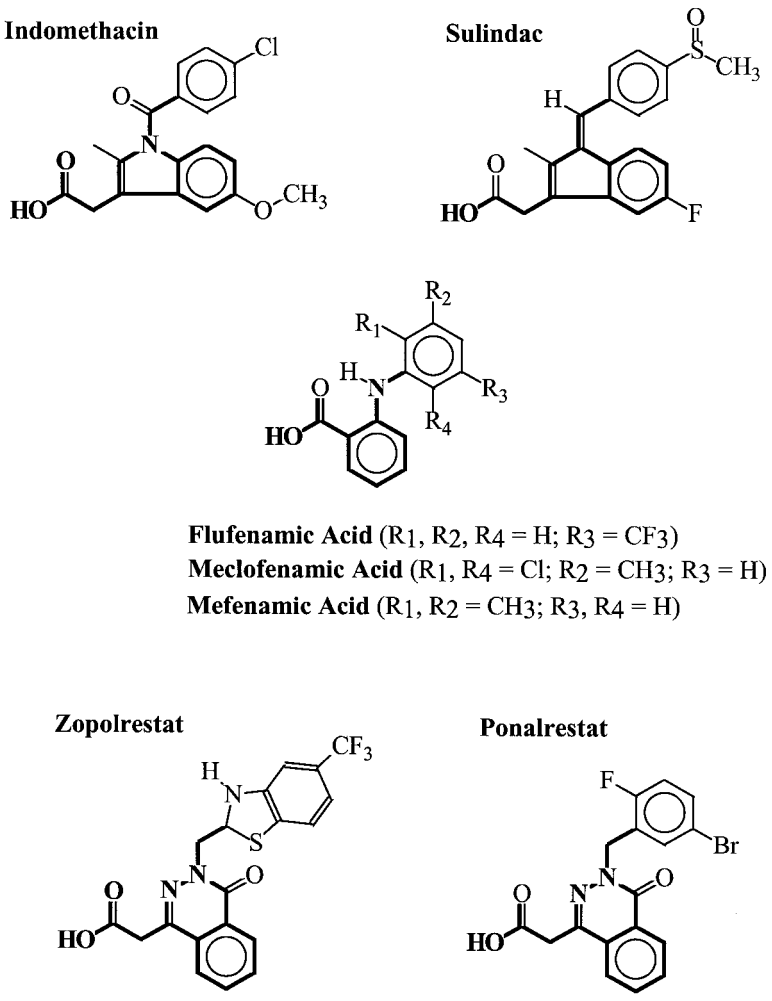

Fig. 11. Structures of nonsteroidal reversible enzyme inhibitors of AKRs. The common pharmacophore in NSAIDs and aldose reductase inhibitors is indicated in bold.

of a spectrum of reversible nonsteroidal inhibitors that fill different portions of the binding site.

4.3. Nonsteroidal mechanism based inactivators for 3α-HSD

Nonsteroidal inhibitors can bind to 3α-HSD with reasonable affinity which suggests that it is possible to develop mechanism-based inactivators for HSDs in the AKR superfamily based on nonsteroids. We synthesized allylic and acetylenic alcohols and cyano- and bromo-hydrins based on *p*-nitroacetophenone as mechanism-based inactivators [57], Fig. 11. In each instance enzymatic oxidation of the alcohol would yield an activated ketone which could undergo nucleophilic attack to produce inactivated enzyme. The specificity of these compounds would depend on the ability of only the target enzyme to produce the activated ketone, and the length of their effect would be defined by the time required to synthesize new enzyme (Fig. 12).

Of the compounds synthesized the most promising were the vinyl and acetylenic alcohols **1** and **3**, which were screened as mechanism based inactivators against rat liver 3α-HSD (an AKR), rat ovarian 20α-HSD (an AKR) and *Streptomyces* 3α,20β-HSD (an SDR). It was found that 200 μM allylic alcohol **1** inactivated 3α-HSD in a time dependent manner yielding a $t_{1/2}$ of less than 1.5 min provided $NADP^+$ was included [57], Table 2. No evidence could be obtained for an $NADP^+$-dependent inactivation of either rat ovarian 20α-HSD or *Streptomyces* 3α/20β-HSD. The enzyme generated vinyl ketone **2** (24 μM) inactivated 3α-HSD with a $t_{1/2}$ life of less than 1.0 min. It was found that the vinyl ketone actually acted as a stoichiometric inactivator of 3α-HSD, suggesting that very little vinyl alcohol needs to be oxidized to inactivate the target enzyme. By contrast nearly 500 μM vinyl ketone **2** was required to inactivate rat ovarian 20α-HSD with a $t_{1/2} = 5.0$ min these properties gave a selectivity quotient ($[I]/t_{1/2}$) of 100-fold for the preferential inactivation of rat 3α-HSD over rat 20α-HSD by the vinyl ketone. Similarly, inactivation of *Streptomyces* 3α,20β-

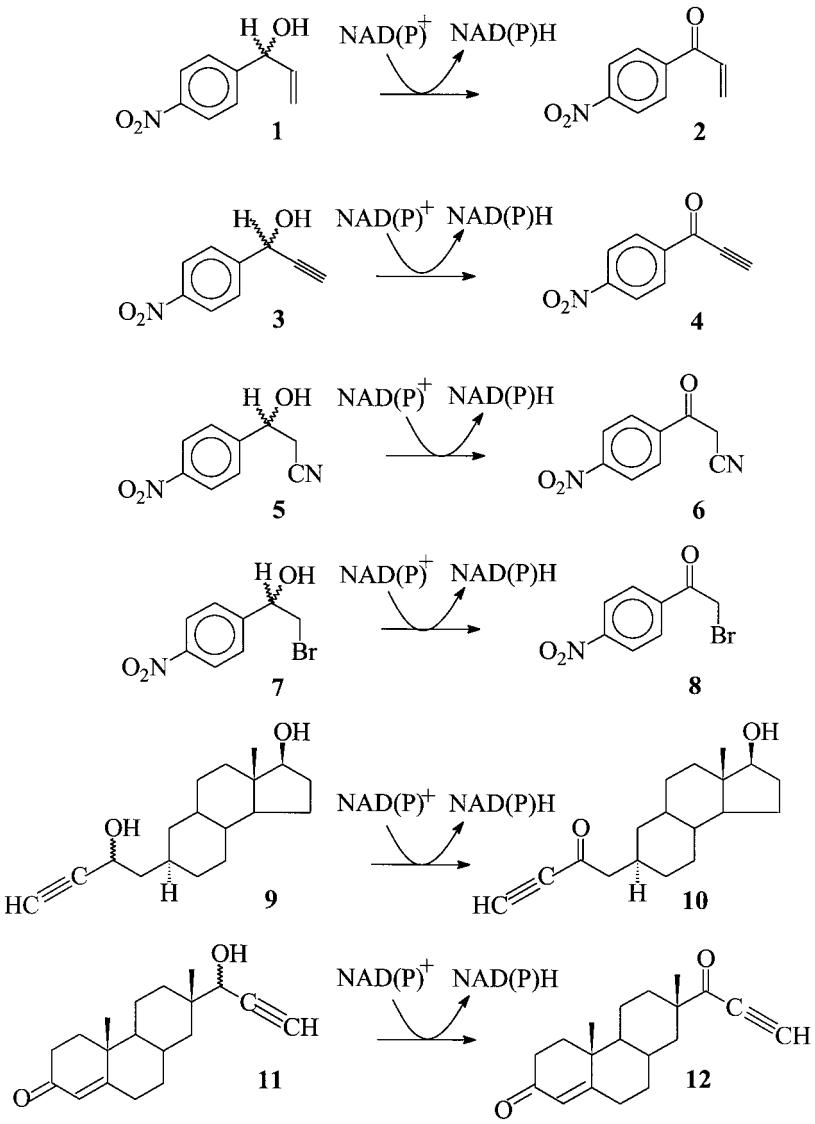

Fig. 12. Nonsteroidal and steroidal mechanism based inactivators can be oxidized by 3α-HSD to yield reactive ketones that will covalently modify this enzyme.

Table 2
Time dependent inactivation of HSDs by nonsteroidal mechanism based inactivators

Enzyme	Family	Nucleotide[a]	Vinyl alcohol (**1**) (mM)	$t_{1/2}$[b] (min)	Acetylenic alcohol (**3**) (mM)	$t_{1/2}$[b] (min)
3α-HSD: Rat liver	AKR	NAD^+	0.2	1.5	1.0	25
20α-HSD: Rat ovary	AKR	$NADP^+$	0.2	ND	1.0	ND
3α,20β-HSD: *Streptomyces*	SDR♯b	NAD^+	0.9	ND	1.2	ND
Enzyme	Family	Nucleotide[c]	Vinyl ketone (**2**) (mM)	$t_{1/2}$[b] (min)	Acetylenic ketone (**4**) (mM)	$t_{1/2}$[b] (min)
3α-HSD: Rat liver	AKR	None	0.024	1.0	0.024	< 1.0
20α-HSD: Rat ovary	AKR	None	0.50	5.0	0.66	6.0
3α,20β-HSD: *Streptomyces*	SDR	None	0.53	12.0	0.37	38.0

[a] Nucleotide is required for the time dependent inactivation by the vinyl and acetylenic alcohols.
[b] $t_{1/2}$ life of the enzyme.
[c] No nucleotide is required to observe enzyme inactivation with these activated ketones.

HSD required 530 μM vinyl ketone **2** and gave a $t_{1/2}$ for the enzyme of 12.0 min providing a 240-fold selectivity quotient for the inactivation of rat 3α-HSD. Thus selective nonsteroidal mechanism-based inactivators can be designed for HSDs in the AKR superfamily.

4.4. Secosteroid mechanism based inactivators

As an alternative, consideration was given to generate allylic and acetylenic alcohols as mechanism-based inactivators based on steroids. However, the introduction of these latent groups at the C3 or C17 position of the steroid necessitated the synthesis of secosteroid based ligands **9–12**, [58]. Thus the synthetic routes to these compounds are demanding and unlike nonsteroidal compounds the structural variants are limited. We evaluated A- and D-ring secosteroid acetylenic alcohols **9** and **11**, as mechanism based inactivators of 3α-HSD. Unfortunately, both compounds caused time dependent inactivation of rat liver 3α-HSD provided $NADP^+$ was present. These data suggest that the D-ring secosteroid **11** can bind into the active site backwards and is oxidized to the acetylenic ketone **12**. Thus secosteroid mechanism-based inactivators are likely to be broader based AKR inhibitors and could inactivate both 3α- and 20α-HSDs. Such compounds might be useful in maintaining pregnancy if they showed specificity for uterine 3α-HSD and ovarian 20α-HSD.

5. Conclusions

AKRs are a rapidly growing gene superfamily which contain an increasing number of steroid transforming enzymes (HSDs and 5β-reductases). Many of these HSDs have the potential to regulate the occupancy of nuclear and membrane bound steroid hormone receptors and are drug targets. 3α-HSD is the most thoroughly characterized HSD in the AKR gene superfamily and, based on structure-function studies, a number of important lessons have emerged that could be exploited to develop potent and selective inhibitors of steroid transforming AKRs. First, transition state analogs for HSDs should mimic the proposed tetrahedral oxyanion transition state, and this could be achieved using steroid boronates or alternately steroid carboxylates. In fact bile acids inhibit Type 3 3α-HSD with nanomolar affinity [36]. Second, transition state analogs for 5β-reductases should mimic the proposed carbocation transition state, and would be best designed on steroid heterocycles—e.g., A-ring containing piperidines with A/B-ring *cis*-fused ring junctions. Third, in designing reversible inhibitors to fill the steroid-binding pockets of AKRs it must be remembered that there is ligand induced movement of loop-B and the C-terminal loop. Thus diverse nonsteroidal based ligands could be developed to fill portions of the pocket. The identification of these ligands would lend itself to the power of combinatorial chemistry since there may be an array of ligand binding modes within a given AKR pocket. These ligands in all probability need to contain a carboxyl group to interact with the oxyanion hole. Fourth, in developing mechanism-based inactivators, the key would be to use nonsteroidal-based scaffolds. These have ease of synthesis, and are less likely to have affinities for steroid hormone receptors. The vinyl alcohols developed on *p*-nitroacetophenone for 3α-HSD show that selective nonsteroidal mechanism-based inactivators is an achievable goal. It is likely that the potency and selectivity of such compounds could be enhanced by coupling chemistries to alter their steric bulk. Fifth, mechanism-based inactivators targeting several HSDs in the AKR superfamily are likely to be achieved via secosteroids.

Acknowledgements

These studies were supported by DK47015 awarded to TMP. The contributions of the following investigators are gratefully acknowledged: Drs Mitchell Lewis, Melanie Bennett and Susan Hoog (for the determination of 3α-HSD crystal structures); Joseph Jez, John Pawlowski and Brian Schlegel (for site-directed mutagenesis studies); and Dr Douglas F. Covey and Joseph Ricigliano (for enzyme inhibition studies).

References

[1] H-K. Lin, J.M. Jez, B.P. Schlegel, D.M. Peehl, J.A. Pachter, T.M. Penning, Expression and characterization of recombinant type 2 3α-hydroxysteroid dehydrogenase (HSD) from human prostate. Demonstration of bifunctional 3α/17β-HSD activity and cellular distribution, Mol. Endocrinol. 11 (1997) 1971–1984.

[2] J.W. Funder, P.T. Pearce, R. Smith, A.I. Smith, Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated, Science 242 (1998) 583–585.

[3] P.M. Stewart, C.B. Whorwood, 11β-hydroxysteroid dehydrogenase activity and corticosteroid hormone action, Steroids 59 (1994) 90–95.

[4] A.L. Albiston, V.R. Obeyesekere, R.E. Smith, Z.S. Krozowski, Cloning and tissue distribution of the human 11-HSD type 2 enzyme, Mol. Cell. Endocrinol. 105 (1994) R11–R17.

[5] E.F. Adams, N.G. Coldham, V.H. James, Steroidal regulation of oestradiol-17β dehydrogenase activity of the human breast cancer cell line MCF-7, J. Endocrinol. 118 (1988) 149–154.

[6] M.J. Reed, A. Singh, M.W. Ghilchik, N.G. Coldham, A. Purohit, Regulation of oestradiol 17β-hydroxysteroid dehydrogenase in breast tissues: the role of growth factors, J. Steroid Biochem. Mol. Biol. 39 (1991) 791–798.

[7] V. Luu-The, C. Labrie, H.F. Zhao, J. Couet, Y. Lachance, J. Simard, G. Leblanc, J. Cote, D. Berube, R. Gagne, F. Labrie,

Characterization of cDNAs for human estradiol 17β-dehydrogenase and assignment of the gene to chromosome 17: evidence of two mRNA species with distinct 5′-termini in human placenta, Mol. Endocrinol. 3 (1989) 1301–1309.
[8] S. Andersson, W.M. Geissler, S. Patel, L. Wu, The molecular biology of androgenic 17β-hydroxysteroid dehydrogenases, J. Steroid Biochem. Mol. Biol. 53 (1995) 37–39.
[9] F. Labrie, V. Luu-The, S-X. Lin, C. Labrie, J. Simard, R. Breton, A. Belanger, The key role of 17β-hydroxysteroid dehydrogenases in sex steroid biology, Steroids 62 (1997) 148–158.
[10] J.D. Taurog, R.J. Moore, J.D. Wilson, Partial characterization of the cytosol 3α-hydroxysteroid: $NAD(P)^+$ oxidoreductase of rat ventral prostate, Biochemistry 14 (1975) 810–817.
[11] G.H. Jacobi, R.J. Moore, J.D. Wilson, Characterization of the 3α-hydroxysteroid dehydrogenase of dog prostate, J. Steroid Biochem. 8 (1977) 719–723.
[12] G.H. Jacobi, J.D. Wilson, The formation of 5α-androstane-3α,17β-diol by dog prostate, Endocrinology 99 (1976) 602–610.
[13] G.H. Jacobi, R.J. Moore, J.D. Wilson, Studies on the mechanism of 3α-androstanediol-induced growth of the dog prostate, Endocrinology 102 (1978) 1748–1758.
[14] J.T. Isaacs, Changes in dihydrotestosterone metabolism and the development of benign prostatic hyperplasia in the aging beagle, J. Steroid Biochem. 18 (1983) 749–757.
[15] M.G. Biswas, D.W. Russell, Expression cloning and characterization of oxidative 17β- and 3α-hydroxysteroid dehydrogenases from rat and human prostate, J. Biol. Chem. 272 (1997) 15,959–15,966.
[16] M.D. Majewska, N.L. Harrison, R.D. Schwartz, J.L. Barker, S.M. Paul, Steroid hormone metabolites are barbiturate-like modulators of the $GABA_A$ receptor, Science 232 (1986) 1004–1007.
[17] M.D. Majewska, Neurosteroids: endogenous bimodal modulators of the $GABA_A$ receptor. Mechanism of action and physiological significance, Prog. Neurobiol. 38 (1992) 379–395.
[18] J.J. Lambert, J.A. Peters, N.C. Sturgess, T.G. Hales, Steroid modulation of the $GABA_A$ receptor complex: electrophysiological studies, in: Steroids and Neuronal Activity. Ciba Foundation Symposium, vol. 153, Wiley, Chichester, 1990, pp. 56–82.
[19] J.J. Lambert, D. Belelli, C. Hill-Venning, J.A. Peters, Neurosteroids and $GABA_A$ receptor function, Trends Pharmacol. Sci. 16 (1995) 295–303.
[20] M. Mahendroo, K.M. Cala, D.W. Russell, 5α-Reduced androgens play a key role in murine parturition, Mol. Endocrinol. 10 (1996) 380–392.
[21] W.G. Wiest, R.B. Wilcox, Purification and properties of rat ovarian 20α-hydroxysteroid dehydrogenase, J. Biol. Chem. 236 (1961) 2425–2428.
[22] M. Mori, W.G. Wiest, Purification of rat ovary 20α-hydroxysteroid dehydrogenase by affinity chromatography, J. Steroid Biochem. 11 (1979) 1443–1449.
[23] H. Jornvall, B. Persson, M. Krook, S. Atrian, R. Gonzalez-Duarte, J. Jeffery, D. Ghosh, Short-chain dehydrogenases/reductases (SDR), Biochemistry 34 (1995) 6003–6013.
[24] M.E. Baker, D.D. Fanestil, Licorice, computer-based analyses of dehydrogenase sequences, and the regulation of steroid and prostaglandin action, Mol. Cell. Endocrinol. 78 (1991) C99–102.
[25] M.E. Baker, Unusual evolution of 11β-hydroxysteroid and retinol dehydrogenases, Bioessays 18 (1996) 63–70.
[26] A. Azzi, P.H. Rehse, D-W. Zhu, R. Campbell, F. Labrie, S-X. Lin, Crystal structure of human estrogenic 17β-hydroxysteroid dehydrogenase complexed with 17β-estradiol, Nature Structure Biology 3 (1996) 665–668.
[27] R. Breton, D. Housset, C. Mazza, J.C. Fontecilla-Camps, The structure of a complex of human 17β-hydroxysteroid dehydrogenase with estradiol and $NADP^+$ identifies two principal targets for the design of inhibitors, Structure 4 (1996) 905–915.
[28] J.E. Pawlowski, M. Huizinga, T.M. Penning, Cloning and sequencing of the cDNA for rat liver 3α-hydroxysteroid/dihydrodiol dehydrogenase, J. Biol. Chem. 266 (1991) 8820–8825.
[29] A. Stolz, M. Rahimi-Kiani, D. Ameis, E. Chan, M. Ronk, J.E. Shively, Molecular structure of rat hepatic 3α-hydroxysteroid dehydrogenase. A member of the oxidoreductase gene family, J. Biol. Chem. 266 (1991) 15,253–15,257.
[30] K.C. Cheng, P.C. White, K.N. Qin, Molecular cloning and expression of rat liver 3α-hydroxysteroid dehydrogenase, Mol. Endocrinol. 5 (1991) 823–828.
[31] M. Khanna, K.N. Qin, R.W. Wang, K.C. Cheng, Substrate specificity, gene structure, and tissue-specific distribution of multiple human 3α-hydroxysteroid dehydrogenases, J. Biol. Chem. 270 (1995) 20,162–20,168.
[32] R. Miura, K. Shiota, K. Noda, S. Yagi, T. Ogawa, M. Takahashi, Molecular cloning of cDNA for rat ovarian 20α-hydroxysteroid dehydrogenase (HSD1), Biochem. J. 299 (1994) 561–567.
[33] W.R. Lacy, K.J. Washenick, R.G. Cook, B.S. Dunbar, Molecular cloning and expression of an abundant rabbit ovarian protein with 20α-hydroxysteroid dehydrogenase activity, Mol. Endocrinol. 7 (1993) 58–66 (published erratum appears in Mol. Endocrinol. 1993 Sep;7(9):1239).
[34] J.M. Jez, T.G. Flynn, T.M. Penning, A new nomenclature for the aldo-keto reductase superfamily, Biochem. Pharmacol. 53 (1997).
[35] Y. Deyashiki, A. Ogasawara, T. Nakayama, M. Nakanishi, Y. Miyabe, K. Sato, A. Hara, Molecular cloning of two human liver 3α-hydroxysteroid/dihydrodiol dehydrogenase isoenzymes that are identical with chlordecone reductase and bile-acid binder, Biochem. J. 299 (1994) 545–552.
[36] A. Stolz, L. Hammond, H. Lou, H. Takikawa, M. Ronk, J.E. Shively, cDNA cloning and expression of the human hepatic bile acid-binding protein. A member of the monomeric reductase gene family, J. Biol. Chem. 268 (1993) 10,448–10,457.
[37] J.C. Warren, G.L. Murdock, Y. Ma, S.R. Goodman, W.E. Zimmer, Molecular cloning of testicular 20α-hydroxysteroid dehydrogenase: identity with aldose reductase, Biochemistry 32 (1993) 1401–1406.
[38] Y. Deyashiki, K. Ohshima, M. Nakanishi, K. Sato, K. Matsuura, A. Hara, Molecular cloning and characterization of mouse estradiol 17β-dehydrogenase (A-specific), a member of the aldoketoreductase family, J. Biol. Chem. 270 (1995) 10,461–10,467.
[39] Y. Onishi, M. Noshiro, T. Shimosato, K. Okuda, Molecular cloning and sequence analysis of cDNA encoding Δ^4-ketosteroid-5β-reductase of rat liver, FEBS Letters 238 (1994) 215–218.
[40] K. Kondo, M. Kai, Y. Setoguchi, G. Eggertsen, P. Sjoborn, T. Setoguchi, K. Okuda, I. Bjorkhem, Cloning and expression of cDNA of human Δ^4-3-oxosteroid-5β-reductase and substrate specificity of the expressed enzyme, Eur. J. Biochem. 219 (1994) 357–363.
[41] D.R. Tomlinson, E.J. Stevens, L.T. Diemel, Aldose reductase inhibitors and their potential for the treatment of diabetic complications, Trends in Pharmacol. Sci. 15 (1972) 293–297.
[42] A.J. Turner, J.A. Illingworth, K.F. Tipton, Simulation of biogenic amine metabolism in the brain, Biochem. J. 144 (1972) 353–360.
[43] T.M. Penning, I. Mukharji, S. Barrows, P. Talalay, Purification and properties of a 3α-hydroxysteroid dehydrogenase of rat liver cytosol and its inhibition by anti-inflammatory drugs, Biochem. J. 222 (1984) 601–611.
[44] J.E. Pawlowski, T.M. Penning, Overexpression and mutagenesis of the cDNA for rat liver 3α-hydroxysteroid/dihydrodiol

dehydrogenase. Role of cysteines and tyrosines in catalysis, J. Biol. Chem. 269 (1994) 13,502–13,510.
[45] L.J. Askonas, J.W. Ricigliano, T.M. Penning, The kinetic mechanism catalysed by homogeneous rat liver 3α-hydroxysteroid dehydrogenase. Evidence for binary and ternary dead-end complexes containing non-steroidal anti-inflammatory drugs, Biochem. J. 278 (1991) 835–841.
[46] S.S. Hoog, J.E. Pawlowski, P.M. Alzari, T.M. Penning, M. Lewis, Three-dimensional structure of rat liver 3α-hydroxysteroid/dihydrodiol dehydrogenase: a member of the aldo-keto reductase superfamily, Proc. Natl. Acad. Sci. USA 91 (1994) 2517–2521.
[47] M.J. Bennett, B.P. Schlegel, J.M. Jez, T.M. Penning, M. Lewis, Structure of 3α-hydroxysteroid/dihydrodiol dehydrogenase complexed with $NADP^+$, Biochemistry 35 (1996) 10,702–10,711.
[48] M.J. Bennett, R.H. Albert, J.M. Jez, H. Ma, T.M. Penning, M. Lewis, Steroid recognition and regulation of hormone action: crystal structure of testosterone and $NADP^+$ bound to 3α-hydroxysteroid/dihydrodiol dehydrogenase, Structure 5 (1997) 799–812.
[49] D.K. Wilson, K.M. Bohren, K.H. Gabbay, F.A. Quiocho, An unlikely sugar substrate site in the 1.65 Å structure of the human aldose reductase holoenzyme implicated in diabetic complications, Science 257 (1992) 81–84.
[50] B.P. Schlegel, K. Ratnam, T.M. Penning, Retention of NADPH-linked quinone reductase activity in an aldo-keto reductase following mutation of the catalytic tyrosine, Biochemistry 37 (1998) 11003–11011.
[51] B.P. Schlegel, J.M. Jez, T.M. Penning, Mutagenesis of 3α-hydroxysteroid dehydrogenase reveals a'push-pull' mechanism for proton transfer in aldo-keto reductases, Biochemistry 37 (1998) 3538–3548.
[52] J.M. Jez, T.M. Penning, Engineering steroid 5β-reductase activity into rat liver 3α-hydroxysteroid dehydrogenase, Biochemistry 37 (1998) 9605–9703.
[53] A. Okuda, K. Okuda, Purification and characterization of Δ^4-3-ketosteroid 5β-reductase, J. Biol. Chem. 259 (1984) 7519–7524.
[54] J.M. Jez, B.P. Schlegel, T.M. Penning, Characterization of the substrate binding site in rat liver 3α-hydroxysteroid dehydrogenase: the roles of tryptophans in ligand binding and protein fluorescence, J. Biol. Chem. 271 (1996) 30,190–30,198.
[55] B.L. Mylari, E.R. Larson, T.A. Beyer, W.J. Zembrowski, C.E. Aldinger, M.F. Dee, T.W. Siegel, D.H. Singleton, Novel potent ADR inhibitors 3,4-dihydro-4-oxo-3[(5-(trifluoromethyl)-2-benzothiazoyl)methyl]-1-phthalazineacetic acid (zopolrestat) and its congeners, J. Med. Chem. 34 (1991) 108–122.
[56] W.H.J. Ward, C.M. Sennitt, H. Ross, A. Dingle, D. Timms, D.J. Mirrlees, D.P. Tuffin, Ponalrestat—a potent and specific inhibitor of aldose reductase, Biochem. Pharmacol. 39 (1990) 337–346.
[57] J.W. Ricigliano, T.M. Penning, Synthesis and evaluation of nonsteroidal mechanism-based inactivators of 3α-hydroxysteroid dehydrogenase, Biochem. J. 269 (1989) 139–149.
[58] B.P. Schlegel, J.E. Pawlowski, Y. Hu, D.M. Scolnick, D.F. Covey, T.M. Penning, Secosteroid mechanism-based inactivators and site-directed mutagenesis as probes for steroid hormone recognition by 3α-hydroxysteroid dehydrogenase, Biochemistry 33 (1994) 10,367–10,374.
[59] W.W. Cleland, Statistical analysis of enzyme kinetic data, Methods Enz. 63 (1979) 103–138.