Characterization of the Substrate Binding Site in Rat Liver 3α-Hydroxysteroid/Dihydriodiol Dehydrogenase

THE ROLES OF TRYPTOPHANS IN LIGAND BINDING AND PROTEIN FLUORESCENCE*

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Rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD), a member of the aldo-ketoreductase superfamily, inactivates circulating steroid hormones using NAD(P)H as cofactor. Despite determination of the 3α-HSD/NADP* binary complex structure, the functional elements that dictate the binding of steroids remain unclear (Bennett, M.J., Schlegel, B.P., Jez, J.M., Penning, T.M., and Lewis, M. (1996) Biochemistry 35, 10702–10711). Two tryptophans (Trp86 and Trp227) near the active site may have roles in substrate binding, and their fluorescence may be quenched upon binding of NADPH. Trp86 is located within an apolar cleft, while Trp227 is found on an opposing loop near the active site. A third tryptophan, Trp148, is on the periphery of the structure. To investigate the roles of these tryptophans in protein fluorescence and ligand binding, we generated three mutant enzymes (W86Y, W148Y, and W227Y) by site-directed mutagenesis. Spectroscopic measurements on these proteins showed that Trp148 contributed the most to the enzyme fluorescence spectra, with Trp227 adding the least. Trp86 was identified as the tryptophan quenched by bound NADPH through an energy transfer mechanism. The W86Y mutant altered binding of cofactor (a 3-fold increase in $K_d$ for NADPH) and steroid (a 7-fold increase in $K_d$ for testosterone). This mutation also dramatically decreased the catalytic efficiency observed with one-, two-, and three-ring substrates and decreased the binding affinity for nonsteroidal anti-inflammatory drugs but had little effect on the binding of aldose reductase inhibitors. Interestingly, mutation of Trp227 significantly impaired steroid binding (a 22-fold increase in $K_d$ for testosterone), but did not alter binding of cofactor, smaller substrates, or inhibitors. Kinetically, the W148Y mutant was similar to wild-type enzyme. Our results demonstrate that Trp86 and the apolar cleft is part of the substrate binding pocket. In addition, we propose a role for Trp227 and its associated loop in binding steroids, but not small substrates or inhibitors, most likely through interaction with the C- and D-rings of the steroid. This work provides the first evidence that tryptophans on opposite sides of the apolar cleft are part of the steroid binding pocket and suggests how the enzyme may discriminate between nonsteroidal anti-inflammatory drugs and aldose reductase inhibitors like zopolrestat. A model of how androstanedione binds in the apolar cleft is developed. These data provide further evidence that loop structures in members of the aldo-ketoreductase superfamily are critical determinants of ligand binding.

Rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD, EC 1.1.1.213) is a representative hydroxysteroid dehydrogenase (HSD) with the principal physiological role of inactivating circulating androgens, prostestins, and glucocorticoids. It also functions as a dihydroidiol dehydrogenase by oxidizing poly cyclic aromatic hydrocarbon trans-dihydriodiol (proximate carcinogens) into ortho-quinones with the production of reactive oxygen species and semiquinone radicals that may contribute to chemical carcinogenesis (1–5). It is very similar (>69% amino acid sequence identity) to its human homologues, including human type I and type II 3α-HSds (4–6). In endocrine target tissues, like the prostate, 3α-HSD converts 5α-dihydrotestosterone (a potent androgen) into 3α-androstanediol (a weak androgen) (7) and may act as a molecular switch by regulating occupancy of the androgen receptor. As the most thoroughly characterized mammalian 3α-HSD, rat liver 3α-HSD serves as an excellent model for investigating the structure and function of these enzymes.

cDNA cloning indicates that the mammalian 3α-HSds are members of the aldo-ketoreductase (AKR) superfamily and are 45–60% identical in amino acid sequence to other AKR proteins, including aldose reductase, aldehyde reductase, 17β-HSD, and 20α-HSD (8–12). This similarity raises the issue of how closely related enzymes recognize different substrates (aldo-keto sugars versus steroid hormones) and is an important concern in developing therapeutic agents against specific protein targets. For example, retinopathic, neuropathic, and nephropathic diabetic complications have been associated with the conversion of glucose to sorbitol catalyzed by aldose reductase (13). As such, aldose reductase inhibitors may be useful therapies for these complications of diabetes, but the specificity of existing compounds has been lacking (14). Similarly, the potential role of 3α-HSds in the regulation of hormone levels in endocrine target tissues and in carcinogen activation makes

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1 The abbreviations and trivial names used are: 3α-HSD, 3α-hydroxysteroid dehydrogenase or 3α-hydroxysteroid-NAD(P)*-oxidoreductase (formerly EC 1.1.1.50 and now EC 1.1.1.213 because of its A-face specificity in hydride transfer); HSD, hydroxysteroid dehydrogenase; AKR, aldo-ketoreductase; NSAID, nonsteroidal anti-inflammatory drug; androsterone, 5α-androstan-3α-ol-17-one; androstanediol, 5α-androstan-3,17-dione; BME, β-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis.

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these enzymes candidate drug targets. Although nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit rat liver 3α-HSD, these compounds are not specific for this protein (15). An understanding of how these enzymes achieve substrate specificity would provide insight into designing effective and specific AKR inhibitors.

The three-dimensional structures of the rat liver 3α-HSD apoenzyme and the E-NADP⁺ binary complex have been solved at 3.0- and 2.7-Å resolutions, respectively (16–17). This protein adopts an (α/β)₉-barrel fold and contains three tryptophans (Trp⁹⁶, Trp¹⁴⁸, and Trp²²⁷). Although the location of the cofactor in the binary complex and site-directed mutagenesis studies have identified the residues involved in catalysis (18), the functional components of the substrate binding site remain unknown. The active site is at the base of an apolar cleft. Projecting toward the center of the barrel, this cleft is about 11 Å deep, is large enough to accommodate steroid ligands, and could be the substrate binding site in rat liver 3α-HSD. Trp⁹⁶ and other apolar residues (Leu¹⁴⁸, Phe¹⁴⁸, and Phe²²⁸) form one side of this cleft and would provide an ideal environment for binding steroid hormones. A flexible loop containing Trp²²⁷ may make part of the opposite side of the substrate binding pocket. However, this loop was disordered in the electron density of the apo-enzyme structure, and in the binary complex structure Trp²²⁷ made a crystal contact with the second molecule in the asymmetric unit. Neither structure clearly indicates the location of Trp²²⁷ or the flexible loop in relation to the putative steroid binding site. Finally, binding of NADPH quenches the fluorescence emission of 3α-HSD, suggesting that either Trp⁹⁶ or Trp²²⁷ may be near cofactor (18, 19). The third tryptophan, Trp¹⁴⁸, should have no function in ligand binding based on its location away from the active site.

In this work, we used site-directed mutagenesis to investigate the role of tryptophans in protein fluorescence and to delineate the structural components of the steroid and inhibitor binding site by constructing the following mutants of rat liver 3α-HSD: W86Y, W148Y, and W227Y. Our results indicate that the contribution of each tryptophan to the overall fluorescence of 3α-HSD is large enough to accommodate steroid ligands, and could be the substrate binding site in rat liver 3α-HSD. However, the active site is at the base of an apolar cleft in which it resides is part of the substrate/inhibitor binding pocket. The mutagenesis data also suggest that Trp²²⁷ plays an important role in binding steroid hormones but has no role in accommodating one-, two-, or three-ring substrates or NSAIDs. Also, mutations of Trp⁹⁶ and Trp²²⁷ had little effect on the binding of aldose reductase inhibitors. A model for binding androstanediol and how the enzyme may discriminate between NSAIDs and aldose reductase inhibitors is proposed. These data provide another example where loop structures in (α/β)₉-barrel proteins are determinants in ligand binding.

**EXPERIMENTAL PROCEDURES**

*Materials*—The DNA Synthesis Service in the Department of Chemistry at the University of Pennsylvania synthesized the primers used for polymerase chain reaction-based site-directed mutagenesis. The DE-52 cellulose was from Baxter; and the Blue Sepharose was purchased from Pharmacia. Goat anti-rabbit IgG-horseradish peroxidase conjugate and 4-chloro-1-naphthol were from Bio-Rad. Smithgall and Penning (20) previously described the preparation of polyclonal rabbit anti-rat 3α-HSD antisera. NAD⁺, NADH, and NADPH were from Boehringer-Mannheim. Androsterone, androstandiolone, and testosterone were obtained from Steraloids. Radiolabeled [H]testosterone (92.5 Ci/mmol) was purchased from NEN DuPont. Zopolrestat and ponalrestat were provided courtesy of Hoechst. Fluorescence excitation and emission spectra were performed in 1-ml systems containing 20 μg of protein and 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM sodium azide using a Gilford 260 spectrophotometer or a Beckman DU-640 spectrophotometer equipped with an ATT 6300 personal computer. The FLUOR program (Softways, Inc.) digitized the spectra. Scans of the fluorescence excitation and emission spectra were performed in 1-m1 systems containing 20 μg of protein and 10 mM potassium phosphate buffer (pH 7.0) buffer. Determination of the energy transfer emission spectra of the apoenzymes, the E-NADP⁺ binary complexes, and the E-NADPH:testosterone ternary complexes of wild-type 3α-HSD and each mutant were performed in the same system with 0.5 mM acetonitril as co-solvent. The binary complex spectra were measured in the presence of 1.5 μM NADPH, while the ternary complex spectra were scanned with 50 μM testosterone added.

Steady-state Enzyme Kinetics—Initial velocities were measured on either a Gilford 260 spectrophotometer or a Beckman DU-640 spectrophotometer by observing the rate of change in absorbance of pyridine nucleotide at 340 nm (ε = 6270 M⁻¹ cm⁻¹) in 1-m1 systems at 25°C using a 1-cm path length. Throughout the purifications, enzyme activity was monitored using a standard assay system containing 100 mM potassium phosphate buffer (pH 7.0), 2.3 mM NAD⁺, and 75 μM androsterone with 4% acetonitril as co-solvent. Measurements of the Kₘ and kcat values for androsterone oxidation were made at 2.3 mM NAD⁺ with varied steroid concentration (11.5–73.0 μM). Kₘ and kcat values for NAD⁺ were determined at 75 μM androsterone by varying the NAD⁺ concentration (0.09–3.3 mM). Kinetic constants for androstanediol reduction were measured at 180 μM NADH with varied steroid concentration (2.4–28.4 μM). Determination of the kinetic constants for NADH oxidation used 30 μM androstanediol and were made by varying the NADH concentration (4.3–177 μM). These measurements all used the standard assay system. Determination of kinetic constants with 4-nitrobenzaldehyde (0.02–20 mM) and 2-decanol (0.2-5 mM) as substrates used the same assay system at pH 6.0 with 200 μM NADH. Assays with 9,10-phenanthrenequinone (1–30 μM) used 200 μM NADPH as cofactor in the pH 6.0 buffer. All reactions were initiated by the addition of the enzyme and were corrected for nonenzymatic rates. Calculation of all kcat values and Km values used the ENZFITTER nonlinear regression analysis program (23) to fit untransformed data with a hyperbolic function, as originally described by Wilkinson (24), yielding estimates of the kinetic constants and the associated standard error.

*Inhibition Studies*—Initial velocities were measured using the standard androsterone assay system as described above with various concen-
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**TABLE I**

| Recombinant 3α-HSD wild type | Volume | Total protein | Total activity | Specific activity | Purification factor | Yield |
|-------------------------------|--------|---------------|----------------|-------------------|--------------------|-------|
| Sonicate (step 1)             |        |               |                |                   |                    |       |
| DE52 cellulose (step 2)       |        |               |                |                   |                    |       |
| Blue Sepharose (step 3)       |        |               |                |                   |                    |       |
| W68Y (step 3)                | 4.4    | 51.2          | 85.55          | 0.167             | 1.00               | 100   |
| W148Y (step 3)               | 5.1    | 17.6          | 15.28          | 0.868             | 5.20               | 17.9  |
| W227Y (step 3)               | 3.0    | 9.78          | 14.64          | 1.50              | 9.10               | 17.1  |

**RESULTS**

Expression and Purification of Recombinant Wild-type and Mutant 3α-HSDs—Wild-type 3α-HSD and the W68Y, W148Y, and W227Y mutants were overexpressed in E. coli DH5α cells and purified (Table I). The specific activities of the purified proteins for androsterone turnover were 1.50, 0.059, 0.674, and 0.080 µmol/min/mg for wild-type enzyme and the W68Y, W148Y, and W227Y mutants, respectively. The wild-type and mutant forms of the 3α-HSD appeared as single homogeneous bands of the same molecular weight on SDS-PAGE gels (Fig. 1A). Each of the mutants was also immunoreactive with rabbit anti-rat 3α-HSD antiserum, as demonstrated by Western blot analysis (Fig. 1B).
FIG. 3. Emission spectra of the apoenzyme, the E-NADPH binary complex, and the E-NADPH-testosterone ternary complex for wild type and the tryptophan mutants. The emission spectra of wild-type 3α-HSD (A) and the W86Y (B), W148Y (C), and W227Y (D) mutant apoenzymes are shown (—). The corresponding emission spectra of the E-NADPH binary complexes in the presence of 1.5 μM NADPH (—) and the emission spectra of the E-NADPH-testosterone ternary complexes in the presence of 1.5 μM NADPH and 50 μM testosterone are shown (— —). All spectra were scanned from 300 to 500 nm with excitation at 290 nm. Scans were performed at 60 nm/min with excitation and emission band pass set at 5 nm each. These experiments used 20 μg of protein in 10 mM potassium phosphate (pH 7.0) buffer with 4% acetonitrile as co-solvent.

**TABLE II**



| Substrate          | Wild type | W86Y   | W148Y  | W227Y  |
|--------------------|-----------|--------|--------|--------|
|                    | kcat (min⁻¹) | Km (μM) | kcat/Km (min⁻¹/μM) | Km (μM) | kcat/Km (min⁻¹/μM) | Km (μM) | kcat/Km (min⁻¹/μM) | Km (μM) | kcat/Km (min⁻¹/μM) | Km (μM) |
| Androsterone       | 65.8 ± 5.5 | 45.4 ± 7.9 | 1.45 | 166 ± 37 | 42.0 ± 6.2 | 1.07 | 408 ± 134 | 0.075 |
| NAD⁺               | 59.4 ± 1.9 | 7.78 ± 0.37 | 34.9 ± 3.0 | 6.37 ± 0.31 |
| Androstanedione    | 18.5 ± 0.5 | 1.83 ± 0.11 | 9.08 ± 0.29 | 24.7 ± 6.76 |
| NADH               | 15.4 ± 0.6 | 3.31 ± 0.52 | 8.56 ± 0.90 | 5.90 ± 0.5 |
| 4-Nitrobenzaldehyde| 0.45 | 0.23 ± 0.1 | 38.4 ± 4.2 | 38.5 ± 2.0 |
| 2-Decalone         | 22.9 ± 1.9 | 4.27 ± 0.51 | 18.8 ± 3.1 | 10.3 ± 1.7 |
| 9,10-Phenanthrenequinone | 236 ± 4 | 70.3 ± 0.4 | 164 ± 9 | 155 ± 10 |

**Fluorescence Excitation and Emission Spectra of Wild-type and Mutant 3α-HSDs**—The 3α-HSD mutants allowed the contribution from each tryptophan to the overall protein fluorescence spectrum to be assessed. Although the fluorescence signals of the tryptophans were not strictly additive, Trp^{148} dominated the spectra, Trp^{86} provided the next largest share, and Trp^{227} contributed the least.

Removal of each tryptophan also shifted the emission $\lambda_{max}$ relative to wild-type enzyme and provided an indication of the local environments of these residues (Fig. 2). Wild-type 3α-HSD had an emission $\lambda_{max} \approx 336$ nm. The W86Y and W227Y mutants were blue-shifted with emission $\lambda_{max}$ of 332 and 331 nm, respectively, and suggested a partly solvent-accessible environment for these tryptophans (27). The W227Y mutant also displayed a noticeable loss of emission in the higher wavelengths, indicating that this residue is highly solvent-exposed.
Finally, mutation of Trp<sup>148</sup> had the opposite effect; it red-shifted the emission $\lambda_{\text{max}}$ to 343 nm, consistent with this residue being buried away from solvent. These mutations showed that the contribution of each tryptophan to the fluorescence spectra corresponds to their solvent exposure in the three-dimensional structure; the more solvent-inaccessible the tryptophan, the greater contribution to the observed spectra. Trp<sup>148</sup> is solvent-inaccessible on the interior of an $\alpha$-helix facing the $\beta$-barrel, and Trp<sup>227</sup>, in the apolar cleft, is partly accessible to solvent. In contrast, Trp<sup>227</sup> is on a flexible loop near the active site, and the potential for extensive solvent quenching would explain its low fluorescence yield.

**Identification of the Tryptophan Involved in Energy Transfer with NADPH**—The emission spectra of the apo-enzyme, the E-NADPH binary complexes, and the E-NADPH-testosterone ternary complexes of wild-type 3α-HSD and each tryptophan mutant clearly identified Trp<sup>86</sup> as the tryptophan quenched by NADPH through an energy transfer mechanism (Fig. 3). The emission spectra of the binary complexes of the wild-type enzyme, the W148Y mutant, and the W227Y mutant each exhibited an energy transfer emission band of approximately the same intensity at 450 nm. The W86Y mutant lacked this band, indicating Trp<sup>86</sup> as the quenched tryptophan. This emission signal was unobservable in controls containing only 1.5 $\mu$M NADPH at the same excitation wavelength (data not shown). The fluorescence spectra of the ternary complexes of wild-type enzyme and each tryptophan mutant showed additional quenching of protein fluorescence emission. With the exception of the W86Y mutant, there was a decrease in emission at 450 nm upon binding of testosterone to the E-NADPH complexes. Since the energy transfer band is assignable to Trp<sup>86</sup>, this decrease in emission indicates that steroid binding interferes with energy transfer between NADPH and Trp<sup>86</sup>.

**Steady-state Kinetic Properties of the Tryptophan Mutants**—The kinetic properties of the recombinant wild-type enzyme and the three tryptophan mutants were compared using four standard substrates: androsterone and NAD<sup>+</sup> (oxidation reaction) and androstanediene and NADH (reduction reaction) (Table II). Mutation of Trp<sup>86</sup> altered both substrate and cofactor kinetics. The W86Y mutant resulted in a 3-fold increase in $K_m$ for androsterone and a 6-fold increase in $K_m$ for androstanediene. Likewise, this mutation increased the $K_m$ for NAD<sup>+</sup> 6-fold and the $K_m$ for NADH 8-fold. In addition, of the three mutant enzymes, the W86Y mutant exhibited the lowest catalytic efficiency for all four substrates. The W227Y mutant displayed significantly impaired kinetics for each steroid substrate with 9- and 43-fold increases in $K_m$ for androsterone and androstanediene, respectively, but only slight differences with NADH (4-fold increase in $K_m$) and none with NAD<sup>+</sup>. The inability to saturate the W227Y mutant due to solubility limitations of the steroid substrates emphasizes the importance of this residue in steroid binding and contributes to the low turnover numbers observed with this mutant. As expected from its position in the three-dimensional structure, the W148Y mutant gave only modest changes in kinetic constants compared with wild-type enzyme for all substrates.

**Determination of the Binding Constants for NADPH and Testosterone**—To directly evaluate the roles of each tryptophan in cofactor binding, we measured the $K_a$ values of each mutant for NADPH by fluorescence titration. For these experiments, NADPH is preferred over NADH, since its low $K_a$ value ensures saturation before interference from the inner filter effect occurs. Fig. 4A shows a typical titration curve of wild-type enzyme with NADPH and demonstrates saturation at the highest ligand concentrations. Titration of each tryptophan mutant indicated only minor changes in $K_a$ for cofactor (Fig. 4B). Both wild-type enzyme and the W148Y mutant had identical binding constants, 141 ± 15 nM and 141 ± 21 nM, respectively. The W86Y mutant had a $K_a$ of 363 ± 32 nM, and the W227Y mutant had a $K_a$ of 252 ± 34 nM.

Equilibrium dialysis experiments quantitated the ability of each tryptophan mutant to bind radiolabeled testosterone to the E-NADPH complex (Fig. 5). In these studies it was necessary to add NADH, since 3α-HSD displays an ordered bi-bi mechanism in which cofactor binds first (28). NADH was used in these experiments because it is more stable than NADPH over the time required to reach binding equilibrium. Radiolabeled testosterone was chosen as the steroid for these experiments because it is bound by the enzyme, is not a substrate, and binding can be directly quantitated by scintillation counting. The binding constants for the wild-type enzyme and the W148Y mutant were similar, 4.2 ± 0.8 and 6.6 ± 0.9 $\mu$M, respectively. The effects on steroid binding in the W86Y and W227Y mutants reflected the changes observed in the steady-state kinetic analysis. The W86Y mutant displayed a 7-fold increase in $K_a$ (31 ± 8.6 $\mu$M), and the W227Y mutant had a 22-fold increase in $K_a$ (92 ± 32 $\mu$M) for testosterone, indicating that both residues play an important role in steroid binding.
Effect of the W86Y and W227Y Mutants on the Turnover of One-, Two-, and Three-ring Substrates—To elucidate the involvement of Trp86 and Trp227 in substrate recognition, the W86Y and W227Y mutants were used to turnover substrates of varying ring size: 4-nitrobenzaldehyde (one ring); 2-decalone (two rings); and 9,10-phenanthrenequinone (three rings) (Table II). The W227Y mutant was kinetically similar to wild-type enzyme, yielding similar $K_m$ values with 4-nitrobenzaldehyde and 2-decalone, and had a slightly elevated $K_m$ for 9,10-phenanthrenequinone. However, mutation of Trp86 altered the catalytic efficiency of the enzyme for each substrate, and the effects were related to the size of the molecule. Determination of the catalytic efficiency with 4-nitrobenzaldehyde was not possible for the W86Y mutant, since this was a very poor substrate with a $k_{cat}$ value 300-fold less than wild-type enzyme. This mutant gave a 65- and 14-fold reduction in catalytic efficiency for the turnover of 2-decalone and 9,10-phenanthrenequinone, respectively. As expected, the W148Y mutant had no effect on the turnover of one-, two-, and three-ring substrates.

Effect of W86Y and W227Y Mutations on the Affinity of 3α-HSD Inhibitors—To further investigate the effects of Trp86 and Trp227 on ligand binding, we measured the ability of various 3α-HSD inhibitors to block androsterone oxidation catalyzed by the W86Y and W227Y mutants (Fig. 6). Table III summarizes the $IC_{50}$ values obtained for various inhibitors. Values obtained for NSAIDs with recombinant wild-type enzyme were similar to those reported by Penning and Talalay for the native rat liver enzyme (15). Each mutant bound cofactor with nanomolar affinity. Since the cofactor binding site extends from the core of the barrel to the periphery of the structure and involves contacts with at least 12 amino acids, it is unlikely that these mutations caused gross structural changes in the three-dimensional structure of the protein. It was found that bound NADPH quenches the fluorescence of Trp86 by an energy transfer mechanism. In the 3α-HSD-NADP$^+$ binary complex structure, Trp86 is 10 Å away from the nicotinamide ring and is near enough for energy transfer to occur (29). Although removal of Trp86 abolishes the energy transfer peak, some quenching of fluorescence emission is evident in the W86Y mutant and may result from collisional quenching of other residues in the cofactor binding pocket. A similar energy transfer mechanism occurs in aldose reductase.

### DISCUSSION

We have described the first functional studies on amino acids located in the presumptive substrate binding site of rat liver 3α-HSD. We used site-directed mutagenesis to probe the contributions of the three tryptophans in 3α-HSD to protein fluorescence and the roles of each in cofactor, substrate, and inhibitor binding. Our results provide a model for steroid binding in 3α-HSD and give insight into how different inhibitors may be accommodated in the apolar cleft.

Tryptophans, Protein Fluorescence, and Cofactor Binding—Quenching of intrinsic protein fluorescence upon NADPH binding has been observed in 3α-HSD and used to calculate binding affinities for cofactor (18–19). Mutation of each tryptophan produced only marginal changes in the $K_d$ for NADPH, and each mutant bound cofactor with nanomolar affinity. Since the cofactor binding site extends from the core of the barrel to the periphery of the structure and involves contacts with at least 12 amino acids, it is unlikely that these mutations caused gross structural changes in the three-dimensional structure of the protein. It was found that bound NADPH quenches the fluorescence of Trp86 by an energy transfer mechanism. In the 3α-HSD-NADP$^+$ binary complex structure, Trp86 is 10 Å away from the nicotinamide ring and is near enough for energy transfer to occur (29). Although removal of Trp86 abolishes the energy transfer peak, some quenching of fluorescence emission is evident in the W86Y mutant and may result from collisional quenching of other residues in the cofactor binding pocket. A similar energy transfer mechanism occurs in aldose reductase,
but the quenched tryptophan remains unidentified (30–31).

The mechanism of fluorescence quenching in aldose reductase also involves a conformational change upon binding of cofactor (30). The structure of the aldose reductase apoenzyme showed that the tryptophan analogous to Trp227 was packed against another loop of the protein, and that subsequent binding of NADPH disrupts this packing, thereby providing additional fluorescence quenching (32). In 3α-HSD, it is not known if similar changes in the environment of Trp227 occur upon cofactor binding, since the loop in which it resides was disordered in the apoenzyme structure and formed a crystal contact in the binary complex structure (16, 17).

Tryptophans as Components of the Steroid Binding Pocket—Comparison of the fluorescence spectra of the binary and ternary complexes also provided evidence for the location of the steroid binding site. The emission spectra for the binary and ternary complexes also provided evidence for the location of the steroidal cofactor (30). The structure of the aldose reductase apoenzyme showed that the tryptophan analogous to Trp227 was packed against another loop of the protein, and that subsequent binding of NADPH disrupts this packing, thereby providing additional fluorescence quenching (32). In 3α-HSD, it is not known if similar changes in the environment of Trp227 occur upon cofactor binding, since the loop in which it resides was disordered in the apoenzyme structure and formed a crystal contact in the binary complex structure (16, 17).

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**Fig. 6. Structures of 3α-HSD inhibitors.**

rat liver 3α-HSD is the large apolar cleft near the catalytic tetrad (Asp50, Tyr55, Lys84, and His117). The W86Y mutation affected the $K_m$ values for NAD$^+$ and NADH and significantly altered the $K_m$ values for steroid substrates. In addition, our studies revealed that one- and two-ring substrates, which may occupy the same space as the A- and B-rings of the steroid, were turned over poorly by the W86Y mutant but that catalytic efficiency increased with substrate size. Turnover of 4-nitrobenzaldehyde was too low for accurate determination of a $K_m$ value, while the $k_{cat}/K_m$ for 2-decalone was reduced 65-fold relative to wild type and was reduced even less for 9,10-phenanthrenequinone and steroids. These data indicate that although the W86Y mutant may alter the topology of the apolar surface, larger substrates may compensate by interacting with a greater surface area of the cleft. The fluorescence data and kinetic studies on the W86Y mutant provide evidence that Trp227 is near the A- and B-rings of bound steroid.

Unexpectedly, the $K_m$ values for androstanediol and androstenedione and the $K_4$ for testosterone were increased dramatically in the W227Y mutant. Interestingly, this mutation did not affect the kinetic constants for smaller substrates. This size-specific effect implies that Trp227 (and its associated loop) interacts with the C- and/or D-rings of steroid ligand.

Studies on aldose reductase suggest that the analogous tryptophan is part of the apolar cleft and is involved in substrate and inhibitor binding. In the three-dimensional structure of aldose reductase complexed with zopolrestat, the inhibitor occupies the apolar cleft, and this tryptophan makes van der Waals contacts with the ligand (35). Aldose reductase also catalyzes the reduction of the C21 aldehyde in isocorticosteroids (36). Modeling studies indicate that this tryptophan may interact with the A- and B-rings of the isocorticosteroid, which in this orientation would occupy the same space as the C- and D-rings of a 3-ketosteroid in 3α-HSD (37). Also, in other (α/β)$_h$-barrel proteins, loops on the C-terminal side of the barrel near the active site contribute to substrate binding (38–40).

Our results would be consistent with the following model for steroid binding in 3α-HSD (Fig. 7). The C3 position of bound steroid points toward the catalytic tetrad (Asp50, Tyr55, Lys84, and His117) so that 4-pro-R-hydrider transfer from the nicotinamide cofactor can occur. Orientation of the steroid α-face toward the side of the apolar cleft containing Trp227 would preserve the known stereochemistry of hydride transfer. Also, a steroid in this position would interfere with energy transfer between Trp227 and bound cofactor, as observed in spectra of the E$^\gamma$/NADPH-testosterone complex. In addition, the length of the substrate would allow surface interactions between amino acids of the apolar cleft (Leu384, Trp386, Phe388, and Phe129) would provide an ideal environment for interaction with steroid substrates. Previous studies with affinity labels and mechanism-based inactivators have targeted this general vicinity of the structure as the location of steroid binding by tagging a cysteine near but not in the proposed binding site (33–34). The results of the kinetic studies with the W86Y mutant support the assertion that the steroid binding pocket of

**TABLE III**

| Inhibitor Recombinant | Recombinant | W86Y | W227Y |
|-----------------------|-------------|------|-------|
| Indomethacin          | 3.02        | 44.0 | 2.21  |
| (E/Z)-Sulindac        | 6.12        | 96.0 | 10.2  |
| Flufenamic acid       | 8.52        | 121  | 9.60  |
| Mefenamic acid        | 11.6        | 83.4 | 8.00  |
| Meclofenamic acid     | 2.89        | 84.2 | 2.19  |
| Zopolrestat           | 46.3        | 123  | 60.8  |
| Ponalrestat           | 59.0        | 113  | 85.8  |
| 1,10-Phenanthroline   | 9090        | 8300 | 41,100|
| 1,7-Phenanthroline    | 23,800      | 24,600 | 130,000|
| Hexestrol             | 4.73        | 52.7 | 14.9  |
acids along this side of the apolar cleft and the α-face of the steroid. Finally, Trp²²⁷ (and its associated loop) could interact with either the edge or the β-face of the C- and/or D-rings of the steroid to form the opposite side of the binding cleft.

Tryptophans analogous to Trp⁸⁶ and Trp²²⁷ are present in other members of the AKR superfamily, including 17β-HSD, 20α-HSD, aldose reductase, and aldehyde reductase (8–12). Based on the conservation of these residues in the superfamily and our site-directed mutagenesis data, these tryptophans are predicted to form basic components of the substrate binding pocket in the AKR proteins but probably do not determine specificity between steroid and sugar substrates.

**Inhibitor Specificity: Discrimination between NSAIDs and Aldose Reductase Inhibitors**—Inhibition of 3α-HSD by NSAIDs is significant because of the high correlation that exists between the inhibition constants observed and the rank order of potency of these drugs (15). Since this rank order is essentially identical to that for prostaglandin H synthase (cyclooxygenase), the accepted target for NSAIDs, 3α-HSD may also be a target for these agents. Importantly, NSAIDs are equipotent inhibitors of 3α-HSD and prostaglandin H synthase. In contrast, aldose reductase inhibitors, e.g. zopolrestat and ponalrestat, are weak inhibitors of 3α-HSD but potent inhibitors of aldose reductase. The ability of aldose reductase inhibitors to discriminate between 3α-HSD and aldose reductase indicates that selective inhibition of AKR superfamily members is an achievable goal. Inhibition data obtained with the tryptophan mutants provides information on the nature of this specificity.

Structural elements of NSAIDs that are important in determining binding to 3α-HSD and prostaglandin H synthase include 1) a primary aromatic region separated from an electrophilic group by 2.8–3.8 Å; 2) a secondary lipophilic group containing one or two electronegative groups (hydroxyl or halogen); and 3) the ability to form a charge transfer complex with a polarized carbonyl, implying that an anionic binding site is required. These descriptors would predict that compounds like zopolrestat should inhibit 3α-HSD and aldose reductase equally well, but zopolrestat inhibits aldose reductase with an IC₅₀ of 3 μM (43) and 3α-HSD with an IC₅₀ of 46 μM.

One explanation for this finding is that the apolar clefts of 3α-HSD and aldose reductase are different. Zopolrestat binds to the aldose reductase-NADPH complex with 110 protein-ligand contacts (35). In this complex, the carboxylate of zopolrestat occupies the anionic binding site formed by the active site residues, in accord with the model discussed above. But zopolrestat’s binding avidity results from van der Waals interactions between four key residues (Trp²⁶⁰, Trp¹¹¹, Phe¹²², and Leu³⁰⁰) and the lipophilic moieties of the inhibitor. Over half of the protein-ligand contacts in this ternary complex are made with these four residues. The thalazine ring is packed against Trp²⁶⁰, and a W20A mutant of aldose reductase dramatically impairs zopolrestat binding (31), while the benzothiazolyl ring is bound among Trp¹¹¹, Phe¹²², and Leu³⁰⁰. In 3α-HSD the anion binding site is conserved, but the residues that contact zopolrestat are substituted by Thr²⁴⁴, Phe¹¹⁸, Phe¹²⁹, and Asn³⁰⁶, respectively. Structurally, these changes result in the elimination of one indole ring (Trp²⁶⁰) and the substitution of another indole ring (Trp¹¹¹) by a phenyl ring. The ring bulk in the apolar cleft is thereby reduced, decreasing the number of protein-ligand interactions. As a consequence, the binding of zopolrestat is reduced to micromolar affinity.

The issue remains why NSAIDs and aldose reductase inhib-
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The aldose reductase-NADPH-zopolrestat complex, tryptophans analogous to Trp<sub>30198</sub> and Trp<sub>327</sub> contact the inhibitor (35). Mutation of Trp<sub>30198</sub> in 3α-HSD slightly increases the IC<sub>50</sub> value for both zopolrestat and ponalrestat but dramatically increases the IC<sub>50</sub> values for NSAIDs. Also, the W227Y mutant had no effect on inhibition by either the NSAIDs or aldose reductase inhibitors, suggesting that this residue does not interact with either type of inhibitor. Although the carboxylate moiety from both classes of inhibitors may bind at the anionic binding site formed by the active site residues, it is likely that the pthalazine ring of an aldose reductase inhibitor and the indole or benzyl ring of an NSAID that contain the carboxyl substituent fit into the apolar cleft differently.

We also used inhibitors that did not conform to either the NSAID or aldose reductase pharmacophore models. The planar 1,7- and 1,10-phenanthrolines, which lack any electronnegative group, inhibit the wild type and tryptophan mutants extremely poorly, presumably because these compounds lack the substituents required for binding at the anionic site. In contrast, it is proposed that inhibitors, such as hexestrol, bind tightly because they are phenolic and contain the appropriate electronnegative group for access to the anionic binding site.

These data suggest that the selective design of AKR inhibitors must not only take into account the need to accommodate the anionic binding site present in superfamily members, but in order to gain specificity it will be necessary to take advantage of differences in the apolar cleft. This would necessitate using multiring structures, like NSAIDs and the aldose reductase inhibitors, to ensure they occupy only the cleft of the targeted AKR protein.

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