Elucidation of a Complete Kinetic Mechanism for a Mammalian Hydroxysteroid Dehydrogenase (HSD) and Identification of All Enzyme Forms on the Reaction Coordinate

THE EXAMPLE OF RAT LIVER 3α-HSD (AKR1C9)*

Received for publication, April 24, 2007, and in revised form, September 7, 2007. Published, JBC Papers in Press, September 11, 2007, DOI 10.1074/jbc.M703414200

William C. Cooper1, Yi Jin1, and Trevor M. Penning2

From the Center of Excellence in Environmental Toxicology, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084

Hydroxysteroid dehydrogenases (HSDs) are essential for the biosynthesis and mechanism of action of all steroid hormones. We report the complete kinetic mechanism of a mammalian HSD using rat 3α-HSD of the aldo-keto reductase superfamily (AKR1C9) with the substrate pairs androstane-3,17-dione and NADP (reduction) and androsterone and NADP+ (oxidation). Steady-state, transient state kinetics, and kinetic isotope effects reconciled the ordered bi-bi mechanism, which contained 9 enzyme forms and permitted the estimation of 16 kinetic constants. In both reactions, loose association of the NADP(H) was followed by two conformational changes, which increased cofactor affinity by >86-fold. For androstane-3,17-dione reduction, the release of NADP+ controlled kcat, whereas the chemical event also contributed to this term. kcat was insensitive to [3H]NADPH, whereas kcat/Km, and the Dkcat/Km (ratio of the maximum rates of single turnover) were 1.06 and 2.06, respectively. Under multiple turnover conditions partial burst kinetics were observed. For androstanediol oxidation, the rate of NADPH release dominated kcat whereas the rates of the chemical event and the release of androstane-3,17-dione were 50-fold greater. Under multiple turnover conditions full burst kinetics were observed. Although the internal equilibrium constant favored oxidation, the overall Keq favored reduction. The kinetic Hal dane and free energy diagram confirmed that Keq was governed by ligand binding terms that favored the reduction reagents. Thus, HSDs in the aldo-keto reductase superfamily thermodynamically favor ketosteroid reduction.

Mammalian hydroxysteroid dehydrogenases (HSDs)3 play pivotal roles in steroid hormone biosynthesis and metabolism.

* This work was supported by National Institutes of Health Grants R01-DK47015 and P30 ES013508 to T. M. P. (T. M. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

□ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Dept. of Pharmacology, University of Pennsylvania, 3620 Hamilton Walk, Philadelphia, PA 19104-6084. Tel.: 215-573-2236; Fax: 215-573-2236; E-mail: penning@pharm.med.upenn.edu.

3 The abbreviations used are: HSD, hydroxysteroid dehydrogenase; AKR, aldo-keto reductase; AKR1C2, human type 3α-HSD; AKR1C9, rat liver 3α-HSD; androstanediol, androstane-3,17-dione; androsterone, 17β-hydroxy-5α-androstan-3-one; 5α-DHT, 5α-dihydrotestosterone; KIE, kinetic isotope effect; SDR, short-chain dehydrogenase reductase; NADPD, [3H]NADPH.
Complete Kinetic Mechanism for 3α-HSD

FIGURE 1. Structural changes in AKR1C9 associated with ligand binding revealed by comparing the crystal structures of the apoenzyme E, the E-NADP⁺ binary complex, and the E-NADP⁺-testosterone ternary complex. The crystal structures were overlaid to identify conformational changes that occur. Portions of loop structures are colored for the apoenzyme (A), for the binary complex (B), and for the ternary complex (C) according to the following scheme: loop A in cyan, loop B in blue, loop C in orange, and the β-α, loop in pink. Ligands in the complexes are shown in green (ball and stick presentation). The E-NADP⁺ binary complex is equivalent to the kinetically observed E**-NADP⁺, and the E-NADP⁺-testosterone complex is equivalent to the kinetically observed E***-NADP⁺-testosterone (see “Results” section).

EXPERIMENTAL PROCEDURES

Materials—Cofactors were purchased from Roche Diagnostics. Steroids were purchased from Steraloids. Deuterated compounds for the synthesis of deuterated NADPH were purchased from Cambridge Isotope Laboratories. NADP⁺-specific alcohol dehydrogenase from Thermoanaerobium brockii was purchased from Sigma. Aldehyde dehydrogenase was purchased from Roche Biochemicals. The expression of pET16b-3α-HSD in Escherichia coli DE3 cells and purification of recombinant AKR1C9 have been described previously (25). A specific activity of 1.6 μmol of androsterone oxidized/min/mg of protein was determined. All other reagents were of ACS grade or better. Unless stated otherwise, experiments were carried out at 25 °C in 10 mM potassium phosphate buffer (pH 7.0) and 4% acetonitrile.

Steady-state Kinetic Analysis—Initial velocities were measured on a Hitachi F-4500 fluorescence spectrophotometer by monitoring NADPH depletion or production by the loss or gain of emission at 450 nm upon excitation at 340 nm.

Double reciprocal plots of families of lines in which the initial velocities were plotted against substrate concentrations, androstenedione 0.07–7 μM and NADPH 0.07–8 μM for reduction, and androsterone 0.1–30 μM and NADP⁺ 0.39–27 μM for oxidation, were constructed (26). Data were fitted to the minimal rate equation for an ordered bi-bi reaction, Equation 1 (also see Scheme 1),

$$ v = V_m AB / (K_{mA}K_{mB} + K_{mB} + K_{mA}A + AB) $$

(Eq. 1)

with the Cleland FORTRAN program and analyzed for a sequential mechanism. Patterns from plots of families of lines (see “Results”) using the SEQUEN program were judged to be the best fit according to the criteria of Cleland (27).

The individual rate constants described in Scheme 1 were obtained using the measured steady-state kinetic parameters based on the definitions of these parameters in terms of rate constants for an ordered bi-bi reaction where the isomerization of the central complex is included in the reaction sequence (28). $k_{AB}$, $k_{BA}$, $k_{Qr}$, and $k_{Qb}$, which describe cofactor binding and release, were

scopic rate constants for conformational changes of all enzyme forms involved in ligand binding, and the chemistry of transformation have yet to be described for any HSD in real-time. Knowledge of the number of enzyme forms that exist is a pre-requisite to identify all the steps that could be intercepted by HSD inhibitors.

The hydride transfer reaction catalyzed by HSDs is reversible in vitro. The seemingly directional preference of an individual HSD, either an AKR or an SDR, seen in transfection studies is believed to be governed by the cofactor ratio and cofactor preference. In the cytosol, the relationships are [NADPH] > [NAD⁺] and [NAD⁺] >> [NADH] (21, 22). Thus enzymes that have high affinity for NADPH will act as reductases, and those that preferentially utilize NADH/NAD will act as oxidases. AKRs have nanomolar affinity for the NADPH/NADP⁺ pair. We found that the NADPH-dependent reduction of 5α-DHT catalyzed by human type 3 3α-HSD (AKR1C2) occurred unimpeded in the presence of 1 mM NAD⁺ but that the NAD⁺-dependent oxidation of 3α-androstanediol oxidation catalyzed by AKR1C2 was potently inhibited by low micromolar concentrations of NADPH, suggesting that the reaction is unidirectional under normal cellular redox conditions (5). Recently, transfection studies with HSDs showed that a functional pseudo-equilibrium was reached such that the rates of the forward and reverse reactions were identical (23, 24). Thus, directionality and/or reversibility of an HSD are also related to the equilibrium constant of the reaction, which has generally not been reported for mammalian HSDs.

In this study we elucidate the complete kinetic mechanism for a mammalian HSD at physiological pH using AKR1C9. We find that the reaction sequence is governed by 9 enzyme forms, and 16 kinetic constants were estimated. We also find that the internal equilibrium constant for the reaction favors oxidation but this is not reflected in the overall $K_{eq}$ which favors reduction. The kinetic Haldane and the energy diagram confirmed that $K_{eq}$ was governed by ligand binding terms that favored reactants in the reduction direction. Our findings are discussed within a physiological context.
Complete Kinetic Mechanism for 3α-HSD

E + NADPH \[\overset{k_{\text{Ar}}}{\rightleftharpoons}\] E\(\cdot\)NADPH + Androstanedione

(E\(\cdot\)NADPH\(\cdot\)Androstanedione \[\overset{k_{1\beta}}{\rightleftharpoons}\] E\(\cdot\)NADPH + Androsterone \[\overset{k_{5}}{\rightarrow}\] E + NADP⁺

SCHEME 1. Sequential ordered bi-bi mechanism for AKR1C9.

E + NADPH \[\overset{1/K_{1\text{NADPH}}}{\rightleftharpoons}\] E\(\cdot\)NADPH \[\underset{k_{3}}{\rightarrow}\] E\(\cdot\)NADPH + Androsterone \[\underset{k_{4}}{\rightarrow}\] E\(\cdot\)NADPH + Androstanedione \[\overset{k_{6}}{\rightarrow}\] E\(\cdot\)NADPH + Androsterone

SCHEME 2. Three-step model for NADPH binding to AKR1C9.

directly calculated. However, the remaining rate constants could only be derived by iteratively fitting six independent algebraic equations for each of the six steady-state parameters that were measured (i.e. \(k_{\text{catRed}}/K_{m\text{NADPH}}\), \(k_{m\text{Androstanedione}}/k_{\text{catCoxP}}\), \(K_{m\text{NADP}^+}\), and \(K_{m\text{Androsterone}}\)). Initial estimates and constraints of rate constants were made based on results of transient kinetic experiments. (see “Transient State Kinetic Analysis”). Values of the rate constants were adjusted until all the fits converged simultaneously, i.e. the calculated values of all six steady-state parameters matched the measured values.

The synthesis of 4-pro-\(R\)-[\(^2\)H]NADPH (NADPD) was performed as described by Hermes et al. (29) and Viola et al. (30) with minor modifications. Analysis of the NADPD product and assessment of deuterium incorporation was determined at 2.9 ppm by [\(^1\)H]NMR. The [\(^1\)H] content at the 4-pro-\(R\) position was estimated to be >99%. For estimates of primary KIEs, initial velocities were performed as described previously by substituting NADPD in the assays. The nomenclature of Northrop (31) was used where \(\Delta k_{\text{cat}}/K_{m}\) is the ratio of \(k_{\text{cat}}\) determined in the presence of NADPH versus NADPD, and \(\Delta k_{\text{cat}}/K_{m}\) is the ratio of \(k_{\text{cat}}/K_{m}\) determined in the presence of NADPH versus NADPD. Measurement of Equilibrium Constant—The equilibrium constant of the reaction was determined experimentally using a method described by Talalay and Levy (32). In this method, the equilibrium was approached from both the reduction and oxidation directions. Experiments were performed at different cofactor and steroid concentrations. In a typical experiment, the initial reaction mixture contained 4.1 \(\mu\)g of AKR1C9, 22.6 \(\mu\)M androstanedione, 34.6 \(\mu\)M androsterone, and 23 \(\mu\)M NADPH (in 100 mM phosphate buffer, pH 7.0, 5% methanol at 25 °C). When the equilibrium was reached, 8.5 nmol of NADP⁺ was added to force the oxidative reaction and establish a new equilibrium. The changes in absorbance at 340 nm were monitored to calculate the concentrations of reactants and products at each equilibrium end-point permitting the calculation of \(K_{\text{eq}}\).

Transient State Kinetic Analysis—Transient state kinetic assays were performed on an Applied Photophysics SX.18MV-R stopped-flow reaction analyzer instrument fitted with a 20-\(\mu\)l flow cell and a dead time of ~1 ms. All concentrations are given as final.

Kinetics of Binary Complex Formation—The binding of NADPH to AKR1C9 was monitored in real-time using stopped-flow spectroscopy by exciting the protein at 290 nm and capturing the emission of the energy transfer band at 450 nm through a 10-nm band-pass filter. The energy transfer band is produced as a result of the interaction of NADPH with Trp-86 (33). Consecutively, to monitor the binding of NADP⁺, the decrease in protein fluorescence emission at 330 nm was recorded upon quenching the excited tryptophan residues at 290 nm. In a typical experiment, AKR1C9 (0.25 \(\mu\)M) was mixed with NADPH (2.0–11 \(\mu\)M). For NADP⁺ binding, enzyme (0.70 \(\mu\)M) was mixed with NADP⁺ (4.4–36 \(\mu\)M). The time courses of the fluorescence increase or decrease at each concentration of ligand were best fitted to double exponential equation (Equation 2),

\[
F_t = (\Delta F)_1 e^{-k_{\text{obs1}} t} + (\Delta F)_2 e^{-k_{\text{obs2}} t} + F_{\text{eq}}
\]

where \(F_t\) is the fluorescence at time \(t\), \(\Delta F\) is the amplitude of the fluorescence increase or decrease, \(k_{\text{obs1}}\) and \(k_{\text{obs2}}\) are the apparent first-order rate constants for the fast and slow phases, respectively, and \(F_{\text{eq}}\) is the fluorescence at equilibrium. Data were analyzed based on a three-step model for cofactor binding (Scheme 2). Estimates of the kinetic constants \(K_{1\text{NADPH}}\) and \(k_{3}−k_{5}\) were obtained by the method of Stone and Le Bonniec (34). \(K_{1\text{NADPH}}\) is the apparent dissociation constant of the initial loose complex. The plot of \(k_{\text{obs2}}\) versus [NADPH] provided the estimate of \(k_{5}\). Values of \(K_{aNADPH}\) were determined by steady-state fluorescence titration (25), and estimates of \(K_{1}\) and \(k_{5}−k_{5}\) were substituted into Equation 3 to solve for \(k_{6}\).

\[
K_d = K_{1\text{NADPH}} k_{6}/(k_{5}k_{6} + k_{4}k_{6} + k_{3}k_{6})
\]

Similarly, estimates of kinetic constants for the binding of NADP⁺ (\(k_{13}−k_{16}\) and \(K_{1\text{NADP}^+}\)) were obtained.

Ligand Chase Experiments—Enzyme (0.4 \(\mu\)M) was incubated with NADPH (12 \(\mu\)M) in one syringe and mixed with NADP⁺ (40 \(\mu\)M) from a second syringe. The decrease of the energy transfer band corresponding to dissociation of NADPH from the binary complex was monitored at 450 nm. Transients were best fitted to a bi-exponential (Equation 2).

Kinetics of Ternary Complex Formation—A solution containing enzyme (0.4 \(\mu\)M) and NADPH (12 \(\mu\)M) from one syringe was mixed with increasing concentrations of testosterone (3.60–6.15 \(\mu\)M) from a second syringe. Decreases in fluoros-
cence at 450 nm were monitored with excitation at 295 nm. Fluorescence traces were marked by a single exponential decay. Five transient traces were averaged for each steroid concentration, and values for $k_{obs}$ were fitted to Equation 4 to solve for $k_{on}$ and $k_{off}$ (35).

$$k_{obs} = k_{on} [T] + k_{off} \quad (Eq. 4)$$

**Transient State Turnover Experiments**—For single turnover experiments, AKR1C9 (0.5 μM) was incubated with stoichiometric amounts of NADPH (0.3 μM) in one syringe and mixed with a varying excess of androstanedione (4–15.0 μM) added from a second syringe in the stopped-flow instrument. Similarly for single turnover of androsterone oxidation, a solution of AKR1C9 (0.8 μM) and NADP$^+$ (0.6 μM) from one syringe were mixed with an excess of androsterone ranging from 7.0 to 30 μM from a second syringe. Reactions were monitored fluorimetrically (excitation at 340 nm and emission at 450 nm). Data were fitted to a single exponential equation. Plots of $k_{obs}$ versus steroid concentration were fitted to the hyperbolic equation (Equation 5), where $A$ is the steroid concentration, $K_{lim}$ is the maximum rate of single turnover, and $K_d$ is a quasi dissociation constant for steroid substrate (34).

$$k_{obs} = k_{lim}[A]/[A] \quad (Eq. 5)$$

Values of $K_{lim}$ provided the lower limits for the $k_{off}$ and $K_d$ and initial constraints on $k_{on}$, $k_{off}$, $k_{on}$, and $k_{off}$ were set based on values of $K_d$ during steady-state fitting analysis.

To study multiple turnover of androstanedione reduction, a solution of AKR1C9 (1.5 μM) and NADPH (25 μM) was mixed with androstanedione (13.5 μM). A complete concentration series with androstanedione was not possible due to steroid solubility constraints. Kinetic transients of NADPH depletion at 450 nm were collected. Experiments were repeated in the absorbance mode. Similarly, multiple turnover experiments of androsterone oxidation were conducted in which AKR1C9 (1 μM) and NADP$^+$ (40 μM) from one syringe were mixed with increasing concentrations of androsterone (10–35 μM) added from a second syringe. Transient traces were fitted to an equation (Equation 6) that contained both burst and steady-state terms,

$$F_t = Amp \cdot e^{-k_{off}t} + v_{ss}t + c \quad (Eq. 6)$$

where $F_t$ equals fluorescence at time $t$. $Amp$ equals the amplitude of the burst, $k_{obs}$ is the apparent rate constant of the burst in units of $s^{-1}, v_{ss}$ is the steady-state rate in units of $\Delta F/\Delta t$, and $c$ is the fluorescence at equilibrium (35). The maximum value of $k_{obs}$ yielded $k_{burst}$, and the maximum value of $v_{ss}$ was, subsequently, mathematically transformed into micromolar/second and divided by enzyme concentration to yield $K_{eq}$ (s$^{-1}$).

**RESULTS**

Dissection of the complete kinetic mechanism for AKR1C9 was conducted using androstanedione and NADPH, and androsterone and NADP$^+$, as substrate pairs at physiological pH. Androstanedione and androsterone were chosen, because these steroids have been standards with which all other assays on this enzyme in our laboratory have been compared. NADP(H) cofactors were chosen, because bound NADP$^+$ was present in the crystal structures of the binary and ternary complexes. Thus, the existence of kinetically inferred complexes could be related to structural changes in the enzyme.

**Steady-state Initial Velocity Studies**—Initial velocity studies for androstanedione reduction and androsterone oxidation using NADP(H) were performed. Double reciprocal plots of the initial velocity data (see supplemental data) were characterized by a fan of lines that converged to an intersecting point to the left of the origin and above or on the x-axis. This is indicative of a sequential mechanism defined by the requirement of the cofactor and substrate to bind to the enzyme to form a ternary complex before any product can be produced (28). The sequential mechanism was previously shown to be an ordered bi-bi reaction as seen in Scheme 1. Fits of family of lines were generated through the Cleland SEQUEN program to yield steady-state kinetic parameters given in Table 1.

It was found that $k_{cat}$ was 0.82 s$^{-1}$ for the oxidation direction was 2-fold greater than the reduction direction, $k_{cat} = 0.42$ s$^{-1}$, whereas $K_{m,NADPH}$ was smaller than $K_{m,NADP^+}$ by >18-fold. $K_i$ values for the cofactors were determined to be 0.17 μM and 1.51 μM for NADPH and NADP$^+$, respectively, and were in reasonable agreement with the $K_d$ values of 0.14 μM and 0.32 μM determined directly by fluorescence titration (25). The internal consistency of the kinetic parameters in Table 1 was checked with the kinetic Haldane relationship for an ordered bi-bi mechanism (28) by Equation 7.

$$K_{eq} = V_{maxRed}K_{mAndrosterone}/V_{maxOx}K_{mNADPH}K_{mAndrostanedione} \quad (Eq. 7)$$

The steady-state parameters in Table 1 gave a value of 22.7 for
Complete Kinetic Mechanism for 3α-HSD

this expression where the forward direction is reduction. This is in good agreement with a value of 20.9 ± 1.8 determined experimentally using a method described by Talalay and Levy (32). The kinetic Haldane revealed that the $K_{\text{m}}$ is dominated by sub-micromolar $K_s$ and $K_m$ values for NADPH and androstanediol, respectively.

Rate Constants Obtained in the Steady State—The individual rate constants described in Scheme 1 were calculated and derived from the measured steady-state parameters (Table 2). In the reduction direction these constants showed that the binding of NADPH ($k_{\text{ADP}}$) was slow and did not occur at the diffusion limit. The binding of androstanediol ($k_{\text{AD}}$) to the binary complex occurred 5-times faster than $k_{\text{ADP}}$ and the rate of conversion of the central complex ($k_{\text{p}}$) was 6.6 s$^{-1}$. The release of the steroid product androsterone ($k_{\text{Pr}}$) occurred at a comparable rate of 10.5 s$^{-1}$. The main contribution to the overall $k_{\text{cat}}$ of 0.42 s$^{-1}$ came from the release of the NADP$^+$ product, $k_{\text{Ox}} = 0.65$ s$^{-1}$.

In the oxidation direction, macroscopic rate constants showed that the binding of NADP$^+$ ($k_{\text{OB}}$) was 10-fold slower than the binding of NADPH ($k_{\text{OA}}$), and the binding of androsterone ($k_{\text{PB}}$) was 100-fold slower than the binding of androstanediol ($k_{\text{OB}}$). However, the conversion of the central complex ($k_{\text{p}}$ of 43.2 s$^{-1}$) was >50-fold faster than the $k_{\text{cat}}$ of oxidation (0.82 s$^{-1}$). The release of steroid product was also fast ($k_{\text{Pr}}$ of 55.2 s$^{-1}$), and the reaction was rate-controlled by the release of NADPH ($k_{\text{Ar}}$ of 0.86 s$^{-1}$). Because these rate constants may reflect more than one event, the reaction was further dissected using transient kinetic approaches.

Transient Kinetics of Cofactor Binding—The binding of NADPH to AKR1C9 is associated with the quenching of the intrinsic tryptophan fluorescence of the protein and yields a kinetic fluorescent kinetic transient that can be measured by stopped-flow kinetics (25). This kinetic transient was abolished by the R276M mutant and therefore assigned to cofactor anchoring by the guanidinium group of Arg-276. A two-step binding model was proposed that involved rapid formation of a loose complex followed by conformational changes to yield a tight binding complex that occurred as a result of the anchoring event. In re-examining this kinetic transient we found that these fluorescence kinetic transients can be best fitted to a bi-exponential decay rather than to a mono-exponential decay based on the shape and size of the residuals (Fig. 2). The differences in the apparent rate constants $k_{\text{obs1}}$ and $k_{\text{obs2}}$ for the fast and slow phases were ~10-fold, and the amplitudes of the slow phase contributed to 25–30% of the total fluorescence change. Both $k_{\text{obs1}}$ and $k_{\text{obs2}}$ showed saturation kinetics versus the ligand concentration, thus a three-step binding model was proposed, which invoked the formation of a loose enzyme cofactor complex followed by two conformational changes (Scheme 2).

The kinetic rate constants defined in Scheme 2 were estimated using the transient data and are listed in Table 3. The dissociation constants for the initial loose complexes were determined to be 12 μM and 35 μM for E-NADPH and E-NADP$^+$, respectively. Compared with the apparent $K_d$ values determined experimentally by fluorescence titration, the sequential conformational change steps resulted in 86- and 110-fold increases in affinity for NADPH and NADP$^+$ binding, respectively.

Ligand exchange experiments were performed on the premixed enzyme and NADPH solution by chasing the bound NADPH with excess NADP$^+$ and measuring the decay in amplitude at 450 nm of the energy transfer band (see supplemental data). The kinetic traces were best fitted to a bi-exponential equation consistent with the three-step model for the release of cofactor.

Kinetics of Ketosteroid Binding—Because it is not possible to directly measure the kinetics of the binding of 3-ketosteroid steroid substrates we conducted experiments with testosterone, a competitive inhibitor and related 3-ketosteroid. Testosterone is also the ligand present in the crystal structure of the ternary complex. Quenching of the energy transfer band upon testosterone binding to the enzyme NADPH binary complex was marked by a decrease in fluorescence at 450 nm in the steady state and yielded a $K_d$ value of...
The complete set of experimentally derived kinetic constants for the reduction of androstanediol by NADPH and oxidation of androstenedione by NADP$^+$

| Kinetic constant | Value | Kinetic constant | Value |
|------------------|-------|------------------|-------|
| $K_{\text{NADPH}}$ ($\mu M$) | $12 \pm 4$ | $K_{\text{NADPH}}$ ($\mu M$) | $0.14$ |
| $k_1$ ($s^{-1}$) | $590 \pm 190$ | $k_1$ ($s^{-1}$) | $220 \pm 22$ |
| $k_2$ ($s^{-1}$) | $170 \pm 38$ | $k_2$ ($s^{-1}$) | $6.4$ |
| $k_3$ ($\mu M^{-1}s^{-1}$) | $27.8$ | $k_3$ ($s^{-1}$) | $55.2$ |
| $k_4$ ($s^{-1}$) | $6.6 (4.5)^a$ | $k_4$ ($s^{-1}$) | $43.2 (39.2)^a$ |
| $k_5$ ($s^{-1}$) | $10.5$ | $k_5$ ($s^{-1}$) | $0.26$ |
| $k_6$ ($s^{-1}$) | $2.9$ | $k_6$ ($s^{-1}$) | $60 \pm 9$ |
| $k_7$ ($s^{-1}$) | $58 \pm 15$ | $k_7$ ($s^{-1}$) | $280 \pm 70$ |
| $K_{\text{NADPH}}$ ($\mu M$) | $35 \pm 13$ | $K_{\text{NADPH}}$ ($\mu M$) | $0.32$ |

$^a$ $K_d$ is the dissociation constant of the initial loose complex and was determined from transient cofactor binding experiments (see Materials and Methods). $K_d$ is the apparent dissociation constant of the final tight complex, which was determined by independent steady-state fluorimetric titration (25).

$^b$ Values obtained from iterative fits of equations for steady state parameters.

$^c$ Values obtained from transient kinetic experiments of cofactor binding.

$^d$ Values of $k_{\text{lim}}$ from transient single turnover experiments are in parenthesis.

TABLE 3

FIGURE 3. Transient kinetics of testosterone binding. A, a representative fluorescence transient trace for the quenching of the energy transfer band at 450 nm upon mixing a preincubated solution of AKR1C9 (0.4 $\mu M$) and saturating NADPH (12 $\mu M$) with testosterone (3.6 $\mu M$); B, the dependence of $k_{\text{obs}}$ on varying testosterone concentrations.

$0.5 \mu M$ obtained by fluorescence titration.$^4$ Stopped-flow experiments were then performed to determine whether steroid binding was accompanied with a fluorescence kinetic transient. Each transient trace was fitted to a single exponential equation (Fig. 3). At high testosterone concentrations the amplitude decayed too rapidly and the observable rate constant could not be discerned. As a result, saturation kinetics was not detected. At lower steroid concentrations, the concentration dependence on $k_{\text{obs}}$ was linear. The microscopic association and dissociation rate constants were estimated to be $95.7 \pm 4.8 \mu M^{-1}s^{-1}$ and $14.1 \pm 1.9 s^{-1}$, respectively, and gave a $K_d$ of $0.15 \mu M$.

Single Turnover Experiments—Transient kinetic traces of a decrease in NADPH fluorescence over time were recorded for androstanediol reduction catalyzed under single turnover conditions (Fig. 4). Rate constant $k_{\text{obs}}$ values were obtained from fitting transients to a single exponential equation. As predicted, the amplitude of the signal was directly proportional to the concentration of NADPH oxidized. Subsequently, $k_{\text{obs}}$ values were plotted against [androstanediol]. The plot was characterized by a hyperbola whereby: 1) the plateau yielded a $k_{\text{lim}}$ value that estimates a maximal rate of single turnover and 2) the dependence of $k_{\text{lim}}$ on androstanediol concentration under single turnover conditions (Fig. 4, $B$). The dependence of $k_{\text{lim}}$ on androstanediol concentration under single turnover conditions approximated a value of $4.5 s^{-1}$.

Multistep Turnover Reduction Experiments—Transient state multistep turnover experiments were also performed to see whether androstanediol reduction was accompanied by burst-phase kinetics. Transient traces were characterized by a

$^4$ W. C. Cooper, unpublished results.

FIGURE 4. Reduction of androstanediol by NADPH or NADPD and oxidation of androstenedione by NADP$^+$ under single turnover conditions. A, a representative fluorescence transient trace at 450 nm for the single turnover of androstanediol reduction obtained by mixing a solution of AKR1C9 (0.5 $\mu M$) and NADPH (lower trace) or NADPD (upper trace) (0.3 $\mu M$) with androstanediol (10 $\mu M$). The transient was fitted to a mono-exponential function to obtain $k_{\text{obs}}$; B, the dependence of $k_{\text{obs}}$ on androstanediol concentration with NADPH (●) and NADPD (○); C, a representative transient kinetic trace for the single turnover of androstanediol oxidation obtained by mixing a solution of enzyme (0.8 $\mu M$) and cofactor (0.6 $\mu M$) with androstanediol (20 $\mu M$). The transient was fitted to a mono-exponential function to obtain $k_{\text{obs}}$, $D$, dependence of $k_{\text{obs}}$ on substrate concentration for androstanediol oxidation.
Complete Kinetic Mechanism for 3α-HSD

TABLE 4
| Kinetic constants from single and multiple turnover experiments and their associated KIEs | Reduction | Oxidation |
|---|---|---|
| Steady state | | |
| $k_{cat}^0$ (s⁻¹) | 0.42 ± 0.02 | 0.82 ± 0.04 |
| $k_{cat}/K_m^0$ (μM⁻¹ s⁻¹) | 1.08 ± 0.10 ($n$ = 5) | ND⁺ |
| $k_{cat}/K_m^0$ | 0.65 ± 0.04 | 0.20 ± 0.01 |
| $k_{cat}/K_m^0$ | 1.06 ± 0.13 ($n$ = 3) | ND |
| Single turnover | | |
| $k_{lim}$ (s⁻¹) | 4.5 ± 0.1 | 35.7 ± 0.8 |
| $k_{lim}$ (μM) | 17 ± 1.1 | 32 ± 4 |
| $k_{lim}/K_m$ (μM⁻¹ s⁻¹) | 2.6 | 1.3 |
| $k_{lim}/K_m$ | 2.29 ± 0.22 ($n$ = 3) | ND |
| Multiple turnover | | |
| $k_{lim}$ (s⁻¹) | 5.5 ± 0.27 | 55.8 ± 2.8 |
| $k_{lim}$ (μM) | 2.07 ($n$ = 1) | ND |
| $k_{lim}$ (μM) | 0.41 ± 0.07 | 0.77 ± 0.06 |
| $K_m^0$ | ND | 3.5 ± 0.3 |
| $K_m^0$ | 0.93 ($n$ = 1) | ND |

⁺ND, not determined.
* Denotes apparent values because $k_{cat}$ could not be obtained over a range of substrate concentration to achieve pseudo-first order conditions.

small but yet discernable burst that was not stoichiometric with enzyme concentration (Fig. 5A). To validate this observation multiple turnover experiments for androstanedione reduction were also performed in the absorbance mode. A transient trace obtained over 5 s was characterized by a burst phase of distinguishable amplitude followed by a steady-state phase as illustrated in Fig. 5B. Fitting the traces to Equation 6 gave an apparent $k_{lim}$ of 4.5 s⁻¹ and $k_{lim}$ of 0.42 s⁻¹, respectively. Transient-state multiple turnover experiments were also performed to determine whether androsterone oxidation was accompanied by burst-phase kinetics. Androsterone oxidation was characterized by a pronounced burst that yielded a $k_{lim}$ of 5.6 s⁻¹ and $k_{lim}$ of 0.41 s⁻¹, which agreed well with a $k_{lim}$ of 4.2 s⁻¹ and a $k_{lim}$ of 0.42 s⁻¹, respectively.

Primary KIEs on Steady-state and Transient State Kinetic Parameters—To confirm that the chemical step was not rate-determining for androstanedione reduction, primary KIE values were performed using NADPD. There was no significant $D_{cat}$ nor $D_{cat}/K_m$ and values of 1.08 and 1.06 were obtained (Table 4). By contrast under single turnover conditions, $D_{lim}$ = 2.06 was observed (Fig. 4). Under multiple turnover conditions a $D_{lim}$ equal to 2.07 was noted and was reduced to 0.93 for $D_{lim}$ (Fig. 5A). The data were internally consistent with steps other than chemistry being rate-determining in the reduction of androstanedione.

DISCUSSION

A complete kinetic mechanism for a mammalian HSD has been presented (see Table 3 and Scheme 3). The mechanism contains 9 enzyme forms, and 16 individual kinetic constants were assigned. The steady-state kinetic parameters for the mechanism were reconciled with rate constants obtained from the transient-state kinetic measurements. This mechanism can be used as a guide for studies on other HSDs.

Rate-determining Steps in AKR1C9 Catalysis—Our study focused on the NADP(H)-dependent interconversion of andro-
Complete kinetic mechanism for AKR1C9.

\[
\begin{align*}
\text{E} + \text{NADPH} &\xrightarrow{k_1} \text{E} \cdot \text{NADPH} \xrightarrow{k_3} \text{E}^* \cdot \text{NADPH} \xrightarrow{k_5} \text{E}^{**} \cdot \text{NADPH} + \text{Androstanedione} \\
&\text{E}^{**} \cdot \text{NADPH} + \text{Androsterone} \xrightarrow{k_7} \text{E}^{**} \cdot \text{NADP}^+ + \text{Androsterone}
\end{align*}
\]

SCHEME 3. Complete kinetic mechanism for AKR1C9.

We have previously shown that the NADPH reduction of 5α-DHT catalyzed by AKR1C9 had a distinctively different kinetic profile (36). In this reaction, there was no burst-phase kinetics in multiple turnover experiments, and a presence of significant primary KIEs provided evidence that chemistry was a major rate-determining step. Thus AKR1C9 can catalyze identical reactions on two highly related 3-ketosteroids (5α-DHT and androstanedione) with different rate-determining profiles. This is likely due to the different positioning of the steroid at the active site and changes in proximity between the reactants. We have previously shown using alanine-scanning mutagenesis that subtle changes in the binding of steroids to AKR1C9 can alter the rate-determining step of the reaction (37).

Loop Dynamics—Fluorescence kinetic transients were observed for the binding of NADPH and the competitive inhibitor testosterone, and these may report the conformational changes that are observed upon ligand binding in the crystal structures of AKR1C9 and its complexes. The binding of E**-NADPH complex, the on rate is ~20 fold higher than the off rate. This step may be related to the formation of the cofactor tunnel that involves the residues on loop β1-α1 and loop B, the nicotinamide ring being sandwiched between Trp-86 and Tyr-216, and the formation of hydrogen bonds with the C3 carboxamide group that enable the head group to orient correctly. The lower off rates suggest that the disruption of the tunnel and binding of the head group is slow.

To progress from the binary complex to the ternary complex, structural data imply that additional ordering of the loops occurs (19). Kinetic transients were captured possibly reflecting these changes for the binding of testosterone to AKR1C9. The microscopic association and dissociation rate constants for testosterone binding were estimated to be 95.7 μM⁻¹s⁻¹ and 14.1 s⁻¹ and approached those for androstanedione obtained in the steady-state solution, which were 55.2 μM⁻¹s⁻¹ and 27.8 s⁻¹. Significantly, these values differ from the association and dissociation rate constants for androsterone obtained from steady-state analysis, which were 0.26 μM⁻¹s⁻¹ and 10.5 s⁻¹, respectively. This reflects a 80-fold decrease in the $K_d$ value for androstanedione. Considering the structural differences between androstanedione (substrate) and androsterone (product) in the reduction direction, it is apparent that the enzyme has evolved to favor 3-ketosteroid binding, i.e. the binding of reductive substrates.

Equilibrium Constants and Free Energy Diagram—The concept that HSDs work in pairs to act as either reductases or oxidases and thereby regulate ligand access to steroid hormone receptors is based to a large extent on transfection assays using radiotracers. In transfection paradigms HSDs are forced to use the prevailing concentrations of reduced or oxidized cofactor, because the fast phase of the kinetic transient can be eliminated by the R276M mutant (25). In the second conformational change step that yields the tight cofactor to AKR1C9 was dissected into a three-step process that can be related to conformational changes. The formation of the initial loose complex $E$-NADPH was followed by two subsequent conformational changes that result in a tight binding complex. The first conformational change may be attributed to the formation of an electrostatic linkage between Arg-276 and the 2'-phosphate of AMP, because the fast phase of the kinetic transient can be eliminated by the R276M mutant (25). In the second conformational change step that yields the tight complex $E**$-NADPH, the on rate is ~20 fold higher than the off rate. This step may be related to the formation of the cofactor tunnel that involves the residues on loop β1-α1 and loop B, the nicotinamide ring being sandwiched between Trp-86 and Tyr-216, and the formation of hydrogen bonds with the C3 carboxamide group that enable the head group to orient correctly. The lower off rates suggest that the disruption of the tunnel and binding of the head group is slow.

To progress from the binary complex to the ternary complex, structural data imply that additional ordering of the loops occurs (19). Kinetic transients were captured possibly reflecting these changes for the binding of testosterone to AKR1C9. The microscopic association and dissociation rate constants for testosterone binding were estimated to be 95.7 μM⁻¹s⁻¹ and 14.1 s⁻¹ and approached those for androstanedione obtained in the steady-state solution, which were 55.2 μM⁻¹s⁻¹ and 27.8 s⁻¹. Significantly, these values differ from the association and dissociation rate constants for androsterone obtained from steady-state analysis, which were 0.26 μM⁻¹s⁻¹ and 10.5 s⁻¹, respectively. This reflects a 80-fold decrease in the $K_d$ value for androstanedione. Considering the structural differences between androstanedione (substrate) and androsterone (product) in the reduction direction, it is apparent that the enzyme has evolved to favor 3-ketosteroid binding, i.e. the binding of reductive substrates.

Equilibrium Constants and Free Energy Diagram—The concept that HSDs work in pairs to act as either reductases or oxidases and thereby regulate ligand access to steroid hormone receptors is based to a large extent on transfection assays using radiotracers. In transfection paradigms HSDs are forced to use the prevailing concentrations of reduced or oxidized cofactor, because the fast phase of the kinetic transient can be eliminated by the R276M mutant (25). In the second conformational change step that yields the tight complex $E**$-NADPH, the on rate is ~20 fold higher than the off rate. This step may be related to the formation of the cofactor tunnel that involves the residues on loop β1-α1 and loop B, the nicotinamide ring being sandwiched between Trp-86 and Tyr-216, and the formation of hydrogen bonds with the C3 carboxamide group that enable the head group to orient correctly. The lower off rates suggest that the disruption of the tunnel and binding of the head group is slow.
5α-DHT catalyzed by AKR1C2, which allows calculation of a $K_{eq}$ of 8.0 for its reduction reaction (39). When combined with the data reported here, it is clear that all AKR1C isoforms will preferentially act as reductases. Thus the human enzymes will function as 3-, 17-, and 20-ketoreductases. The unexpected finding of this study is the $K_{eq}$ does not appear to be governed by cofactor, because the same $K_{eq}$ is observed irrespective of whether reduction is coupled to NADPH/NADP$^+$ or NADH/NAD$. Rather, the $K_{eq}$ is governed by favorable binding terms for the reduction reactants. Also, the $K_{eq}$ is not overwhelmingly in favor of reduction. The $K_{eq}$ values reported are rather modest and indicate that under some conditions the oxidative reaction may be observed. Two observations suggest that this can happen. Transfection of AKR1C9 into HEK293 cells showed that a pseudo-equilibrium was reached in which the product to substrate ratio was 97:3 (24). At this equilibrium the forward and reverse reaction rates were identical. Second, in studies on the metabolism of 3α-hydroxysteroids in human hepatoma cells we noted that, although the favored metabolic route was formation of steroid conjugates, a small amount of epimerization (<5%) to the 3β-hydroxysteroid metabolites was observed that could only be explained by oxidation back to the 3-ketosteroid.

**CONCLUSIONS**

AKR1C9, an HSD, is a member of the steroid hormone-transforming AKRs that regulate ligand occupancy of steroid receptors. Understanding the number of enzyme forms and their effects on rate determination of steroid transformation identifies steps for interception by inhibitors and regulation of these reactions. This study revealed that in the reaction sequence there are nine enzyme forms and six contain AKRs, the overall $K_{eq}$ of AKR1C9 clearly favors oxidation, which is unique among AKRs, the overall $K_{eq}$ favors reduction. This difference in AKRs is achieved by slow product release steps that control the reaction in both directions. The $K_{eq}$ that favors reduction is dominated by ligand binding terms for the reduction reactants.

**Acknowledgments**—We thank Jason E. Drury and Carol A. Shultz for providing purified AKR1C9 and Vladi V. Heredia for helpful discussions.

**REFERENCES**

1. Penning, T. M. (1997) *Endocr. Rev.* **18**, 281–305
2. Payne, A. H., and Hales, D. B. (2004) *Endocr. Rev.* **25**, 947–970
3. Funder, J. W., Pearce, P. T., Smith, R., and Smith, A. I. (1988) *Science* **242**, 583–585
4. Draper, N., and Stewart, P. M. (2005) *J. Endocrinol.* **186**, 251–271
5. Rizner, T. L., Lin, H.-K., Peehl, D. M., Steckelbroeck, S., Bauman, D. R., and Penning, T. M. (2003) *Endocrinology* **144**, 2922–2932
6. Bauman, D. R., Steckelbroeck, S., Williams, M. V., Peehl, D. M., and Penning, T. M. (2006) *Mol. Endocrinol.* **20**, 445–458
7. Penning, T. M., Bauman, D. R., Jin, Y., and Rizner, T. L. (2007) *Mol. Cell. Endocrinol.* **265**, 97–72
8. Liden, M., Tryggvason, K., and Eriksson, U. (2003) *Mol. Aspects of Med.* **24**, 403–409
9. Jez, J. M., Bennett, M. J., Schlegel, B. P., Lewis, M., and Penning, T. M. (1997) *Biochem. J.* **326**, 625–638
10. Jörnvall, H., Persson, B., Krook, M., Atrian, S., González-Duarte, R., Jef-
frey, J., and Ghosh, D. (1995) Biochemistry 34, 6003–6013
11. Penning, T. M. (2003) Hum. Reprod. Update 9, 193–205
12. Ogg, D., Elleby, B., Norstrom, C., Stefansson, K., Abrahamsen, L., Opp-
ermann, U., and Svensson, S. (2005) J. Biol. Chem. 280, 3789–3794
13. Hosfield, D. I., Wu, Y., Skene, R. I., Hilgers, M., Jennings, A., Snell, G. P.,
and Aertgeerts, K. (2005) J. Biol. Chem. 280, 4639–4648
14. Penning, T. M., Burczynski, M. E., Jez, J. M., Hung, C.-F., Lin, H.-K., Ma,
H., Moore, M., Palackal, N., and Ratnam, K. (2000) Biochem. J. 351, 67–77
15. Steckelbroeck, S., Jin, Y., Gopishetty, S., Oyesanmi, B., and Penning, T. M.
(2004) J. Biol. Chem. 279, 10784–10795
16. Ricigliano, J. W., and Penning, T. M. (1990) Biochem. J. 269, 749–755
17. Askonas, L. J., Ricigliano, J. W., and Penning, T. M. (1991) Biochem. J. 278,
835–841
18. Hoog, S. S., Pawlowski, J. E., Alzari, P. M., Penning, T. M., and Lewis, M.
(1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2517–2521
19. Bennett, M. J., Schlegel, B. P., Jez, J. M., Penning, T. M., and Lewis, M.
(1996) Biochemistry 35, 10702–10711
20. Bennett, M. I., Albert, R. H., Jez, J. M., Ma, H., Penning, T. M., and Lewis,
M. (1997) Steroids 5, 799–812
21. Veech, R. L., Eggleston, I. V., and Krebs, H. A. (1969) Biochem. J. 115,
609–619
22. Williamson, D. H., Lund, P., and Krebs, H. A. (1967) Biochem. J. 103,
514–527
23. Khun, N., Sharma, K. K., Andersson, S., and Auchus, R. J. (2004) Arch.
Biochem. Biophys. 429, 50–59
24. Papari-Zareei, M., Brandmaier, A., and Auchus, R. J. (2006) Endocrinology
147, 1591–1597
25. Ratnam, K., Ma, H., and Penning, T. M. (1999) Biochemistry 38,
7856–7864
26. Cleland, W. W., (1979) Methods Enzymol. 63, 103–138
27. Cleland, W. W. (1977) Adv. Enzymol. 45, 273–387
28. Segel, I. H. (1993) Enzyme Kinetics: Behavior and Analysis of Rapid Equi-
librium and Steady-State Enzyme Systems, Wiley & Sons, New York, pp.
274–574
29. Hermes, J. D., Morrical, S. W., O’Leary, M. H., and Cleland, W. W. (1984)
Biochemistry 23, 5479–5488
30. Viola, R. E., Cook, P. F., and Cleland, W. W. (1979) Anal. Biochem. 96,
334–340
31. Northrop, D. B. (1982) Methods Enzymol. 87, 607–625
32. Talalay, P., and Levy, H. R. (1959) Ciba Foundation Study Group No. 2:
Steric Course of Microbiological Reactions, pp. 64–66, Little, Brown & Co.,
Boston
33. Jez, J. M., Schlegel, B. P., and Penning, T. M. (1996) J. Biol. Chem. 271,
30190–30198
34. Stone, S. R., and Le Bonniec, B. F. (1997) J. Mol. Biol. 265, 344–362
35. Johnson, K. A. (1992) The Enzymes 20, 1–62
36. Heredia, V. V., and Penning, T. M. (2004) Biochemistry 43, 12028–12037
37. Heredia, V. V., Cooper, W. C., Kruger, R. G., Jin, Y., and Penning, T. M.
(2004) Biochemistry 43, 5832–5841
38. Grimschaw, C. E., Bohren, K. M., Lai, C.-J., and Gabbay, K. H. (1995) Bio-
chemistry 34, 14356–14365
39. Jin, Y., and Penning, T. M. (2006) Biochemistry 45, 13054–13063
40. Steckelbroeck, S., Oyesanmi, B., Jin, Y., Lee, S. H., Kloosterboer, H. J., and
Penning, T. M. (2006) J. Pharmacol. Exp. Ther. 316, 1300–1309