Purification from rat liver of a novel constitutively expressed member of the aldo-keto reductase 7 family that is widely distributed in extrahepatic tissues

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Antiserum raised against human aflatoxin B₁ aldehyde reductase 1 (hAFAR1) has been used to identify a previously unrecognized rat aldo-keto reductase (AKR). This novel enzyme is designated rat aflatoxin B₁ aldehyde reductase 2 (rAFAR2) and it characteristically migrates faster during SDS/PAGE than does the archetypal ethoxyquin-inducible rAFAR protein (now called rAFAR1). Significantly, rAFAR2 is essentially unreactive with polyclonal antibodies raised against rAFAR1. Besides its distinct electrophoretic and immunochemical properties, rAFAR2 appears to be regulated differently from rAFAR1 as it is expressed in most rat tissues and does not appear to be induced by ethoxyquin. Multiple forms of rAFAR2 have been identified. Anion-exchange chromatography on Q-Sepharose, followed by adsorption chromatography on columns of Matrex Orange A and Cibacron Blue, have been employed to purify rAFAR2 from rat liver cytosol. The Q-Sepharose chromatography step resulted in the resolution of rAFAR2 into three peaks of AKR activity, two of which were purified and shown to be capable of catalysing the reduction of 2-carboxybenzaldehyde, succinic semialdehyde, 4-nitrobenzaldehyde and 9,10-phenanthrenequinone. The two most highly purified rAFAR2-containing preparations eluted from the Cibacron Blue column were 91 and 98 % homogeneous.

Analysis of these by SDS/PAGE indicated that the least anionic (peak CBA5) comprised a polypeptide of 37.0 kDa, whereas the most anionic (peak CBA6) contained two closely migrating polypeptides of 36.8 and 37.0 kDa; by contrast, in the present study, rAFAR1 was estimated by SDS/PAGE to be composed of 38.0 kDa subunits. Final purification of the 37 kDa polypeptide in CBA5 and CBA6 was accomplished by reversed-phase HPLC. Partial proteolysis of the two preparations of the 37 kDa polypeptide with *Staphylococcus aureus* V8 protease yielded fragments of identical size, suggesting that they represent the product of a single gene. Furthermore, the peptide maps from CBA5 and CBA6 differed substantially from that yielded by rAFAR1, indicating that they are genetically distinct from the inducible reductase. A peptide generated by CNBr digestion of the 37 kDa polypeptide from CBA6 was shown by Edman degradation to share 88 % sequence identity with residues Tyr¹⁶⁸–Leu¹⁸³ of rAFAR1. This provides evidence that the rat protein identified by its cross-reactivity with anti-hAFAR1 serum is an additional member of the AKR7 family.

Key words: carboxybenzaldehyde, γ -hydroxybutyrate, phenanthrenequinone, succinic semialdehyde, tissue-specific expression.

INTRODUCTION

The aldo-keto reductase (AKR) superfamily comprises soluble NAD(P)(H)-dependent oxidoreductases that typically catalyse the reduction of carbonyl-containing compounds and/or the oxidation of alcohols [1,2]. Endogenous substrates for AKR include monosaccharides [3,4], prostaglandins [5,6] and steroids [7], neurotransmitter aldehydes produced by monoamine oxidase [8] and reactive α,β -unsaturated carbonyls arising from lipid peroxidation [9,10]. In addition, AKR metabolizes numerous harmful compounds of foreign origin, including cytotoxic therapeutic drugs, polycyclic aromatic hydrocarbons, mycotoxins, isoflavonoid phytoalexins, herbicides and pesticides [11–14].

Amino acid sequence alignments have allowed division of AKR into seven multigene families [1,2]. The AKR1 family contains all of the better characterized mammalian isoenzymes, including aldehyde reductase, aldose reductase, chlordecone reductase, 3 α -hydroxysteroid dehydrogenase (also called dihydrodiol dehydrogenase), prostaglandin F synthase, and

Δ^4 -3 oxosteroid 5 β -reductase [15]. As indicated by their names, the AKR1 proteins exhibit diverse activities; they are widely distributed and have been identified in human, rat, mouse, cow, pig, rabbit, frog and barley. The AKR2–5 families comprise reductases from bacteria, yeast and plants, and do not appear to be represented in mammalian species [1,2]. Proteins placed in the AKR6 family are β -subunits of the *Shaker*-related voltage-gated K⁺ channels isolated from human, rat and cow [1,16–18]. It has been proposed that β -subunits mediate the regulation of the K⁺ channel by intracellular chemical signals, presumably carbonyl groups, though it remains to be established whether β -subunits function as enzymes. Finally, the AKR7 family has been identified in rat and human [19–22]. Whilst only distantly related to other AKR families, the mammalian AKR7 isoenzymes catalyse the reduction of many of the aromatic aldehydes and α -dicarbonyls which are metabolized by aldehyde reductase and aldose reductase [21–23].

Protein crystallography has shown that human aldose reductase (AKR1B1), rat 3 α -hydroxysteroid dehydrogenase

Abbreviations used: AFAR, aflatoxin B₁-aldehyde reductase; hAFAR, human AFAR; rAFAR, rat AFAR; AFB₁, aflatoxin B₁; AKR, aldo-keto reductase; 2-CBA, 2-carboxybenzaldehyde; CBA1–6, chromatography peaks 1–6 containing reductase activity towards 2-carboxybenzaldehyde; DTT, dithiothreitol; ECL[®], enhanced chemiluminescence (Amersham); GABA, γ -aminobutyric acid; GHB, γ -hydroxybutyric acid; 4-NBA, 4-nitrobenzaldehyde; NQO1, NAD(P)H:quinone oxidoreductase 1; 9,10-PQ, 9,10-phenanthrenequinone; SSA, succinic semialdehyde; UTR, untranslated region.

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(AKR1C9) and mouse fibroblast-growth-factor-1-induced protein (AKR1B8) are monomeric proteins and share a common (α/β)₈-barrel three-dimensional fold [24–26]. Such studies have demonstrated that the active centres of the AKR1 enzymes are structurally similar. Moreover, their catalytic mechanism is distinct from that employed by either the long-chain alcohol dehydrogenase or the short-chain dehydrogenase/reductase superfamilies, some of which can metabolize compounds that are also AKR substrates [15]. The crystal structure of the rat voltage-gated K⁺-channel β -subunit 2 (Kv β 2 or AKR6A2) has also been solved [27]. This has shown that, although AKR6A2 possesses a (α/β)₈-barrel fold similar to that of the AKR1 family members, and can bind nicotinamide cofactor, it is capable of forming tetramers. This is an important observation, because it means that the quaternary structure of AKR proteins is variable.

The body of literature describing the biological activities of AKR7 isoenzymes is relatively small. The founding member of this family (AKR7A1) was discovered in rat liver as an aflatoxin B₁-aldehyde reductase (rAFAR1) that was induced by feeding rodents on diets containing the synthetic antioxidant 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin) [19]. Subsequent work revealed that rAFAR1 is induced by a range of xenobiotics, including butylated hydroxyanisole, coumarin, 1,2-dithiole-3-thione, β -naphthoflavone, oltipraz and *trans*-stilbene oxide [28–30], most of which are believed to transcriptionally activate gene expression through the antioxidant responsive element [31]. Following characterization of this inducible rat AKR, two related human reductases, called hAFAR1 (or AKR7A2) [22] and hAFAR2 (or AKR7A3) [32], were isolated by molecular-cloning techniques. Functional studies of the rat and human AKR7 isoenzymes have shown that, under *in vitro* conditions, aflatoxin B₁-aldehyde is not a particularly good substrate for this family of reductases [21,22]. Both rAFAR1 and hAFAR1 have substantial activity towards succinic semialdehyde (SSA) [21,22], suggesting that this compound, which is derived from γ -aminobutyric acid, may represent an important physiological substrate for the AKR7 enzymes. In addition to exhibiting SSA reductase activity, rAFAR1 and hAFAR1 are able to metabolize 2-carboxybenzaldehyde (2-CBA) [22], a compound that is structurally related to SSA and has been shown to be reduced *in vivo* by rats and man [33]. Among aldo-keto reductases, 2-CBA appears to be a relatively specific substrate for AKR7 family members [23].

In the light of the finding that humans possesses two AFAR isoenzymes, we have been prompted to consider whether the rat also possesses multiple AFARs. In the present study polyclonal antibodies raised against hAFAR1 have been used to reveal the existence of an immunochemically related protein in numerous rat tissues, which is not induced by ethoxyquin and is not recognized by anti-rAFAR1 serum. The novel immunoreactive protein exists in several forms, and these have been purified from rat liver. As described below, the purified protein has been designated rAFAR2 because it is catalytically active with the carbonyl-containing substrates 2-CBA and SSA. Though rAFAR2 is distinct from rAFAR1, it is structurally related and is a member of the AKR7 family.

MATERIALS AND METHODS

Reagents

Chemicals were of the highest grade and were readily available commercially. Matrex Orange A chromatography gel was obtained from Amicon Ltd. (Stonehouse, Gloucestershire, U.K.). The pre-packed HiTrap Blue columns as well as the Q-Sepharose and Sephadex G-150 chromatography gels, and *Staphylococcus*

aureus V8 protease, were purchased from Sigma Chemical Co (Poole, Dorset, U.K.).

Buffers

These were prepared at 20 °C, and the pH values quoted are those obtained at ambient temperature.

Animals

The initial study into possible existence of multiple AFAR proteins used as tissue source livers from 12 week old male Fischer 344 rats fed on either a control diet or on a diet containing 0.5% (w/w) ethoxyquin for the 14 days immediately before sacrifice. The Fischer rats for this analysis were purchased from Harlan Olac Ltd. (Bicester, Oxon., U.K.), and the feeding experiments performed by colleagues at the MRC Toxicology Unit, University of Leicester, Leicester, U.K. (for further details see [34]). In all other studies, 12-week-old male Sprague–Dawley rats (from Imperial Cancer Research Fund, Biological Resource, Clare Hall, Potters Bar, Herts, U.K.) were used as the tissue source. Upon removal, the organs from Fischer 344 and Sprague–Dawley rats were snap-frozen, shipped to Dundee on solid CO₂, and stored at –70 °C until use.

Human material

Several samples of normal human liver cytosol were examined during the course of the study to allow comparison of hepatic polypeptides in rat and man that cross-react with antibodies against various AFAR proteins. The liver sample examined was from patient 387 who died from a cerebral infarct; the specimen has been characterized previously [35].

Cell culture

Rat H4IIE hepatoma cells were from the European Collection of Animal Cell Cultures. The cells were seeded at a density of 2×10^4 into T-75 flasks containing minimal essential medium with Earle's salts and glutaMAX-1[™] (Eagle) supplemented with 1% (v/v) non-essential amino acids, 10% (v/v) foetal-bovine serum and 50 units of penicillin/streptomycin. Cells were maintained in an atmosphere of 5% CO₂ at 37 °C. Cells were harvested at 80% confluency by scraping them into a Universal sterile tube containing 10 ml of PBS.

Molecular cloning and expression of rat AKR1 family members

Complementary DNAs were amplified by reverse-transcription (RT)-PCR using total rat liver RNA as source of message for aldehyde reductase (AKR1A3), 3 α -hydroxysteroid dehydrogenase (AKR1C9) and Δ^4 -3-ketosteroid 5 β -reductase (AKR1D2). Aldose reductase (AKR1B4) was cloned using RNA from rat kidney as template for RT-PCR. In these reactions, the oligonucleotide primers used to amplify the cDNA were designed to create an *Nde*I restriction site at the initiation codon, either a *Eco*RI or *Kp*NI restriction site in the 5'-untranslated region (UTR) and either a *Bam*HI or *Xho*I restriction site in the 3'-UTR. The primers, along with the cDNA sequences used in their design, were as follows:

AKR1A3[36]:

5'-CCGGATTTCATATGACGGCCTCCAGTG-3' (sense) and

5'-CGCGGATCCTACGGGCCTCAGTATG-3' (antisense)

AKR1B4 [37]:

5'-CCGGATTCATATGGCTAGCCATCTGGA-3' (sense) and

5'-CCGCTCGAGCCACAGCTTCAGACTTC-3' (antisense)

AKR1C9 [38]:

5'-CGGGGTACCATATGGATTCCATATCTCTGC-3' (sense)

and

5'-CGCGGATCCCACCATGTTACTATTTCATC-3' (antisense)

AKR1D2 [39]:

5'-CCGGATTCATATGAACCTCAGCACTGC-3' (sense)

and

5'-CGCGGATCCATGTTCAGTATTCGTCATG-3' (antisense)

The amplified cDNAs were purified from the reaction mixtures (using QIAquick PCR purification kits) before being subjected to double digestion with either *Eco*RI or *Kp*NI and either *Bam*HI or *Xho*I. The restriction-digest products were resolved by agarose-gel electrophoresis, and the fragments of interest were ligated into pTZ19r that had been treated with the same restriction enzymes using standard methods. Following propagation of these cDNA clones in pTZ19r, the coding regions for AKR1A3, AKR1C9 and AKR1D2 were excised with *Nde*I and *Bam*HI and that for AKR1B4 was excised with *Nde*I and *Xho*I to allow them to be subcloned into the pET15b expression vector. This was achieved by ligation of the cDNAs both in-frame and downstream of the region in pET15b that encodes an N-terminal polyhistidine tag. The ligation products were transformed into *Escherichia coli* BL21 pLysS cells. Recombinant rat AKR isoenzymes were purified from soluble bacterial extracts by nickel chelate chromatography as described previously [20,22].

Antibodies

These were all raised in female New Zealand White rabbits. The antiserum employed against rAFAR1 was raised previously against the native ethoxyquin-inducible reductase purified from rat liver [19]. Antiserum for hAFAR1 was raised by Ireland et al. [22] against the recombinant protein expressed in *E. coli*; the RW143 serum was used, and this displays specificity for the C-terminal portion of the protein [22]. Production of antibodies against rat NAD(P)H:quinone oxidoreductase 1 (NQO1) is described elsewhere [30]. During the present project antibodies were raised against bacterially expressed polyhistidine-tagged AKR1A3, AKR1B4, AKR1C9 and AKR1D2 using standard methods.

Biochemical analyses

Protein concentrations were measured by the method of Bradford [40].

Reductase activity was measured at 25 °C in 100 mM sodium phosphate buffer, pH 7.0, using either 2-CBA (1 mM), SSA (1 mM), 4-nitrobenzaldehyde (1 mM) or 9,10-phenanthrene-quinone (0.05 mM) as substrates. Both NADPH and NADH were employed as cofactors (at a concentration of 0.2 mM) and the initial rate of reaction was monitored using a Cobas Fara centrifugal analyser (Hoffmann-La Roche Ltd., Basel, Switzerland) at 340 nm.

Discontinuous SDS/PAGE was performed by the method of Laemmli [41], and proteins were stained with Coomassie Blue. For most analyses the Bio-Rad Mini-Protean II Cell apparatus was used (Bio-Rad Laboratories, Hemel Hempstead, Herts, U.K.). However, when maximal resolution between the multiple AFAR proteins was required, electrophoresis was carried out in

the larger Bio-Rad Protean II xi Cell. In many electrophoretic runs, bacterially expressed rAFAR1 was included as a purified standard to facilitate identification of proteins. For this purpose, the cDNA coding region for the reductase was subcloned into pET17b and expressed in *E. coli* as the full-length rat protein without an N-terminal polyhistidine tag. The heterologously expressed rAFAR1 was purified from bacterial lysates by Q-Sepharose chromatography, essentially as described by Ellis et al. [28].

For Western blotting, electrophoretic transfer of polypeptides from SDS/PAGE gels to nitrocellulose membranes was carried out with Bio-Rad equipment that was appropriate for the size of the PAGE gel. Protein binding sites on membranes were blocked by placing them overnight in 20 mM Tris/HCl/150 mM NaCl/0.25% (v/v) Tween, pH 7.4, which contained 10% (w/v) semi-skimmed milk (buffer A). The blots were then incubated for 1 h at room temperature with antisera against AKR isoenzymes (at 1:2000 dilution in buffer A). 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, at 1:3000 dilution) in buffer A. The antibody complexes were detected by enhanced chemiluminescence (ECL*, Amersham) and revealed after exposure to Blue-sensitive X-ray film (Genetic Research Instrumentation, Braintree, Essex, U.K.) [28].

Protein purification

The chromatography methods used to isolate the rat AFAR proteins were adapted from those used to purify hAFAR1 [22]. All steps were performed on ice or at 4 °C. The protocol is described fully in the Results section below. Briefly, rat liver 100000 g supernatant (cytosol) was prepared in 10 mM Tris/HCl, pH 8.2 (buffer B), containing 1 mM dithiothreitol (DTT) and dialysed extensively against the same buffer before being subjected to chromatography on Q-Sepharose. This anion-exchange step resulted in resolution of rAFAR1 from the additional AFAR forms in rat liver. The various AFAR-containing peaks were collected separately, and each was subjected sequentially to chromatography on columns of Matrex Orange A and HiTrap Blue (i.e. Cibacron Blue).

Protein chemistry

Portions (100 µg protein) of the AFAR preparations obtained from the HiTrap Blue step were subjected to reversed-phase HPLC on a 4.6 mm × 250 mm Brownlee C₁₈ column (7 µm particle size and 300 Å pore size). This column was developed using a linear 35–70% acetonitrile gradient which was formed in 0.1% (v/v) trifluoroacetic acid over 90 min using two model 510 pumps from Waters Associates (Instruments), Northwich, Cheshire, U.K. The column was eluted at 1.0 ml/min, and the entire chromatography run took 100 min to complete; it included a 10 min isocratic 'loading' period during which the flow rate was increased from 0.1 to 1.0 ml/min. The protein-containing peaks were collected manually (about 1.5 ml) and freeze-dried twice to remove all traces of trifluoroacetic acid. The dried material was dissolved in about 100 µl of electrophoresis sample buffer (0.1% (v/v) SDS in 10 mM Tris/HCl, pH 7.0) and subjected to a final SDS/PAGE purification step in 12%-(w/v)-polyacrylamide resolving gels. Western blotting was used to confirm that the correct polypeptide had been isolated.

Peptide maps were prepared by examination of partial digestion of the AFAR preparations with *S. aureus* V8 protease. Electrophoretically purified AFAR proteins were located within PAGE gels by rinsing them briefly with ice-cold water followed

by immersion in aq. 250 mM KCl containing 1 mM DTT. The polypeptide band of interest was cut out of the polyacrylamide gel using a razor blade, and the gel slice obtained was rinsed twice for a total of 10 min with 3.0 ml ice-cold aq. 1 mM DTT before being freeze-dried. A single freeze-dried gel slice was allowed to rehydrate for 5 min in the sample well of a pre-cast slab SDS/PAGE gel (comprising a 16% polyacrylamide resolving gel). This was allowed to occur in a solution of protein elution buffer [i.e. 0.1% (v/v) SDS/1 mM EDTA/2.5 mM DTT/125 mM Tris/HCl, pH 6.8], which was placed into the sample well immediately before the gel slice. Once rehydrated, the gel slice was overlaid with a 5 μ l aliquot of protein elution buffer containing 20% (v/v) glycerol. On top of this was layered a 5 μ l aliquot of a 100 μ g/ml solution of *S. aureus* V8 protease in 125 μ M Tris/HCl, pH 6.8, containing 1 mM EDTA, 0.1% (w/v) SDS, 10% (v/v) glycerol and 0.001% (w/v) Bromophenol Blue. Immediately following these additions, the electrophoresis run was begun. Digestion of the AFAR proteins by V8 protease was allowed to proceed within the gel by briefly stopping electrophoresis for 30 min once the sample had migrated about two-thirds the way through the 3% polyacrylamide stacking gel, at which time the components appeared to be maximally concentrated.

CNBr digestion of AFAR was performed on material that had been purified by HPLC and SDS/PAGE, as described above. Freeze-dried polyacrylamide-gel slices, which contained about 2 μ g of AFAR, were rehydrated for 10 min in ice-cold aq. 70% (v/v) formic acid. After this period of equilibration (carried out in a microcentrifuge tube), the acid was removed, two crystals of CNBr were added along with 0.5 ml of fresh 70% (v/v) formic acid, and the mixture was flushed with N₂ gas. The reaction was allowed to proceed in the dark for 30 min at room temperature. Digestion was stopped by removing the solution and washing the gel twice with 1 ml of ice-cold water for 5 min, followed by two changes of ice-cold 1 M Tris/HCl buffer, pH 6.8, also for 5 min. The gel slices were placed in the wells of preformed slab SDS/PAGE gels, and the cleavage products were resolved by electrophoresis in 16%-(w/v)-polyacrylamide gels. The fragments were either stained with Coomassie Blue or transferred onto Problott[®] membrane for amino acid sequencing. This was performed as described previously [42].

RESULTS

Identification of a novel 37 kDa-AFAR protein in rat tissues and rat cell line

The molecular mass of rAFAR1, as estimated by SDS/PAGE, has been variously reported to lie between 36 and 40 kDa [19,22,28]. During the present study it was estimated by SDS/PAGE to be 38 kDa.

Western blotting with anti-rAFAR1-serum has previously shown that the reductase is present at low levels in livers of rats fed on control diet, but is induced 15-fold in the livers of rats fed on an ethoxyquin-containing diet [28]. Figure 1(A) shows that anti-rAFAR1 serum cross-reacts with a single electrophoretic band in both rat and human liver cytosol. Surprisingly, in the reverse experiment, the antibody raised against hAFAR1 detected two polypeptides in liver cytosols from rats fed on a control diet and those fed on an ethoxyquin-containing diet (Figure 1B). Among these two cross-reacting bands the polypeptide that migrated more slowly during SDS/PAGE corresponded to the inducible rat protein and was estimated to have a molecular mass of 38 kDa. The additional polypeptide with the faster electrophoretic mobility, which was not detected with anti-rAFAR1 serum, was found to co-migrate with the human

Figure 1 A novel 37 kDa polypeptide in rat liver cross-reacts with anti-hAFAR1 serum

Portions (5 μ g of protein) of hepatic cytosol were subjected to Western blotting. The samples were loaded in an identical fashion on to two separate polyacrylamide-gels as follows: 1, liver from Fischer 344 rats fed on control diet; 2, liver from rats fed on an ethoxyquin-containing diet; 3, normal human liver. Once the SDS/PAGE run was complete, the proteins were transferred electrophoretically to immobilon-P membranes and probed with either anti-rAFAR1 serum (A) or anti-hAFAR1 serum (B). Cross-reacting polypeptides were revealed by ECL[®]. The position of mobility of 37 kDa and 38 kDa polypeptides is indicated. Abbreviations: Con., control; EQ, ethoxyquin.

AKR7A2 immunogen (L. S. Ireland and J. D. Hayes, unpublished work). In these experiments its molecular mass was estimated to be 37 kDa, and as this polypeptide did not appear to be inducible by ethoxyquin, it was thought to be distinct from rAFAR1. The fact that it cross-reacted with hAFAR1 antiserum suggests that it is likely to be a further member of the AKR7 family. It was postulated that the 37 kDa polypeptide which is immunochemically related to hAFAR1 might represent rAFAR2. However, until this hypothesis was substantiated (see below) the putative rAFAR2 isoenzyme was called 37 kDa-AFAR.

Immunoblotting of extracts prepared from rat H4IIE cells with anti-hAFAR1-serum also showed the presence of two cross-reacting polypeptides (Figure 2). The two bands observed in H4IIE-cell extracts co-migrated with those in rat liver, and the slower electrophoretic band had the same mobility as bacterially expressed rAFAR1. The 37 kDa-AFAR rat protein was not recognized with antibodies raised against rAFAR1.

The expression of 37 kDa-AFAR was examined in extrahepatic rat organs, not only to confirm that it is regulated differently from rAFAR1, but also to identify the best tissue source for the

Figure 2 Identification of 37 kDa-AFAR in a rat hepatoma cell line

Portions (5 µg of protein) of postnuclear supernatants from the livers of rats fed on control diet and from H4IIE cells, along with heterologously expressed purified rAFAR1 (0.02 µg of protein), were subjected to Western blotting as described in the text. SDS/PAGE, performed in a Bio-Rad Protean II xi Cell apparatus, was loaded as follows: 1, rAFAR1; 2, Sprague-Dawley rat liver cytosol; 3, soluble extract from untreated H4IIE cells. Blots of the resolved proteins were prepared and probed with anti-hAFAR1 serum.

novel protein. Western blotting of organs from rats fed on a control diet showed that rAFAR1 has a limited tissue distribution. Among the organs studied, significant amounts of rAFAR1 were observed in only kidney, liver, testis and pancreas. By contrast, 37 kDa-AFAR was found to possess a broader tissue distribution than rAFAR1, with substantial levels being observed in kidney, vas deferens, testis, pancreas, brain, adrenal gland and lung (Figure 3). Significant amounts of this novel protein were also detected in liver, heart, skeletal muscle, epididymus, ovary, spleen and stomach. The Western blots also revealed the presence of a fast-migrating cross-reacting protein in kidney, liver and spleen that was estimated to be about 36.8 kDa.

Resolution of rat carbonyl-reducing enzymes from liver cytosol

Liver was chosen as the source of 37 kDa-AFAR because immunoblotting demonstrated it to be relatively abundant in hepatic cytosol; also the liver is a large organ and our laboratory has previous experience of analysing AKR from this tissue

[28,43]. Dialysed rat liver cytosol was applied to a column of Q-Sepharose that was developed with a linear 0–250 mM NaCl gradient in buffer B containing 1 mM DTT. Monitoring the column eluate with the substrates SSA, 4-nitrobenzaldehyde (4-NBA) and 9,10-phenanthrenequinone (9,10-PQ) showed that this anion-exchange-chromatography step gave rise to seven peaks of carbonyl-reducing activity. These have been designated peaks Ia, Ib, Ic, II, III, V and VI (Figure 4a) in accordance with the nomenclature introduced by Ellis et al. [28]. The highly anionic material recovered in peak VI, which was eluted by the final 1 M NaCl step, did not possess activity towards 4-NBA and 9,10-PQ with NADPH as cofactor. Peak VI did, however, exhibit activity towards 4-NBA and 9,10-PQ with NADH as cofactor (results not shown). It should be noted that, in the present study, the use of a larger column resulted in greater resolution than was achieved previously, and as a consequence three separate activities were detected in peak I (Ia, Ib and Ic). The enzyme activity designated peak IV by Ellis et al. [28] has not been recovered as a separate entity in the present chromatographic fractionation (Figure 4a).

Examination of eluate from Q-Sepharose with 2-carboxy-benzaldehyde as substrate revealed a unique set of activities which could not be readily aligned with activity for SSA, 4-NBA or 9,10-PQ. As shown in Figure 4(b), a total of six 2-CBA reductase peaks (CBA1–CBA6) were distinguished across the profile. The first of these, CBA1, did not bind the anion-exchange column and was eluted during the washing stage between fractions 27 and 35, whereas CBA2 was eluted by the salt gradient at a similar position to peak Ic. Fractions representing CBA3 correspond to peak III. The three remaining peaks of reductase activity towards 2-CBA were designated CBA4, CBA5 and CBA6 and eluted in fractions 130–142, 145–158 and 162–176 respectively. Although CBA4 appears to be eluted with peak V, neither CBA5 nor CBA6 was co-eluted with previously described AKR chromatography peaks.

The identity of some of the enzymes in the peaks from Q-Sepharose could be inferred from substrate activities. Peak Ia displays activity towards SSA, 4-NBA and 9,10-PQ, and on this basis could contain a protein related to carbonyl reductase, as carbonyl reductase itself has been found to be absent from rat liver [44]. Aldehyde reductase is likely to contribute to the enzyme activity in peak II because it exhibits significant activity towards SSA and 4-NBA. Also, the absence of NADH-dependent reductase activity in this peak (results not shown) is consistent

Figure 3 Tissue-specific expression of rAFAR1 and the 37 kDa-AFAR in the rat

Extracts from approx. 100 mg of fresh frozen tissue were prepared as described previously. Portions (20 µg of protein) of 12 000 g supernatants from tissues of Sprague-Dawley rats were subjected to SDS/PAGE using a Bio-Rad Protean II xi Cell to ensure complete resolution of the 37 and 38 kDa immunoreactive polypeptides. As a marker for rAFAR1, a 0.02 µg portion of the purified protein was also applied. The identity of the organs analysed is shown, and the Western blot was probed with anti-hAFAR1 serum.

Figure 4 Resolution of AKR from rat liver by anion-exchange chromatography

Rat liver cytosol (approx. 9 g of protein) that had been dialysed against 10 mM Tris/HCl buffer, pH 8.2 (buffer B), containing 1 mM DTT, was applied to a 2.6 cm × 72 cm column of Q-Sepharose equilibrated with the same buffer. The Q-Sepharose column was eluted at 32 ml/h and fractions (8 ml each) were collected. The anion-exchanger was developed with a linear 0–250 mM NaCl gradient formed in buffer B containing 1 mM DTT that was applied to the column between fractions 35 and 185. Lastly, highly anionic proteins were recovered by elution with 1 M NaCl in buffer B containing 1 mM DTT. Reductase activity in column eluate towards SSA (●), 4-NBA (□) and 9,10-PQ (▲) with NADPH was measured, and is shown in (A). AKR activity towards 2-CBA with either NADPH (■) or NADH (○) was measured (B). Note that peak IV, as described by Ellis et al. [28], was not recovered in this chromatography step.

with this assumption. The elution position and preferential activity of peak III towards SSA and 9,10-PQ suggests it contains rAFAR1. Peak V possesses significant activity with 9,10-PQ and

could therefore contain either a hydroxysteroid dehydrogenase or NQO1, or both.

The proteins eluted within the peaks of enzyme activity from Q-Sepharose were probed with antibodies to allow identification of the reductases present. Antibodies against AKR1A3, AKR1B4, AKR1C9, AKR1D2 and rat NQO1 cross-reacted with proteins in peaks Ia, II, III, and VI (Table 1). Each peak containing reductase activity towards 2-CBA was probed with antisera against rAFAR1 and hAFAR1. The two minor peaks, CBA1 and CBA2, did not contain any protein species that could be detected by these antisera. Western blotting using anti-rAFAR1 serum showed that, in addition to cross-reacting with a 38 kDa polypeptide in CBA3, the same protein was also detectable in large amounts in CBA4, and in lesser amounts in CBA5. As expected, Western blotting of column fractions with anti-hAFAR1 serum identified two polypeptides of 37 and 38 kDa. The distribution of the cross-reacting 38 kDa polypeptide among the column fractions was identical with that observed with the rAFAR1 antibodies (Table 1). By contrast with the 38 kDa protein, the cross-reacting 37 kDa polypeptide appeared to be more tightly bound by the anion-exchange column. Relatively small amounts of this polypeptide were observed in CBA3. Quantitatively, the major fraction of the immunoreactive 37 kDa protein recovered was found to be eluted equally between CBA4, CBA5 and CBA6. Thus Q-Sepharose showed that the 37 and 38 kDa polypeptides in rat liver that cross-react with anti-hAFAR1 serum are chromatographically separate, and that both exhibit activity towards 2-CBA.

Purification of 37 kDa-AFAR

Following Q-Sepharose fractionation, the peaks of reductase activity towards 2-CBA were combined as indicated in Figure 4(b) and dialysed against 10 mM sodium phosphate buffer, pH 8.0, containing 1 mM DTT. Each peak was loaded on to an identical Matrex Orange A column that was developed using a standardized procedure with a linear 0–1.5 M NaCl gradient. Succinic semialdehyde and 2-CBA were used to detect rAFAR1 and 37 kDa-AFAR. The CBA3 pool was resolved by Matrex

Table 1 Immunochemical properties of AKR chromatographic peaks obtained from Q-Sepharose

Western blotting was carried out on the fractions eluted from the Q-Sepharose anion-exchanger shown in Figure 4 as described in the text.

Column fraction	Peak	Cross-reactivity with antibodies against:						
		AKR1A3	AKR1B4	AKR1C9*	AKR1D2	rAFAR1	hAFAR1†	NQO1
50	Ia	—	—	++	—	—	—	—
57	Ib	—	—	—	—	—	—	—
89	Ic/CBA2	—	—	—	—	—	—	—
101	II	+++	—	—	—	—	—	—
113	III/CBA3	++	+++	—	—	+++	+	—
129	V/CBA4	—	++	+++	—	+++	+	+++
133	CBA4	—	—	+++	+	+++	+++	—
150	CBA5	—	—	++	+++	+	+++	—
169	CBA6	—	—	—	—	—	+++	—
187	VI	—	—	—	—	—	+	—

* The protein in peak Ia which cross-reacted with anti-AKR1C9 serum was 31 kDa, whereas the cross-reacting protein in fractions 129–150 was 37 kDa; as the immunogen is 37 kDa, the polypeptide in peak Ia that cross-reacts with the antibody is not AKR1C9, but is a structurally distinct protein.

† Anti-hAFAR1 serum cross-reacted with a 37 and a 38 kDa polypeptide: the 37 kDa protein was detected in fractions 133–187; the 38 kDa protein was detected in fractions 113–150.

Figure 5 Fractionation of major hepatic anionic 2-CBA reductases by Matrex Orange A

The 2-CBA reductases in rat liver cytosol were resolved by anion-exchange chromatography. The four major forms (CBA3–CBA6) were isolated as shown in Figure 4 and dialysed against 10 mM sodium phosphate buffer, pH 8.0 (buffer C), containing 1 mM DTT. Each peak was diluted to 120 ml volume before being applied to 1.6 cm × 1.4 cm columns of Matrex Orange A. The columns were eluted at 30 ml/h and developed with 400 ml linear 0–1.5 M NaCl gradients in buffer C containing 1 mM DTT. The salt gradients were initiated at fraction 32, and the fraction volume was 6 ml. The absorbance of the eluate at 280 nm (◇), and AKR activity towards 2-CBA (■) and SSA (●) with NADPH as cofactor was examined. The elution profiles of CBA3, CBA4, CBA5 and CBA6 are shown in (a), (b), (c) and (d) respectively.

Orange A into two peaks of reductase activity with SSA as substrate (Figure 5a). The first SSA reductase peak to be eluted from this column lacked reductase activity towards 2-CBA and is therefore assumed to represent AKR1A3 or AKR1B4. The

second peak to be eluted exhibited activity towards 2-CBA and was therefore considered to be rAFAR1, a conclusion supported by Western-blotting data (results not shown). Furthermore, this second peak exhibited approx. 6-fold higher 2-CBA reductase activity with NADH as cofactor than with NADPH (for simplicity, only 2-CBA activity with NADPH is shown in Figure 5). The CBA4, CBA5 and CBA6 pools from Q-Sepharose were each eluted from Matrex Orange A as essentially a single peak of reductase activity towards SSA and 2-CBA (Figures 5b–5d). Furthermore, it was also noted that the CBA5 and CBA6 reductases did not bind the Matrex Orange A adsorption gel as tightly as did rAFAR1 (CBA3).

Each enzyme-containing peak eluted from Matrex Orange A was subjected to SDS/PAGE. This revealed that the CBA3 preparation was homogeneous and the single polypeptide comigrated with the bacterially expressed rAFAR1 protein of 38 kDa molecular mass. By contrast, the reductase-containing fractions from CBA4, CBA5 and CBA6 contained multiple protein bands.

Further purification of CBA4, CBA5 and CBA6 was performed on the ADP-analogue matrix Cibacron Blue, using pre-packed HiTrap Blue columns. Fractions from Matrex Orange A were combined as shown in Figure 5, and each pool was applied directly to a HiTrap Blue column. The AKR protein in these preparations bound tightly to the Cibacron Blue matrix with insignificant quantities of the reductase activity being eluted in the flow-through fractions. Displacement of active protein from Cibacron Blue was achieved using a 1 mg/ml solution of NADPH in buffer B. The material eluted from the HiTrap Blue columns with reductase activity was examined by SDS/PAGE. This showed that the CBA4 preparation still contained significant amounts of a contaminating 58 kDa protein. However, both CBA5 and CBA6 were found to be comparatively pure, and both contained a 37 kDa protein which was demonstrated to cross-react with anti-hAFAR1 serum (results not shown). Densitometric scanning indicated that CBA5 was about 92% pure, and Western blotting suggested that the major contaminant was rAFAR1. Although CBA6 was estimated to be 98% pure, close examination of the electrophoresis gel indicated the presence of two partially resolved bands in the preparation.

The purification scheme resulted in excellent recovery of 2-CBA reductase activity from rat liver. Typically, from roughly 9 g of cytosolic protein, between 0.5 and 1.5 mg of purified protein was obtained in the final CBA5 and CBA6 pools eluted from HiTrap Blue. The purification method is highly efficient and allowed a total recovery of about 15% of the total reductase activity between CBA5 and CBA6, and a further 9% in CBA3. A summary of the purification is shown in Table 2.

The specific activities of CBA5 and CBA6 towards AKR substrates were determined. Table 3 shows that under 'standard' assay conditions, CBA5 exhibits activities similar to those of CBA6 towards 2-CBA and SSA. However, CBA5 possesses higher activity than CBA6 towards 4-NBA and 9,10-PQ. Furthermore, comparison of the activity of these preparations with that of rAFAR1 revealed significant differences in specificity under the conditions used. For example, rAFAR1 exhibited greater activity for both 4-NBA and 9,10-PQ than either CBA5 or CBA6. This survey also revealed that, under the assay conditions used, rAFAR1 uniquely possesses about 7-fold greater activity towards 2-CBA with NADH as cofactor than with NADPH; by contrast, with SSA, 4-NBA and 9,10-PQ as substrates, rAFAR1 shows a preference for NADPH as cofactor. Neither CBA5 nor CBA6 displayed higher activity towards 2-CBA with NADH as cofactor than with NADPH as cofactor (Table 3).

Table 2 Purification of 2-carboxybenzaldehyde reductases from rat liver

Reductase activity was monitored using 2-CBA and NADPH at 25 °C, and protein concentration was measured by the method of Bradford [40].

Fraction	Volume (ml)	Total Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)	Purification (fold)
Cytosol	210	9240	34400	3.7	100	1
CBA3 Q-Sepharose	92	1060	5340	5	15	1.4
CBA3 Matrex Orange	81	8	3110	390	9	105
CBA4 Q-Sepharose	102	350	3640	10.5	11	2.8
CBA4 Matrex Orange	86	9	1270	141	4	38
CBA4 HiTrap Blue	10	0.3	710	2370	2	640
CBA5 Q-Sepharose	119	285	5300	18.6	15	5
CBA5 Matrex Orange	94	14	3980	285	12	77
CBA5 HiTrap Blue	10	1.2	3690	3075	11	830
CBA6 Q-Sepharose	128	224	4550	20	13	5.4
CBA6 Matrex Orange	92	10.1	2820	280	8	76
CBA6 HiTrap Blue	10	0.5	1850	3700	5	1000

Table 3 Specific activities of the AFAR preparations from rat liver

The enzyme assays were performed in triplicate at 25 °C. Activities towards the aldehyde-containing substrates were measured at a concentration of 1 mmol/l, and activity towards 9,10-PQ was determined at 0.05 mmol/l.

Protein	Substrate ... Cofactor ...	Specific activity (nmol/min per mg of protein)				
		2-CBA	SSA	4-NBA	9,10-PQ	
		NADH	NADPH	NADPH	NADPH	NADPH
rAFAR1		2980 ± 42	390 ± 25	345 ± 20	1080 ± 20	4520 ± 70
CBA5		1840 ± 20	3075 ± 40	2245 ± 20	890 ± 15	3320 ± 90
CBA6		1735 ± 25	3700 ± 50	2990 ± 30	490 ± 20	1790 ± 70

Demonstration that rAFAR1 and 37 kDa-AFAR are structurally distinct

The two electrophoretically distinguishable rat polypeptides that cross-react with anti-hAFAR1-serum were resolved by reversed-phase HPLC on a Brownlee C₁₈ column as described in the Materials and methods section. Using this method, the 37 kDa-AFAR protein was eluted from the Brownlee C₁₈ column at 67 min, whereas the 38 kDa rAFAR1 was eluted at 70 min. This chromatographic approach was therefore adopted to remove traces of rAFAR1 from CBA5, and to purify CBA6 further (Figure 6). The polypeptides eluted from the reversed-phase HPLC column were freeze-dried and their identity confirmed by Western blotting with antibodies against rAFAR1 and against hAFAR1 before further analysis.

Peptide fingerprinting by partial proteolysis was performed on the 37 kDa-AFAR polypeptide present in both CBA5 and CBA6 to establish whether they might be identical. The fingerprints obtained were also compared directly with that generated from a digest of rAFAR1, performed in parallel (from CBA3), to help establish their relationship. Figure 7 shows SDS/PAGE of the partial proteolysis products obtained from these proteins following digestion with *S. aureus* V8 protease. Examination of the gel shows that the 37 kDa polypeptide from CBA5 and the 37 kDa polypeptide from CBA6 yield digestion products with essentially identical electrophoretic mobilities. The gel also demonstrates that these digestion products are distinct from those obtained from rAFAR1. In particular, major proteolysis

products of 21 kDa, 12 kDa and 10 kDa were recovered from the CBA5 and CBA6 protein that were not observed in digests of rAFAR1.

Evidence to support the designation of 37 kDa-AFAR as rAFAR2

The 37 kDa-AFAR obtained by reversed-phase HPLC of CBA6 was subjected to amino acid sequencing to establish its relationship with rAFAR1. Attempts to obtain sequence data by subjecting the 37 kDa AFAR directly to Edman degradation were unsuccessful, suggesting that the protein has a blocked N-terminus. However, following CNBr digestion of 37 kDa-AFAR by the 'in-gel' protocol described in the Materials and methods section, four peptide fragments were recovered. The smallest of these was about 14 kDa. Automated Edman degradation of this fragment yielded the sequence:

YNATTRQVETELLPC

which aligned with amino acids 168 and 183 of rAFAR1 (Figure 8). Although the amino acid sequence from 37 kDa-AFAR is closely similar to that of rAFAR1, differences occur at positions equivalent to Ile¹⁷¹ and Phe¹⁸⁰ of rAFAR1; at these positions the 37 kDa-AFAR protein contains threonine and leucine. These data indicate 37 kDa-AFAR has a primary structure that is distinct from that of rAFAR1. The 37 kDa protein is therefore designated rAFAR2.

Figure 6 SDS/PAGE analysis of AFAR proteins resolved by reversed-phase HPLC

The CBA4, CBA5 and CBA6 pools of 2-CBA reductase activity were prepared by sequential chromatography on Q-Sepharose, Matrex Orange A and HiTrap Blue. Portions (100 µg of protein) of material from the HiTrap Blue column were applied to a 4.6 mm × 250 mm Brownlee C₁₈ column and proteins resolved by using a 35–70% acetonitrile gradient in aq. 0.1% trifluoroacetic acid as described in the Materials and methods section. The protein-containing peaks were collected and tested for cross-reactivity towards anti-hAFAR1 serum. Those peaks which reacted with this antibody were analysed by SDS/PAGE. The gel was loaded as follows: 1, molecular-mass standards; 2, 0.2 µg of recombinant rAFAR1; 3, HiTrap Blue-purified material from CBA4 (A), CBA5 (B) and CBA6 (C) respectively; 4, material from CBA4, CBA5 and CBA6 that was eluted at 67 min from the reversed-phase HPLC column; 5, material from CBA4, CBA5 and CBA6 that was eluted at 70 min from the reversed-phase HPLC column.

Figure 7 Peptide maps of 37 kDa-AFAR and rAFAR1 proteins

Cleavage of the 37 kDa-AFAR and rAFAR1 with *S. aureus* V8 protease was performed 'in-gel' as described in the text. The peptide products were examined by SDS/PAGE. The polyacrylamide gel was loaded as follows: 1, molecular-mass standards; 2, 2 µg rAFAR1 (from CBA3); 3, 2 µg of 37 kDa-AFAR from CBA5; 2 µg of 37 kDa-AFAR from CBA6.

rAFAR2	(M) YNAT <u>T</u> RQVETELLPCL
rAFAR1	M YNAITRQVETELFPCL-183
hAFAR1	M YNAT <u>T</u> RQVETELFPCL-186
hAFAR2	M YNAITRQVETELFPCL-187
AKR1A3	E CHPYLAQNELIAHCQA-202
AKR1B4	E CHPYLTQEKLIEYCHC-202
AKR1C9	E CHLYLNQSKMLDYCKS-208
AKR1D2	E CHPYFTQTKLLEVSAS-210

Figure 8 Sequence alignment of CNBr peptide from rAFAR2 with other AKRs

The alignments are based on the work of Jez et al. [1,2]. The residues in rAFAR2 that differ from those in rAFAR1 are underlined. Sequences of hAFAR1 and hAFAR2 are from [22] and [32].

DISCUSSION

Identification of AFAR proteins

The present study describes the purification of a hitherto unidentified member of the AKR7 family. The novel protein is designated rAFAR2, denoting the fact that it is the second AFAR protein to be identified in the rat. Thus the inducible rat AFAR protein described in earlier studies is now referred to as rAFAR1. The rAFAR1 isoenzyme has also been called AKR7A1 [1], but in the present paper we have chosen to retain our rAFAR nomenclature since the 'r' (rat) and 'h' (human) prefixes help to avoid confusion about the source of the immunogen used to raise antibodies.

The detection of rAFAR2 has been greatly facilitated by the use of an antibody that was raised against recombinant hAFAR1 and which cross-reacts with the rat protein. Fortunately, rAFAR2 possesses a faster mobility during SDS/PAGE than rAFAR1, and hence Western blotting using the appropriate antiserum can readily identify the 'new' isoenzyme. In addition, the task of monitoring the eluate of columns used to purify rAFAR2 was made easier by employing the substrate 2-CBA.

The aromatic aldehyde 2-CBA was originally chosen for study as a possible AFAR substrate by Ireland et al. [22] while searching for compounds with similar properties to the dialdehydic form of AFB₁-8,9-dihydrodiol. As a substrate, 2-CBA has certain of the chemical features favoured by members of the AFAR family, including an aromatic ring structure and a negatively charged group removed four carbon atoms from the carbonyl group that is catalytically reduced. The method employed to measure 2-CBA reductase activity is convenient for monitoring column eluate as it is a spectrophotometric assay and is thus easier to perform than the HPLC-based assay used to determine AFAR activity [13,45]. Recent examination of human AKRs suggests 2-CBA is not utilised as a substrate by AKR1 members [23]. It is therefore possible that this aldehyde will prove to be a useful reagent in the discovery of additional AFAR isoenzymes.

Catalytic properties of the two rat AFAR proteins

Whereas rAFAR2 utilizes some of the same substrates as

rAFAR1, catalytic differences appear to exist between the two reductases. The two principal rAFAR2-containing preparations (CBA5 and CBA6) both seem to possess significantly higher SSA reductase activity than rAFAR1. The specific activity of rAFAR1 towards SSA recovered in the present study (Table 3) is lower than that we have observed previously, presumably because of differences in the method of purification employed. However, this does not alter the conclusion that rAFAR2 is probably more active than rAFAR1 towards this physiological carbonyl. Preliminary data indicate that rAFAR2 has a significantly lower K_m for SSA than rAFAR1 (V. P. Kelly and J. D. Hayes, unpublished work), but further work is required to determine precisely the kinetic properties of rAFAR2 towards SSA and cofactor. Without knowledge of K_m and V_{max} , comments about catalytic differences between the two AFAR isoenzymes should be treated with caution, because it is likely that the 'standard' assay conditions adopted in the present study do not saturate rAFAR1 and rAFAR2 to an equal extent. Unfortunately, because of paucity of material, this task will be laborious until methods are established for large-scale purification of rAFAR2.

An interesting cofactor-specific difference between the activities of the two AFAR proteins towards 2-CBA was apparent throughout the purification (see Figure 4). With NADH as cofactor, rAFAR1 displayed an approx. 7-fold higher specific activity under 'standard' assay conditions than with NADPH as cofactor. By contrast, rAFAR2 exhibited lower activity towards 2-CBA with NADH as cofactor than with NADPH. The high activity of rAFAR1 towards 2-CBA with NADH is surprising given that previous work [21] has shown that this reductase has a markedly lower K_m for NADPH than for NADH, at least with 4-NBA as substrate. The possibility that rAFAR1-catalysed reduction of 2-CBA with NADPH as cofactor is subject to product inhibition, whereas rAFAR2 is not inhibited, requires evaluation. Further investigation is required to clarify the basis for the catalytic differences between the two rat AFARs.

Biological function of rAFAR2

The rAFAR1 and rAFAR2 proteins differ markedly in their distribution in rat tissues (see Figure 3). The expression of rAFAR1 is restricted to liver, kidney, testis and pancreas, and is highly inducible in the liver by metabolizable synthetic antioxidants such as ethoxyquin. With the possible exception of expression of the reductase in the pancreas, this pattern of regulation is consistent with rAFAR1 playing a role in detoxification. By contrast, rAFAR2 is more widely distributed than rAFAR1, and is not obviously induced by ethoxyquin. The fact that rAFAR2 is present in pancreas, brain, adrenal gland, heart, stomach and skeletal muscle, organs which are not particularly associated with drug metabolism, suggests that this member of the AKR7 family performs a constitutive 'house-keeping' role. It is noteworthy that rAFAR2 has a tissue distribution that is similar to that of hAFAR1 [22,23].

The work of Ireland et al. [22] identified SSA, a metabolite of the major inhibitory neurotransmitter γ -aminobutyric acid (GABA), as a likely *in vivo* substrate of the hAFAR1 protein. Support for this suggestion has been more recently obtained by finding that purification and cloning of the major SSA reductase from human brain resulted in isolation of hAFAR1 [46]. In the present study it has been found that rAFAR2 also exhibits SSA reductase activity. Thus it seems likely that a major biological function for many of the AKR7 family members is that of synthesis of γ -hydroxybutyric acid (GHB).

SSA produced catalytically from GABA by a transaminase may be catabolized by SSA dehydrogenase to succinic acid [47].

However, SSA can also be converted into GHB by SSA reductase. In many rat tissues this step could be catalysed by rAFAR2. Rather than being an unwanted metabolite, GHB has important physiological properties. At low concentrations, GHB modulates dopamine (3,4-dihydroxyphenethylamine) synthesis and release, whereas at high concentrations it binds to GABA_B receptors and causes a reversal of the effects produced at low concentrations [48,49]. It has also been proposed that GHB helps protect cells against death during periods of anoxia and during periods of excessive metabolic demand [50]. Thus the AFAR proteins may contribute to cell survival during physiological stress.

Multiple forms of rAFAR2

The chromatographic profiles obtained during purification of rAFAR2 suggested that the enzyme exists in several different forms. In particular, Q-Sepharose chromatography yielded three peaks of 2-CBA reductase activity which cross-reacted with hAFAR1 antibodies; these were called CBA4, CBA5 and CBA6 according to their order of elution. Lack of material prevented CBA4 from being characterized and, although the most highly purified preparation contained substantial amounts of a 37 kDa polypeptide that cross-reacted with anti-hAFAR1 serum, the relationship between CBA4 and CBA5 and between CBA4 and CBA6 has not been rigorously established. The 37 kDa-AFAR polypeptides in CBA5 and CBA6 have, however, been purified to homogeneity, and peptide mapping indicated that they represent closely similar, if not identical, proteins. The reason for resolution of the protein into several chromatographic peaks is uncertain, but it is an important issue, because the CBA5 and CBA6 preparations displayed different activities for SSA and other substrates. It appears unlikely that oxidation is responsible for the generation of three peaks, since all the buffers employed for protein purification contained DTT. It is probable that rAFAR2 is modified post-synthetically, possibly by phosphorylation, glycosylation, glutathiolation or proteolysis. Close examination of SDS/PAGE analysis of CBA6 indicated that, in addition to the major 37 kDa polypeptide, a 36.8 kDa band could also be discerned. Although it seems highly likely that a product-precursor relationship exists between the polypeptides of apparent 36.8 and 37 kDa molecular mass, this heterogeneity does not provide an explanation of why the 37 kDa-AFAR subunit is recovered in both CBA5 and CBA6.

In view of the fact that AFAR proteins are more closely related to the tetrameric voltage-gated K⁺-channel β -subunits than to the monomeric AKR1 enzymes, the possibility has been considered that rAFAR2 may be capable of self-associating. This hypothesis has been tested by subjecting CBA4, CBA5 and CBA6 preparations (post Q-Sepharose fractions shown in Figure 4b) to gel-filtration chromatography on Sephadex G-150. Following calibration of the column with β -amylase, alcohol dehydrogenase, BSA, carbonic anhydrase and cytochrome *c*, it was found that CBA4, CBA5 and CBA6 were eluted from Sephadex G-150 with apparent native molecular masses of 59, 70 and 65 kDa (results not shown). Since the estimated molecular masses of the reductase in these preparations determined by gel-filtration chromatography greatly exceeds that determined by SDS/PAGE, it appears likely that rAFAR2 is engaged in some type of protein-protein interaction. Dimerization of rAFAR2 would be expected to yield a complex of 74 kDa. This value slightly exceeds that estimated by chromatography. It is therefore concluded that rAFAR2 may exist as a homodimer, but that the size of the complex is underestimated, either because the association

between the subunits is reversible, or because the dimer has a propensity to interact with the column matrix.

At this stage it is unclear why rAFAR2 should exist as a dimeric protein in the cell. If the sole purpose of rAFAR2 is to act as an enzyme, it would not be required to exist as a dimer, since the AKR1 members are both monomeric and catalytically active. It is therefore possible that rAFAR2 serves an additional role besides that of reducing carbonyls. It may dimerize in order to regulate enzyme activity or intracellular localization. Our gel-filtration-chromatography experiments indicate that, in its native state, rAFAR2 does not exist as a single subunit, but that it is able to form complexes either with itself or with another protein. It requires to be established whether rAFAR2 can dimerize with rAFAR1.

Concluding comments

This paper reports the identification of a hitherto unrecognized AFAR isoenzyme in the rat, and its purification from rat liver cytosol. The first member of the AFAR family to be described was discovered because of its marked inducibility by ethoxyquin and unique catalytic activity towards aflatoxin B₁. The rAFAR2 protein is notable because of its broad distribution in rat tissues. Rather than contributing to detoxification of xenobiotics, the 'new' protein may play an important *in vivo* role in the synthesis of GHB in neural and peripheral tissues. The rAFAR2 protein appears to be able to form dimers. Its further molecular characterization will expand our knowledge of the physiological roles played by the AKR7 proteins in general.

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