Major differences exist in the function and tissue-specific expression of human aflatoxin B₁ aldehyde reductase and the principal human aldo-keto reductase AKR1 family members

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Complementary DNA clones encoding human aflatoxin B₁ aldehyde reductase (AKR7A2), aldehyde reductase (AKR1A1), aldose reductase (AKR1B1), dihydrodiol dehydrogenase 1 (AKR1C1) and chlordecone reductase (AKR1C4) have been expressed in *Escherichia coli*. These members of the aldo-keto reductase (AKR) superfamily have been purified from *E. coli* as recombinant proteins. The recently identified AKR7A2 was shown to differ from the AKR1 isoenzymes in being able to catalyse the reduction of 2-carboxybenzaldehyde. Also, AKR7A2 was found to exhibit a narrow substrate specificity, with activity being restricted to succinic semialdehyde (SSA), 2-nitrobenzaldehyde, pyridine-2-aldehyde, isatin, 1,2-naphthoquinone (1,2-NQ) and 9,10-phenanthrenequinone. In contrast, AKR1A1 reduces a broad spectrum of carbonyl-containing compounds, displaying highest specific activity for SSA, 4-carboxybenzaldehyde, 4-nitrobenzaldehyde, pyridine-3-aldehyde, pyridine-4-aldehyde, 4-hydroxynonenal, phenylglyoxal, methylglyoxal, 2,3-hexanedione, 1,2-NQ, 16-ketoestrone and D-glucuronic acid. Comparison between the kinetic properties of AKR7A2 and AKR1A1 showed that both recombinant enzymes exhibited roughly similar k_{cat}/K_m values for SSA, 1,2-NQ and 16-ketoestrone. Many of the compounds which are substrates for AKR1A1 also serve as substrates for AKR1B1, though the latter enzyme was shown to display a specific activity significantly less than that of AKR1A1 for most of the aromatic and aliphatic aldehydes studied. Neither AKR1C1 nor AKR1C4 was found to possess high reductase activity towards aliphatic aldehydes, aromatic aldehydes, aldoses or dicarbonyls. However, unlike AKR1A1 and AKR1B1, both AKR1C1 and AKR1C4 were able to catalyse the oxidation of 1-acenaphthenol and, in addition,

AKR1C4 could oxidize di- and tri-hydroxylated bile acids. Specific antibodies raised against AKR7A2, AKR1A1, AKR1B1, AKR1C1 and AKR1C4 have been used to show the presence of all of the reductases in human hepatic cytosol; the levels of AKR1B1 and AKR1C1 were markedly elevated in livers with alcohol-associated injury, and indeed AKR1B1 was only detectable in livers with evidence of alcoholic liver disease. Western blotting of extracts from brain, heart, kidney, liver, lung, prostate, skeletal muscle, small intestine, spleen and testis showed that AKR7A2 is present in all of the organs examined, and AKR1B1 is similarly widely distributed in human tissues. These experiments revealed however, that the expression of AKR1A1 is restricted primarily to brain, kidney, liver and small intestine. The AKR1C family members proved not to be as widely expressed as the other reductases, with AKR1C1 being observed in only kidney, liver and testis, and AKR1C4 being found in liver alone. As human kidney is a rich source of AKR, the isoenzymes in this organ have been studied further. Anion-exchange chromatography of human renal cytosol on Q-Sepharose allowed resolution of AKR1A1, AKR1B1, AKR1C1 and AKR7A2, as identified by substrate specificity and Western blotting. Immunohistochemistry of human kidney demonstrated that AKR7A2 is expressed in a similar fashion to the AKR1 family members in proximal and distal convoluted renal tubules. Furthermore, both AKR7A2 and AKR1 members were expressed in renal carcinoma cells, suggesting that these groups of isoenzymes may be engaged in related physiological functions.

Key words: alcohol abuse, enzyme induction, kidney, quinones, succinic semialdehyde.

INTRODUCTION

Carbonyl-containing compounds are widely distributed in nature and pose a serious threat to living organisms because of their ability to react with cellular macromolecules. Aldehydes and ketones are harmful to cells because they can react covalently with secondary amine groups and thiol groups in proteins [1]. Furthermore, certain aldehydes are mutagenic as they can

interact with DNA, principally by covalent modification of guanine through the N-1 atom and the C-2 amino group [2,3].

Examples of commonly encountered toxic carbonyl compounds are provided by formaldehyde and acrolein, which are produced by photo-oxidation of hydrocarbons in polluted air. Humans are also exposed to numerous noxious environmental contaminants like benzene, which do not themselves contain carbonyl moieties but are subject to oxidation reactions in the

Abbreviations used: AFAR, aflatoxin B₁ aldehyde reductase; AKR, aldo-keto reductase; 2-CBA, 2-carboxybenzaldehyde; 3-CBA, 3-carboxybenzaldehyde; 4-CBA, 4-carboxybenzaldehyde; DD, dihydrodiol dehydrogenase; DTT, dithiothreitol; ECL, enhanced chemiluminescence; HSD, hydroxysteroid dehydrogenase; Igpal, (octylphenoxy)polyethoxyethanol; IPTG, isopropyl β -D-thiogalactoside; NBA, nitrobenzaldehyde; 1,2-NQ, 1,2-naphthoquinone; Pefabloc, 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride; 9,10-PQ, 9,10-phenanthrenequinone; SSA, succinic semialdehyde; UTR, untranslated region.

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body that can generate derivatives such as benzoquinone and muconaldehyde [4]. Monoamine oxidase catalyses the formation of carbonyl-containing compounds from biogenic amines [5], cytochromes P450 catalyse the formation of quinone-containing compounds from oestrogen [6] and alcohol dehydrogenase produces acetaldehyde from ethanol [7]. The lipid peroxidation products 4-hydroxynonenal and malondialdehyde are quantitatively important reactive carbonyls which are produced normally in the cell under conditions of oxidative stress [8]. Carbonyl groups are also present in a number of therapeutic drugs, such as the anticancer agents doxorubicin and daunomycin [9], the cysteine protease inhibitor *N*-acetyl-leucyl-leucyl-norleucinal [10], the diuretic ethacrynic acid [11] and the pesticide chlordecone [12]. The presence of carbonyl moieties in these compounds is a factor which may contribute to their toxicity.

Within the cell, protection is provided against reactive carbonyl compounds by several families of detoxification enzymes, including aldo-keto reductase (AKR), aldehyde dehydrogenase, aldehyde oxidase, alcohol dehydrogenase and glutathione *S*-transferase [4]. Little is known about the relative importance of these different families of enzymes in conferring resistance against noxious carbonyls or converting carbonyls into biologically active compounds. It is anticipated that selectivity for different carbonyl substrates and tissue-specific expression will be major factors determining the contribution of different reductases, dehydrogenases and transferases to the detoxification of xenobiotics.

Most of the enzymes which detoxify reactive carbonyls are relatively well described [13]. However, the AKR superfamily has not been as thoroughly characterized as other families of carbonyl-metabolizing enzymes. Information from molecular cloning has allowed this superfamily to be divided, on the basis of sequence identity, into seven families (AKR1–AKR7) [14]. The majority of the mammalian AKRs share greater than 40% identity and have therefore been placed together as members of the AKR1 family [14]. Knowledge of the biochemical properties and physiological roles of these reductases has not kept pace with the molecular cloning experiments. The two best described AKR enzymes are aldehyde reductase (AKR1A1) and aldose reductase (AKR1B1); these enzymes exhibit broad overlapping substrate specificities, though AKR1A1 preferentially catalyses the NADPH-dependent reduction of aliphatic aldehydes, aromatic aldehydes and biogenic amines, whereas AKR1B1 is better at catalysing the NADPH-dependent reduction of aldopentoses and aldohexoses [15–18]. Less well understood, at least in man, is dihydrodiol dehydrogenase (DD), which catalyses the oxidation of non-K-region *trans*-dihydrodiols of polycyclic aromatic hydrocarbons, steroid hormones and bile acids [19]. Whereas the catalytic properties of the rodent DD isoenzymes have been thoroughly investigated [20–22], the human DD proteins have attracted relatively little attention [23,24]. In man, at least four different DD isoenzymes exist, namely, AKR1C1 [also called DD1 or 20 α -hydroxysteroid dehydrogenase (HSD)] [25,26], AKR1C2 (also called DD2 or bile acid-binding protein) [25,26], AKR1C3 (also called type II 3 α -HSD) [27], and AKR1C4 (also called DD4, chlordecone reductase or type I 3 α -HSD) [27,28]. The paucity of biochemical information about the human AKR1C family is probably due, in part, to the fact that certain of the forms are difficult to resolve from each other by conventional chromatographic methods, and that research groups have commonly experienced difficulty in obtaining full-length cDNAs for these reductases [28–30].

We have recently reported the molecular cloning of an aflatoxin B₁ aldehyde reductase (AFAR) from both rat [31] and human liver [32], which is only distantly related to other human AKRs;

the rat and human AFAR are 78% identical but share less than 25% sequence identity with AKR1 family members. Following the recommendations of Jez et al. [14], we have designated rat and human AFAR as AKR7A1 and AKR7A2 respectively.

In an attempt to establish whether AKR7 enzymes may serve different roles from those of other members of this superfamily, we have compared the substrate specificities of human AKR7A2 with those of AKR1A1, AKR1B1, AKR1C1 and AKR1C4. In these experiments the level of expression of the various AKR proteins in different human organs has also been examined to determine whether the isoenzymes might possess tissue-specific functions.

MATERIALS AND METHODS

Chemicals and enzymes

The *Nde*I restriction endonuclease was purchased from NBL Gene Sciences Ltd. (Cramlington, Northumberland, U.K.). All other restriction enzymes and T₄ DNA ligase were from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). The T₄ polynucleotide kinase was obtained from Promega Ltd. (Southampton, U.K.). Cloned Pfu DNA polymerase was procured from Stratagene Ltd. (Cambridge, U.K.). Nickel-agarose and HiTrap Blue columns were from Pharmacia Biotech (Milton Keynes, Herts., U.K.). The AKR substrates 2-carboxybenzaldehyde (2-CBA) and 3-CBA were from Aldrich (Gillingham, Dorset, U.K.). All other chemicals were provided by Sigma (Poole, Dorset, U.K.). Pefabloc [4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride] was purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.). Both the Multilink and streptavidin peroxidase label were purchased as a Biogenex StrAviGen Multilink[™] kit from Biogenex Laboratories (San Ramon, CA, U.S.A.).

Human tissues

Human tissues were obtained with formal consent (for full autopsy and the removal of tissues for teaching and research). Organs were available from five male individuals [33]: patient 145 was 73 years old with an aortic aneurysm; patient 386 was 73 years old with bronchopneumonia and atherosclerosis; patient 387 was 52 years old with cerebral infarct; patient 388 was 65 years old with fractured pelvis, alcoholic cirrhosis and fibrosing alveolitis; patient 396 was 38 years old with aspiration pneumonia, bleeding oesophageal varices and alcoholic liver disease. The organs were obtained between 6 and 12 h post mortem and stored at –70 °C until use.

Bacterial strains, plasmids, library and cDNA clones

The *Escherichia coli* strains NM522 and BL21pLysS and the expression vector pET15b were from Novagen Inc. (Witney, Oxon, U.K.). The cloning vector pBluescript (–) and a human Unizap liver cDNA library were obtained from Stratagene Ltd.

The cDNA clones for human AKR were provided by the following fellow scientists: AKR1A1 (in pGEM-3) was a gift from Dr. Kurt M. Bohren and Dr. Kenneth H. Gabbay (Baylor College of Medicine, Houston, TX, U.S.A.) [34]; AKR1B1 (in pGEM-3) was given by Dr J. Mark Petrash (Washington University in St Louis, St Louis, MO, U.S.A.) [35]; AKR1C1 (in pBluescript (–), also called c32, was a gift from Dr. Paul J. Ciaccio and Dr. Ken D. Tew (Fox Chase Cancer Center, Philadelphia, PA, U.S.A.) [30]. The cDNA for AKR7A2 (in pET15b and called pLI19) has been cloned previously by Ireland et al. [32].

Figure 1 Cloning strategy for the construction of plasmids for recombinant AKR1C4 expression

Molecular cloning of human AKR1C4

Complementary DNA encoding human AKR1C4 was obtained by the PCR from 1 μ l of a human liver cDNA library using the two oligonucleotides 5'-ATCCCAAATATCAGCGTGTAGAGCTCAATGATGGTC-3' (sense) and 5'-GACCATGGATAGATCTAGCTAGCTGAAGTTGCC-3' (antisense) to introduce a 5' *Sst*I and a 3' *Bgl*II site; the sense oligonucleotide corresponds to nucleotides +5 to +40, and the antisense oligonucleotide to nucleotides +1124 to +1092 of the AKR1C4 cDNA described by Khanna et al. [27]. PCR was performed in a total volume of 50 μ l of reaction buffer containing 100 ng of template DNA, 200 ng of each of the two oligonucleotides, 0.2 mM each of the four dNTPs and 1.25 units of cloned Pfu DNA polymerase. During the first two cycles of amplification, the cDNA library was denatured at 94 °C for 1 min, annealed to the primers at 48 °C for 2 min and extended at 72 °C for 2 min. During the second stage of amplification (cycles 3–34), the DNA template was denatured at 94 °C for 1 min, annealed at 58 °C for 2 min and extended at 72 °C for 3 min. In the final cycle (cycle 35), the DNA was extended at 72 °C for 7 min to yield the first amplification product.

To allow the AKR1C4 cDNA to be propagated it was cloned into pBluescript (–). The cloning was facilitated by performing a second PCR reaction on the first amplification product, using the oligonucleotides 5'-CGGGGTACCATATGCCCGTATT-

GGGATTGGCA-3' (sense) and 5'-ATGTCGTGCAGGATCCTCTATGCTAATATTC-3' (antisense) to introduce 5' *Kpn*I and *Nde*I sites and a 3' *Bam*HI site; the 22 nucleotides in the 3' portion of the sense oligonucleotide represent nucleotides +46 to +67 of the AKR1C4 cDNA and the nine nucleotides in the 3' portion of the antisense oligonucleotide represent nucleotides +972 to +964 of the AKR1C4 cDNA. The same PCR conditions described above were employed to amplify the cDNA, except that the annealing temperature during the first two cycles was increased to 60 °C and the second stage of amplification was performed for 33 cycles. The second amplification product was digested with *Kpn*I and *Bam*HI before being ligated into pBluescript (–) that had been treated with the same restriction enzymes. The ligation reaction mixture was used to transform *E. coli* NM522 cells. Several clones containing the AKR1C4 cDNA (pTOC1) were identified by blue/white selection after plating the bacteria on Luria–Bertani agar plates containing X-galactosidase and isopropyl β -D-thiogalactoside (IPTG). The AKR cDNA insert in pTOC1 was excised by digestion with *Nde*I and *Bam*HI endonucleases before ligation into similarly treated pET15b (to yield pTOC2) and transformation into *E. coli* NM522.

The cloning strategy described above for AKR1C4 was based on the cDNA sequence described by Winters et al. [28] and resulted in the expression of a truncated form of the reductase that initiated translation from an internal methionine. As shown in Figure 1, the expression of full-length protein was achieved by ligation of a double-stranded synthetic oligonucleotide into pTOC2 through the unique *Nde*I site at the translational start site. Two complementary oligonucleotides 5'-TATGGATCCCAAATATCAGCGTGTAGAGCTCAATGATGGTCACTT-3' and 5'-TAAAGTGACCATCATTGAGCTCTACACGCTGATATTTGGGATCCA-3' were synthesized that allowed both the formation of double-stranded DNA with *Nde*I overhangs and the introduction of a single point mutation (underlined) which caused the generation of an *Sst*I restriction site without altering the sequence of the encoded protein. A ligation was performed with pTOC2, linearized by digestion with *Nde*I, and a 200-fold molar excess of both of the 45-mer oligonucleotides. Ligated products were transformed into *E. coli* strain XL-blue and, following extraction of plasmid DNA, the constructs were treated with *Sst*I to remove possible multiple oligonucleotide inserts through the *Nde*I site. The *Sst*I-treated construct was religated to yield pET15b containing the entire coding region of AKR1C4 (pTOC3).

Generation of bacterial expression constructs for human AKR isoenzymes

Human AKR cDNA clones contained in pGEM-3 or pBluescript (–) were linearized by restriction digestion with either *Hind*III or *Sst*I. Linearized plasmids were purified by agarose gel electrophoresis and the DNA was extracted by the QIAquick gel extraction method (Qiagen Inc.). The resulting DNA preparations were used as templates for PCRs that were performed in order to create restriction sites for sequential cloning into pBluescript and pET15b: the sense oligonucleotide was designed to introduce either an *Xba*I or *Eco*RI site into the 5' untranslated region (UTR) as well as an *Nde*I site at the ATG initiation codon of the AKR cDNAs; the antisense oligonucleotide was designed to introduce either an *Xho*I or *Bam*HI site into the 3' UTR of the AKR cDNAs. The human AKR1A1 was amplified using 5'-GCTCTAGACATATGGCGGCTTCCTGTGTTCTAC-3' (sense) and 5'-GGAAGGGAGGCCAGGATCCTGTGGTCTCAG-3' (antisense). AKR1B1 was amplified using 5'-GGGAATTCATATGGCAAGCCGTCTCTCTGCTC-3' (sense) and

5'-GGTATAGGTCACCTGGGCTCGAGCAGGCAACCAC-3' (antisense). AKR1C1 was amplified using 5'-GCTCTAGACATATGGATTGAAATATCAGTGTG-3' (sense) and 5'-GTCCAGTCACCAGCCTCGAGCCATCCTCTGTG-3' (antisense). Amplification of these cDNAs was carried out essentially as described above for PCR of AKR1C4 from the human liver cDNA library, with the exception that the second stage was reduced from 33 cycles to 22 cycles. The PCR products were cloned into pBluescript using the following restriction enzymes: AKR1A1, *Xba*I and *Bam*HI; AKR1B1, *Eco*RI and *Xho*I; AKR1C1, *Xba*I and *Xho*I. After propagation in *E. coli* NM522, the AKR-encoding inserts were excised from pBluescript by digestion with *Nde*I and either *Bam*HI or *Xho*I and cloned into pET15b.

DNA sequencing

The AKR cDNAs cloned into pET15b were each sequenced to verify the accuracy of amplification using the fmol[™] DNA Sequencing System (Promega Ltd.). Each construct was sequenced in the 5' → 3' orientation using both vector- and cDNA-specific oligonucleotide primers to generate overlapping sequence data. PCR was performed in a total of 16 µl of reaction buffer, containing 30 fmol of template DNA, 5 µl of fmol[™] sequencing 5 × buffer and 1.5 pmol of ³²P-labelled primer. In the first stage of PCR, the DNA was denatured at 94 °C for 2 min. In the second stage (30 cycles), the DNA was denatured at 94 °C for 30 s, annealed at 42 °C for 1 min and extended at 70 °C for 1 min.

Expression of recombinant protein

The various AKR cDNAs in pET15b were transformed into *E. coli* BL21pLysS and expressed under the control of an IPTG-inducible T7 polymerase. Bacterial cultures were grown at 37 °C in liquid broth containing a final concentration of 0.2 mg/ml (w/v) ampicillin and 0.034 mg/ml (w/v) chloramphenicol until an attenuation of 0.4 was reached, whereupon IPTG was added to a final concentration of 1 mmol/l and the cells were left to grow for a further 2.5 h. After this period of induction, the culture was cooled on ice for a period of 30 min before the cells were harvested by centrifugation (3200 g, 10 min, 4 °C). The resulting bacterial cell pellets were stored at -70 °C.

Purification of recombinant AKR

Each of the human AKR isoenzymes was expressed in *E. coli* from pET15b as a polyhistidine fusion protein that contained an additional six histidine residues and a thrombin cleavage site at the N-terminus. Recombinant protein was isolated from bacterial lysates by chelation chromatography on a nickel-agarose column. AKR isoenzymes were routinely prepared from frozen bacterial pellets obtained from an equivalent of either 100 ml or 200 ml of culture. Cells were resuspended in 10 ml of 5 mM imidazole/0.5 M NaCl/20 mM Tris/HCl, pH 7.9, containing 0.01 % (v/v) ipegal (buffer A) at 25 °C, and were lysed by treatment with 0.5 mg of lysozyme for 10 min at 37 °C. To ensure maximum bacterial lysis, the resuspended lysozyme-treated cells were refrozen in liquid nitrogen before being thawed quickly in a 37 °C water-bath by the addition of 40 ml of pre-warmed buffer A. After thawing, cells were sonicated at 16 µm amplitude for three separate 30 s bursts and debris was removed by centrifugation (12000 g, 20 min, 4 °C). The supernatant was filtered under vacuum through a 0.45 µm Nalgene polyethersulfone membrane bottle-top filter disc and applied to a 1 ml nickel-agarose chelating column at a flow rate of 4 ml/min; the supernatant and buffers

were kept on ice. In order to remove non-specifically bound material, the column was washed with buffer A containing increasing concentrations of imidazole as follows: 5 ml of 5 mM imidazole, 30 ml of 30 mM imidazole, 5 ml of 50 mM imidazole, and 1 ml of 80 mM imidazole. Finally, the AKR was eluted from nickel-agarose with 2 ml of buffer A containing 250 mM imidazole. After addition of glycerol to a final concentration of 25 % (v/v), the polyhistidine-tagged AKR isoenzymes were stored at -70 °C until required for characterization of catalytic activity.

Recombinant AKR proteins lacking the N-terminal polyhistidine tag (for use as immunogens) were obtained by cleavage with thrombin of the protein eluted from the nickel-agarose; AKR was recovered from the digest by affinity chromatography on HiTrap Blue columns. Before addition of thrombin (2 units of proteinase/mg of AKR), the nickel-agarose eluate was adjusted to 2.5 mM CaCl₂, 150 mM NaCl, 50 mM Tris/HCl, pH 8.0, and 75 mM imidazole. Thrombin cleavage of the polyhistidine-tag was allowed to proceed for approx. 72 h at 25 °C before the protein was loaded on to a HiTrap Blue column that had been pre-equilibrated with 20 ml of 50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl. After washing with 10 ml of the same buffer used to equilibrate the column, AKR protein was eluted with 2 ml of 50 mM Tris/HCl/2 M NaCl buffer, pH 8.0, followed by 3 ml of the same buffer containing 2 mg/ml NADP⁺. Finally, the enzyme-containing eluate was pooled and concentrated by centrifugation (1000 g, 30 min, 20 °C) from 5 ml to 1 ml using a 15 ml Vivaspin Concentrator (Greiner).

Discontinuous SDS/PAGE

Discontinuous SDS/PAGE was performed as described previously [32] in 12 % polyacrylamide resolving gels using a Mini-Protein II Cell apparatus (Bio-Rad Laboratories, Hemel Hempstead, Herts, U.K.).

Enzyme and protein assays

AKR activity was measured at 25 °C using a Cobas Fara centrifugal analyser (Hoffmann-La Roche Ltd., Basel, Switzerland) by following the initial rate at 340 nm of either the oxidation of NADPH or the reduction of NADP⁺. The 'standard' assay for measurement of reduction of aldehydes or ketones was carried out with 0.2 mM NADPH as co-factor in 250 µl of a reaction mixture containing 100 mM sodium phosphate buffer, pH 7.0. The majority of the carbonyl-containing substrates examined were analysed, as described previously, at a concentration of 1 mM [16,24,36-38]; the exceptions were acrolein (10 mM), benzil (0.1 mM), doxorubicin (0.25 mM), glucose (10 mM), D-glucuronic acid (10 mM), 4-hydroxynonenal (4 mM), 16-ketoestrone (0.2 mM), 1,2-naphthoquinone (1,2-NQ) (0.05 or 0.10 mM), 9,10-phenanthrenequinone (9,10-PQ) (0.05 mM or 0.10 mM) and D-xylose (10 mM). The oxidation reactions were studied with 0.2 mM NADP⁺ and either 0.25 mM androsterone, 0.25 mM cholic acid, 0.25 mM chenodeoxycholic acid, 0.25 mM lithocholic acid or 1 mM 1-acenaphthenol as substrate in 100 mM glycine buffer, pH 9.0. In these enzyme assays, the concentration of organic solvent (methanol, acetonitrile or dimethylsulphoxide), which was sometimes required to dissolve the substrate, was kept below 4 % (v/v) in the final reaction mixture; on such occasions, the solvent was included at the same concentration in all reactions for a given substrate. The enzyme activity was not interfered with under these assay conditions. For all reactions, the non-enzymic rate was subtracted from the observed initial reaction velocity. Each assay was carried out in triplicate and was performed on at least three

separate occasions using different batches of recombinant enzyme. Kinetic parameters were determined by employing the Ultrafit curve-fitting software (Biosoft, Cambridge, U.K.) to fit the Michaelis–Menten equation using the Marquardt algorithm [32].

Protein concentrations were determined by the Coomassie dye-binding method using BSA to calibrate the assay [32].

Western blotting

Antibodies against purified recombinant AKR which lacked the N-terminal polyhistidine-tag (see above) were raised in female New Zealand White rabbits by methods described previously [39].

Tissue extracts (approx. 150 mg) were prepared in 500 μ l of ice-cold 50 mM sodium phosphate buffer, pH 7.0, containing 0.05 % (v/v) Igepal [(octylphenoxy)polyethoxyethanol] and 2.5 mg/ml Pefabloc proteinase inhibitor. Extracts were centrifuged twice, each for 10 min at 11 000 g (4 °C) and the supernatant was collected. Aliquots (10 μ g of protein) of the resulting material were subjected to SDS/PAGE and transferred electrophoretically on to nitrocellulose membrane using a Bio-Rad Mini Trans-Blot apparatus. Protein binding sites on the membrane were blocked by placing them overnight in 20 mM Tris/HCl/150 mM NaCl/0.1 % (v/v) Tween, pH 7.4 (buffer B) which contained 10 % (w/v) semi-skimmed dried milk. The blots were incubated with antisera raised against AKR isoenzymes (1:5000 dilution) in buffer B containing 10 % (w/v) semi-skimmed dried milk. After washing, bound antibody was allowed to react for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG) (1:3000 dilution) in buffer B which also contained 10 % (w/v) semi-skimmed dried milk. The antibody complexes were detected by enhanced chemiluminescence (ECL) and visualized following exposure to Hyperfilm-ECL [40].

Resolution of AKR isoenzymes in human kidney

AKRs in human kidney were resolved by anion-exchange chromatography on Q-Sepharose to allow an examination of the relative amounts of the different isoenzymes and to allow a comparison with the profile obtained from the human hepatic enzymes. The protocol used to resolve the reductases on the anion-exchanger was essentially identical with that used previously to isolate human AKR7A2 [32]. Briefly, 38 g of kidney from patient 145 was homogenized in approx. 100 ml of ice-cold 10 mM Tris/HCl buffer, pH 8.2. Renal extracts were centrifuged at 12 000 g for 20 min (4 °C) before the supernatant was collected and recentrifuged at 100 000 g for 90 min (4 °C). The 100 000 g supernatant was dialysed at 4 °C for 18 h against two changes, each of 2 litres, of 10 mM Tris/HCl, pH 8.2, containing 2 mM dithiothreitol (DTT) (buffer C). The dialysed material was applied (32 ml/h) to a 2.2 cm $\times$ 36 cm column of Q-Sepharose that was equilibrated with buffer C at 4 °C. The column was washed with 200 ml of buffer C before a linear 0–250 mM NaCl gradient, formed in 1.2 litres of the same buffer, was employed to separate AKR isoenzymes; chromatography was carried out at 4 °C. Finally, highly anionic material was eluted by the step-wise application of 1 M NaCl in buffer C.

Immunohistochemistry of human kidney

Formalin-fixed paraffin sections of 5 μ m thickness were prepared to allow localization of AKR proteins in macroscopically normal tissue. Furthermore, sections containing a renal adenocarcinoma surrounded by normal kidney were also included in the study.

Sections were mounted on AAS (3-aminopropyltriethoxysilane) slides and processed using standard methodology. Sections were blocked in normal serum before being incubated with primary rabbit anti-AKR antibodies (at a dilution of 1:200 in PBS) for 60 min at 20 °C. After incubation with primary antibody, the slides were washed and re-incubated with Multilink biotinylated goat anti-rabbit IgG antibody (at a dilution of 1:25 in PBS) for 20 min at 20 °C. The excess secondary antibody was removed by washing in PBS and the immune complexes were allowed to react with biotin–streptavidin–horseradish peroxidase conjugate before being visualized by reaction with 3,3'-diaminobenzidine in an aqueous solution of 0.1 % (v/v) H_2O_2 [32].

RESULTS

Molecular cloning of AKR1C4

The first cDNA to be isolated for AKR1C4 (called chlordecone reductase) represented a partial-length clone [28]. Our early attempts to isolate this cDNA from a human liver library by PCR involved the use of oligonucleotides that generated a product lacking the ATG initiator codon. Furthermore, as it was assumed that Met¹⁶ represented the transcriptional start site, the first pET15b expression construct we produced (pTOC2) encoded a truncated protein lacking 15 N-terminal amino acids that was found to be highly insoluble when expressed in *E. coli* BL21pLysS. A literature search revealed that Qin et al. [41] had isolated an AKR1C4 cDNA, called HAKRa, which contains a 45 nt longer 5' coding sequence than the original clone for chlordecone reductase. By designing two complementary oligonucleotides, which encoded the missing N-terminal amino acids and formed *Nde*I overhangs upon hybridization to each other, an expression vector (pTOC3) was generated that encodes the entire AKR1C4 protein. DNA sequencing confirmed that the coding region for the protein was correct, though a single silent A $\rightarrow$ C point mutation was deliberately introduced into the cDNA at position +30 to create an *Sst*I restriction site which, as described in the Materials and methods section, served to prevent multiple oligonucleotide inserts into pTOC2. The strategy used to generate the expression vector for full-length AKR1C4 is shown in Figure 1.

Expression of human AKR isoenzymes in bacteria

Bacterial expression constructs for AKR1A1, AKR1B1 and AKR1C1, based on pET15b, were made as described above.

Figure 2 Electrophoretic properties of purified recombinant human AKR isoenzymes

Heterologously expressed human AKRs purified by nickel-agarose chromatography were examined by SDS/PAGE. The gel was loaded either with size standards (Std, 1 μ g of protein) or recombinant reductase (0.5 μ g of protein) as indicated.

Figure 3 Structures of AKR substrates

Chemical structures of compounds used to characterize human AKRs in Tables 1–5.

Table 1 Catalytic activities of human AKRs towards aromatic and aliphatic aldehydes

Enzyme assays were performed at 25 °C in 100 mM sodium phosphate buffer, pH 7.0, using 0.2 mM NADPH and 1 mM of the aldehyde of interest. Assays were carried out in triplicate and the values shown represent means $\pm$ S.E.M. Examination of activity with each substrate was carried out on three separate occasions and the data presented are typical results. The chemical structures of the substrates are shown in Figure 3.

Substrate	Chemical structure	Enzyme ...	Specific activity (nmol/min per mg of protein)				
			AKR1A1	AKR1B1	AKR1C1	AKR1C4	AKR7A2
Succinic semialdehyde	1		4983 $\pm$ 330	54 $\pm$ 6	< 5	< 5	1441 $\pm$ 131
2-Carboxybenzaldehyde	2		< 5	8 $\pm$ 1	< 5	< 5	1704 $\pm$ 156
3-Carboxybenzaldehyde	3		162 $\pm$ 18	78 $\pm$ 4	< 5	5 $\pm$ 1	29 $\pm$ 3
4-Carboxybenzaldehyde	4		2794 $\pm$ 351	93 $\pm$ 6	< 5	< 5	43 $\pm$ 4
2-Chlorobenzaldehyde	5		134 $\pm$ 6	430 $\pm$ 29	5 $\pm$ 1	5 $\pm$ 1	391 $\pm$ 6
3-Chlorobenzaldehyde	6		3269 $\pm$ 171	701 $\pm$ 65	11 $\pm$ 1	30 $\pm$ 2	162 $\pm$ 1
4-Chlorobenzaldehyde	7		508 $\pm$ 13	416 $\pm$ 37	6 $\pm$ 1	20 $\pm$ 3	37 $\pm$ 1
2-Nitrobenzaldehyde	8		204 $\pm$ 15	62 $\pm$ 3	7 $\pm$ 1	9 $\pm$ 1	946 $\pm$ 15
3-Nitrobenzaldehyde	9		1842 $\pm$ 197	175 $\pm$ 11	< 5	8 $\pm$ 1	443 $\pm$ 49
4-Nitrobenzaldehyde	10		8366 $\pm$ 419	249 $\pm$ 24	< 5	< 5	308 $\pm$ 13
Pyridine-2-aldehyde	11		112 $\pm$ 4	468 $\pm$ 27	8 $\pm$ 1	7 $\pm$ 1	1044 $\pm$ 51
Pyridine-3-aldehyde	12		3460 $\pm$ 49	317 $\pm$ 23	9 $\pm$ 1	11 $\pm$ 1	69 $\pm$ 3
Pyridine-4-aldehyde	13		2927 $\pm$ 127	242 $\pm$ 8	26 $\pm$ 1	14 $\pm$ 2	269 $\pm$ 10
<i>N</i> -Acetyl-leucyl-leucyl-norleucinal	14		11 $\pm$ 1	34 $\pm$ 2	< 5	< 5	< 5

Table 2 Catalytic activities of AKRs towards aromatic ketones and α,β -unsaturated carbonyls

Reductase activity was measured at 25 °C with 0.2 mM NADPH, as described in the text. Results shown are means $\pm$ S.E.M. from three determinations. The chemical structures of the substrates are shown in Figure 3.

Substrate*	Chemical structure	Enzyme ...	Specific activity (nmol/min per mg of protein)				
			AKR1A1	AKR1B1	AKR1C1	AKR1C4	AKR7A2
2-Nitroacetophenone	15		8 $\pm$ 1	18 $\pm$ 1	16 $\pm$ 2	62 $\pm$ 9	< 5
3-Nitroacetophenone	16		12 $\pm$ 1	48 $\pm$ 5	20 $\pm$ 2	45 $\pm$ 4	< 5
4-Nitroacetophenone	17		37 $\pm$ 1	64 $\pm$ 2	7 $\pm$ 1	28 $\pm$ 1	< 5
Ethacrynic acid	18		15 $\pm$ 2	< 5	5 $\pm$ 1	< 5	6 $\pm$ 1
Acrolein	19		268 $\pm$ 18	277 $\pm$ 25	56 $\pm$ 3	67 $\pm$ 1	45 $\pm$ 3
4-Hydroxynonenal	20		1426 $\pm$ 160	189 $\pm$ 13	5 $\pm$ 1	9 $\pm$ 1	21 $\pm$ 2
Doxorubicin	21		11 $\pm$ 1	24 $\pm$ 2	< 5	< 5	< 5
Daunomycin	22		120 $\pm$ 3	109 $\pm$ 10	4 $\pm$ 1	9 $\pm$ 1	6 $\pm$ 1

* Acrolein and 4-hydroxynonenal were measured at concentrations of 10.0 and 4.0 mM respectively. All other substrates were used at a concentration of 1.0 mM.

DNA sequencing of the inserts confirmed that PCR amplification of the cDNAs had not resulted in the introduction of any mutations into the coding regions for any of the enzymes. These three constructs, and that for AKR1C4 (pTOC3), gave high levels of expression of the human reductases in *E. coli*. Furthermore, nickel-agarose chelation chromatography resulted in a high recovery of each of the four AKR1 members, and it was estimated that approx. 1 mg of purified protein was recovered from 100 ml of bacterial culture for each of the four reductases.

In addition to the four constructs encoding AKR1 family members, we have recently reported a bacterial expression construct for human AKR7A2 [32]. All of the cDNAs for these reductases are expressed from pET15b, and therefore the recombinant proteins all contain identically modified N-termini (specifically, they each contain the additional 1.8 kDa peptide MGSSHHHHHSSGLVPRGSH). Electrophoretic examination of the five bacterially expressed recombinant human AKR proteins isolated by nickel-agarose chelation chromatography showed that they all possessed similar mobilities during SDS/PAGE. Amongst the proteins studied, AKR1A1 had the slowest mobility and AKR1B1 had the fastest mobility (Figure 2). By

this method, the apparent molecular masses of the polyhistidine-tagged AKR1A1, AKR1B1, AKR1C1, AKR1C4 and AKR7A2 proteins were estimated to be 39.0 kDa, 37.5 kDa, 38.3 kDa, 38.0 kDa and 38.0 kDa respectively; these results are in good agreement with the calculated molecular masses of 38.4 kDa, 37.7 kDa, 38.6 kDa, 38.9 kDa and 38.5 kDa for the polyhistidine-tagged AKR1A1, AKR1B1, AKR1C1, AKR1C4 and AKR7A2 proteins respectively.

Catalytic activity of recombinant human AKR1 family members

The activity of the purified polyhistidine-tagged AKR isoenzymes was studied using a wide range of carbonyl-containing compounds, the structures of which are shown in Figure 3. The results obtained demonstrate that each of the different family members studied possesses distinct catalytic activity. Although some overlap exists in the substrate specificity of certain of the isoenzymes studied, no universal substrate was found that all AKR isoenzymes metabolize efficiently. Indeed, each AKR possesses unique catalytic features that facilitates their identification.

Table 3 Catalytic activities of human AKRs towards dicarbonyls

The ability of the AKR isoenzymes to reduce various dicarbonyls was measured at 25 °C as described in the Materials and methods section. The chemical structures of the substrates are shown in Figure 3.

Substrate*	Chemical structure	Enzyme ...	Specific activity (nmol/min per mg of protein)				
			AKR1A1	AKR1B1	AKR1C1	AKR1C4	AKR7A2
Phenylglyoxal	23		3163 ± 178	187 ± 5	13 ± 1	12 ± 1	350 ± 9
Methylglyoxal	24		4273 ± 274	380 ± 18	16 ± 1	12 ± 1	429 ± 39
Glyoxal	25		117 ± 5	233 ± 17	14 ± 1	26 ± 2	37 ± 2
Benzil	26		34 ± 1	19 ± 1	37 ± 3	29 ± 2	9 ± 2
2,3-Hexanedione	27		3446 ± 295	398 ± 24	49 ± 3	30 ± 1	79 ± 8
1,2-Cyclohexanedione	28		274 ± 27	134 ± 7	40 ± 4	8 ± 1	28 ± 1
1,2-Naphthoquinone	29		6300 ± 210	397 ± 33	< 5	< 5	2700 ± 139
9,10-Phenanthrenequinone	30		722 ± 73	215 ± 14	55 ± 5	41 ± 3	3100 ± 107
Isatin	31		< 5	306 ± 20	11 ± 1	60 ± 9	1728 ± 51
16-Ketoestrone	32		2545 ± 121	349 ± 5	26 ± 3	10 ± 1	1137 ± 58

* Benzil, 1,2-naphthoquinone, 9,10-phenanthrenequinone and 16-ketoestrone were measured at 0.10, 0.05, 0.10 and 0.20 mM concentrations respectively. All other substrates were measured at 1.0 mM concentrations.

Table 4 Catalytic activities of AKRs towards aldoses and aldehydic acids

Enzyme assays were performed in triplicate at 25 °C in 100 mM sodium phosphate buffer, pH 7.0, with 0.2 mM NADPH as co-factor. The chemical structures of the substrates are shown in Figure 3.

Substrate*	Chemical structure	Enzyme ...	Specific activity (nmol/min per mg of protein)				
			AKR1A1	AKR1B1	AKR1C1	AKR1C4	AKR7A2
D,L-Glyceraldehyde	33		1263 ± 56	441 ± 14	7 ± 1	7 ± 1	15 ± 2
D-Xylose	34		33 ± 3	69 ± 5	5 ± 1	< 5	< 5
D-Glucose	35		11 ± 1	38 ± 5	< 5	< 5	< 5
D-Glucuronic acid	36		5562 ± 343	54 ± 5	< 5	5 ± 1	< 5

* The substrate D,L-glyceraldehyde was measured at a concentration of 1 mM, whereas D-xylose, D-glucose and D-glucuronic acid were all measured at concentrations of 10 mM.

Under 'standard' assay conditions, AKR1A1 was shown to demonstrate characteristically high specific activity towards many aromatic and aliphatic aldehydes, including succinic semialdehyde (SSA), 4-CBA, 3-CBA, 3-nitrobenzaldehyde (3-NBA), 4-NBA, pyridine-3-aldehyde and pyridine-4-aldehyde (Table 1). With α,β -unsaturated carbonyls as substrates, AKR1A1 exhibited high activity with 4-hydroxynonenal (Table 2), and with dicarbonyls it possessed highest activity with phenylglyoxal, methylglyoxal, 2,3-hexanedione, 1,2-NQ and 16-ketoestrone (Table 3). With aldoses and aldehydic acids as substrates, AKR1A1 could reduce both D,L-glyceraldehyde and D-glucuronic acid (Table 4).

It was found that recombinant AKR1B1 exhibited a similarly broad substrate specificity to AKR1A1. Caution must however be taken when comparing AKR1B1 with AKR1A1 because the former reductase generally possesses a lower K_m value than the latter enzyme for many of the aldehydes tested. Therefore the concentration of substituted benzaldehydes used in the assay (1 mM) probably results in saturating kinetics for AKR1B1 but may fail on occasions to saturate AKR1A1 [42,43]. Despite this reservation, the specific activity of AKR1B1 towards many of the substrates examined was significantly lower than that of AKR1A1. A few substrates, such as 4-chlorobenzaldehyde (Table 1), acrolein and daunomycin (both Table 2), were metabolized equally effectively by AKR1A1 and AKR1B1.

The results presented in Tables 1–4 show that the two human

AKR1C family members studied possess relatively poor reductase activity for most of the aldehyde- and ketone-containing compounds: AKR1C1 and AKR1C4 exhibit a small amount of reductase activity towards the aromatic ketones 2-nitroacetophenone and 3-nitroacetophenone, the α,β -unsaturated carbonyl acrolein (Table 2) and the α -dicarbonyls 2,3-hexanedione, 9,10-PQ and isatin (Table 3). It is possible that the concentration of some of these carbonyl substrates in the standard assay is not sufficient to saturate the AKR1C isoenzymes. Although this remains to be established, it seems unlikely from our results that the AKR1C enzymes will make a substantial contribution in human tissues to the reduction of most carbonyls, even at high substrate concentrations. However, both human AKR1C1 and AKR1C4 demonstrate high oxidase activity towards 1-acenaphthenol (Table 5). In addition, AKR1C4 also catalyses the oxidation of androsterone and cholic acid, compounds which are apparently poor substrates for AKR1C1 (Table 5).

Substrate specificity of AKR7A2 and comparison with that of AKR1 isoenzymes

The substrate specificity of AKR7A2 is distinct from that of the AKR1 family members, and from this study it appears to be more restricted in its activity than either AKR1A1 or AKR1B1 (Tables 1–4). It is noteworthy that, unlike AKR1A1 and AKR1B1, AKR7A2 exhibited only low reductase activity to-

Table 5 Oxidation of steroids, bile acids and alcohols by AKRs

The oxidation of AKR substrates was measured at 25 °C in 100 mM glycine buffer, pH 9.0, using 0.2 mM NADP⁺ as co-factor. The chemical structures of the substrates are shown in Figure 3.

Substrate*	Chemical structure	Enzyme ...	Specific activity (nmol/min per mg of protein)				
			AKR1A1**	AKR1B1	AKR1C1	AKR1C4	AKR7A2
1-Acenaphthenol†	37		32 ± 2	19 ± 3	761 ± 46	741 ± 28	< 5
Androsterone‡	38		38 ± 5	20 ± 1	< 5	436 ± 58	< 5
Lithocholic acid§	39		26 ± 1	8 ± 1	32 ± 4	85 ± 2	< 5
Chenodeoxycholic acid	40		45 ± 5	25 ± 2	24 ± 1	540 ± 34	11 ± 1
Cholic acid¶	41		61 ± 4	23 ± 2	< 5	473 ± 19	< 5

*The substrate 1-acenaphthenol was used in the assay at a concentration of 1 mM, whereas all other substrates were used at a concentration of 0.25 mM.

†At 37 °C, AKR1C1 and AKR1C4 were found to have specific activities of 1645 ± 35 and 1951 ± 49 nmol/min per mg of protein.

‡At 37 °C, AKR1C4 was found to have a specific activity of 636 ± 59 nmol/min per mg of protein.

§At 37 °C, AKR1C4 was found to have a specific activity of 201 ± 8 nmol/min per mg of protein.

||At 37 °C, AKR1C1 and AKR1C4 were found to have specific activities of 548 ± 5 and 1133 ± 168 nmol/min per mg of protein.

¶At 37 °C, AKR1C4 was found to have a specific activity of 1074 ± 99 nmol/min per mg of protein.

**The oxidation reactions catalysed by AKR1A1, AKR1B1 and AKR7A2 were only examined at 25 °C.

wards aromatic ketones and α,β -unsaturated aldehydes (Table 2) and aldoses and aldohydro acids (Table 4). Also, AKR7A2 was found to be almost entirely incapable of catalysing the oxidation of hydroxyl groups on steroid ring structures (Table 5). Human AKR7A2 and AKR1A1 were shown to catalyse the reduction of many of the aromatic aldehydes included in this study. On the basis of electron-withdrawing abilities of the substituted benzaldehydes contributing to the reactivity of the carbonyl group, it might be expected that NBAs would be better substrates than CBAs, and in turn CBAs would be better substrates than chlorobenzaldehydes. This predicted trend was not observed. Both AKR7A2 and AKR1A1 have higher catalytic efficiencies (k_{cat}/K_m) for CBAs than for NBAs, and therefore presumably substrate binding to these AKRs is a more important factor during enzyme-catalysed reduction than the inherent reactivity of the carbonyl group (Table 6). It should be noted however that AKR7A2 and AKR1A1 have higher k_{cat}/K_m values for CBAs and NBAs than for chlorobenzaldehydes, possibly indicating that both reductases prefer substrates having a highly negatively charged substitution-group (Table 6). The substitution-group electron-withdrawing profile was not observed with the other reductases studied (Table 1).

Many of the aromatic aldehydes that are reduced by AKR7A2 are substrates for AKR1A1. Examination of the series of substituted benzaldehydes, however, revealed major differences between the specificity of AKR7A2 and AKR1A1 (Table 6). It has already been noted that AKR7A2 has high activity with 2-CBA, and essentially no activity with 3-CBA and 4-CBA [32]. Tables 1 and 6 show that, in contrast, AKR1A1 has high activity with 4-CBA and low activity with both 3-CBA and 2-CBA. None of the chlorobenzaldehydes was found to be a good substrate for AKR7A2 or AKR1A1. Amongst the compounds studied, AKR7A2 has activity that is only modest with just 2-chlorobenzaldehyde, whereas AKR1A1 is most active with 3-chlorobenzaldehyde. Likewise, most of the NBAs examined were poor substrates for AKR7A2 and AKR1A1. It was found that AKR7A2 is most active with 2-NBA, for which its activity is low (Table 6). In contrast, AKR1A1 exhibits reasonable activity towards 4-NBA, with incremental decreases being observed in activity with 3-NBA and 2-NBA (Table 6). Thus, AKR7A2 shows a preference for CBA, chlorobenzaldehyde and NBA when the carboxy-, chloro- and nitro-groups are on the 2- (*ortho*)

position of the benzene ring. In contrast, AKR1A1 shows a preference for these benzaldehydes when the substituted groups are on either the 4- (*para*) or 3- (*meta*) positions of the benzene ring.

Examination of a series of pyridine aldehydes as possible substrates for AKR7A2 again revealed a distinct selectivity for certain isomers, in that the reductase had modest activity with pyridine-2-aldehyde, lower activity with pyridine-4-aldehyde and essentially no activity with pyridine-3-aldehyde (Table 6). In contrast, AKR1A1 had reasonable levels of activity with both pyridine-3-aldehyde and pyridine-4-aldehyde, but only low activity with pyridine-2-aldehyde (Table 6). Thus, there appears to exist a complementarity between AKR7A2 and AKR1A1 in their abilities to reduce aromatic aldehydes.

AKR7A2 displayed reasonably high levels of activity with certain, relatively bulky, α -diketones. It was found that AKR7A2 strongly favours such compounds when they contain at least two ring structures, and when the carbonyls are situated on a ring structure. For example, 1,2-NQ, 9,10-PQ, isatin and 16-ketoestrone are reasonable substrates for the reductase, whereas benzil, which is closely related to 9,10-PQ, does not serve as a substrate (Tables 3 and 6). AKR7A2 was shown to possess essentially no activity towards the relatively small α -diketones 2,3-hexanedione and 1,2-cyclohexanedione (Table 3), and only modest activity with the α -oxo aldehydes phenylglyoxal and methylglyoxal (Table 3). The possibility that failure of AKR7A2 to reduce 1,2-cyclohexanedione or benzil results from either insufficient substrate in the standard assay to saturate the enzyme or product inhibition cannot be excluded, but this appears to be unlikely, as the observed activity signal was almost undetectable. At present, the structural features required for a dicarbonyl to serve as an AKR7A2 substrate are uncertain, though the differences in size between the α -diketones and α -oxo aldehydes, which are substrates for this enzyme, suggest that these two groups of compounds are recognized by the reductase in a distinct fashion. It is interesting to note that AKR7A2 appears to be inactive with benzil (Table 3), an observation which suggests that redox-cycling *per se* is not essential for reduction of dicarbonyls. Table 6 shows that although there is some overlap between the α -dicarbonyls reduced by AKR7A2 and those reduced by AKR1A1, significant differences in specificity exist. For example, only AKR7A2 can catalyse the reduction of isatin,

Table 6 Kinetic properties of recombinant human AKR1A1 and AKR7A2

The activities of polyhistidine-tagged AKR1A1 and AKR7A2 were measured as described in the text. The assays were carried out on at least two separate occasions, and Simple Fit with the Ultrafit curve-fitting program was employed to calculate K_m and k_{cat} values from the initial reaction velocities obtained at 20 or more different substrate concentrations; the values shown represent means $\pm$ S.E.M. Abbreviation: ns, no significant activity.

Substrate	Chemical structure	Enzyme	K_m (μ M)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{M}^{-1}$)
Succinic semialdehyde	1	AKR1A1	231 $\pm$ 19	560 $\pm$ 33	2.5 $\times 10^6$
		AKR7A2	20 $\pm$ 1	113 $\pm$ 9	5.7 $\times 10^6$
2-Carboxybenzaldehyde	2	AKR1A1	ns	ns	ns
		AKR7A2	17 $\pm$ 1	95 $\pm$ 6	5.8 $\times 10^6$
3-Carboxybenzaldehyde	3	AKR1A1	ns	ns	ns
		AKR7A2	ns	ns	ns
4-Carboxybenzaldehyde	4	AKR1A1	24 $\pm$ 1	418 $\pm$ 41	1.7 $\times 10^7$
		AKR7A2	ns	ns	ns
2-Chlorobenzaldehyde	5	AKR1A1	657 $\pm$ 21	9 $\pm$ 1	2.0 $\times 10^4$
		AKR7A2	8584 $\pm$ 269	149 $\pm$ 4	2.0 $\times 10^4$
3-Chlorobenzaldehyde	6	AKR1A1	2413 $\pm$ 232	442 $\pm$ 7	2.0 $\times 10^5$
		AKR7A2	1913 $\pm$ 249	9 $\pm$ 1	5.0 $\times 10^3$
4-Chlorobenzaldehyde	7	AKR1A1	3066 $\pm$ 99	102 $\pm$ 9	3.0 $\times 10^4$
		AKR7A2	ns	ns	ns
2-Nitrobenzaldehyde	8	AKR1A1	54 $\pm$ 4	3 $\pm$ 0.1	5.0 $\times 10^4$
		AKR7A2	1060 $\pm$ 130	95 $\pm$ 7	9.0 $\times 10^4$
3-Nitrobenzaldehyde	9	AKR1A1	1008 $\pm$ 6	101 $\pm$ 1	1.0 $\times 10^5$
		AKR7A2	979 $\pm$ 84	33 $\pm$ 3	3.0 $\times 10^4$
4-Nitrobenzaldehyde	10	AKR1A1	191 $\pm$ 19	417 $\pm$ 5	2.2 $\times 10^6$
		AKR7A2	2742 $\pm$ 320	47 $\pm$ 4	2.0 $\times 10^4$
Pyridine-2-aldehyde	11	AKR1A1	134 $\pm$ 16	3 $\pm$ 0.1	3.0 $\times 10^4$
		AKR7A2	616 $\pm$ 1	60 $\pm$ 2	1.0 $\times 10^5$
Pyridine-3-aldehyde	12	AKR1A1	597 $\pm$ 15	371 $\pm$ 12	6.0 $\times 10^5$
		AKR7A2	259 $\pm$ 4	2 $\pm$ 0.1	6.0 $\times 10^3$
Pyridine-4-aldehyde	13	AKR1A1	794 $\pm$ 69	372 $\pm$ 20	5.0 $\times 10^5$
		AKR7A2	1718 $\pm$ 118	42 $\pm$ 1	2.0 $\times 10^4$
1,2-Naphthoquinone	29	AKR1A1	144 $\pm$ 17	881 $\pm$ 42	6.2 $\times 10^6$
		AKR7A2	102 $\pm$ 10	435 $\pm$ 27	4.2 $\times 10^6$
9,10-Phenanthrenequinone	30	AKR1A1	152 $\pm$ 9	50 $\pm$ 4	3.0 $\times 10^5$
		AKR7A2	8 $\pm$ 0.1	187 $\pm$ 22	2.5 $\times 10^7$
Isatin	31	AKR1A1	ns	ns	ns
		AKR7A2	156 $\pm$ 6	166 $\pm$ 9	1.1 $\times 10^6$
16-Ketoestrone	32	AKR1A1	213 $\pm$ 2	425 $\pm$ 5	2.0 $\times 10^6$
		AKR7A2	55 $\pm$ 6	49 $\pm$ 3	9.0 $\times 10^5$

and it is significantly more efficient than AKR1A1 at metabolizing 9,10-PQ. Conversely, only AKR1A1 can catalyse the reduction of 2,3-hexanedione.

Organ distribution of AKR isoenzymes in man

Polyclonal antibodies have been raised against each of the bacterially expressed human AKR proteins. Western blotting demonstrated that the antibodies obtained each cross-reacted only with the immunogen and not with the other four recombinant AKR members included in the study. Initial experiments were undertaken using liver extracts from four different cadavers to establish whether inter-individual differences in the levels of AKR proteins commonly occur. Immunoblotting of the hepatic 11000 g supernatants from patients 386, 387, 388 and 396 described in the Materials and methods section revealed substantial variation in the amount of certain of the AKR isoenzymes. Whereas AKR1A1 was present in large amounts in livers 386 and 387, it was essentially absent from specimen 388 and was observed at reduced levels in specimen 396 (Figure 4). Immunoreactive AKR1B1 protein was barely detectable in livers 386 and 387 but was present in modest amounts in 388 and significantly greater amounts in 396 (Figure 4). Similarly, lower levels of AKR1C1 were found in specimens 386 and 387 than in 388 and 396. Immunoblotting using antibodies against AKR1C4

and AKR7A2 showed that the levels of these reductases were not highly variable amongst the liver specimens. It is concluded that the apparent decrease in the amount of AKR1A1 in livers 388 and 396, as well as the elevated amounts of AKR1B1 and AKR1C1 in these two specimens, is probably attributable to alcohol-related pathophysiological responses rather than to genetic polymorphisms, because column chromatographic analyses of reductases in human liver have previously demonstrated the presence of significant amounts of AKR1A1, but not AKR1B1, in normal tissue [24,32,44].

Using the antibodies described above in immunoblotting experiments, major differences were demonstrated in the expression of the various AKR isoenzymes in cerebrum, heart, kidney, liver, lung, prostate, skeletal muscle, small intestine, spleen and testes. Amongst these organs, kidney and testis were found to contain most of the reductases. With the exception of AKR1B1 (see above), the other reductases were also detected in the liver. Cerebrum was also found to express substantial amounts of AKR1A1, AKR1B1 and AKR7A2, but AKR1C1 and AKR1C4 did not appear to be represented in this tissue. It was apparent that the expression of the AKR1C family (i.e. 1C1 and 1C4) was more restricted than the expression of AKR1A1, AKR1B1 and AKR7A2. Figure 5 shows that high levels of AKR1A1 are present in kidney and liver, and moderate levels of this reductase are found in cerebrum, small intestine and testis.

Figure 4 Variable expression of AKR1 family proteins in human liver

Soluble protein extracts (11000 *g* supernatant fraction) were prepared from specimens of human liver [33]. These were subjected to SDS/PAGE and examined by Western blotting as described in the Materials and methods section. In each of the panels the sample loaded in the left-hand lane (Std) represents the his-tagged AKR immunogen (0.05 μ g of protein) appropriate to the antibody employed. The remaining samples loaded in lanes 2–5 of the gel represent hepatic extract (10 μ g of protein) from liver specimens 386, 387, 388 and 396 as indicated above the blots. The resolved proteins were probed separately with AKR antibodies as indicated in the left-hand margin.

Relatively small amounts of AKR1A1 were observed in lung, prostate and spleen. Also, this protein was detected in neither heart nor skeletal muscle. In contrast, AKR1B1 was observed in greatest amounts in skeletal muscle and testis, though substantial levels of the reductase were found in all other organs examined except liver (at least from the two cadavers who died from non-alcohol-related disease). Immunoblotting showed that the expression of AKR1C1 was restricted to kidney, liver and testis, and that the expression of AKR1C4 was limited to liver. Lastly, immunoblots for AKR7A2 showed that it is the most widely distributed of all the members of the AKR superfamily (Figure 5). This enzyme was found in highest amounts in cerebrum, kidney, liver, small intestine and testis, though substantial amounts were also seen in heart, prostate, skeletal muscle and spleen, and low levels were observed in lung. AKR7A2 was detected at low levels in pancreas, and therefore it appears to be expressed in all of the organs investigated.

Characterization of human kidney AKR isoenzymes

As immunoblotting indicated that human kidney contains high levels of most AKR isoenzymes, an attempt was made to resolve the reductases from this organ in order to establish the relative amounts of the different enzymes. Anion-exchange chromatography on Q-Sepharose was carried out using essentially identical

Figure 5 Tissue-specific expression of AKR in the human

Extracts of human tissues obtained from patient 386 were prepared in ice-cold sodium phosphate buffer, pH 7.0, containing 0.5% (v/v) Igepal and 2.5 mg/ml Pefabloc [32]. In each panel the SDS/PAGE gel was loaded in lane 1 (Std) with 50 ng of purified recombinant His-tagged AKR immunogen. All other lanes contained tissue extract (10 μ g of protein), the origin of which is indicated at the top of the blots. Once transferred to immobilon-P, the proteins were probed with antibodies as indicated in the left-hand margin.

conditions with those employed by Ireland et al. [32] during the purification of AFAR from human liver. The Q-Sepharose elution profile of renal AKR activities towards 9,10-PQ, 4-NBA, SSA and 2-CBA (Figure 6) is broadly similar to that reported for the human liver enzymes. On the basis of substrate specificity and elution position, the human renal AKR activity, which was recovered in fractions 7–17, 50–57, 62–67, 132–139, 170–177 and 183–186, appears to be equivalent to the liver AKR peaks 1, 4, 5, 6, 8 and 9 respectively [32]; thus, these peaks of renal AKR activity from Q-Sepharose were called K1, K4, K5, K6, K8 and K9 (see Figure 6a). The eluate obtained from the anion-exchange column was analysed using methylglyoxal as a substrate, and the major portion of methylglyoxal activity was recovered in peak K5 (it closely mirrored reductase activity with SSA). In addition, oxidase activity towards 1-acenaphthenol was also studied, and the majority of activity was recovered in K4 and the fractions immediately preceding K4 (i.e. K2 and K3).

The reductase activity towards 2-CBA in human kidney was resolved by anion-exchange chromatography into four peaks, which eluted at Na^+ concentrations of 35, 140, 165 and 900 mmol/l. In contrast, the 2-CBA reductase activity in human liver is recovered from Q-Sepharose columns principally in a single peak, which under the same chromatography conditions employed in this study eluted at an Na^+ concentration of 165 mmol/l. One of these additional renal peaks of activity eluted from Q-Sepharose in fractions 115–119, between K5 and K6, and was designated K6'. The other two renal peaks of 2-CBA activity eluted in K4 and K9.

Figure 6 Resolution of AKR activity in human kidney by anion-exchange chromatography

Renal cytosol (85 ml, 2.4 g of protein) which had been dialysed for 18 h at 4 °C against buffer C was applied to a 2.2 cm × 36.0 cm column of Q-Sepharose. After applying the sample, the column was washed with 200 ml of buffer C before a linear 1200 ml gradient of 0–250 mM NaCl was used to resolve the retained AKR activity. The highly anionic bound protein was finally eluted by the application of 1 M NaCl in buffer C. Fractions of 8 ml were collected and AKR activity towards 2-CBA (▲), SSA (●), 4-NBA (□), 9,10-PQ (◇), methylglyoxal (■), and 1-acenaphthenol (△) was measured. The Na⁺ concentration (○) was determined using an ion-selective electrode.

An additional chromatographic peak of 9,10-PQ activity that had not been observed in the examination of liver extracts was found in kidney. This peak eluted from Q-Sepharose in fractions 58–63, between K4 and K5, and was designated K5'.

Western blotting of the Q-Sepharose eluate was performed to allow identification of the AKR isoenzymes in each of the peaks. The results obtained (Table 7) indicate that AKR1A1 elutes in

K5 and AKR1B1 elutes in K5'. These immunoblotting experiments revealed the presence of a major and a minor form of AKR1B1 that differed in mobility during SDS/PAGE. The major form had an apparent molecular mass of 36.0 kDa and was found predominantly in peaks K5' and K5 from Q-Sepharose. The minor form had a molecular mass of 35.0 kDa and eluted earlier in peak K4. It was found that the proteins

Table 7 Immunochemical properties of AKR chromatographic peaks obtained from Q-Sepharose

The peaks of human kidney AKR activity eluted from Q-Sepharose were examined by Western blotting as described in the text. Each of the antibodies was found to cross-react with more than one peak of carbonyl-reducing activity, and the relative amount of immunoreactivity is indicated by plus signs as follows: + + +, maximal reactivity; + +, strong reactivity; +, modest reactivity; —, no reactivity.

Q-Sepharose peak	Fractions	Cross-reactivity with antibodies against				
		AKR1A1	AKR1B1	AKR1C1	AKR1C4	AKR7A2
K1	7–17	—	—	—	—	—
K2	39–46	—	—	+ +	—	—
K3	47–50	—	—	+ +	—	—
K4	51–57	—	(+)*	+	—	—
K5'	59–62	+	+ + +	—	—	—
K5	63–67	+ + +	+ + +	—	—	—
K6'	115–119	—	—	—	—	+ + +
K6	132–139	—	—	—	—	+ +
K8	170–177	—	—	—	—	—
K9	183–186	—	—	—	—	+

* The polypeptide that reacted with antibody against AKR1B1 in fractions 53–57 had a lower molecular mass than that which cross-reacted with AKR1B1 in peak K5' or K5.

Figure 7 Immunohistochemical localization of AKR in human kidney

Formalin-fixed paraffin sections of kidney were reacted with: **A** (top left) anti-AKR7A2 serum; **B** (top right) anti-AKR1A1 serum; **C** (bottom left) anti-AKR1B1 serum; and **D** (bottom right) negative control. Cross-reacting protein was visualized using avidin-conjugated peroxidase and 3,3'-diaminobenzidine. Abbreviations: B, Bowman's capsule; T, tubules; G, glomerulus.

which cross-reacted with antibodies raised against AKR1C1 were eluted in K2, K3 and K4, and presumably these peaks contain both AKR1C1 and AKR1C2, since it is highly unlikely that the polyclonal antisera can discriminate between these

highly homologous proteins which share 98% amino acid sequence identity [30]. Proteins with identical electrophoretic mobilities (37 kDa) which reacted with anti-AKR7A2 IgG were identified in K6' (i.e. fraction 117), K6 (i.e. fraction 134) and K9

Figure 8 Immunohistochemical localization of AKR in renal adenocarcinoma

Formalin-fixed paraffin sections of tumour-containing kidney were reacted with: **A** (top left) anti-AKR7A2 serum; **B** (top right) anti-AKR1A1 serum; **C** (bottom left) anti-AKR1B1 serum; and **D** (bottom right) negative control. Cross-reacting protein was visualized using avidin-conjugated peroxidase and 3,3'-diaminobenzidine. Abbreviation: A, adenocarcinoma.

(i.e. fraction 184). It is particularly noteworthy that antibodies against AKR7A2 did not cross-react with protein in K4 (fractions 53, 54 and 55), despite the fact that this chromatographic peak contained substantial amounts of 2-CBA reductase activity.

Immunohistochemical localization of AKR in human kidney

Immunohistochemistry with antibodies against AKR7A2, AKR1A1 and AKR1B1 showed expression in proximal and distal tubules, endothelial cells lining the Bowman's capsules and some cysts (Figure 7). The distal tubules were more strongly stained than the proximal tubules. In contrast, neither intraglomerular cells nor blood vessels showed significant staining. As shown in Figure 8, renal adenocarcinoma stained positively with all antisera raised against AKR isoenzymes. Furthermore, immunoreactivity was homogeneous with all tumours staining.

DISCUSSION

The present study was undertaken to determine whether AKR7A2 is catalytically distinct from other human AKRs, and whether its tissue distribution differs from other members of the superfamily. This paper demonstrates that AKR7A2 is functionally unique and that, unlike most AKRs, it is essentially ubiquitously expressed in human organs.

Substrate specificity of AKR7A2 and comparison with other human AKRs

It has been shown previously that both rat and human AKR7 (previously designated rAFAR and hAFAR, and more recently called AKR7A1 and AKR7A2 respectively) exhibit activity towards certain aromatic aldehydes, anionic aliphatic aldehydes and α -dicarbonyls [32,45]. We have now extended the series of potential substrates investigated and compared the substrate specificity of AKR7A2 with that of AKR1A1, AKR1B1, AKR1C1 and AKR1C4. For the purpose of this study, enzyme assays were performed under previously described 'standard' conditions (i.e. 1 mM carbonyl-containing compound [16,24,36–38]) or at the maximum concentration of substrate permitted by its aqueous solubility; the choice of a 1 mM concentration of AKR carbonyl substrate in the assay is reasonable for the study of xenobiotic metabolism, since this level of foreign compound is unlikely to be exceeded *in vivo*.

The present investigation has revealed significant differences in the specificities of AKR isoenzymes. Under the assay conditions employed, AKR7A2 exhibits a higher specific activity with 9,10-PQ, 2-NBA, 2-CBA and isatin than any of the human AKR1A members studied. AKR7A2 also possesses substantial activity with SSA. However, AKR7A2 does not appear to catalyse the reduction of a broad spectrum of carbonyls, and evidence from studying substituted benzaldehydes suggests that the enzyme

possesses a relatively limited catalytic specificity. In contrast with AKR1A1, AKR7A2 was found to have low specific activity (i.e. < 10% of the activity of AKR1A1) with 4-CBA, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde, 4-NBA, pyridine-3-aldehyde, 4-hydroxynonenal and 2,3-hexanedione. In contrast with AKR1A1 and AKR1B1, AKR7A2 showed little activity towards D,L-glyceraldehyde and D-glucuronic acid, and unlike AKR1C1 and AKR1C4, it was not able to catalyse the oxidation of either 1-acenaphthenol or bile acids.

In broad terms, AKR7A2 was found to display an activity towards aromatic aldehydes that is in certain respects complementary to that of AKR1A1. Although in the present project both enzymes catalysed the reduction of aromatic aldehydes, AKR7A2 and AKR1A1 were shown to display selectivity for different isomers. Thus, AKR7A2 is active with 2-CBA, but not 4-CBA, whereas AKR1A1 lacks activity towards the former CBA, but exhibits high activity with the latter. Similarly, AKR7A2 is most active with 2-NBA, and least active towards 4-NBA, whereas AKR1A1 is unable to reduce the former compound but is active with the latter compound. Despite this apparent complementary activity towards aromatic aldehydes, fundamental differences seem to exist between the catalytic properties of the two enzymes towards other types of carbonyls. For example, AKR7A2 lacks activity with α,β -unsaturated carbonyls and D-glucuronic acid compounds, which serve as excellent substrates for AKR1A1.

It should be noted that AKR1A1 is generally a high- K_m aldehyde reductase, whereas AKR1B1 is a low- K_m aldehyde reductase [42,43]. Some of the specific activity values in Tables 1–4, for example with the aromatic aldehydes, methylglyoxal and D,L-glyceraldehyde, may represent saturating kinetics for AKR1B1, but not for AKR1A1. A compilation of some literature values for AKR1A1 and AKR1B1 kinetics has been drawn up (Table 8) to allow comparison between our data and those of others. Table 8 shows that the kinetic values obtained for AKR1A1 during the present study (particularly for 4-CBA and 4-NBA) reflect those published previously. Table 8 also shows that AKR1B1 has significantly lower K_m values for the aromatic aldehydes than either AKR1A1 or AKR7A2. Comparison between Table 6 and Table 8 also reveals that although the NBAs and chlorobenzaldehydes are in general poor substrates for AKR1A1 and AKR7A2, they are relatively good substrates for AKR1B1.

As a consequence of contrasting the activity of AKR7A2 with the activities of other AKRs, we have characterized two members of the AKR1C family in humans whose functional properties have been poorly described hitherto. It was found that both AKR1C1 and AKR1C4 have either extremely low or undetectable reductase activity with most dicarbonyls, aromatic and aliphatic aldehydes, α,β -unsaturated carbonyls, ketones and aldoses (Tables 1–5). However, it was shown that both AKR1C family members can catalyse the oxidation of 1-acenaphthenol, and also that at 25 °C AKR1C4, but not AKR1C1, is able to oxidize androsterone, cholic acid, chenodeoxycholic acid and lithocholic acid.

Physiological roles of AKR7A2 and AKR1 family members in human tissues

Western blotting experiments demonstrated marked differences in the tissue-specific expression of the AKR isoenzymes. AKR7A2 was found to be the most widely distributed of the human reductases studied, and although it was not expressed at exceptionally high levels in any organ, it was detected in all tissues studied. This observation suggests that AKR7A2 is likely to have

additional biological roles besides its putative involvement in drug metabolism. In particular, this enzyme can reduce the γ -aminobutyric acid metabolite SSA to the sedative 4-hydroxybutyrate, and it may therefore play a neurophysiological role [32]. The expression profile for AKR7A2, obtained by Western blotting, is generally in close agreement with that described previously by Northern blotting [32]. However, the Northern analysis showed low levels of AKR7A2 in brain and high levels in pancreas [32], in contrast to this study which showed high levels of the reductase in brain and low levels in pancreas. The possibility that altered transcriptional versus translational expression of AKR7A2 or differences in protein stability may occur in brain and pancreas requires further study.

The expression of AKR1A1 and AKR1B1 in different tissues is similar to that described previously [42,43]. Levels of AKR1A1 in liver and kidney have been reported to be ten times higher than in brain. In agreement with the present study, a number of previous reports have indicated that human liver expresses mainly AKR1A1 and little or no AKR1B1 [24,44,51]. The blots in Figure 5 show AKR1A1 to be expressed primarily in brain, kidney, liver and small intestine, a fact which, taken together with its broad substrate specificity, suggests it acts principally as a detoxification enzyme. In contrast, AKR1B1 is widely distributed in human organs, an observation which suggests that it may have additional roles besides that of detoxification of foreign compounds, such as the production of sorbitol and fructose from glucose [52] and osmoregulation in the kidney [53].

Both AKR1A1 and AKR1B1 may act as local scavengers of osones and methylglyoxal, and may protect cells and tissues against sugar-mediated damage [18,54]. Furthermore, the abilities of AKR1A1 and AKR1B1 to reduce the lipid peroxidation product 4-hydroxynonenal suggests that these reductases serve as a protection mechanism against oxidative stress, particularly AKR1B1 in skeletal muscle because of its remarkably high expression level in this tissue. It is interesting that the testis expressed most of the AKR isoenzymes, and in particular contained high levels of AKR1B1. In addition, AKR1B1 is expressed in the ovaries of the female reproductive system which are responsible for producing ova [47]. Gametes (spermatozoa and ova) carry half the genes determining the genetic make-up of the potential offspring. Therefore, exposure of either the testes or ovaries to oxidative stress or harmful xenobiotics may result in germ-line mutations affecting the gametes. AKR1B1 is possibly expressed in these reproductive organs as a protection mechanism against oxidative stress and chemical insult.

Among the organs studied, AKR1C1 was only observed in kidney, liver and testis, whereas AKR1C4 was restricted to the liver. These results are in good agreement with those in the literature [27,55]. The narrow catalytic activities of the AKR1C enzymes and their limited distribution in human tissues suggests that they possess specific detoxification functions which possibly differ from those of other members of this superfamily. It is possible that human liver AKR1C4, and AKR1C1 present in liver, kidney and testis, function to inactivate and maintain the correct levels of steroid hormones. They may also bind bile acids and facilitate their intracellular transport [25,56].

Multiple forms of AKRs in human kidney

Examination of renal AKRs revealed the presence of several chromatographically distinct forms of AKR7. The two major AKR7-containing peaks resolved by Q-Sepharose anion-exchange chromatography of kidney cytosol appeared to be functionally similar, as they both exhibited activity with 2-CBA and 9,10-PQ. Furthermore, the AKR in both peaks possessed

Table 8 Kinetic properties of human AKR1A1 and AKR1B1

The following kinetic values represent a compilation of previously published data and those obtained during the present investigation.

Substrate	Enzyme	K_m (μ M)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{M}^{-1}$)	Ref.
Succinic semialdehyde	AKR1A1	170	222	1.3×10^6	[46]
	AKR1A1	231	560	2.5×10^6	This study
4-Carboxybenzaldehyde	AKR1A1	25	438	1.8×10^7	[42]
	AKR1A1	24	418	1.7×10^7	This study
2-Chlorobenzaldehyde	AKR1B1	12	437	3.6×10^7	[47]
	AKR1B1	14	138	9.9×10^6	[48]
4-Chlorobenzaldehyde	AKR1A1	940	96	1.0×10^5	[42]
	AKR1A1	3066	102	3.0×10^4	This study
3-Nitrobenzaldehyde	AKR1B1	7	160	2.3×10^7	[48]
	AKR1A1	2400	282	1.2×10^5	[42]
4-Nitrobenzaldehyde	AKR1A1	1008	101	1.0×10^5	This study
	AKR1B1	14	343	2.5×10^7	[47]
Pyridine-3-aldehyde	AKR1A1	193	300	1.6×10^6	[16]
	AKR1A1	191	417	2.2×10^6	This study
Acrolein	AKR1B1	4	25	6.3×10^6	[16]
	AKR1B1	2	618	3.1×10^8	[47]
4-Hydroxynonenal	AKR1B1	80	87	1.0×10^6	[48]
	AKR1B1	22	102	5.0×10^6	[48]
Phenylglyoxal	AKR1B1	4	60	1.5×10^7	[48]
	AKR1A1	1300	438	3.4×10^5	[42]
Methylglyoxal	AKR1B1	8	142	1.8×10^7	[48]
	AKR1B1	514	154	3.0×10^5	[48]
Glyoxal	AKR1A1	3400	560	1.7×10^5	[49]
	AKR1B1	7	109	1.6×10^7	[49]
D-Xylose	AKR1A1	711 000	75	1.1×10^2	[38]
	AKR1B1	5000	31	6.2×10^3	[50]
D-Glucose	AKR1A1	2 500 000	246	9.8×10^1	[49]
	AKR1B1	70 000	64	9.1×10^2	[49]
D-Glucuronic acid	AKR1A1	3600	1250	3.5×10^5	[49]
	AKR1B1	2800	81	2.9×10^4	[49]
NADPH	AKR1A1	4	66	3.3×10^7	[16]
	AKR1B1	2	23	1.2×10^7	[16]

similar mobilities during SDS/PAGE, and both cross-reacted equally during Western blotting with antibodies raised against the recombinant protein. As shown in Figure 6, the two peaks from Q-Sepharose were called K6' and K6 and these were eluted from the anion-exchanger at Na^+ concentrations of 140 and 165 mmol/l respectively. The former peak, K6', appears to be the principal form in kidney. Previous work has shown that AFAR in human liver elutes from Q-Sepharose as a single peak at an Na^+ concentration of 165 mmol/l, and it is therefore assumed that the renal AKR7 protein eluted from Q-Sepharose in peak K6 is equivalent to the hepatic form of the enzyme. The final 1 M NaCl stepped elution of Q-Sepharose allowed the recovery of a further peak (K9) of reductase activity which also cross-reacted with antibodies against recombinant AKR7A2. It remains to be established whether the reductases in peak K6', peak K6 and peak K9 are genetically distinct, but it is apparent that the three ionically distinct forms of the enzyme are catalytically similar. After submission of the present study for publication, Knight et al. [57] reported the cloning of a cDNA for a second human member of the AKR7 family. The deduced amino acid sequence of this additional reductase (hAFAR2 or AKR7A3) shares 88 % identity with the human AFAR(1) isoenzyme described by Ireland et al. [32]. It remains to be determined whether hAFAR2/AKR7A3 is present in K6' or K9.

The use of Q-Sepharose to resolve AKRs has revealed the existence of a novel, relatively neutral, 2-CBA and 9,10-PQ reductase in human kidney. This enzyme was eluted early on the salt gradient and was recovered between fractions 52 and 60

within peak K4 in the chromatogram shown in Figure 6. Western blotting data indicates that this reductase is immunochemically separate from AKR1A1, AKR1B1 and AKR7A2. This enzyme also appears to be expressed in human liver (peak 4) [32]. The identity of the enzyme responsible for this 2-CBA and 9,10-PQ activity is not known, however, it could be due to carbonyl reductase or another member of the short-chain dehydrogenase/reductase family. Alternatively, since AKR1C1 antibodies were immunoreactive with the protein in this peak, and because multiple dihydrodiol dehydrogenase isoenzymes exist in humans [24], it is possible that the unidentified human reductase is a novel AKR1C member with an activity similar to that of rat 3α -HSD.

Western blotting of column eluate from chromatography of kidney cytosol on Q-Sepharose revealed that in addition to the major 36 kDa form of AKR1B1 (peak 5') a less abundant immunochemically related polypeptide of 35 kDa was also recovered in peak 4. Although a number of aldose reductase-related proteins have been described in the literature [14,47], the possibility exists that the 35 kDa polypeptide represents a post-synthetically modified form of AKR1B1. This possibility was not pursued, as the 35 kDa polypeptide was not a major reductase in the kidney.

Regulation of AKR isoenzymes and contribution to drug resistance

The expression of AKRs has been examined in a small number of liver specimens, both with and without obvious evidence of alcohol-induced disease. This study revealed that the amount of

AKR1B1 protein can be highly variable (Figure 4). In agreement with previous enzyme-based investigations [32], hepatic cytosols from individuals with histologically normal livers were found to contain no detectable AKR1B1, whereas hepatic cytosols from individuals who died from disease associated with alcohol abuse contained elevated amounts of the enzyme. These results suggest that the expression of aldose reductase in human liver is inducible either by alcohol and its associated metabolites, such as acet-aldehyde [13], or by the products of lipid peroxidation, such as 4-hydroxynonenal, that are elevated in livers exposed to ethanol [58]. The hypothesis that AKR1B1 is inducible by chemicals has been confirmed in colon cancer HT-29 cells using the redox-cycling agent *t*-butylhydroquinone and the α,β -unsaturated carbonyl diethylmaleate (N. Pandit, T. O'Connor, L. I. McLellan and J. D. Hayes, unpublished work). It is therefore postulated that overexpression of AKR1B1 represents an adaptive response of human liver to the aldehydes generated by exposure to ethanol. Significantly, aldose reductase in rat smooth muscle is inducible by H_2O_2 and 4-hydroxynonenal [59]. It is possible that in both humans and the rat, aldose reductase is regulated by reactive oxygen species and reactive carbonyl-containing compounds; such inducing agents are likely to regulate gene expression through the antioxidant responsive element (for a review, see [60]). It has been found that 29% of human hepatocellular carcinomas overexpress AKR1B1 [47], an observation which suggests that this enzyme is not only induced by chemicals but might also be regulated by cytokines and growth factors produced in response to tissue injury. This possibility merits further investigation.

Besides the inducibility of aldose reductase, our study has shown that AKR1C1 is also overexpressed in the livers of the individuals who drank excessive amounts of alcohol. The possibility that AKR1C1 is inducible by ethanol and its metabolites appears highly likely because this enzyme can be induced in human HT-29 cells by *t*-butylhydroquinone and ethacrynic acid [61]. Significantly, AKR1C1 is also inducible in HepG2 human hepatoma cells by the same compounds plus metabolizable polycyclic aromatic hydrocarbons and the redox-active dimethoxy-1,4-naphthoquinone [62]. The present work, along with that of others, suggests that several human AKR are co-ordinately regulated in response to stress caused by the actions of reactive oxygen species and thiol-active compounds.

Ciacco et al. [30,61] have shown that AKR1C1 is also overexpressed in an ethacrynic acid-resistant human colon cell line which was derived from HT-29 cells. Our finding that AKR1C1 is unable to metabolize ethacrynic acid suggests that overexpression of the reductase is probably not responsible for resistance to the α,β -unsaturated carbonyl. The most likely explanation for tolerance of ethacrynic acid in the resistant colon cells is that, in addition to overexpression of AKR1C1, they also overexpress the class Pi glutathione S-transferase P1-1, an enzyme that efficiently detoxifies α,β -unsaturated carbonyls [63].

Immunohistochemical localization of AKR isoenzymes in human kidney

AKR1B1 reduces glucose to the non-charged, non-diffusible osmolyte sorbitol. In kidney, the collecting duct cells [64] and medullary tubules of the loop of Henle are bathed in hypertonic interstitial fluid [65]. Graded increments in the tonicity of this fluid cause increases in the expression of AKR1B1 and subsequent increases in sorbitol formation [66]. Sorbitol, in combination with other osmolytes, such as *myo*-inositol, betaine and glycerophosphorylcholine, balance the extracellular hypertonicity and protect cells against high salt concentrations and urea [67].

AKR1B1 is therefore believed to play a role in osmoregulation and its gene is transcriptionally regulated through an osmotic response element by hyperosmotic stress [68]. The presence of AKR1A1 and AKR7A2 in the tubules of the kidney indicates that these enzymes may play a similar role to AKR1B1 in regulating the osmolarity of kidney cells.

Previous studies have shown that AKR1B1 is expressed in the cortex and in the medulla of kidney [69], showing highest expression in the medulla where cells are exposed to salt and urea concentrations that may reach molar levels [67]. AKR1A1 has also been shown previously to be expressed in the cortex and the medulla [69], with levels of expression exceeding those of AKR1B1 [70]. AKR1B1 is induced by osmotic stress in glomerular endothelial cells [71]. This study has shown that AKR1A1 and AKR7A2, like AKR1B1, are expressed in glomerulus. It remains to be established whether AKR1A1 and AKR7A2 isoenzymes are osmoregulated.

Figure 8 shows that AKR1A1, AKR1B1 and AKR7A2 are expressed in human kidney adenocarcinoma. Renal tumours are generally intrinsically resistant to cancer chemotherapy, and it is possible that the expression of AKR contributes to clinical drug resistance.

Conclusion

This study has revealed that major differences exist in both the function and regulation of AKR7A2 and the other members of the AKR superfamily. Although AKR7A2 is the most widely expressed of the AKRs we have studied it possesses a limited substrate specificity, facts which suggest that it performs some physiological role besides that of detoxification. In human liver, the levels of AKR7A2 show little inter-individual variation, suggesting that the hepatic enzyme is not highly inducible. However, evidence is presented that AKR1B1 and AKR1C1 are inducible in human liver.

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REFERENCES

- 1 Stadtman, E. R. and Berlett, B. S. (1997) *Chem. Res. Toxicol.* **10**, 485–494
- 2 Wiseman, H. and Halliwell, B. (1996) *Biochem. J.* **313**, 17–29
- 3 Henle, E. S. and Linn, S. (1997) *J. Biol. Chem.* **272**, 19095–19098
- 4 Snyder, R. and Andrews, L. S. (1996) in Casarett and Doull's *Toxicology: the Basic Science of Poisons*, 5th Edn. (Klaassen, C. D., ed.), pp. 737–771, McGraw-Hill, New York
- 5 Tipton, K. F. (1980) in *Enzymatic Basis of Detoxication*, Vol. I (Jakoby, W. B., ed.), pp. 355–370, Academic Press, New York
- 6 Cavalieri, E. L., Stack, D. E., Devanesan, P. D., Todorovic, R., Dwivedy, I., Higginbotham, S., Johansson, S. L., Patil, K. D., Gross, M. L., Gooden, J. K. et al. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10937–10942
- 7 McMahon, R. E. (1982) in *Metabolic Basis of Detoxication* (Jakoby, W. B., ed.), pp. 91–104, Academic Press, New York
- 8 Zollner, H., Schaur, R. J. and Esterbauer, H. (1991) in *Oxidative Stress: Oxidants and Antioxidants* (Sies, H., ed.), pp. 337–369, Academic Press, New York
- 9 Powis, G. (1994) in *Anticancer Drugs: Reactive Metabolism and Drug Interactions* (Powis, G., ed.), pp. 273–386, Pergamon Press Ltd, Oxford
- 10 Inoue, S., Sharma, R. C., Schimke, R. T. and Simoni, R. D. (1993) *J. Biol. Chem.* **268**, 5894–5898
- 11 O'Dwyer, P. J., LaCreta, F., Nash, S., Tinsley, P. W., Schilder, R., Clapper, M. L., Tew, K. D., Panting, L., Litwin, S., Comis, R. L. and Ozols, R. F. (1991) *Cancer Res.* **51**, 6059–6065
- 12 Molowa, D. T., Shayne, A. G. and Guzelian, P. S. (1986) *J. Biol. Chem.* **261**, 12624–12627

- 13 Parkinson, A. (1996) in Casarett and Doull's Toxicology: The Basic Science of Poisons, 5th Edn. (Klaassen, C. D., ed.), pp. 113–186, McGraw-Hill, New York
- 14 Jez, J. M., Bennett, M. J., Schlegel, B. P., Lewis, M. and Penning, T. M. (1997) *Biochem. J.* **326**, 625–636
- 15 Petrash, J. M. and Srivastava, S. K. (1982) *Biochim. Biophys. Acta* **707**, 105–114
- 16 Bohren, K. M., Page, J. L., Shanker, R., Hendry, S. P. and Gabbay, K. H. (1991) *J. Biol. Chem.* **266**, 24031–24037
- 17 Vander Jagt, D. L., Robinson, B., Taylor, K. K. and Hunsaker, L. A. (1992) *J. Biol. Chem.* **267**, 4364–4369
- 18 Feather, M. S., Flynn, T. G., Munro, K. A., Kubiseski, T. J. and Walton, D. J. (1995) *Biochim. Biophys. Acta* **1244**, 10–16
- 19 Penning, T. M., Pawlowski, J. E., Schlegel, B. P., Jez, J. M., Lin, H.-K., Hoog, S. S., Bennett, M. J. and Lewis, M. (1996) *Steroids* **61**, 508–523
- 20 Smithgall, T. E., Harvey, R. G. and Penning, T. M. (1986) *J. Biol. Chem.* **261**, 6184–6191
- 21 Nakagawa, M., Tsukada, F., Nakagawa, T., Matsuura, K., Hara, A. and Sawada, H. (1989) *J. Biochem.* **106**, 633–638
- 22 Klein, J., Seidel, A., Frank, H., Oesch, F. and Platt, K. L. (1991) *Chem.-Biol. Interact.* **79**, 287–303
- 23 Deyashiki, Y., Taniguchi, H., Amano, T., Nakayama, T., Hara, A. and Sawada, H. (1992) *Biochem. J.* **282**, 741–746
- 24 Ohara, H., Miyabe, Y., Deyashiki, Y., Matsuura, K. and Hara, A. (1995) *Biochem. Pharmacol.* **50**, 221–227
- 25 Stolz, A., Hammond, L., Lou, H., Takikawa, H., Ronk, M. and Shively, J. E. (1993) *J. Biol. Chem.* **268**, 10448–10457
- 26 Hara, A., Matsuura, K., Tamada, Y., Sato, K., Miyabe, Y., Deyashiki, Y. and Ishida, N. (1996) *Biochem. J.* **313**, 373–376
- 27 Khanna, M., Qin, K.-N., Wang, R. W. and Cheng, K.-C. (1995) *J. Biol. Chem.* **270**, 20162–20168
- 28 Winters, C. J., Molowa, D. T. and Guzelian, P. S. (1990) *Biochemistry* **29**, 1080–1087
- 29 Deyashiki, Y., Ogasawara, A., Nakayama, T., Nakanishi, M., Miyabe, Y., Sato, K. and Hara, A. (1994) *Biochem. J.* **299**, 545–552
- 30 Ciaccio, P. J. and Tew, K. D. (1994) *Biochim. Biophys. Acta* **1186**, 129–132
- 31 Ellis, E. M., Judah, D. J., Neal, G. E. and Hayes, J. D. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10350–10354
- 32 Ireland, L. S., Harrison, D. J., Neal, G. E. and Hayes, J. D. (1998) *Biochem. J.* **332**, 21–34
- 33 Sherratt, P. J., Pulford, D. J., Harrison, D. J., Green, T. and Hayes, J. D. (1997) *Biochem. J.* **326**, 837–846
- 34 Bohren, K. M., Bullock, B., Wermuth, B. and Gabbay, K. H. (1989) *J. Biol. Chem.* **264**, 9547–9551
- 35 Petrash, J. M., Harter, T. M., Devine, C. S., Olins, P. O., Bhatnagar, A., Liu, S.-Q. and Srivastava, S. K. (1992) *J. Biol. Chem.* **267**, 24833–24840
- 36 Ram, D., Tulsiani, P. and Touster, O. (1977) *J. Biol. Chem.* **252**, 2545–2550
- 37 Sawada, H., Hara, A., Nakayama, T. and Kato, F. (1980) *J. Biochem. (Tokyo)* **87**, 1153–1165
- 38 Bohren, K. M., Grimshaw, C. E. and Gabbay, K. H. (1992) *J. Biol. Chem.* **267**, 20965–20970
- 39 Hayes, J. D. and Mantle, T. J. (1986) *Biochem. J.* **233**, 779–788
- 40 Ellis, E. M., Judah, D. J., Neal, G. E., O'Connor, T. and Hayes, J. D. (1996) *Cancer Res.* **56**, 2758–2766
- 41 Qin, K.-N., New, M. I. and Cheng, K.-C. (1993) *J. Steroid Biochem. Mol. Biol.* **46**, 673–679
- 42 Wermuth, B., Munch, J. D. B. and von Wartburg, J.-P. (1977) *J. Biol. Chem.* **252**, 3821–3828
- 43 Grimshaw, G. E. and Mathur, E. J. (1989) *Anal. Biochem.* **176**, 66–71
- 44 von Wartburg, J.-P. and Wermuth, B. (1980) *Methods Enzymol.* **89**, 506–513
- 45 Ellis, E. M. and Hayes, J. D. (1995) *Biochem. J.* **312**, 535–541
- 46 Barski, O. A., Gabbay, K. H., Grimshaw, C. E. and Bohren, K. M. (1995) *Biochemistry* **34**, 11264–11275
- 47 Cao, D., Fan, S. T. and Chung, S. S. M. (1998) *J. Biol. Chem.* **273**, 11429–11435
- 48 Vander Jagt, D. L., Kolb, N. S., Vander Jagt, T. J., Chino, J., Martinez, F. J., Hunsaker, L. A. and Royer, R. E. (1995) *Biochim. Biophys. Acta* **1249**, 117–126
- 49 Vander Jagt, D. L., Hunsaker, L. A., Robinson, B., Strangebye, L. A. and Deck, L. M. (1990) *J. Biol. Chem.* **265**, 10912–10918
- 50 Wermuth, B. (1982) in *Enzymology of Carbonyl Metabolism* (Weiner, H. and Wermuth, B., eds.), pp. 261–274, Alan R. Liss, Inc., New York
- 51 Wirth, H. P. and Wermuth, B. (1985) in *Enzymology of Carbonyl Metabolism*, 2nd Edn. (Flynn, T. G. and Weiner, H., eds.), pp. 231–239, Alan R. Liss, Inc., New York
- 52 Bagnasco, S., Uchida, S., Balaban, R., Kador, P. and Burg, M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1718–1720
- 53 Smardo, Jr., F. L., Burg, M. B. and Garcia-Perez, A. (1992) *Am. J. Physiol.* **262**, C776–C782
- 54 Monnier, V. M., Sell, D. R., Nagaraj, R. H. and Miyata, S. (1991) *Gerontology* **37**, 152–165
- 55 Penning, T. M., Bennett, M. J., Smith-Hoog, S., Schlegel, B. P., Jez, J. M. and Lewis, M. (1997) *Steroids* **62**, 101–111
- 56 Hara, A., Matsuura, K., Tamada, Y., Sato, K., Miyabe, Y., Deyashiki, Y. and Ishida, N. (1996) *Biochem. J.* **313**, 373–376
- 57 Knight, L. P., Primiano, T., Groopman, J. D., Kensler, T. W. and Sutter, J. R. (1999) *Carcinogenesis* **20**, 1215–1223
- 58 Chen, J. J., Schenker, S. and Henderson, G. I. (1997) *Hepatology* **25**, 142–147
- 59 Spycher, S. E., Tabataba-Vakili, S., O'Donnell, V. B., Palomba, L. and Azzi, A. (1997) *FASEB J.* **11**, 181–188
- 60 Hayes, J. D., Ellis, E. M., Neal, G. E., Harrison, D. J. and Manson, M. M. (1999) in *Cellular Responses to Stress* (Downes, C. P., Wolf, C. R. and Lane, D. P., eds.), *Biochem. Soc. Symp.* **64**, pp. 141–168, Portland Press Ltd., London
- 61 Ciaccio, P. J., Jaiswal, A. K. and Tew, K. D. (1994) *J. Biol. Chem.* **269**, 15558–15562
- 62 Burczynski, M. E., Lin, H.-K. and Penning, T. M. (1999) *Cancer Res.* **59**, 607–614
- 63 Hayes, J. D. and Pulford, D. J. (1995) *Crit. Rev. Biochem. Mol. Biol.* **30**, 445–600
- 64 Jans, A. W., Grunewald, R. W. and Kinne, R. K. (1988) *Biochim. Biophys. Acta* **971**, 157–162
- 65 Tomlinson, D. R., Stevens, E. J. and Diemel, L. T. (1994) *Trends Pharmacol. Sci.* **15**, 293–297
- 66 Bagnasco, S. M., Murphy, H. R., Bedford, J. J. and Burg, M. B. (1988) *Am. J. Physiol.* **254**, 788–792
- 67 Burg, M. B. (1995) *Am. J. Physiol.* **268**, F983–F996
- 68 Ruepp, B., Bohren, K. M. and Gabbay, K. H. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8624–8629
- 69 Ansari, N. H. and Srivastava, S. K. (1993) *Biochem. Mol. Biol. Int.* **30**, 37–44
- 70 Robinson, B., Hunsaker, L. A., Strangebye, L. A. and Vander-Jagt, D. L. (1993) *Biochim. Biophys. Acta* **1203**, 260–266
- 71 Hohman, T. C., Carper, D., Dasgupta, S. and Kaneko, M. (1991) *Adv. Exp. Med. Biol.* **284**, 139–152

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