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Fjord-region Benzo[g]chrysene-11,12-dihydrodiol and Benzo[c]phenanthrene-3,4-dihydrodiol as Substrates for Rat Liver Dihydrodiol Dehydrogenase (AKR1C9):

Structural Basis for Stereochemical Preference

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Abstract

This study demonstrates that benzo[g]chrysene-11,12-dihydrodiol (B[g]C-11,12-dihydrodiol) derived from the *fjord*-region parent hydrocarbon B[g]C is oxidized by rat AKR1C9 with a k_{cat}/K_m 100 times greater than that observed with the commonly studied *bay*-region benzo[a]pyrene-7,8-dihydrodiol (B[a]P7,8-dihydrodiol). Conversely, despite its strikingly similar structure to B[g]C-11,12-dihydrodiol, benzo[c]phenanthrene-3,4-dihydrodiol (B[c]Ph-3,4-dihydrodiol) is consumed by AKR1C9 at sluggish rates comparable to those observed with B[a]P-7,8-dihydrodiol. CD spectroscopy revealed that only the (+)-B[g]C-11,12-dihydrodiol stereoisomer was oxidized, while AKR1C9 oxidized both stereoisomers of B[a]P7,8-dihydrodiol and B[c]Ph-3,4-dihydrodiol. The (+)-*S,S*- and (-)-*R,R*-stereoisomers of B[g]C-11,12-dihydrodiol were purified by chiral RP-HPLC. The 11*S*,12*S*-stereoisomer was oxidized at the same rate as the racemate. The 11*R*,12*R*-stereoisomer did not act as an inhibitor to AKR1C9, indicating that the (-)-*R,R*-stereoisomer was excluded from the active site. To understand the basis of stereochemical preference, we screened alanine-scanning mutants of active site residues of AKR1C9. These studies revealed that in comparison to the wild type, F129A, W227A, and Y310A enabled the oxidation of both the B[g]C-11*S*,12*S*-dihydrodiol and the B[g]C-11*R*,12*R*-dihydrodiol. Molecular modeling revealed that unlike B[a]P-7,8-dihydrodiol and B[c]Ph-3,4-dihydrodiol, B[g]C-11,12-dihydrodiol enantiomers are significantly bent out of plane. As a consequence, the (-)-*R,R*-stereoisomer was prevented from binding to the active site because of unfavorable interactions with F129, W227, or Y310. Additionally, LC/MS validated that the product of the reaction of B[g]C-11,12-dihydrodiol oxidation catalyzed by AKR1C9 was B[g]C-11,12-dione, which was trapped *in vitro* with the nucleophile 2-mercaptoethanol. The similarity between rates of *trans*-dihydrodiol oxidation by the rat and human liver specific AKRs (AKR1C9 and AKR1C4) implicate these enzymes in hepatocarcinogenesis in rats observed with the *fjord*-region PAH.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants created during incomplete combustion and pyrolysis (1,2) that require metabolic activation in order to elicit their carcinogenic effects (3-5). Human exposure to these carcinogens occurs from a myriad of sources including cigarette smoke (6), coal soot, charbroiled meats, fossil fuel

exhaust, and petroleum refining (7,8). Annually, 900-1300 tons of PAH are emitted in the U.S. leading to contamination of soil, water (9), and air in the form of fine particulate matter. A representative sample of PAH contained in samples of particulate size $2.5\ \mu\text{m}$ collected from St. Louis, MO showed that the most studied PAH, the *bay*-region benzo[*a*]pyrene (B[*a*]P), a Group I carcinogen, is present at $3.1\ \mu\text{g/g}$ (10). Group 1 carcinogens refer to compounds ranked as known human carcinogens by the World Health Organization, IARC (11). Comparatively, *ffjord*-region PAHs, such as benzo[*g*]chrysene (B[*g*]C) and benzo[*c*]phenanthrene (B[*c*]Ph), are present at concentrations of 0.07 and $0.9\ \mu\text{g/g}$ respectively (10). Despite the lower occurrence of the *ffjord*-region PAH, these compounds are of interest because *bay*-region substitution of PAH (methylation) or introduction of a *ffjord*-region is known to result in an increase in mutagenicity and carcinogenicity over the unsubstituted PAH (12). Because less is known about these PAH, they are currently ranked as Group 2B; “insufficient data to identify as human carcinogens” (13). If more data were available on their mode of activation, a reclassification could occur.

Two major pathways of PAH activation are possible, starting from racemic B[*g*]C-11,12-dihydrodiol. This compound is anticipated to undergo monooxygenation by P450s to yield (+)- and (-)-*anti*-diol-epoxide products (14,15), which can form *N2*-deoxyguanosine adducts with DNA (16,17) and act as a potential human carcinogens (18) (Scheme 1). Studies in mouse epidermis have identified P4501A1 as playing a major role in the bioactivation of B[*a*]P, B[*g*]C, and B[*c*]Ph (19).

An alternative fate involves *trans*-dihydrodiol oxidation catalyzed by aldo-keto reductases (AKRs) to yield a ketol that spontaneously rearranges into a catechol. The unstable catechol undergoes two one-electron oxidations, first forming an *o*-semiquinone (20) anion radical and H_2O_2 (21) followed by the formation of an *o*-quinone (22) with consequential superoxide anion production. In turn, H_2O_2 undergoes Fenton chemistry to produce a hydroxyl radical, another reactive oxygen species (ROS) (21).

At this point, the fate of the *o*-quinone bifurcates, where one option is that the *o*-quinone can be reduced back to the catechol and undergo futile redox cycling in the presence of NAD(P)H to amplify ROS (21,23). In turn, ROS can inflict large amounts of oxidative damage on cells, causing strand scission (20) and DNA lesions, for example, 8-oxo-2'-deoxyguanosine (23, 24). The second option is that *o*-quinones are highly reactive Michael acceptors and can react with deoxyguanosine to form bulky or depurinating DNA adducts (25-27). Both oxidative and covalent DNA adducts may lead to G to T transversions in DNA, which are commonly observed in the tumor suppressor *p53* in lung cancer (28). Previously, rat liver AKR1C9 oxidized racemic B[*a*]P-7,8 dihydrodiol to form an *o*-quinone product, B[*a*]P-7,8-dione, both *in vitro* and in transfected MCF-7 cells (22,29).

The reasons for the high mutagenic potential of the *ffjord*-region PAH have not been fully explained. In this study, we demonstrate that B[*g*]C-11,12-dihydrodiol is oxidized by AKR1C9 with a k_{cat}/K_m over 100 times greater than that observed with the well-characterized *bay*-region B[*a*]P-7,8-dihydrodiol. When the oxidation of racemic B[*g*]C-11,12-dihydrodiol is run to completion, only the *S,S*-stereoisomer, which corresponds to the (+)-stereoisomer, is consumed. Despite the fact that B[*g*]C-11,12-dihydrodiol has one additional ring compared to B[*c*]Ph-3,4-dihydrodiol, B[*c*]Ph-3,4-dihydrodiol is consumed by AKR1C9 at a sluggish rate comparable to that observed with B[*a*]P-7,8-dihydrodiol. Alanine-scanning mutagenesis of contact residues in the AKR1C9 active site revealed that in comparison to the wild type, the F129A, W227A, and Y310A mutants permit oxidation of B[*g*]C-11*R*,12*R*-dihydrodiol, which corresponds to the (-)-stereoisomer; thus, these mutants oxidize both stereoisomers of B[*g*]C-11,12-dihydrodiol. The structural basis for stereochemical preference is examined. The

toxicological consequence of oxidizing B[g]C-11,12-dihydrodiol efficiently by AKRs is discussed.

Experimental Procedures

Caution

PAHs are potentially hazardous chemicals and should be handled with care in accordance with the NIH Guidelines for Use of Chemical Carcinogens.

Chemicals and Reagents

B[c]Ph-3,4-dihydrodiol was kindly provided by Dr. Mahesh K. Lakshman, The City College and The City University of New York (138th Street at Convent Avenue, New York, New York 10031). B[a]P-4,5-dihydrodiol, B[g]C-11,12-dihydrodiol, and B[g]C-11,12-dione were synthesized according to published methods (30). All PAHs were analyzed by LC/MS for identity and purity prior to use. NAD⁺ and NADP⁺ were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). AMPSO and Chromasolv plus DMSO were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO). MOPS was purchased from Fisher Chemicals (Fairlawn, NJ). All other chemicals were of the highest grade available, and all solvents for LC/MS were of HPLC grade. The molar extinction coefficient for B[g]C-11,12-dione was determined to be $\epsilon_{262} = 48,553 \text{ M}^{-1} \text{ cm}^{-1}$ in ethanol.

Expression and Purification of Recombinant Wild type and Mutant of AKR1C9

Wild type homogeneous recombinant AKR1C9 was purified as described (31). Enzyme solutions were titered by measuring the oxidation of androsterone to androstanedione under standard assay conditions (31). The specific activity of the aliquots was $1.6 \mu\text{mol/min/mg}$ at 25 °C. Alanine-scanning mutants used were T24A, L54A, F118A, F129A, T226A, W227A, N306A, and Y310A and have been purified and characterized previously (32).

Determination of the Steady State Kinetic Parameters of B[g]C-11,12-dihydrodiol and B[c]Ph-3,4-dihydrodiol Oxidation via Absorbance Spectroscopy

Initial velocities for the oxidation of the PAH *trans*-dihydrodiols catalyzed by AKR1C9 were measured using a range of *trans*-dihydrodiol concentrations (5 to 60 μM) and 2.3 mM NADP⁺ in 50 mM AMPSO buffer (pH 9.0) in the presence of 8% v/v DMSO as described in Palackal et al. (33). The resultant v versus $[S]$ plots were linear and revealed that it was not possible to observe saturation kinetics because of the poor solubility of the *trans*-dihydrodiol. By running subsequent reactions at 20 μM , we ensured that reactions were run under pseudo-first-order conditions so that $v/[S]$ provides an estimate of $V_{\text{max}}/[K_m]$. Control reactions were performed in which one of the following were absent: NADP⁺, *trans*-dihydrodiol, or enzyme. Initial velocity measurements were corrected for the 33% inhibition of AKR1C9 by DMSO. Therefore, the velocities measured were multiplied by 1.33 to obtain the true initial velocity.

Determination of the Kinetic Parameters for the Oxidation of B[g]C-11,12-dihydrodiol and B[c]Ph-3,4-dihydrodiol Catalyzed by AKR1C9 Using RP-HPLC Methods

A RP-HPLC method was established to detect the consumption of B[g]C-11,12-dihydrodiol *in vitro*. Reactions containing 20 μM *trans*-dihydrodiol and 2.3 mM NADP⁺ in 50 mM AMPSO buffer (pH 9.0) in the presence of 8% v/v DMSO were preincubated at 37 °C for 5 min. The reaction was initiated by the addition of AKR1C9 and quenched upon mixing with ice-cold acetone. Racemic B[a]P-4,5-dihydrodiol was added to the quenched reaction as an internal standard. Remaining unconsumed *trans*-dihydrodiol was extracted with ethyl acetate. Samples were dried under vacuum and resuspended in methanol. Aliquots were injected onto a RP-HPLC Column (Zorbax-ODS C18, 5 μm , 4.6 mm $\times$ 250 mm; Agilent, Santa Clara, CA) on a

Waters Alliance 2690 HPLC system with an in line photodiode array detector (PDA). Organic soluble metabolites were eluted at 0.5 mL/min with a water/methanol linear gradient from 60 to 90% methanol over 60 min. PAH metabolites were identified by comparison of retention time to those obtained with authentic synthetic standards. Consumption of the *trans*-dihydrodiol was monitored directly at 270.9 nm ($\epsilon = 60,000 \text{ M}^{-1} \text{ cm}^{-1}$). The loss of *trans*-dihydrodiol during sample workup was corrected for by normalization to the internal standard. Initial velocities were obtained from plots of *trans*-dihydrodiol consumed versus time.

Identification of the Stereoisomer of B[g]C-11,12-dihydrodiol Oxidized by AKR1C9 Using CD Spectroscopy

To identify the stereoisomer of B[g]C-11,12-dihydrodiol oxidized by AKR1C9, a 50 mL reaction containing 50 μM *trans*-dihydrodiol and NADP^+ was run to completion. Aliquots of the reaction were removed over time to monitor the progress of the reaction via RP-HPLC. At the end point of the reaction (corresponding to 50% substrate depletion), the reaction was quenched by the addition of ethyl acetate, and unreacted *trans*-dihydrodiol was extracted. The sample was dried with anhydrous sodium sulfate, and ethyl acetate was removed under vacuum. Subsequently, samples were purified by thin layer chromatography on $20 \times 20 \text{ GF}$ (Analtech) plates using a 55:45 ethyl acetate/hexanes (v/v) solvent system. The unreacted stereoisomer was removed from the plate, purified, and subjected to CD spectroscopy on an AVIV CD spectrometer to determine the Cotton effect of the remaining stereoisomer. The (-)-stereoisomer corresponds to the 11*S*,12*S* configuration, and the (-)-stereoisomer corresponds to the 11*R*,12*R* configuration (34).

Separation of the B[g]C-11*S*,12*S*-dihydrodiol and B[g]C-11*R*,12*R*-dihydrodiol Enantiomers Using a Chiralcel OD-RH Column

A chiral RP-HPLC method was used to separate B[g]C-11*S*,12*S*-dihydrodiol and B[g]C-11*R*,12*R*-dihydrodiol from the racemic B[g]C-11,12-dihydrodiol mixture. Aliquots of racemic *trans*-dihydrodiol were dissolved in methanol and injected onto a Chiralcel OD-RH column (5 μm , 2.1 mm $\times$ 150 mm; Chiral Technologies, Inc., West Chester, PA) on a Waters Alliance 2690 HPLC system with an in line PDA. The two enantiomers were eluted at 0.2 mL/min with a water/methanol linear gradient from 60 to 90% methanol over 30 min. The peak at 26 min was identified as B[g]C-11*R*,12*R*-dihydrodiol by coelution with B[g]C-11*R*,12*R*-dihydrodiol purified from an enzymatic reaction run to completion and identified by CD spectroscopy.

Inhibition Studies with B[g]C-11*R*,12*R*-dihydrodiol

The enzymatic oxidation of 20 μM B[g]C-11*S*,12*S*-dihydrodiol was monitored while the concentration of B[g]C-11*R*,12*R*-dihydrodiol was increased from 0 to 35 μM . The initial velocity (v) in each case was determined as a fraction of the initial velocity (v_o), observed in the absence of the *R,R*-stereoisomer.

Preparation of the 2-Mercaptoethanol Conjugates of B[g]C-11,12-dione

Authentic thio-ether conjugate standards were synthesized by incubating 20 μM B[g]C-11,12-dione, 2.3 mM NADP^+ , and 400 μM 2-mercaptoethanol in 50 mM MOPS buffer at pH 7.0 at 37 °C for 12 h. The *o*-quinone products formed by the enzymatic oxidation of racemic B[g]C-11,12-dihydrodiol were isolated under similar conditions except that reactions containing *trans*-dihydrodiol substrates were initiated by the addition of AKR1C9. Conjugates were analyzed via a RP-HPLC system with the water/methanol gradient described above. The *o*-quinone thio-ether conjugates from the reaction displayed different retention times and UV/vis spectra (compared to those of *trans*-dihydrodiols).

LC/MS Analysis of Thio-Ether Conjugates

The identity of thio-ether conjugates were validated by LC/MS. Mass spectrometric data were acquired on a Finnigan LCQ ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA) equipped with an electrospray ionization (ESI) source in the positive ion mode. Instrument operating conditions were as follows: capillary voltage 7 V and capillary temperature at 180 °C, with a needle voltage of 4.5 kV, applied to the ESI source. Nitrogen was used as the sheath gas (65 psi) and auxiliary (15 arbitrary units) gas to assist with nebulization. Full scanning analyses were performed in the range of m/z 150-700. Collision-induced dissociation (CID) experiments coupled with multiple tandem mass spectrometry (MS) employed argon as the collision gas. The relative collision energy was set at 20-40% of the maximum. Online chromatography was performed using a Waters Alliance 2690 HPLC system. A reversed-phased column was used at a flow rate of 0.5 mL/min. Solvent A was 5 mM ammonium acetate in water containing 0.01% formic acid, and solvent B was 5 mM ammonium acetate in methanol containing 0.01% formic acid. Organic soluble metabolites were eluted at 0.5 mL/min with the water/methanol gradient described above.

Results

Oxidation of B[g]C-11,12-dihydrodiol and B[c]Ph-3,4-dihydrodiol by AKR1C9

To further elucidate the role of AKRs in PAH activation, racemic *ffjord*-region *trans*-dihydrodiols of B[g]C-11,12-dihydrodiol and B[c]Ph-3,4-dihydrodiol were tested as substrates for AKR1C9. Initial velocity measurements were indirectly determined using continuous UV spectrophotometric assays to monitor NADPH formation, which is formed stoichiometrically during *trans*-dihydrodiol oxidation by AKR1C9. Initial velocity measurements were also directly determined using a discontinuous assay via RP-HPLC to monitor the disappearance of *trans*-dihydrodiol over time. Initial rates obtained from spectrophotometric assays agreed with initial velocities obtained from the RP-HPLC assays.

Initial velocities for the oxidation of racemic B[g]C-11,12-dihydrodiol and racemic B[c]Ph-3,4-dihydrodiol were compared with that obtained for the oxidation of racemic B[a]P-7,8-dihydrodiol (Table 1). It was found that racemic B[g]C-11,12-dihydrodiol was oxidized with a k_{cat}/K_m of 1520 min⁻¹ mM⁻¹, which is more than 100 times greater than that observed with B[a]P-7,8-dihydrodiol. Surprisingly, racemic B[c]Ph-3,4-dihydrodiol, which differs from racemic B[g]C-11,12-dihydrodiol by having one less ring, gave a k_{cat}/K_m of 39 min⁻¹ mM⁻¹.

Determination of the End Point of Enzymatic Oxidation of Racemic *trans*-Dihydrodiols by AKR1C9

Using RP-HPLC methods, the AKR1C9 dependent disappearance of racemic *trans*-dihydrodiol substrates was monitored over time to determine whether AKR1C9 oxidized both stereoisomers of racemic *trans*-dihydrodiols. Reactions were carried out until no further change in peak area was observed. Time courses of racemic B[a]P-7,8-dihydrodiol and racemic B[c]Ph-3,4-dihydrodiol oxidation validated that AKR1C9 utilized both stereoisomers. By contrast, the reaction of AKR1C9 and racemic B[g]C-11,12-dihydrodiol failed to reach 100% completion (Figure 1). RP-HPLC assays showed that the peak area corresponding to racemic B[g]C-11,12-dihydrodiol only decreased to 50%. One explanation is that the putative *o*-quinone product covalently modifies and inactivates the free enzyme, thus causing the reaction to halt. For example, naphthalene-1,2-dione leads to the time and concentration dependent irreversible inactivation of AKR1C9 via alkylation (35). However, once the oxidation of racemic B[g]C-11,12-dihydrodiol reached an end point, the addition of more enzyme caused no further oxidation of the *trans*-dihydrodiol, indicating that the enzyme was not being inactivated (results not shown). An alternative explanation is that AKR1C9 stereospecifically oxidizes either the *S,S*- or *R,R*-enantiomer.

Identification of the Stereoisomer of B[g]C-11,12-dihydrodiol Preferentially Oxidized by AKR1C9

CD spectroscopy was employed to determine the stereoisomer of B[g]C-11,12-dihydrodiol preferentially oxidized by AKR1C9. The unreacted stereoisomer was purified after monitoring a large scale enzymatic reaction of racemic B[g]C-11,12-dihydrodiol oxidation to completion. The negative Cotton effect obtained from the unreacted B[g]C-11,12-dihydrodiol substrate showed that the (-)-stereoisomer remained, revealing that only the (+)-stereoisomer is consumed by AKR1C9 (Figure 2). For B[a]P-7,8-dihydrodiol, B[c]Ph-3,4-dihydrodiol, and B[g]C-11,12-dihydrodiol, the (-)-isomer would correspond to the *R,R*-configuration and the (+)-isomer would correspond to the *S,S*-configuration (34,36-38).

Examination of 11*S*,12*S*- and 11*R*,12*R*-Enantiomers of Racemic B[g]C-11,12-dihydrodiol as Substrates and Inhibitors of AKR1C9

To validate that B[g]C-11*S*,12*S*-dihydrodiol was oxidized by AKR1C9, B[g]C-11*S*,12*S*-dihydrodiol and B[g]C-11*R*,12*R*-dihydrodiol were purified from racemic B[g]C-11,12-dihydrodiol using a Chiracel OD-RH column. The purification yielded two distinct peaks with retention times of 17 and 26 min (Figure 3A). The peak containing B[g]C-11*R*,12*R*-dihydrodiol was identified by coelution with unreacted B[g]C-11*R*,12*R*-dihydrodiol identified in the CD experiment. The (-)-stereoisomer corresponded to the peak at 26 min (Figure 3B).

We compared the k_{cat}/K_m for the oxidation of 20 μM *S,S*-stereoisomer and 20 μM racemic B[g]C-11,12-dihydrodiol by AKR1C9. Oxidation of the *S,S*-stereoisomer went to 100% completion, whereas the oxidation of the racemic *trans*-dihydrodiol went to 50% completion (Figure 3C). Additionally, 20 μM B[g]C-11*S*,12*S*-dihydrodiol was oxidized twice as fast as the 20 μM racemic *trans*-dihydrodiol since the effective substrate concentration was 2-fold higher. This implies that the unreacted B[g]C-11*R*,12*R*-dihydrodiol does not act as an inhibitor of AKR1C9.

To confirm that B[g]C-11*R*,12*R*-dihydrodiol is excluded from the active site, we analyzed the ability of the 11*R*,12*R*-enantiomer to block the oxidation of B[g]C-11*S*,12*S*-dihydrodiol (Figure 3D). Using a concentration range from 0 to 35 μM B[g]C-11*R*,12*R*-dihydrodiol, no change in initial velocity for B[g]C-11*S*,12*S*-dihydrodiol oxidation was observed. These findings suggest that the *R,R*-stereoisomer is not an inhibitor for AKR1C9 and that it does not bind to the active site.

Effects of Alanine-Scanning Mutations on B[g]C-11,12-dihydrodiol Oxidation Catalyzed by AKR1C9

Previously, alanine-scanning mutagenesis of active site residues in the ligand-binding cavity of AKR1C9 was used to dissect their role in steroid hormone binding and catalysis (32,39). In the earlier studies, by far, the largest changes for the oxidation of androsterone were observed in k_{cat} rather than K_m . As a result, these changes were reflected in catalytic efficiency, k_{cat}/K_m (Table 2). The smallest change in k_{cat}/K_m was observed when alanine was used to substitute hydrophilic residues, for example, T24A and T226A. Conversely, W227A, F118A, and F129A revealed the largest changes in k_{cat}/K_m , while L54A, N306A, and Y310A showed intermediate changes in k_{cat}/K_m versus wild type. The changes in k_{cat} observed were explained by the incorrect positioning of the substrate with respect to the catalytic residues (39). In this study, we used these alanine mutants to determine which residues are important in the exclusion of B[g]C-11*R*,12*R*-dihydrodiol from the AKR1C9 active site.

Similar to the results observed with androsterone, the smallest change in k_{cat}/K_m was with T24A and T226A, Table 2. T226A showed no change in comparison to that of the wild type, implying that the side chain of T226 does not affect the positioning of reactants. The alanine

mutants that suffered the largest decline in k_{cat}/K_m were L54A, F118A, and F129A. When the k_{cat}/K_m for androsterone and racemic B[g]C-11,12-dihydrodiol were compared, the largest change was observed for the F129A mutant.

Identification of the Stereoisomer of B[g]C-11,12-dihydrodiol Oxidized by AKR1C9 Alanine Mutants

Oxidation of racemic B[g]C-11,12-dihydrodiol by AKR1C9 alanine mutants was monitored over time to determine whether the mutants retained the stereospecificity of the wild type enzyme. Results showed that reactions with the racemic *trans*-dihydrodiol went to 50% completion with the T24A, T226A, and N306A mutants (Table 2). Next, using the purified enantiomers of B[g]C-11,12-dihydrodiol, we tested to see if the alanine mutants retained the stereochemical preference for B[g]C-11*S*,12*S*-dihydrodiol. The T24A, T226A, and N306A mutants solely utilized B[g]C-11*S*,12*S*-dihydrodiol. Conversely, W227A, F129A, and Y310A were also able to oxidize both 11*S*,12*S*- and B[g]C-11*R*,12*R*-dihydrodiols, eliminating the stereochemical preference of the enzyme for the *S,S*-stereoisomer (Figure 4).

The oxidation of the purified B[g]C-11*S*,12*S*-dihydrodiol and B[g]C-11*R*,12*R*-dihydrodiol isomers by the F129A, W227A, and Y310A mutants was monitored over time (Table 3). When the W227A and Y310A mutants were used to oxidize the *R,R*-stereoisomer, initial velocities accounted for 14.5% and 22.0%, respectively, of the initial velocities observed when the racemic B[g]C-11,12-dihydrodiol was oxidized. Since wild type AKR1C9 oxidized only the *S,S*-stereoisomer, the data show that both the W227A and Y310A mutants removed the preference for the enantiomeric starting material. When the F129A mutant was used to oxidize the *R,R*-stereoisomer, the initial velocity accounted for 66.9% of the initial velocity observed when the racemic B[g]C-11,12-dihydrodiol was oxidized. This implies that F129A alters the stereochemical preference from oxidizing predominantly the *S,S*-stereoisomer to oxidizing predominantly the *R,R*-stereoisomer.

Identification of the Product of AKR1C9-Mediated Oxidation of B[g]C-11,12-dihydrodiol

The products of the AKR-mediated oxidation of PAH *trans*-dihydrodiols are reactive *o*-quinones and can be trapped *in vitro* with the nucleophile 2-mercaptoethanol. To trap the products of *ffjord*-region *trans*-dihydrodiol oxidation, B[g]C-11,12-dione thio-ether conjugates formed with 2-mercaptoethanol were synthesized to aid their identification. LC/MS data from the reaction mixture revealed the formation of two products (Figure 5). The peak corresponding to the unreacted *o*-quinone was detected at 41 min. The appearance of two product peaks revealed that *ffjord*-region B[g]C-11,12-dione formed *mono*- and *bis*-conjugates, as identified by the ion chromatograms and MS data. Similarly, LC/MS data showed that the enzymatic oxidation of B[g]C-11,12-dihydrodiol in the presence of 2-mercaptoethanol yielded two product peaks. These peaks coeluted with the synthetically prepared *mono*- and *bis*-thio-ether conjugates and gave MS spectra of $m/z = 384$ [MH^+] and $m/z = 461$ [MH^+], respectively.

Discussion

Our studies demonstrate that *ffjord*-region B[g]C-11,12-dihydrodiol is a superior substrate for AKR1C9. Although racemic B[g]C-11,12-dihydrodiol has a k_{cat}/K_m of $1,520 \text{ min}^{-1} \text{ mM}^{-1}$, AKR1C9 only oxidizes the (+)-*S,S*-stereoisomer. [^{32}P]-Post labeling studies in mouse skin identified that 75% of the adducts were derived from B[g]C-11*R*,12*R*-dihydrodiol, suggesting that B[g]C-11*S*,12*S*-dihydrodiol is the minor metabolic product (40). In this study, we observed that AKR1C9 catalyzes the NADP⁺-dependent oxidation of racemic B[g]C-11,12-dihydrodiol 100 times greater than racemic B[a]P-7,8-dihydrodiol and 40 times faster than racemic B[c]Ph-3,4-dihydrodiol.

***Fjord*-region *trans*-dihydrodiols are Oxidized by AKRs to Form *o*-Quinones**

Our studies provide evidence for the metabolic activation of B[g]C-11*S*,12*S*-dihydrodiol to an *o*-quinone product. Although B[g]C-11*S*,12*S*-dihydrodiol is the minor metabolic isomer formed *in vivo* (41), the deleterious effects of this minor isomer may be increased by the high k_{cat}/K_m values observed for B[g]C-11*S*,12*S*-dihydrodiol oxidation with AKRs. Additionally, AKRs would prevent the conversion of B[g]C-11*S*,12*S*-dihydrodiol to (+)-*syn*-diol-epoxides catalyzed by P450 isoforms.

Comparison of Steady State Kinetics of AKR1C9 and AKR1C4 (the Liver Specific Human AKR)

AKR1C4 is the human AKR most similar to AKR1C9. Both enzymes are hepatospecific and display similar kinetic parameters for B[g]C-11,12-dihydrodiol, B[a]P-7,8-dihydrodiol, and B[c]Ph-3,4-dihydrodiol. For example, the k_{cat}/K_m of AKR1C4 for B[a]P-7,8-dihydrodiol is 3.7 min⁻¹ mM⁻¹, for B[c]Ph-3,4-dihydrodiol is 8.2 min⁻¹ mM⁻¹, and for B[g]C-11,12-dihydrodiol is 160 min⁻¹ mM⁻¹ (42). It is striking that similar to AKR1C9, the k_{cat}/K_m of AKR1C4 for B[g]C-11,12-dihydrodiol is much greater than that for B[a]P-7,8-dihydrodiol, especially because these enzymes are predicted to share only three of the same substrate binding residues within the active site: L54, F118, and W227 (43). The similarities in k_{cat}/K_m imply that although the actual active site composition is very different, both of these liver specific AKRs recognize *trans*-dihydrodiols in a similar manner.

B[g]C-11,12-dihydrodiol and Liver Tumorigenicity

Several studies have identified PAH as causing liver tumors in rodents (44,45) and causing hepatotoxicity. Importantly, *fjord*-region B[g]C-diol-epoxide induces more liver tumors than B[c]Ph-diol-epoxide in male mice, both of which were found to be significantly more tumorigenic than B[a]P-diol-epoxide in this end organ (46). When contact-inhibited WB-F344 rat liver epithelial cells were treated with 1 μM B[g]C, P4501A1/1B1 and AKR1C9 mRNA levels were induced, implying that both P450s and AKRs may be involved in liver toxicity (47). The ultimate genotoxic effects caused by P450 and AKR upregulation results in increases in p53 phosphorylation, accumulation of cells in the S phase, and formation of B[g]C-DNA adducts detected by [³²P]-postlabeling. These findings may implicate the P450 and AKR pathways in the hepatocarcinogenesis observed with the parent PAH (47).

Role of F129, W227, and Y310 in B[g]C-11,12-dihydrodiol Oxidation by AKR1C9

We previously conducted alanine-scanning mutagenesis of the AKR1C9 steroid binding pocket (32,39). In this study, we used these mutants to determine their influence on both the turnover of *fjord*-region *trans*-dihydrodiols and stereochemical preference (Tables 2 and 3). We found that F129, W227, and Y310 all play roles in the stereochemical preference for B[g]C-11*S*,12*S*-dihydrodiol substrate.

By replacing the bulky side chains of the residues with small methyl side chains, the active site could accommodate B[g]C-11*R*,12*R*-dihydrodiol. As a result, B[g]C-11*R*,12*R*-dihydrodiol occupies the space previously taken up by the side chain of F129, W227, and Y310, thus allowing the mutant to bind this *trans*-dihydrodiol and permit oxidation. The decrease in k_{cat}/K_m for F129A, W227A, and Y310A mutants in comparison to that in the WT suggests that these mutants allow for more wobble of the stereoisomer within the active site.

On the basis of the alanine-scanning mutagenesis study with B[g]C-11,12-dihydrodiol, it is hypothesized that L54 and F118 are important to catalysis by forming critical contacts with B[g]C-11*S*,12*S*-dihydrodiol. Without these residues, the mutants display no detectable oxidation of B[g]C-11*S*,12*S*-dihydrodiol or B[g]C-11*R*,12*R*-dihydrodiol. Previous studies have shown

that L54A and F118A mutants retained their ability to oxidize androsterone to androstanedione (39). L54A and F118A play different roles when oxidizing androsterone versus B[g]C-11S, 12S-dihydrodiol, which can be attributable to positioning the functional groups. In comparison to androsterone, which has only one reactive hydroxyl group at the C3 position, B[g]C-11S, 12S-dihydrodiol has two (at the C11 and C12 positions). This implies that in reference to the general acid, Y55, there is only one mode of binding for androsterone, but B[g]C-11S, 12S-dihydrodiol may have several binding modes, and the correct mode may require more guidance from L54 or F118.

Molecular Modeling

We used molecular modeling to understand the structural basis for why wild type AKR1C9 used both stereoisomers of B[c]Ph-3,4-dihydrodiol and B[a]P-7,8-dihydrodiol, while the enzyme was stereoselective for B[g]C-11S, 12S-dihydrodiol. Since the X-ray/Cartesian coordinates for B[g]C-11, 12-dihydrodiol, B[c]Ph-3,4-dihydrodiol, or B[a]P-7,8-dihydrodiol are unavailable, molecular models of these PAHs were constructed using Chem3D. Previously, Chem3D was used to build a molecule of 7,12-dimethylbenz[a]anthracene, which yielded a structure almost identical to what was found in the Cambridge Crystallographic Database (Jin, Y., personal communication). Molecular modeling of the *trans*-dihydrodiols revealed that *bay*-region B[a]P-7,8-dihydrodiol is planar (except for the terminal benzo ring). The presence of a *ffjord*-region in B[c]Ph-3,4-dihydrodiol and B[g]C-11, 12-dihydrodiol caused deviation from planarity upon energy minimization. The ring responsible for the *ffjord*-region in B[g]C-11, 12-dihydrodiol creates an extra twist in the molecule, which is absent in B[c]Ph-3,4-dihydrodiol (Figure 6). Importantly, models of the *S,S*- and *R,R*-stereoisomers of B[g]C-11, 12-dihydrodiol show that the structures are twisted in opposite directions.

Models of B[c]Ph-3S,4S-dihydrodiol and B[c]Ph-3R,4R-dihydrodiol, B[a]P-7S,8S-dihydrodiol and B[a]P-7R,8R-dihydrodiol, and B[g]C-11S, 12S-dihydrodiol and B[g]C-11R, 12R-dihydrodiol were manually docked into the active site of the AKR1C9-NADP⁺-testosterone ternary complex crystal structure (48) (Figure 7). For each *trans*-dihydrodiol, the carbon bonded to the presumptive axial reactive hydroxyl group was aligned with the C3 position of testosterone, and the carbons of the terminal benzo-ring of the *trans*-dihydrodiol were superimposed over the A ring of testosterone. Studies on steroid substrate specificity with AKR1C9 have previously shown that only the 3 α -axial hydroxyl group is oxidized (49). These alignments caused the functional groups of the *trans*-dihydrodiols and testosterone to assume similar spatial orientations to Y55 and the NADP⁺ cofactor. These models showed that B[c]Ph-3S,4S-dihydrodiol and B[c]Ph-3R,4R-dihydrodiol, B[a]P-7S,8S-dihydrodiol, B[a]P-7R, 8R-dihydrodiol, and B[g]C-11S, 12S-dihydrodiol do not clash with active site residues, suggesting that these *trans*-dihydrodiols bind to the active site and will be oxidized. For example, B[g]C-11S, 12S-dihydrodiol was accommodated into the active site so that the aromatic ring that forms the *ffjord*-region is positioned parallel to F129, Y310, and W227.

Conversely, when the presumptive reactive 12 α -axial alcohol of B[g]C-11R, 12R-dihydrodiol is aligned with the carbonyl of C3 on testosterone, the contorted structure of B[g]C-11R, 12R-dihydrodiol caused the ring that forms the *ffjord*-region to directly clash with both F129 and Y310. To align the 11 α -hydroxyl axial alcohol of B[g]C-11R, 12R-dihydrodiol with the carbonyl of C3 of testosterone, the *trans*-dihydrodiol was flipped 180° in the active site. This caused the *R,R*-stereoisomer to clash with Y310 and neighboring F311. This suggests that neither the 12 α - or 11 α -hydroxyl group of B[g]C-11R, 12R-dihydrodiol can assume a binding mode that permits oxidation because of direct clashing with active site residues. The modeling is supported by alanine scanning mutagenesis, where F129A and Y310A mutants can accommodate the *R,R*-stereoisomer because they provide more room for the ring that forms the *ffjord*-region to now bind, resulting in the oxidation of the *S,S*- and *R,R*-stereoisomers.

Alanine-scanning mutagenesis data suggests that W227 plays an important role in preventing the oxidation of B[g]C-11*R*,12*R*-dihydrodiol. We hypothesize that W227 can rotate in the active site and come in closer contact with B[g]C-11*R*,12*R*-dihydrodiol. This is based on the crystal structure of human AKR1C2. In this crystal structure, W227 is rotated 120° around the C α bond to allow a competitive inhibitor to bind (43). Docking experiments using AKR1C2 also revealed that W227 and L54 form the narrowest part of the active site (50). Therefore, if an analogous movement occurs with W227 in the active site of AKR1C9, this could prevent oxidation of the *R,R*-stereoisomer. By contrast, the W227A mutant may be able to oxidize both stereoisomers of B[g]C-11,12-dihydrodiol because of active site widening.

Summary

Our studies provide the first evidence of catalysis of *fford*-region *trans*-dihydrodiols B[g]C-11,12-dihydrodiol and B[c]Ph-3,4-dihydrodiol by AKR1C9 to form *o*-quinone products that may contribute to PAH carcinogenesis. AKR1C9 oxidized B[g]C-11,12-dihydrodiol with a higher catalytic efficiency than other AKRs tested to date. The enzyme was stereoselective for B[g]C-11*S*,12*S*-dihydrodiol, but metabolized both stereoisomers of B[c]Ph-3,4-dihydrodiol. Mutation of F129, W227, and Y310 to alanine provides more room to bind for the rings that create the *fford*-region, thus permitting oxidation of both *S,S*- and *R,R*-stereoisomers. AKR1C4 is the human AKR most similar to AKR1C9. Both enzymes are hepatospecific and display similar kinetic parameters for B[g]C-11,12-dihydrodiol, B[a]P-7,8-dihydrodiol, and B[c]Ph-3,4-dihydrodiol. This suggests that both of these liver specific AKRs recognize *trans*-dihydrodiols similarly even though their binding pocket residues differ. These findings may implicate the AKRs in the hepatocarcinogenesis observed with the parent PAH.

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Figure 1. AKR1C9 preferentially oxidizes one stereoisomer of racemic B[g]C-11,12-dihydrodiol. Progress curve for the oxidation of racemic B[g]C-11,12-dihydrodiol with recombinant AKR1C9. Racemic *trans*-dihydrodiol (20 μ M) was incubated with 0.125 μ g of AKR1C9 and 2.3 mM NADP⁺ in 50 mM AMPPO at pH 9.0 at 37 °C. Initial velocities were determined by taking tangents to the progress curve.

Figure 2.

AKR1C9 only metabolizes B[g]C-11*S*,12*S*-dihydrodiol. The unreacted stereoisomer was purified via TLC from a 50 mL reaction containing 40 μ M racemic B[g]C-11,12-dihydrodiol AKR1C9 and NADP⁺. Both racemic (in blue) and unreacted *trans*-dihydrodiol (in green) were dissolved in methanol and recorded in 0.5 nm steps from 240 to 400 nm. The negative Cotton effect observed from the unreacted B[g]C-11,12-dihydrodiol shows that the (-)-*R,R*-stereoisomer remains, revealing that only the (+)-*S,S*-stereoisomer is consumed by AKR1C9.

Figure 3.

Demonstration that B[g]C-11R,12R-dihydrodiol is neither a substrate nor an inhibitor of AKR1C9. (A) Separation of (+)-S,S- and (-)-R,R isomers of B[g]C-11,12-dihydrodiol from racemic *trans*-dihydrodiol by chiral RP-HPLC. (B) Coelution of one peak from the racemate with the R,R-isomer identified by CD-spectroscopy. (C) Progress curve for the enzymatic oxidation of 20 μ M racemic-B[g]C-11,12-dihydrodiol (red) and 20 μ M B[g]C-11S,12S-dihydrodiol (blue) by recombinant AKR1C9. Each reaction was incubated with AKR1C9 in the presence of 2.3 mM NADP⁺ in 50 mM AMPPO buffer (pH 9.0) at 37 °C. Initial velocities were determined by taking tangents to the progress curves. (D) Effect of increasing concentrations of B[g]C-11R,12R-dihydrodiol on the AKR1C9 mediated oxidation of B[g]

C-11*S*,12*S*-dihydrodiol. v/v_o was measured by dividing v , the initial velocity, in the presence of B[*g*]C-11*R*,12*R*-dihydrodiol, by v_o , the initial velocity in the absence of B[*g*]C-11*R*,12*R*-dihydrodiol.

Figure 4.

Oxidation of 11S,12S- and B[g]C-11R,12R-dihydrodiols by AKR1C9 alanine-scanning mutants. Alanine-scanning mutagenesis was performed for the contact residues in the AKR1C9 active site. With each mutant, the oxidation of (+)-11S,12S- and (-)-11R,12R-isomers of B[g]C-11,12-dihydrodiol (20 μ M) was monitored spectrophotometrically over time with 2.3 mM NADP⁺ in 50 mM AMPPO at pH 9.0 at 37 °C. Initial velocities were determined by taking tangents to the progress curve and converted into k_{cat}/K_m using the relationship $v/[S] \rightarrow V_{max}/K_m$ when $[S] \rightarrow K_m$. Nine replicates were performed in each case, and the standard error was <10% of the mean value.

Figure 5.

LC/MS analysis of the products of B[g]C-11,12-dihydrodiol oxidation. (A) UV trace of reaction mixtures containing thio-ether conjugates obtained from enzymatic reactions of racemic B[g]C-11,12-dihydrodiol with AKR1C9 in the presence of 2-mercaptoethanol. The peak corresponding to the unreacted *trans*-dihydrodiol is at 49.5 min. (B) TIC of the of *mono*-conjugate at 35 min and *bis*-conjugate at 22 min. (C) Ion chromatogram for mass range 460-461. The peak corresponds to the *bis*-thio-ether conjugate at 22 min, (D) Ion chromatogram for mass range 384-385. The peak corresponds to the *mono*-thio-ether conjugate at 35 min. (E) Ion chromatogram for mass range 311-312. The peak corresponding to the unreacted stereoisomer of B[g]C-11,12-dihydrodiol is at 48.8 min. (F) UV trace of reaction mixtures containing thio-ether conjugates formed by reacting B[g]C-11,12-dione with 2-mercaptoethanol. The peak corresponding to unreacted *o*-quinone is at 41.5 min. (G) TIC of *mono*- (at 35 min) and *bis*-conjugates (at 22 min) of B[g]C-11,12-dione. (H) Ion chromatogram for mass range 460-461. The peak corresponds to the *bis*-thio-ether conjugate at 22 min. (I) Ion chromatogram for mass range 384-385. The peak corresponds to the *mono*-thio-ether conjugate at 35 min. (J) Ion chromatogram for mass range 309-310. The peak corresponding to the unreacted stereoisomer of B[g]C-11,12-dione is at 41 min. (K) Identification of the

mono-thio-ether conjugate ($m/z = 385$ [MH^+]) from 35 min. (L) Identification of the *bis*-thio-ether conjugate ($m/z = 461$ [MH^+]) from 22 min.

Figure 6.

Molecular Models of (+)-*S,S*- and (-)-*R,R*-stereoisomers of B[a]P-7,8-dihydrodiol, B[c]Ph-3,4-dihydrodiol, and B[g]C-11,12-dihydrodiol. PAH *trans*-dihydrodiol substrates were built in Chem3D, and energy was minimized using the MM2 program. Structures are shown in ball and stick form minus hydrogen atoms. The starting models for the dihydrodiols were predominately planar, and minimization introduced the twist. The columns show the *S,S*- and *R,R*-stereoisomer of each *trans*-dihydrodiol, respectively. In each panel, the *trans*-dihydrodiol is shown from the top as well as from the side. The *trans*-dihydrodiols are (A) B[a]P-7*S*,8*S*-dihydrodiol, (B) B[c]Ph-3*S*,4*S*-dihydrodiol, (C) B[g]C-11*S*,12*S*-dihydrodiol, (D) B[a]P-7*R*,8*R*-dihydrodiol, (E) B[c]Ph-3*R*,4*R*-dihydrodiol, and (F) B[g]C-11*R*,12*R*-dihydrodiol.

Figure 7.

Docking of PAH *trans*-dihydrodiols in the active site of AKR1C9. Molecular modeling was carried out using crystal structure 1AFS, the ternary complex of AKR1C9-NADP⁺-testosterone (48), in Swiss PDB viewer. The stereoisomers of B[g]C-11,12-dihydrodiol were built as described in Figure 6 and manually docked into the active site. Amino acid residues and *trans*-dihydrodiols are shown in stick representation. The catalytic tetrad is shown in red and other substrate contact residues are shown in magenta. The *trans*-dihydrodiols are (A) B[a]P-7S,8S-dihydrodiol (lavender), (B) B[a]P-7R,8R-dihydrodiol (cyan), (C) B[c]Ph-3S,4S-dihydrodiol (orange), (D) B[c]Ph-3R,4R-dihydrodiol (green), (E) B[g]C-11S,12S-dihydrodiol (yellow), and (F) B[g]C-11R,12R-dihydrodiol (white). With the exception of B[g]C-11R,12R-dihydrodiol, none of the PAH *trans*-dihydrodiols clash with active site residues of AKR1C9. The presence of the ring that forms the *fiord*-region of B[g]C-11R,12R-dihydrodiol causes clashes with active site residues F129, W227, and Y310A (panel F).

Scheme 1.
Proposed Competing Pathways for the Metabolic Oxidation of 11S,12S- and B[g]C-11R,12R-dihydrodiols

Table 1
Oxidation of Structurally Diverse PAH racemic *trans*-Dihydrodiols by AKR1C9

PAH	racemic BP-7,8-dihydrodiol	racemic B[g]C-11,12-dihydrodiol	racemic B[c]Ph-3,4-dihydrodiol
Structure
Region k_{cat}/K_m^a	Bay 12.3	Fjord 1520	Fjord 39.0

^a $k_{cat}/K_m = \text{min}^{-1} \text{mM}^{-1}$, which is derived from $v/[S] \rightarrow V_{max}/K_m$ using the enzyme concentration as a conversion factor. Nine replicates were performed in each case, and the standard error is <10% of the mean value.

Table 2
Oxidation of (+/-)-B[g]C-11,12-dihydrodiol by AKR1C9 mutants

Substrate	B[g]C-11,12-dihydrodiol		Stereoisomer		Androsterone	
Enzyme	k_{cat}/K_m^a	WT/MUT			$k_{cat}/K_m^{a,b}$	WT/MUT
WT	1,520	1.00	S,S		7,500	1.00
T24A	1,510	1.00	S,S		4,600	1.63
L54A	ND	ND	ND		290	25.9
F118A	ND	ND	ND		34	220.6
F129A	14.9	100	S,S, R,R		43	174
T226A	1,508	1.00	S,S		4,500	1.67
W227A	44.2	34	S,S, R,R		9.7	773
N306A	209	7.3	S,S		75	100
Y310A	23.1	66	S,S, R,R		160	46.9

^a $k_{cat}/K_m = \text{min}^{-1} \text{mM}^{-1}$, which is derived from $v/[S] \rightarrow V_{max}/K_m$ using the enzyme concentration as a conversion factor. Nine replicates were performed in each case, and the standard error is 10% of the mean value.

^bTaken from ref 32. Reactions were run in 100 mM potassium phosphate at pH 7.0 and 2.3 mM NADP⁺ with 6% acetonitrile at 37 °C.

^cND = no detectable rate.

Table 3

Contribution of the 11*S*,12*S*- and 11*R*,12*R*-enantiomers of B[g]C-11,12-dihydrodiol to the oxidation of the racemic compound

Enzyme	<i>S,S</i>	<i>R,R</i>
WT	100	0
T24A	100	0
L54A	0	0
F118A	0	0
F129A	33.1	66.9
T226A	100	0
W227A	85.5	14.5
N306A	100	0
Y310A	78.0	22.0

^a Enzymatic reactions were run using 20 μ M of either racemic or B[g]C-11*R*,12*R*-dihydrodiol in 50 mM AMPPO (pH 9.0) at 37 °C.