cDNA cloning, expression and activity of a second human aflatoxin B₁-metabolizing member of the aldo-keto reductase superfamily, AKR7A3

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The aflatoxin B₁ (AFB₁) aldehyde metabolite of AFB₁ may contribute to the cytotoxicity of this hepatocarcinogen via protein adduction. Aflatoxin B₁ aldehyde reductases, specifically the NADPH-dependent aldo-keto reductases of rat (AKR7A1) and human (AKR7A2), are known to metabolize the AFB₁ dihydrodiol by forming AFB₁ dialdehyde. Using a rat AKR7A1 cDNA, we isolated and characterized a distinct aldo-keto reductase (AKR7A3) from an adult human liver cDNA library. The deduced amino acid sequence of AKR7A3 shows 80 and 88% identity with rat AKR7A1 and human AKR7A2, respectively. Recombinant rat AKR7A1 and human AKR7A3 were expressed and purified from Escherichia coli as hexa-histidine tagged fusion proteins. These proteins catalyzed the reduction of several model carbonyl-containing substrates. The NADPH-dependent formation of AFB₁ dialdehyde by recombinant human AKR7A3 was confirmed by liquid chromatography coupled to electrospray ionization mass spectrometry. Rabbit polyclonal antibodies produced using recombinant rat AKR7A1 protein were shown to detect nanogram amounts of rat and human AKR7A protein. The amount of AKR7A-related protein in hepatic cytosols of 1,2-dithiole-3-thione-treated rats was 18-fold greater than in cytosols from untreated animals. These antibodies detected AKR7A-related protein in normal human liver tissue samples ranging from 0.3 to 0.8 µg/mg cytosolic protein. Northern blot analysis showed varying levels of expression of AKR7A RNA in human liver and in several extrahepatic tissues, with relatively high levels in the stomach, pancreas, kidney and liver. Based on the kinetic parameters determined using recombinant human AKR7A3 and AFB₁ dihydrodiol at pH 7.4, the catalytic efficiency of this reaction (kcat/Km) equals or exceeds those reported for other enzymes, for example cytochrome P450s and glutathione S-transferases, known to metabolize AFB₁ in vivo. These findings indicate that, depending on the extent of AFB₁ dihydrodiol formation, AKR7A3 may contribute to the protection against AFB₁-induced hepatotoxicity.

Abbreviations: AFB₁, aflatoxin B₁; AFAR, aflatoxin B₁-aldehyde reductase; AKR, aldo-keto reductase; CYP450, cytochrome P450; D3T, 1,2-dithiole-3-thione; ECL, enhanced chemiluminescence; GST, glutathione S-transferase; IPTG, isopropyl-thio-β-D-galactoside; MES, 2-(N-morpholino)ethanesulfonic acid; NTA, nickel nitrilotriacetic acid; ORF, open reading frame; PBS, phosphate-buffered saline; 1× SSC, 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0.

Introduction

Aflatoxin B₁ (AFB₁) is a toxic metabolite produced under conditions of high heat and humidity by common fungal molds of the Aspergillus genus growing on agricultural crops and feeds, for example peanuts and corn (1). In certain developing regions of Africa and Asia, where food storage conditions are inadequate, humans are exposed to high levels of AFB₁ in their diet. Epidemiological evidence indicates that this toxin contributes to the high incidence of primary liver cancer in these populations (1–3). The harmful effects of AFB₁ have been attributed to the metabolism of this mycotoxin to reactive metabolites that can bind to cellular macromolecules. Initially, an epoxidation reaction, catalyzed by certain cytochrome P450 (CYP450) enzymes, especially hepatic CYP1A2 and CYP3A4, converts AFB₁ to the DNA-reactive AFB₁ exo-8,9-oxide (Figure 1; 4,5). Glutathione S-transferases (GSTs) can conjugate this reactive epoxide with reduced glutathione, thus preventing the formation of DNA adducts which can lead to mutations (6). Alternatively, this epoxide can hydrolyze to form the AFB₁ dihydrodiol. Under physiological conditions, AFB₁ dihydrodiol can rearrange, forming a reactive dialdehyde configuration that can bind to primary amine groups in proteins by Schiff base reactions (7–9).

Another detoxication enzyme, designated aflatoxin B₁ aldehyde reductase (AFAR), was isolated and characterized by Judah and colleagues from the livers of rats following administration of the antioxidant ethoxyquin (10). AFAR catalyzes the reduction of AFB₁ dihydrodiol to a dialcohol. Enhancement of this reaction may lead to decreased formation of protein adducts by, and related cytotoxicity of, aflatoxin, although, direct experimental evidence supporting this hypothesis is still lacking.

Studies in several experimental animal models have shown that variations in the activities of AFB₁-activating and detoxifying enzymes can have a dramatic effect on cancer incidence (11). To extrapolate the work with animal models to human risk assessment, it is important to evaluate the activity of human AFB₁-metabolizing enzymes. Human CYP450 and GST enzymes have been expressed as recombinant proteins and their kinetic parameters have been estimated regarding AFB₁ metabolism (12,13). Recently, Ireland et al. (14) have cloned a human AFAR, designated AKR7A2, with a specific activity towards AFB₁ dihydrodiol of 1.24 ± 0.18 nmol/min/mg; however, kinetic parameters of this enzyme were not reported.

In the present study, we report the isolation of a distinct AFAR cDNA (AKR7A3) from a human liver cDNA library. This protein was expressed in Escherichia coli and used to investigate the catalytic activity of this protein towards several carbonyl-containing substrates, especially AFB₁ dihydrodiol. The expression of AKR7A was detected in a series of human liver cytosolic protein samples and in multiple adult RNA tissue samples. The catalytic efficiency of human AKR7A3 was estimated and compared with other AFB₁-metabolizing
enzymes to determine its relative contribution in the detoxification of AF7.

Materials and methods

Chemicals

4-Nitrobenzaldehyde was obtained from Sigma Chemical Co. (St Louis, MO). AF7 was purchased from Aldrich Chemical Co. (Milwaukee, WI). AF8-oxide was synthesized from AF7, by the dimethyldioxirane method (15) and was hydrolyzed by the addition of water to generate AF7-dihydrodiol (ratio of AF7: 8,9-oxide:water 1:20). The final concentration of AF7-dihydrodiol was determined spectrophotometrically (ε = 21 800/M/cm at 365 nm) (16).

Isolation and characterization of rat and human cDNA clones

A partial rat AKR7A1 cDNA, clone 18.11, was isolated as described (17). The clone 18.11 cDNA sequence began at nucleotide 365 nm) (16).

DNA sequencing strategy and analysis

DNA sequencing strategy and analysis

The rat AKR7A1 AAFR coding sequence was determined using primers specific to the Sp6 and T7 sides of the vector plasmid, pCDNA1 (Invitrogen), by the dideoxy chain termination method as implemented (Sequenase v.2.0; US Biochemicals, Cleveland, OH) using [α-32P]dATP (ICN). When overlapping sequence could not be obtained on both DNA strands, specific sense and antisense oligonucleotide primers were used: p1, 5'-AATTCGACCTCGGACCTGG-3'; p2, 5'-TGGAGAGGACGTACCTTCC-3'; 522-539, -; p3, 5'-AGGGTGAAGAGGCGTAAAG-3'; 565-584, -; p4, 5'-GGACTAGTATAGGCCCG-3', 977-997, -. The initial sequence of the human AKR7A-related cDNA clones was determined using a primer specific to the T7 side of the pCDNA3 polyclinker (Invitrogen) and then six additional primers were used to determine the complete DNA sequence in both orientations by automated sequencing using fluorescent dye terminators (Protein/Peptide/DNA Laboratory, Department of Biological Chemistry, Johns Hopkins University School of Medicine); hP2, 5'-AAATTTGATACCAAGGCG-3', 218-234, +; hP4, 5'-CTGTACCTCCTGACAAGA-3', 459-475, +; hP6, 5'-GAGTGTACCTACCTACA-3', 809-825, +; hP7, 5'-GCGTTCTACACCTGGTG-3'; 118-135, --; hP2, 5'-AGGTTTGAAGGCGTAAAGACCC-3', 578-597, --; hP9, 5'-TGGTACAGAAGAACCTTGG-3', 1018-1136, --. DNA sequences obtained from independent cDNA clones were assembled using the GeneWorks program (IntelliGenetics, Mountain View, CA).

Rat (AKR7A1) and human (AKR7A3) AFAR protein expression constructs

The rat AKR7A1 and human AKR7A3 were subcloned into pTrcHis expression vectors (Invitrogen) such that the expressed protein had an N-terminal hexahistidine (His6) peptide tag, thereby allowing affinity purification by metal chelate affinity chromatography.

The preparation of the rat AKR7A1 expression construct (herein referred to as the His6-AKR7A1 plasmid) involved a two step ligation strategy. Initially, a 1.2 kb BamHI-Xhol AKR7A1 cDNA fragment (region –25 to 1176, GenBank accession no. AF045464) was subcloned into the BamHI and HindIII sites of the pTrcHisA plasmid using T4 DNA ligase to initially ligate the cohesives BamHI sites, T4 DNA polymerase to convert the HindIII and Xhol ends to blunt ends and T4 DNA ligase to join these ends. The plasmid was transformed into E.coli DH5α cells, creating AKR7A1.pTrc1. Next, two primers were designed: pTrc1-P2, 5'-GCCGGGATCCAAAGCCCAGGAACCTTGGG-3', –3 to 20, +, which would introduce a unique BamHI restriction site (stalics) to be fused in-frame with the His6-tag without altering the original rat AKR7A1 amino acid sequence: pTrc1-P2, 5'-GCCGGGATCCAAAGCCCAGGAACCTTGGG-3', 262-282, --. This primer pair amplified a 285 bp fragment that contained an internal AvrII restriction site (position +59 by PCR. This 285 bp fragment was digested with BamHI and AvrII, separated in a low melting point agarose gel and then ligated into the BamHI and AvrII sites of AKR7A1.pTrc1 according to the instructions of FMC Bioproducts, to produce plasmid His6-AKR7A1. This final construct was sequenced using primer pTrc to confirm the proper orientation of the His6-AKR7A1 plasmid and to ensure a correct sequence of nucleotides.

Preparation of the human AKR7A3 expression construct (herein referred to as the His6-AKR7A3 plasmid) involved a similar two step ligation strategy. Initially, a 1.2 kb EcoRI-Ncol AKR7A3 cDNA fragment (region –25 to 1176, GenBank accession no. AF040639) was subcloned into the EcoRI and HindIII sites of the pTrcHisC plasmid using T4 DNA ligase to initially ligate the cohesives EcoRI sites, T4 DNA polymerase to convert the HindIII and Ncol sites to blunt ends and then T4 DNA ligase to join the ends. The plasmid was transformed into E.coli DH5α cells, creating AKR7A3.pTrc1. Next, two primers were designed: hTrc1-P1, 5'-GCCGGGATCCAAAGCCCAGGAACCTTGGG-3', –7 to 15,
Expression of rat AKR7A1 and human AKR7A3 protein

Escherichia coli transformed with either rat His6-AKR7A1 or human His6-AKR7A3 constructs were inoculated into Luria–Bertani medium (1.0% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 0.5% w/v NaCl) containing 100 µg/ml ampicillin and grown overnight at 37°C with orbital shaking at 200 r.p.m. This culture (5 ml) was used to inoculate 300 ml of medium and grown at 37°C, with orbital shaking at 200 r.p.m., until the cells were in mid log phase (A600 = 0.7–0.9). Protein expression was induced by the addition of isopropyl-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM. After 3.5 h, 1 ml of the cell suspension was pelleted by centrifugation, resuspended and boiled for 5 min in 200 µl SDS sample dilution buffer (50 mM Tris–HCl, pH 6.8, 1.5% 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol). A 15 µl sample lysate was analyzed by SDS–PAGE (12% acrylamide), to confirm induction of the expressed proteins.

For production of active protein, His6-tagged proteins from the soluble fraction of the bacterial lysates were purified under non-denaturing conditions as described (23). Escherichia coli was induced with 0.1 mM IPTG, as this concentration would reduce expression levels and prevent incorporation of protein into inclusion bodies and thereby increase solubility of protein in the cytoplasm. After 3 h, this culture was collected in 50 ml aliquots by centrifugation at 2300 g for 15 min at 4°C. These cell pellets were resuspended in 5 ml of ice-cold buffer (50 mM Tris, pH 8.0, 1 mM EDTA and 10 mM 2-mercaptoethanol) and lysozyme was added to a final concentration of 0.5 mg/ml. The suspension was rocked for 30 min at 4°C and lysed by two freeze/thaw cycles, alternating in liquid nitrogen and a final concentration of 0.5 mg/ml ampicillin and grown overnight at 37°C for 2 h and terminated by adding 10 µl of formic acid. The post-incubation reaction mixtures were applied to activated Waters C18 Sep-Pak Cartridges (Millipore, Milford, MA), washed twice with 4 ml water and then eluted with 2 ml 100% ethanol. The eluates were concentrated under argon gas.

A Finngan LCQ liquid chromatography mass spectrometry system was used to perform electrospray ionization mass spectrometry in positive ion mode to perform the identification of the AFP β-proteins. A Thermal Systems Products HPLC was used to provide a constant flow of 200 µl/min to a YMC ODS Sphere M-80 column (2 × 250 mm). A gradient starting at 4% acetonitrile, 2% methanol and finishing at 13% acetonitrile, 12% methanol over 60 min was used to separate the aflatoxin B1 metabolites. A buffer consisting of 0.1% formic acid, pH 2.5, was used throughout the run. The HPLC column was maintained at 55°C and the column effluent directed through a UV detector (365 nm) and into the electrospray ionization interface on the mass spectrometer. The instrument was scanned from 200 to 600 a.m.u. at 1 s/scan. A collision energy of 22% was used for collision reaction monitoring during MS/MS analysis.

Immunoblot analysis

Human liver samples were obtained as discarded surgical specimens from the Johns Hopkins Hospital Department of Pathology. Cytosolic protein fractions were prepared (27) and analyzed (25) as described previously. Cytosolic samples were solubilized in sample dilution buffer, separated by denaturing 12% SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane (Hybond ECL; Amersham, Arlington Heights, IL). Incubation with primary antibody (rabbit anti-rat AKR7A1 serum from the final bleed diluted 1:2000 or AKR7A3) was for 1 h at room temperature. Bound antibody was detected by incubation with a horseradish peroxidase-linked secondary antibody (goat anti-rabbit IgG diluted 1:30 000) (Promega, Madison, WI). Immune-reactive protein was detected by enhanced chemiluminescence (ECL) (Supersignal System; Pierce). Amounts of AKR7A-related proteins were estimated using purified rat (2, 4, 8 and 16 ng) and human (10, 20, 40 and 80 ng) recombinant proteins as standards. The density values of the ECL signals were determined using a Fujix BAS1000 Bio-imaging analyzer (Fuji Photo Film Co., Stamford, CT). For the selected exposure (1 min) the linearity of the ECL signals for the rat and human standard curves corresponds to r values of 0.95 and 0.99 and limits of detection of 2 and 10 ng, respectively.

RNA analysis of human tissues samples

Total cellular RNA (10 µg) was isolated from human liver and analyzed as described previously (17). After hybridization, the blot was washed twice in 1× SSC, 0.1% SDS at 50°C for 30 min. The hybridization signals were visualized by autoradiography using Kodak X-Omat AR film (Kodak Eastman Co., Rochester, NY) with a Cronex Lightning Plus™ intensifying screen (DuPont, Wilmington, DE) for 1–2 days at −80°C. Human tissue poly(A)1 mRNA blots were obtained from Clontech (Palo Alto, CA). All specimens were from normal Caucasians. Samples represent either single or pooled RNA and were not matched for sex or age. No information was available concerning smoking history, diet or other potential environmental exposures. Northern blot analysis was performed according to the manufacturer’s instructions, using human cDNA probes for AKR7A3 and β-actin (Clontech). The membranes were washed twice in ExpressHyb solution (Clontech) at 50°C for 40 min and the signals were detected by autoradiography. β-Actin was used as a control for RNA integrity and the transfer and hybridization procedures. Because levels of this RNA vary between tissues, it is not an accurate control for sample loading (28).
Results

cDNA cloning of rat AKR7A1 and human AKR7A3 and AKR7A2

Colonization of a D3T-induced rat liver cDNA library with a partial rat AKR7A1 cDNA, clone 18.11 (17), led to the isolation of three AKR7A1 clones, designated c.7, c.10 and c.13, which were used to determine the complete cDNA sequence in both directions (Figure 2A). Comparison of our three independent AKR7A1 clones with the published AKR7A1 cDNA sequence (18) revealed only two single base

Fig. 2. (A) Alignment of selected cDNA clones and sequencing strategy for the novel human AFAR (AKR7A3) and the recently reported human AFAR (AKR7A2) cDNA. The relative positions of the predicted nucleotide coding regions for overlapping cDNA clones isolated from a human liver cDNA library are indicated by solid lines. The AKR7A3 and AKR7A2 ORFs (open boxes) are shown. Arrows beneath the clones indicate the site and direction of sequencing primers. The specific clone nucleotide locations for AKR7A3 are as follows: c.6, –13 to the poly(A) tail; c.7, –25 to the poly(A) tail; for the AKR7A2 sequence, c.14, –49 to +1207; c.21, –43 to the poly(A) tail. (B) Nucleotide sequence of human AKR7A3 and alignment of the deduced human AKR7A3 and AKR7A2 and the rat AKR7A1 amino acid sequences. The bold numbers (top right) correspond to the nucleotide sequence of AKR7A3 c.7, relative to the first A (underlined); the lower numbers identify the positions of amino acid residues. Identical amino acid residues of the AKR7A2 and AKR7A1 sequences that align with the AKR7A3 sequence are indicated by asterisks; gaps are indicated by dashes. The consensus polyadenylation signal sequence of the AKR7A3 cDNA is double underlined. The bold brackets (AKR7A1 amino acid residues 126 and 127 of this report) identify the position of two nucleotide discrepancies in our sequence of rat AKR7A1 and the published rat AKR7A1 cDNA sequence (18). Invariant amino acid residues and residues known to be involved in the catalytic activity (Asp44 and Lys77) and cofactor binding (Asp44, Asn144, Glu169 and Ser291) identified by alignment of the reported sequences of the AKR superfamily are boxed (32). Human AKR7A3, GenBank accession no. AFO40639; human AKR7A2, GenBank accession no. AFO40639; human AKR7A1, GenBank accession no. X74673; rat AKR7A1 isolated in this paper, GenBank accession no. AFO45464.
changes corresponding to differences in two amino acid residues: CAC126 (histidine) to CAG126 (glutamate) and G127TG (valine) to C127TG (leucine) (Figure 2B, AKR7A1 sequence, bold brackets).

Cloned c.10 was used to screen an adult human liver cDNA library under low stringency hybridization conditions. Twenty-four human cDNA clones were isolated. Three clones, designated c.6, c.7 and c.18, were used to determine the complete AKR7A3 cDNA sequence (Figure 2A). The unique human AFAR has been designated AKR7A3 based upon the proposed nomenclature system for the aldo-keto reductase (AKR) superfamily that a sequence be placed in the same AKR family if its similarity to any other family member is &gt;40% identical and in the same AKR subfamily if it is &gt;55% identical (29). The longest open reading frame (ORF), 993 bp, predicted a protein of 331 residues, with an estimated molecular mass of 37 kDa (Figure 2B). The nucleotide sequence of this human AKR7A3 ORF is 80% identical to the corresponding ORF of the recently reported human AKR7A2 cDNA sequence (13); the predicted amino acid sequences are 88% identical. This extensive amino acid identity of AKR7A2 and AKR7A3 (Figure 2B) is striking. Moreover, all 38 of these different amino acid residues result from changes in the third (wobble) codon position. Silent nucleotide differences occur in the third position of an additional 28 codons, resulting in the same amino acid residue.

In addition to the complete sequence of three clones, we characterized the 21 clones not sequenced by restriction endonuclease mapping, using two sites unique to human AKR7A2 (13), XmnI and NheI (Figure 2A). Nineteen of these clones had patterns indicative of the human AKR7A3 cDNA sequence. Two clones, designated c.14 and c.21, showed patterns indicative of AKR7A2 (13). As a follow-up, the complete cDNA sequences of c.14 and c.21 proved to be identical to the reported human AKR7A2 cDNA sequence (Figure 2).

Expression of rat AKR7A1 and human AKR7A3 proteins in E.coli

To study their activities, recombinant rat AKR7A1 and human AKR7A3 proteins were expressed as His6-tagged fusion proteins in E.coli. Bacterial cultures induced with IPTG yielded high expression of both rat and human His6-AKR7A proteins (Figure 3, lanes 3 and 6, respectively) when compared with uninduced cultures (Figure 3, lanes 2 and 5, respectively). Rat and human His6-AKR7A proteins were purified by affinity chromatography, yielding &lt;2 mg fusion protein/300 ml bacterial culture. The purities of these preparations were &gt;95%, as judged by SDS–PAGE and Coomassie brilliant blue staining; the apparent molecular weight of each protein (His6-AKR7A1, 40 kDa and His6-AKR7A3, 42 kDa) was as expected (Figure 3, lanes 4 and 7, respectively).

Kinetic properties of purified rat AKR7A1 and human AKR7A3 proteins

A model substrate for AKRs, 4-nitrobenzaldehyde (30), was initially used to characterize the activities of the rat AKR7A1 and human AKR7A3 proteins. These recombinant proteins are active toward this substrate over a broad pH range (pH 5–8), with an optimum at pH 6.6 (data not shown). The specific activity of our recombinant rat AKR7A1 protein toward 1 mM 4-nitrobenzaldehyde (1.7 μmol/min/mg) is similar to that previously reported for recombinant rat AFAR (18). The kinetic parameters of AKR7A3 and AKR7A1 proteins were determined for 4-nitrobenzaldehyde, 9,10-phenanthrequinone and AFB1 dihydrodiol. The apparent Km and Vmax values for these reactions are presented in Table I. Since previous studies have shown that the concentration of the presumed AKR7A substrate, the AFB1 dialdehyde configuration of AFB1 dihydrodiol, is proportional to pH (pK, 8.2) (31), we determined the activity of AKR7A3 both at the pH optimum of enzyme activity, pH 6.6, and at pH 7.4. While the apparent Km values are similar, the Vmax value at pH 7.4 is ~2-fold greater (Table I).

Detection of the dialcohol product of AFB1 dihydrodiol

AFB1 dihydrodiol has a m/z of 347.0 when monitored by positive ion mass spectroscopy. Using the HPLC conditions described in Materials and methods, the retention time for the AFB1 dialcohol was 26.0 min. In the presence of the recombinant human AKR7A3, a product with a retention time of 22.4 min appeared. The m/z of this metabolite was 351.0 and the MS/MS analysis revealed two major fragmentation products at m/z 333 and 315. These products are the result of the sequential loss of water from the primary metabolite. Both the m/z and MS/MS data indicate that this metabolite is AFB1 dialcohol, with a parent mass identical to previous literature reports (10,14). AFB1 dihydrodiol incubations with recombinant protein lacking NADP+ did not produce this AFB1 dialcohol product.

Expression of AFAR in human liver

To characterize the rabbit polyclonal antibodies produced using recombinant rat AKR7A1 protein as the antigen, we performed immunoblot experiments using hepatic cytosols prepared from control or D3T-treated rats (17). On average, the level of AKR7A-immunoreactive protein was increased 18-fold in the hepatic samples from treated animals (Figure 4A, lanes 4–6), relative to control samples (Figure 4A, lanes 1–3). Using the recombinant protein as a standard, the mean ± SE concentration of AKR7A-immunoreactive protein in control cytosols was determined to be 0.3 ± 0.1 μg/mg total cytosolic protein. Since the recombinant human AKR7A3 protein showed strong crossreactivity towards antibodies raised against the recombinant rat AKR7A1 protein (data not shown), we used these antibodies to determine whether AKR7A is constitutively expressed in adult human liver. Immunoblot analyses of cytosolic protein fractions prepared from liver samples of eight individuals were performed; four of these are presented in Figure 4B. A 37 kDa protein band was detected, consistent with the calculated molecular weight of the deduced amino acid sequences of the known human AKR7A proteins. Based
Table I. Catalytic properties of recombinant human AKR7A3 and rat AKR7A1

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<tr>
<th>Substrate</th>
<th>Human</th>
<th>Rat</th>
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<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (µmol/min/mg)</td>
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<tr>
<td>4-Nitrobenzaldehyde</td>
<td>118.3 ± 16</td>
<td>0.78 ± 0.0</td>
</tr>
<tr>
<td>9,10-Phenanthrenequinone</td>
<td>4.3 ± 0.5</td>
<td>6.40 ± 0.2</td>
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<tr>
<td>AFB1 dihydrodiol (pH 6.6)</td>
<td>9.0 ± 2.1</td>
<td>0.34 ± 0.0</td>
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<tr>
<td>AFB1 dihydrodiol (pH 7.4)</td>
<td>8.6 ± 1.6</td>
<td>0.60 ± 0.0</td>
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*aIncubation mixtures included recombinant soluble rat or human protein, 100 nM sodium phosphate buffer, pH 6.6 or 7.4, 0.2 mM NADPH and varying concentrations of 4-nitrobenzaldehyde (0.2, 0.4, 0.6, 1.0, 1.2, 1.4 or 1.6 mM) or 9,10-phenanthrenequinone (2, 3, 4, 5, 10, 20 or 50 µM) and AFB1 dihydrodiol (1.5, 3, 7.5, 12, 15, 18, 25, 40 or 50 µM). The reaction was initiated by the addition of enzyme and the oxidation of NADPH was measured spectrophotometrically (340 nm). Apparent $K_m$ and $V_{max}$ values are presented as the means ± SE of three separate experiments derived from the computer generated best fit to the Michaelis–Menten equation.

*Values were determined from the computer generated best fit to the Michaelis–Menten equation using four separate experiments.

*Not determined.

Fig. 4. (A) Induction of AKR7A immunoreactive protein in the livers of D3T-treated rats. AKR7A protein was detected by immunoblot analysis as described in Materials and methods using polyclonal rabbit anti-AKR7A antibodies raised against denatured His6-AKR7A1. Shown are representative immunoblots of cytosolic protein (25 µg protein/lane) samples of liver tissue from six individual animals, 24 h after per os administration of either vehicle (lanes 1–3) or 0.5 mmol D3T/kg body wt (lanes 4–6). (B) Detection of AKR7A immunoreactive protein(s) in human liver. A human AKR7A-related protein was detected by immunoblot analysis, as described in Materials and methods, using polyclonal rabbit anti-AKR7A antibodies. Shown are representative immunoblots of cytosolic protein (50 µg protein/lane) samples (lanes 1–4) prepared from four individual liver samples.

Fig. 5. Detection of human AKR7A-related RNA in human liver. Shown are duplicate representative northern blots of four total RNA (10 µg RNA/lane) samples (lanes 1–4). Blots were hybridized with cDNA probes for human AKR7A3 or β-actin.

Discussion

This paper describes the cloning, expression and characterization of specific family-7 AKRs, rat AKR7A1 and human AKR7A3. Independent cDNA clones, isolated from either D3T-treated rat liver (17) or adult human liver libraries, were used to determine the respective full-length sequences. The rat AKR7A1 was shown to correspond to a previously reported cDNA sequence (18), although two nucleotide discrepancies predicting different amino acid residues were noted (Figure 2B). The AKR7A1 sequence reported here was verified for three independent cDNA clones in both orientations, whereas the previously published AKR7A1 sequence was verified for only one clone (18).

Using AKR7A1, we isolated cDNA clones corresponding to the previously characterized human AKR7A2 (14) and to human AKR7A3, which was previously unknown. By aligning the segments of these human cDNA sequences corresponding to their protein coding regions, we observed that all of the differences between their predicted amino acid sequences resulted from nucleotide differences in the third (wobble) codon position. Many silent nucleotide differences were also observed. The distribution of these silent and coding nucleotide differences throughout the AKR7A2 and the AKR7A3 sequences indicates that these distinct cDNAs are likely the products of independent genes rather than mRNA splice variants of a single gene.

In the initial characterization of human AKR7A2, fractionation of human liver cytosol by anion exchange chromatography on Q Sepharose resulted in multiple peaks containing carbonyl reductase activity (14). This observation is consistent with our identification of a second human member of the AKR7A superfamily. Further studies will be required in order to determine the enzyme multiplicity and substrate specificity of the human AKR7A family.

A previous analysis of RNA expression, using an AKR7A2 cDNA probe, identified AKR7A2-related mRNA in liver and several other organs, with especially strong hybridization on the recombinant AKR7A3 standards, the amount of this AFAR-related protein in liver samples from the eight individuals examined ranged from 0.3 to 0.8 µg/mg, with a mean ± SE of 0.5 ± 0.1 µg/mg cytosolic protein. The expression of AKR7A-related mRNA in these eight individual liver samples was further characterized by northern RNA analysis. The same four representative samples are presented (Figure 5). Overall, the levels of AKR7A-related mRNA expression varied and was poorly correlated ($r = 0.2$) with AKR7A-related protein amounts.

Expression of human AFAR in tissues

In a separate analysis, a 1.4 kb AKR7A-related RNA was detected in multiple adult tissue samples (Figure 6). The most intense hybridization signals were observed in the samples of the stomach, pancreas, kidney and liver. Moderate signals were detected in the samples of the small intestine, colon, thyroid, spinal cord and bone marrow. Low signals were detected in samples of the lung and peripheral blood leukocytes. The cellular localization, as well as the functional significance of AKR7A expression in these tissues, remains to be determined.
signals observed for kidney, pancreas, small intestine and skeletal muscle (14). Although the tissue RNA expression reported in this current study, using an AKR7A3 cDNA probe, is in good agreement with the previous study, the high homology of the AKR7A2 and AKR7A3 cDNA sequences (80%) indicates that neither study utilized methods to discriminate specific AKR7A2 and AKR7A3 mRNAs. Further studies using sequence-specific oligonucleotide probes will be required in order to determine the relative expression of each gene and their responses to administration of D3T, ethoxyquin and other chemoprotective agents. Since the amino acid sequences of the AKR7A2 and AKR7A3 proteins do not differ by any stretch of residues greater than four, it appears unlikely that specific antibodies can be produced. However, substrate and inhibitor specificity attributes may help to determine the relative expression of these proteins in different tissues.

Previously, it was shown by others (32) that the apparent $K_m$ and $V_{max}$ values for a variety of carbonyl-containing substrates were comparable for the recombinant AKR7A1 and the native form, purified from rat liver. Although we did not purify the native AKR7A proteins, the kinetic parameters of our recombinant AKR7A1 enzyme, determined for 4-nitrobenzaldehyde and 9,10-phenanthrenequinone, are in good agreement with the literature values (32). The purified recombinant AKR7A3 was also shown to be active towards several model substrates for AKRs. Kinetic analysis of the recombinant AKR7A3 showed a 4-fold lower apparent $K_m$ for 4-nitrobenzaldehyde relative to AKR7A1; the apparent $K_m$ values for 9,10-phenanthrenequinone were similar (Table I). The apparent $K_m$ value of AKR7A3 for 4-nitrobenzaldehyde is considerably lower than that determined for AKR7A2 (14), while that for 9,10-phenanthrenequinone is similar.

The reactive AFB$_1$ 8,9-oxide metabolite plays a central role in the formation of cellular macromolecular adducts (Figure 1). It is well known that the C8 position of the unstable, AFB$_1$ exo-8,9-epoxide isomer forms a covalent bond with the N7 of guanine bases in DNA, which, if not repaired, can lead to mutations (4–6). Another pathway, which follows from the spontaneous hydrolysis of the epoxide to form AFB$_1$ dihydrodiol, is the reversible base-catalyzed conversion of AFB$_1$ dihydrodiol to the ring opened oxynionic form, AFB$_1$ dialdehyde (31). The concentration of these two metabolites in vivo at equilibrium is not known. However, the relative amount of AFB$_1$ dialdehyde formed from solutions of AFB$_1$ dihydrodiol over a range of pH values has been determined (31); the AFB$_1$ dialdehyde metabolite exists at pH $> 6$.0. AFB$_1$ dialdehyde forms Schiff bases with lysine residues in proteins at physiological pH (6–8). As a consequence, the potential of this dialdehyde tautomer of AFB$_1$ dihydrodiol to interact with lysine residues in critical cellular proteins, thereby altering their function, has been considered to contribute to AFB$_1$-induced cytotoxicity. Evidence to support this hypothesis comes largely from in vitro studies, where incubation of AFB$_1$ dihydrodiol with isolated reticulocytes results in direct inhibition of protein synthesis (33). However, additional studies will be required to directly correlate the effects of protein binding, impairment of cellular function and toxicity caused by the dialdehydic form of AFB$_1$ dihydrodiol.

Since previous studies did not report the apparent $K_m$ and $V_{max}$ values for the reduction of AFB$_1$ dihydrodiol, we determined these values for AKR7A1 and AKR7A3 (Table I). At the pH optimum for activity of these enzymes with model substrates (pH 6.6), both AKR7A1 and AKR7A3 had similar kinetic rates for AFB$_1$ dihydrodiol, although AKR7A3 had a 3-fold lower apparent $K_m$. AKR7A3 had a 2-fold higher $V_{max}$ in incubations with AFB$_1$ dihydrodiol at pH 7.4 compared with pH 6.6. This outcome is consistent with the observation of Johnson et al. (31) showing that the relative amount of the dialdehydic form of AFB$_1$ dihydrodiol in solution is pH dependent and increases.

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**Table II.** Kinetic constants of enzymatic and non-enzymatic pathways involved in AFB$_1$ metabolism

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$K$ (μM)</th>
<th>$k_2K$ (per M/s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>32</td>
<td>$1.4 \times 10^5$</td>
<td>11</td>
</tr>
<tr>
<td>GST A1-1b</td>
<td>100</td>
<td>$9.0 \times 10^4$</td>
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</tr>
<tr>
<td>GST A2-2b</td>
<td>100</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>GST M1-1b</td>
<td>30</td>
<td>$1.7 \times 10^3$</td>
<td>12</td>
</tr>
<tr>
<td>AKR7A3</td>
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<td>$4.0 \times 10^2$</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>12</td>
</tr>
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<tr>
<td>AKR7A1b</td>
<td>21</td>
<td>$1.0 \times 10^3$</td>
<td>This paper</td>
</tr>
</tbody>
</table>

*4CYP1A2, AKR7A1 and AKR7A3 predicted data are modeled by Michaelis–Menten kinetics.

*3Mathematical kinetic modeling was employed to estimate rates and binding affinities for GSTs using hopKINSIM software.

*Second order rates of AKR7A proteins were calculated by initially converting $V_{max}$ (μmol/min/mg) to $k_2$ (nmol/min/nmol), expressed in units/min, where 1 mg of AKR7A3 or AKR7A1 = 27 nmol, and then calculating second order rate constants by dividing $k_2$ by $K_m$ ($K_m$ and $V_{max}$ values are from Table I).
under basic conditions (31). The reported rate for AKR7A2 (1.2 nmol/min/mg at pH 7.4; 14) is much lower than the $V_{\text{max}}$ determined for AKR7A3 (340 nmol/min/mg). However, the study of AKR7A2 did not report the concentration of AFB$_1$ dihydrodiol used, which may have been low.

Investigations of the catalytic activities of AFB$_1$-metabolizing enzymes is of interest in determining the sensitivity of humans to the toxic and carcinogenic effects of AFB$_1$. For example, several cytosolic GSTs are known to play critical roles in the detoxification of AFB$_1$ by enzymatically conjugating the AFB$_1$ 8,9-oxide with reduced glutathione. Induction of GSTs by chemopreventive agents can profoundly affect the metabolism and disposition of AFB$_1$ in vivo, both in rats (34) and humans (35). In addition, the 52-fold greater GST activity towards aflatoxin in mice than rats appears to be the basis for the resistance of mice to the hepatocarcinogenic actions of this toxin (36). The catalytic efficiencies of AKR7A1 and AKR7A3 were determined in this study and compared with kinetic values derived from several other studies for specific recombinant rat and human CYP450s (12) and GSTs (13) involved in AFB$_1$ metabolism (Table II). The available information suggests that both AKR7A1 and AKR7A3 have activities comparable with other AFB$_1$-metabolizing enzymes, and that these AKRs have the potential to catalyze the reduction of AFB$_1$ dihydrodiol to AFB$_1$ dialcohol in the milieu of various competing pathways of AFB$_1$ metabolism in vivo. Further comparative studies of these enzymes, singly and in combination, can be used to probe the relative contribution of these potentially competing metabolic pathways to the balance of activation and detoxification of AFB$_1$.

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References


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