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Structure–function relationships in 3α-hydroxysteroid dehydrogenases: a comparison of the rat and human isoforms☆

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Abstract

3α-Hydroxysteroid dehydrogenases (3α-HSDs) inactivate steroid hormones in the liver, regulate 5α-dihydrotestosterone (5α-DHT) levels in the prostate, and form the neurosteroid, allopregnanolone in the CNS. Four human 3α-HSD isoforms exist and correspond to AKR1C1–AKR1C4 of the aldo-keto reductase (AKR) superfamily. Unlike the related rat 3α-HSD (AKR1C9) which is positional and stereospecific, the human enzymes display varying ratios of 3-, 17-, and 20-ketosteroid reductase activity as well as 3α-, 17β-, and 20α-hydroxysteroid oxidase activity. Their k_{cat} values are 50–100-fold lower than that observed for AKR1C9. Based on their product profiles and discrete tissue localization, the human enzymes may regulate the levels of active androgens, estrogens, and progestins in target tissues. The X-ray crystal structures of AKR1C9 and AKR1C2 (human type 3 3α-HSD, bile acid binding protein and peripheral 3α-HSD) reveal that the AKR1C2 structure can bind steroids backwards (D-ring in the A-ring position) and upside down (β-face inverted) relative to the position of a 3-ketosteroid in AKR1C9 and this may account for its functional plasticity. Stopped-flow studies on both enzymes indicate that the conformational changes associated with binding cofactor (the first ligand) are slow; they are similar in both enzymes but are not rate-determining. Instead the low k_{cat} seen in AKR1C2 (50-fold less than AKR1C9) may be due to substrate "wobble" at the plastic active site.

Keywords: 3α-Hydroxysteroid dehydrogenase; Aldo-keto reductase; Ursodeoxycholate; Bile acid

1. Introduction

3α-Hydroxysteroid dehydrogenases work in concert with the 5α/5β-reductases to inactivate circulating steroid hormones in the liver and to form crucial intermediates in bile acid biosynthesis (Fig. 1). In peripheral tissues, they may regulate the amount of 5α-dihydrotestosterone (5α-DHT) available for the androgen receptor. In the prostate, 3α-HSD isoforms convert 5α-DHT (a potent androgen; $K_d = 10^{-11}$ M for the androgen receptor) to 3α-diol (a weak androgen; $K_d = 10^{-6}$ M for the androgen receptor) [1,2]. There is also intense interest in identifying the isoform that can oxidize 3α-diol back to 5α-DHT since this may contribute to the imbalance in 5α-DHT levels that can exist in diseases of the prostate [3,4]. In the CNS, 3α-HSD isoforms are implicated in the conversion of 5α-dihydroprogesterone to allopregnanolone, an allosteric effector of the $GABA_A$ receptor [5–7]. Allopregnanolone increases the affinity of $GABA_A$ for its receptor, increases chloride conductance, and as a result has anxiolytic and anesthetic properties [8]. Thus, 3α-HSD isoforms can regulate the ligand occupancy of nuclear receptors and membrane-bound ion-gated channels.

Interest exists in unequivocally identifying the human isoforms responsible for each of these reactions. We now know that there is one dominant soluble 3α-HSD in rat and at least four soluble 3α-HSDs in human. cDNA cloning shows that the rat enzyme has 69% sequence identity with each of the human enzymes at the amino acid level, and that all five enzymes are members of the aldo-keto reductase (AKR) superfamily [9,10]. The enzymes of interest are rat liver 3α-HSD (AKR1C9); human type 1 3α-HSD (AKR1C4); human type 2 3α-HSD (AKR1C3), also known as type 5 17β-HSD; human type 3 3α-HSD (AKR1C2), also known as bile acid binding protein; and human 20(3α)-HSD (AKR1C1) (for a complete listing visit: http://www.med.upenn.edu/akr).

Considerable structure–function studies have been performed on rat 3α-HSD (AKR1C9) and these are pertinent

Abbreviations: AKR, aldo-keto reductase; 3α-HSD, 3α-hydroxysteroid dehydrogenase (EC. 1. 1. 1. 213: A-face specific, AKR1C1–AKR1C4 and AKR1C9 are all 3α-HSD isoforms); 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 3α-diol, 5α-androstane-3α,17β-diol.

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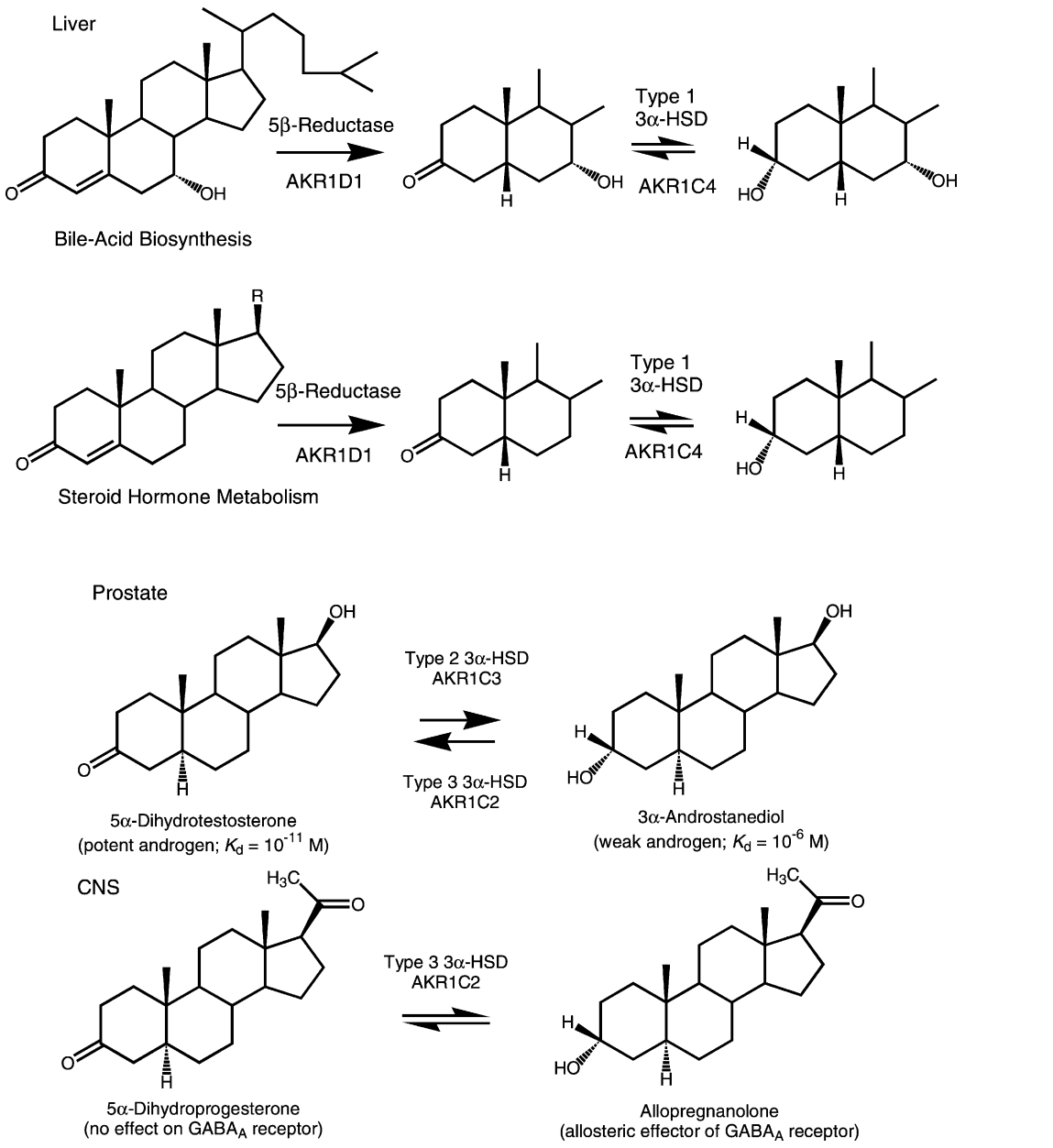

Fig. 1. Reactions catalyzed by human 3α-HSD isoforms.

to the human enzymes because of the high sequence identity that exists. AKR1C9 consists of 322 amino acids and its monomer molecular weight is 37,029 [11]. It catalyzes an ordered bi–bi reaction in which cofactor binds first and leaves last [12]. The binding of cofactor is an obligatory requirement for steroid hormone to bind. The reaction is stereo-specific and involves 4-pro-*R* hydride transfer from the nicotinamide cofactor to the 3-ketosteroid [12]. Crystal structures have been determined for the apoenzyme, the binary complex, and the ternary complex of AKR1C9 [13–15]. The structural motif for the enzyme is an $(\alpha/\beta)_8$ barrel, which is typical for AKR superfamily members. The structures of the binary and ternary complexes reveal that the nicotinamide cofactor lies perpendicular to the steroid ligand and that significant conformational changes occur during the sequential binding of these ligands. These conformational changes could be rate-determining and thus interest exists in deriving rate constants for these steps.

Each of the human 3α-HSD isoforms (AKR1C1–AKR1C4) have been cloned by RT-PCR from human hepatoma (HepG2) cells, and expressed and purified to homogeneity from *E. coli* in milligram amounts [16]. Product profiling showed that each of the purified recombinant proteins were unexpectedly plastic [17]. They will function as 3-, 17- and 20-ketosteroid reductases and will reduce 5α-DHT to 3α-diol, Δ^4-androstene-3,17-dione to

testosterone, estrone to 17β-estradiol and progesterone to 20α-hydroxyprogesterone. They will also function as 3α-, 17β-, and 20α-hydroxysteroid oxidases and oxidize 3α-diol to 5α-DHT, testosterone to Δ^4-androstene-3,17-dione, 17β-estradiol to estrone, and 20α-hydroxyprogesterone to progesterone. It should be emphasized that only AKR1C2 can oxidize 3α-diol to 5α-DHT. In the remaining isoforms, the prevailing 17β-HSD activity oxidized 3α-diol to androsterone which was subsequently converted to 5α-androstane-3,17-dione. Thus, each of the human enzymes will interconvert potent steroid hormones with their cognate inactive metabolites and vice-versa. Depending on their catalytic efficiency, their ability to act as a reductase or oxidase in a cellular context, and their tissue distribution, these enzymes have the potential to regulate ligand occupancy of the androgen, estrogen, and progesterone receptors.

A comparison of the catalytic efficiencies of the rat and human enzymes provides insight into their possible functions, Table 1 [17]. AKR1C4 is predominantly a 3-ketosteroid reductase and its catalytic efficiency is only five-fold less than AKR1C9. AKR1C3 is predominantly a 17-ketosteroid reductase but retains significant 3-ketosteroid reductase activity. AKR1C2 is predominantly a 3-ketosteroid reductase but has a catalytic efficiency 100-fold less than AKR1C9. AKR1C1 is predominantly a 20-ketosteroid reductase.

The functions of AKR1C1–AKR1C4 will also be determined by their tissue distribution. Using an isoform specific RT-PCR protocol, it was found that all four enzymes were expressed in liver [17]. However, AKR1C4 was virtually liver specific. AKR1C2 and AKR1C3 were found in the prostate. AKR1C3 was highly overexpressed in the mammary gland. In the CNS, AKR1C1 and AKR1C2 were both expressed.

Based on their catalytic efficiencies and tissue distribution, we proposed that AKR1C4 is a liver-specific isoform that works in concert with 5β-reductase (also an aldo-keto reductase) in catalyzing steroid hormone inactivation and the biosynthesis of bile acid intermediates. We proposed that AKR1C2 (bile acid binding protein) also acts as a major peripheral 3α-HSD. It is a candidate enzyme for oxidizing 3α-diol to 5α-DHT in the prostate and making allopregnanolone in the CNS. Furthermore, AKR1C3 is a candidate enzyme for generating an estrogenic state in the breast. Its 17-ketosteroid reductase activity would convert Δ^4-androstene-3,17-dione to testosterone which would be aromatized to 17β-estradiol; the same activity would convert estrone to 17β-estradiol. Additionally, its 20-ketosteroid reductase activity will convert progesterone to 20α-hydroxyprogesterone. These activities when combined would provide an environment for the unopposed actions of estrogens in the breast.

Despite this progress, significant unanswered questions remain with regards to AKR1C1–AKR1C4. From a structure–function perspective it is important to understand the structural basis for steroid hormone plasticity and also to know why these enzymes have lower catalytic efficiencies (k_{cat}/K_m) than their rat counterpart. Since the rat and human enzymes have comparable K_m values, the question becomes why is the k_{cat} value of the human enzymes significantly depressed? This paper describes X-ray crystallographic studies and stopped-flow kinetic studies performed on AKR1C2. Our studies reveal that the AKR1C2 structure can bind steroids backwards (D-ring in the A-ring position) and upside down (β-face inverted) relative to the position of 3-ketosteroids in AKR1C9 and this may account for its plasticity. Stopped-flow studies indicate that the conformational changes associated with binding cofactor (the first ligand) are slow but are not rate-determining in either AKR1C9 or AKR1C2. Alanine-scanning mutagenesis of the steroid pocket of AKR1C9 unexpectedly resulted in profound effects on k_{cat} but small effects on K_m. The results suggest that decreases in k_{cat} observed in AKR1C2 may be due to substrate "wobble" imposed by the plastic active site.

2. Materials and methods

2.1. Expression and crystallization of AKR1C2

AKR1C2 cDNA was cloned by RT-PCR from human hepatoma (HepG2) cells and subcloned into the pET16b prokaryotic expression construct [16]. Recombinant AKR1C2 was obtained from *Escherichia coli* in homogeneous form as previously described and crystallized as an E·NADP$^+$·Ursodeoxycholate ternary complex [18].

Table 1
Comparison of the bi-molecular rate constants for 3-, 17-, and 20-ketosteroid reduction catalyzed by human AKRC1 Isoforms

Enzyme	Activity		
	3α-HSD, 5α-DHT reduction (k_{cat}/K_m mM^{-1} min^{-1})	17β-HSD, Androsterone reduction (k_{cat}/K_m mM^{-1} min^{-1})	20α-HSD, Progesterone reduction (k_{cat}/K_m mM^{-1} min^{-1})
AKR1C9 (r3α-HSD)	1000	None	None
AKR1C4 (type 1 h3α-HSD)	231	0.1	0.1
AKR1C3 (type 2 3α-HSD)	6	42	0.1
AKR1C2 (type 3 3α-HSD)	9	0.1	0.1
AKR1C1 (h20α(3α)-HSD)	8	9	109

2.2. Structure-determination of the E·NADP$^+$·Ursodeoxycholate complex

Crystals diffracted to 3.0 Å resolution, the phase-solution was obtained by molecular replacement using the AKR1C9·NADP$^+$·testosterone structure (PDB: 1AFS) as the search model. The final R values were $R_{cryst} = 22.3\%$ and $R_{free} = 27.8\%$.

2.3. Fluorescence stopped-flow studies

The binding of NAD(P)H to recombinant AKR1C2 was measured on an Applied-Photophysics Stopped-Flow SX18MV-R spectrometer (mixing chamber: 20 μl; dead-time: 1–2 ms). One-syringe contained free enzyme (2.0 μM) and the second syringe contained cofactor (0–30 μM). The intrinsic protein fluorescence was excited at 290 nm and the quenching of fluorescence emission was measured in real time upon mixing using a cut-off filter (320 nm). With NADPH an increase in the fluorescence of the energy transfer band was measured in real time using a band-pass filter (450 nm). Fluorescence kinetic transients were fit to mono- and bi-exponentials using the SX18MV-R Software v. 4.46.

2.4. Alanine scanning mutagenesis of the steroid-binding pocket of AKR1C9

Residues implicated in the binding of testosterone to AKR1C9 (except those that comprise the catalytic tetrad), for example, T24, L54, F118, F129, T226, W227, N306, and Y310, were individually mutated to alanine using the QUIKCHANGE procedure (Stratagene) according to the manufacturer's instructions using the pET16b-3α-HSD prokaryotic expression construct as template [19]. The fidelity of the mutants was established by didedoxysequencing, and each of the mutant recombinant proteins were expressed in *E. coli* C41 (DE3) cells following induction with 1 mM IPTG. Each recombinant protein was purified to homogeneity, its steady-state kinetic parameters (k_{cat}, K_m and k_{cat}/K_m) were measured [20]. K_d values for NADPH binding to free enzyme (E) and testosterone binding to E·NADPH (binary complex), were determined by adapting published methods [21,22]. In the case of NADPH binding to free enzyme, a variation of the Scatchard analysis was performed which relates the fractional saturation $\Delta F/\Delta F_{max}$ to the total concentration of free ligand, free enzyme, and K_d [23].

3. Results and discussion

3.1. The human type 3 3α-HSD (AKR1C2) structure

To explain how human 3α-HSDs can act as 3-, 17-, and 20-ketosteroid reductases, we proposed that the conserved position of the cofactor would be invariant in the AKR structure and that it is the orientation of the steroid hormone at the active site that must change (Fig. 2). Furthermore, for AKR1C1–AKR1C4 to display 17-ketosteroid reduction, we proposed that the steroid must bind backwards (A-ring in the D-ring position) and upside down (β-face inverted) relative to the orientation of a 3-ketosteroid substrate (Fig. 2). For 20-ketosteroid reduction, the steroid need only bind backwards. To validate this proposal, we crystallized the AKR1C2·NADP$^+$·Ursodeoxycholate complex. AKR1C2 was chosen since it is the peripheral 3α-HSD most implicated in 5α-DHT and allopregnanolone formation. Ursodeoxycholate was chosen as the steroid ligand since bile acids bind to AKR1C2 with nanomolar affinity.

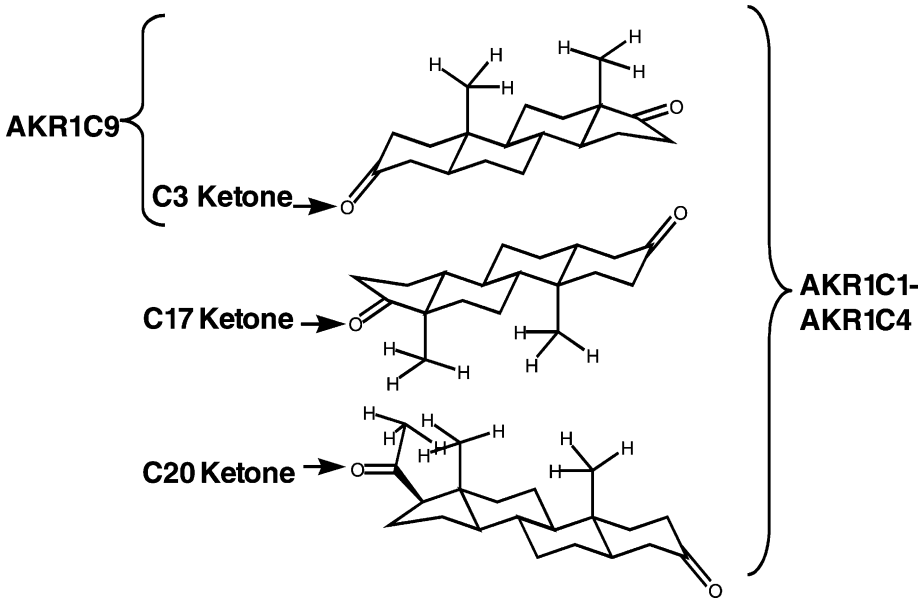

Fig. 2. Different modes of steroid binding at AKR1C active sites.

The structure of the E·NADP$^+$·Ursodeoxycholate complex revealed that it had the general topology of the AKRs [18]. This is characterized by the $(\alpha/\beta)_8$-barrel motif, which contains two additional helices. The barrel consists of an alternating arrangement of α-helices and β-strands, where the β-strands make up the staves of the barrel. NADP$^+$ was bound in an extended conformation in which the nicotinamide ring was *anti*- with respect to its ribose moiety. Ursodeoxycholate was bound in the steroid pocket perpendicular to the cofactor.

The catalytic tetrad is at the base of the pocket and consists of Tyr55, Lys84, His117, Asp50, which collectively form an oxyanion hole. It was found that the C24 carboxylate of the bile acid formed hydrogen bonds with Tyr55 and His117 (which is believed to provide $TyrOH_2^+$ character to the general acid Tyr55) [20]. The 7α-hydroxy group of ursodeoxycholate was also tethered by a hydrogen bond with Tyr24.

3.2. Comparison of the AKR1C2·NADP$^+$·Ursodeoxycholate complex with the AKR1C9·NADP$^+$·testosterone complex

The structure of the AKR1C2·NADP$^+$·Ursodeoxycholate complex [18] was compared with the AKR1C9·NADP$^+$·testosterone complex [15]. Inspection of the overlaid structures showed that ursodeoxycholate was bound upside down relative to testosterone in the rat structure (i.e. the β-face of the steroid was inverted) (Fig. 3). This is achieved in part by a highly conserved Trp227 on loop B rotating 120° to allow the steroid to bind upside-down. To accommodate the inverted angular methyl groups at C18 and C19, the bulky side-chains of L54 and F128 have been replaced with V54 and V128, respectively. This change can also explain how

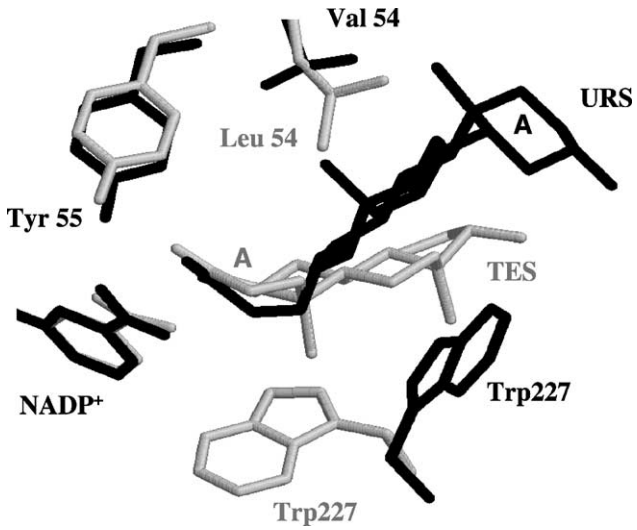

Fig. 3. Comparison of the steroid binding sites of AKR1C2 and AKR1C9. AKR1C2 is in black and AKR1C9 is in gray. URS: ursodeoxycholate and TES: testosterone. The A-rings of the steroid ligands are labeled.

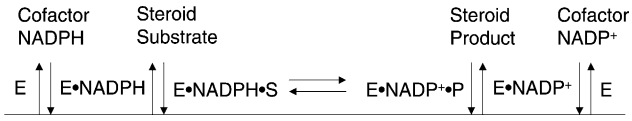

Fig. 4. Ordered bi–bi kinetic mechanisms for AKRs.

AKR1C1, which differs from AKR1C2 by only seven amino acids, can be converted to AKR1C2 by the L54V point mutation [24]. AKR1C1 is predominantly a 20α-HSD in which the 20-ketosteroid needs to bind backwards only. Introduction of valine at position 54 would allow the steroid to bind both backwards and upside down attenuating the 20α-HSD activity.

Ursodeoxycholate is also bound backwards relative to testosterone in AKR1C9 (A-ring in the D-ring position), that is, the A-rings of the respective steroids are at opposite ends of the pocket. This is achieved by substitution of C-terminal loop residues. For example, Y310 is replaced by I310, so that clashing with the C21 methyl group of ursodeoxycholate is prevented which in turn allows the C24 carboxylate to bind at the active site. The ability to bind ursodeoxycholate backwards and upside down in AKR1C2 provides structural validation that this can occur with steroid ligands and may explain why AKR1C1–AKR1C4 can display 3-, 17-, and 20-ketosteroid reductase activity, where these binding modes are required.

3.3. Rate-determining step and meaning of k_{cat} for human AKR1C isoforms

All AKRs catalyze an ordered bi–bi reaction in which k_{cat} will represent the slowest step in the kinetic sequence (Fig. 4). The steady-state kinetic mechanisms for both AKR1C9 and AKR1C2 indicate that the ordered mechanism applies to both enzymes with the cofactor binding first and leaving last [12,25]. For many AKRs, it is the binding and release of cofactor that may be rate-determining overall. For example, in aldose reductase, NADP(H) binds in two steps in which a loose complex E·NAD(P)H isomerizes to a tight complex E^*·NADP(H). In this sequence, the slowest event overall is the release of NADP$^+$ which is governed by the slow rate of conversion of the tight complex E^*·NADP$^+$ to the loose complex E·NADP and is controlled by k_4 (Fig. 5) [26]. It was hypothesized that the rate-determining step in AKR1C9 and AKR1C2 could also be due to slow cofactor release and that the k_{cat} for the human enzymes was so low because the cofactor was bound even more tightly [27].

If cofactor is bound more tightly to the human isoforms, it would be anticipated that the K_d values for NADP$^+$ and

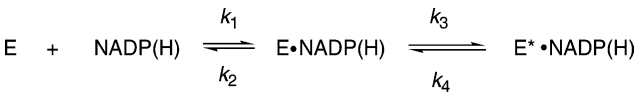

Fig. 5. Kinetic models for the binding of cofactor to AKR1C9 and AKR1C2.

Table 2
Comparison of the K_d values and kinetic constants for $NADP^+$ and NADPH binding to AKR1C2 and AKR1C9

Enzyme	K_d $NADP^+$[a] (nM)	K_d NADPH[a] (nM)	k_{lim}[b] (s^{-1})	k_{cat}[c] (s^{-1})
AKR1C9 (rat 3α-HSD)	268	137	26	0.2
AKR1C2 (type 3 h3α-HSD)	157	117	12	0.004

[a] Titrations were performed by adding increasing concentrations of cofactor [0.1–10 μM NADP(H)] to enzyme (0.25–0.5 μM) following excitation of the protein at 280 nm and by measuring changes in the emission spectrum at 330 nm at pH 7.0. K_d values were computed by a variation of the Scatchard analysis where the fractional saturation $\alpha(\Delta F/\Delta F_{max})$ is related to the total concentration of ligand L_0, the total concentration of enzyme E_0 and the K_d, where $L_0/\alpha = K_d/(1–\alpha) + E_0$ [23].

[b] Estimates of k_{lim} obtained via stopped-flow spectrophotometry in which the k_{obs} for the slow phase was plotted against [$NADP^+$] at pH 7.0.

[c] k_{cat} values obtained for 5α-DHT reduction at pH 7.0 where $NADP^+$ would be the slowest product to leave. AKR1C9 values are taken from Ref. [29] and AKR1C2 values are taken from [17].

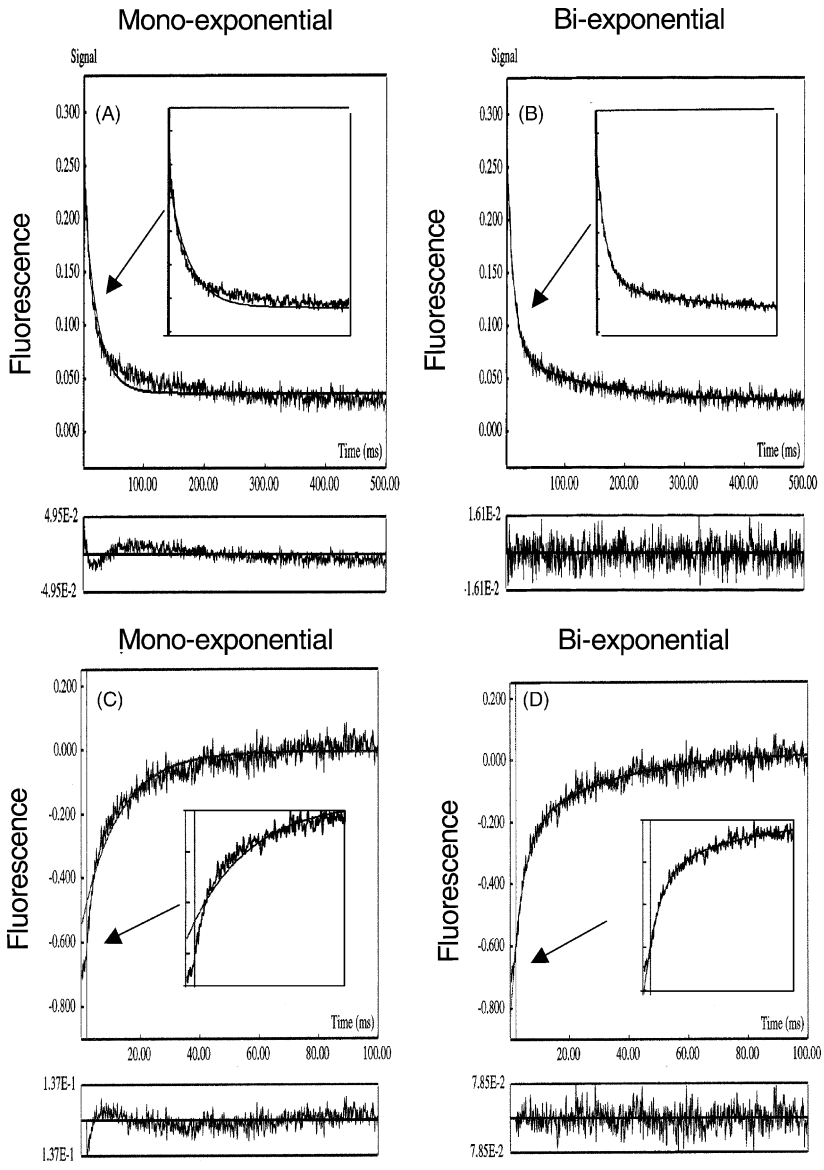

Fig. 6. Fluorescence kinetic transients for AKR1C9 best fit a bi-exponential: (A) shows a representative fluorescence transient observed upon mixing 2.0 μM AKR1C9 with 20 μM $NADP^+$ fit to a single exponential; (B) shows the same data fit to a bi-exponential. The inset and residuals in (B) show a superior fit, studies were performed with a 320 nm cut-off filter; (C) shows a representative fluorescence transient observed upon mixing 6.0 μM AKR1C9 with 20 μM NADPH fit to a single exponential; (D) shows the same data fit to a bi-exponential. The inset and residuals in (D) shows a superior fit, studies were performed with a 450 nm band-pass filter. Higher concentrations of enzyme were used to monitor NADPH binding in order to obtain a kinetic transient associated with the formation of the energy transfer band. Similar results were obtained for AKR1C2.

NADPH determined in the steady state would be lower than that observed for AKR1C9. The binding of cofactor to AKR1C9 and AKR1C2 can be measured by titrating the intrinsic protein fluorescence which is due to three conserved tryptophan residues (Trp 86, Trp 148, and Trp 227). Fluorescence titration of the cofactor binding site showed that while the k_{cat} values may be 50 times lower with AKR1C2, $NADP^+$ or NADPH are bound with similar affinities by the two enzymes (Table 2).

3.4. Fluorescence stopped-flow studies

Previous fluorescence stopped-flow studies showed that the binding of NADP(H) to AKR1C9 was accompanied by a fluorescence kinetic transient indicating that this was a slow event [28]. The fluorescence kinetic transient was best fit to a single mono-exponential which showed saturation kinetics and was best explained by a two step binding model, similar to that seen with aldose reductase [26]. In this model, the fluorescence kinetic transient reflects the slow isomerization event that accompanies the formation of the tight complex $E^*{\cdot}NADP(H)$. Deconvolution of plots of k_{obs} for the transient versus [NADP(H)] provided estimates of k_1, k_2, k_3, and k_4. In this analysis, the slowest step was k_4 for the conversion of $E^*{\cdot}NADP^+$ to $E{\cdot}NADP^+$ but this value was $11\,s^{-1}$, while k_{cat} for the reduction of 3-ketosteroids ranged from 0.2 to $1.0\,s^{-1}$. The kinetic fluorescence transient was assigned to the anchoring of the cofactor tail (2′-phosphate of AMP) by Arg276, since it was eliminated by the R276M mutant [28]. Arg276 is known to form an electrostatic linkage with the 2′ phosphate of AMP of the dinucleotide [14].

We recently obtained a more sensitive stopped-flow instrument with less dead-time, a smaller flow cell of 20 μl and superior software. Binding of NADP(H) to AKR1C9 was now best fit to a bi-exponential and similar results were obtained with AKR1C2 (Fig. 6). The superior fit to the bi-exponential is evident from the absence of the harmonic in the residuals, the smaller size of the residuals, and the decreased variance in the data. This was seen irrespective of whether a 320 nm cut-off filter was used or whether a 450 nm band-pass filter was used.

The slow phase of the bi-exponential showed saturation kinetics, and a plot of k_{obs} versus [NADP(H)] was hyperbolic yielding k_{lim} that governs the formation of the tight complex. Comparison of k_{lim} for the slow phase for both AKR1C9 and AKR1C2 gave similar values indicating that the rate constants that govern the conversion of the loose complex to the tight complex are in agreement in both enzymes. In each case the k_{lim} is significantly greater than k_{cat} for 3-ketosteroid reduction, the direction in which $NADP^+$ would be released as product. These data suggest that no step associated with the binding or release of cofactor is rate-limiting overall in either AKR1C9 or AKR1C2. We predict that k_{cat} in both the rat and human enzymes is governed by a subsequent event. This event is either the binding or release of steroid or the chemical event.

3.5. Alanine scanning mutagenesis of steroid binding site of AKR1C9 shows that steroid wobble decreases k_{cat}

We recently reported the results of an alanine scanning mutagenesis study, in which each of the residues that line the steroid binding pocket of AKR1C9 were individually mutated to alanine [22]. As expected there were no significant changes in the K_d values for NADPH by free enzyme. By contrast, a five-fold change was noted in the K_d value for the binding of testosterone to the $E^*{\cdot}NADPH$ complex (Fig. 7) (Panel A). In general substitution of hydrophobic

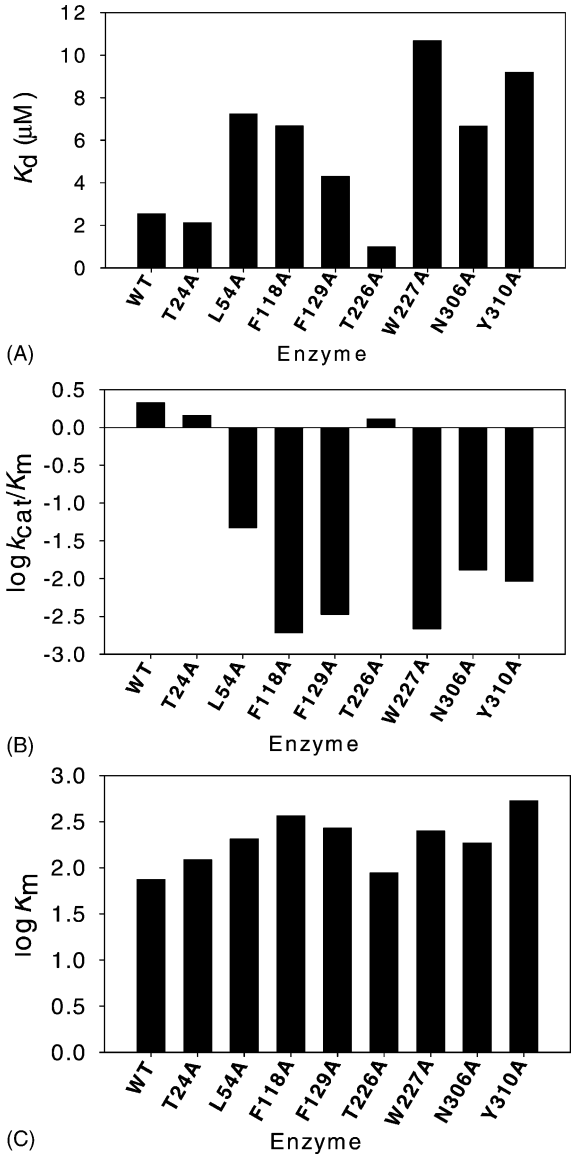

Fig. 7. Alanine-scanning mutagenesis of the steroid binding pocket in AKR1C9. (A), K_d values for the binding of testosterone to the binary complex $E{\cdot}NADP^+$ of rat liver 3α-HSD (AKR1C9) and its alanine mutants; (B) Catalytic efficiencies (shown as $\log k_{cat}/K_m$) for the $NADP^+$-dependent oxidation of androsterone catalyzed by rat liver 3α-HSD and its alanine mutants; and (C) K_m values (shown as $\log K_m$) for the $NADP^+$ dependent oxidation of androsterone catalyzed by rat liver 3α-HSD and its alanine mutants.

residues by alanine led to the largest changes in K_d. However, these changes were dwarfed by the large decrease (up to 700-fold) in $\log k_{cat}/K_m$ for the $NADP^+$-dependent oxidation of androsterone. Plots of $\log K_m$ indicate that the major effect of the alanine mutants is on k_{cat} (Fig. 7). k_{cat} reflects the rate-determining step and since the alanine mutants do not affect cofactor binding, the changes in this value must be due to a subsequent event. These events could be the binding or release of steroid or the chemical event.

Preliminary stopped-flow studies in which the binding of testosterone to the AKR1C9·NADPH complex was measured showed that the binding of steroid hormone was diffusion controlled (William Cooper, unpublished observations). Thus, changes in k_{cat} likely reflect changes in the chemical step. We propose that changes in k_{cat} occurred in the AKR1C9 alanine mutants because the orientation and proximity of the steroid and the catalytic groups (tetrad and cofactor) had been disrupted. These data are best interpreted as being due to the loss of steroid contact residues which introduce substrate wobble. We suggest that an explanation for the low k_{cat} values in the human enzymes is due to steroid "wobble" at the active site that results from a larger binding pocket that accommodates different steroid binding modes.

Acknowledgements

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