Substrate specificity of an aflatoxin-metabolizing aldehyde reductase

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The enzyme from rat liver that reduces aflatoxin B_1-dialdehyde exhibits a unique catalytic specificity distinct from that of other aldo–keto reductases. This enzyme, designated AFAR, displays high activity towards dicarbonyl-containing compounds with ketone groups on adjacent carbon atoms; 9,10-phenanthrenequinone, acenaphthenequinone and camphorquinone were found to be good substrates. Although AFAR can also reduce aromatic and aliphatic aldehydes such as succinic semialdehyde, it is inactive with glucose, galactose and xylose. The enzyme also exhibits low activity towards α,β-unsaturated carbonyl-containing compounds. Determination of the apparent K_m reveals that AFAR has highest affinity for 9,10-phenanthrenequinone and succinic semialdehyde, and low affinity for glyoxal and DL-glyceraldehyde.

INTRODUCTION

Mammalian cells are exposed to a broad spectrum of chemicals that contain potentially toxic ketone and/or aldehyde groups [1]. Not only are these carbonyl-containing compounds widespread in the environment, but they can also be generated in the body through normal catabolic oxidation and deamidation reactions [2].

Many endogenous compounds such as biogenic amines, steroids and other hormones are metabolized through carbonyl intermediates [3,4]. Lipid peroxidation within the cell results in production of acrolein, 4-hydroxynonenal (4-HNE) and malondialdehyde, while oxidative damage to DNA generates base propenals [5,6]. Dietary sources of carbonyl-containing compounds are diverse and include aldehydes found in fruits [7] as well as the breakdown product of ethanol, acetaldehyde. Therapeutic agents such as ethacrynic acid, warfarin, oxisuran, adriamycin, actinomycin D, mitomycin C, metyrapone and *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) represent a further source of exposure to carbonyl-containing compounds [8–10]. The biotransformation of many drugs, exemplified by cyclophosphamide, chlorpheniramine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can produce aldehyde or ketone intermediates [2,11,12]. The herbicide alachlor and the pesticide chlordecone contain ketone groups, and such compounds are encountered as environmental pollutants [13]. Also, the widely distributed potent hepatocarcinogen aflatoxin B_1 (AFB_1), which is produced by the mould *Aspergillus flavus*, is metabolized in the liver to a dialdehyde phenolate anion [14].

Substances that contain carbonyl groups are generally toxic because not only can they form Schiff bases with protein but they are also capable of reacting with thiol groups in protein [14–16]. In addition, some aldehydes can interact with DNA and are mutagenic in *Salmonella* strains used in Ames testing [17]. Because of the reactivity of carbonyl-containing compounds, cells require protection against such compounds: this is achieved through their metabolism by several different detoxification enzymes. For example, aldehydes can be oxidized to carboxylic acids by aldehyde dehydrogenases [18,19] or they can be reduced to alcohols by alcohol dehydrogenases [20] and aldo–keto reductases [1]. Aldose reductase, aldehyde reductase, carbonyl reductase and quinone reductase have all been found to reduce carbonyl groups contained within a diverse range of chemicals [1,21,22]. Collectively, these enzymes are thought to be important in normal metabolic functions such as steroid metabolism [23] as well as the detoxification of potentially mutagenic carbonyl compounds [24]. In common with other detoxification systems, carbonyl-reducing enzymes each display broad overlapping substrate specificities.

The cDNAs encoding several carbonyl-reducing enzymes have been cloned and can be classified on the basis of sequence similarity. Aldose reductase [25,26], aldehyde reductase [26], 3α-hydroxysteroid dehydrogenase (3α-HSD) [27,28], dihydrodiol dehydrogenase (DDD) [29,30], chlordecone reductase [31] and prostaglandin F synthase [32] are all members of the aldo–keto reductase superfamily [26] and they share more than 43% identity at the amino acid level. Members of this superfamily are monomeric NADPH-dependent enzymes, with molecular masses in the range 36–40 kDa. The crystal structure of aldose reductase has been determined and reveals that it consists of an α/β barrel with a large substrate-binding pocket [33]. Sequence analysis has also shown that several of these enzymes, such as 3α-HSD and DDD from various species, are very closely related (more than 90% identity) and share similar substrate specificities [22–31,34]. As a result of the molecular cloning of these enzymes, activities that were previously thought to be due to different proteins are now recognized to be encoded by the same gene; for example, 3-deoxyglucosone reductase is identical to aldehyde reductase [35]. By contrast, carbonyl reductase, which is a member of the short-chain alcohol dehydrogenase family [36], and quinone reductase, a distinct flavoprotein [37,38], are structurally unrelated to the aldo–keto reductases.

We have recently purified an enzyme, called AFAR, from rat-liver cytosol that catalyses the reduction of a dialdehyde metab-

Abbreviations used: ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; AFB_1, aflatoxin B_1; AFAR, aflatoxin B_1-aldehyde reductase; ADR, aldose reductase; DDD, dihydrodiol dehydrogenase; ethoxyquin, 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline; 4-HNE, 4-hydroxynonenal; 3α-HSD, 3α-hydroxysteroid dehydrogenase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 4-NBA, 4-nitrobenzaldehyde; 9,10-PQ, 9,10-phenanthrenequinone: SSA, succinic semialdeyde.

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olite of AFB_1 [39]. Sequencing of the cDNA encoding this enzyme showed that although AFAR is an aldo–keto reductase, the fact that it shares less than 25% identity with aldose reductase and chlordecone reductase suggests that it is only distantly related to other members of this supergene family [40]. We have shown previously that AFAR can metabolize 4-nitrobenzaldehyde (4-NBA), a model substrate for aldehyde reductase, but the ability of this enzyme to reduce carbonyl compounds that can serve as substrates for other members of the aldo–keto reductase family has not been studied.

In this paper we describe the catalytic properties of AFAR and suggest that it could provide protection against a wide spectrum of other noxious carbonyl-containing chemicals besides AFB_1-dialdehyde.

MATERIALS AND METHODS

Chemicals

The 4-HNE was a gift from Dr. Herman Esterbauer, Institute for Biochemistry, University of Graz, Austria. ALLN was purchased from Boehringer Mannheim (Lewes, East Sussex). All other aldehydes and ketones were obtained from Sigma Chemical Co. (Poole, Dorset) or from Aldrich Chemical Co. (Gillingham, Dorset). Nickel agarose was from Qiagen Inc. (Chatsworth, CA), chromatofocusing materials and glutathione–Sepharose were from Pharmacia Biosystems Ltd. (Central Milton Keynes, Bucks.), and matrex gel Orange A was from Amicon Ltd. (Stonehouse, Gloucestershire).

Purification of recombinant AFAR

The cloned cDNA encoding AFB_1-aldehyde reductase (rAFAR) was expressed in *Escherichia coli* from pEE65 (a pET15b-derived expression vector) under the control of an isopropyl β-D-thiogalactoside-inducible T7 polymerase [40]. Recombinant protein was obtained from the *E. coli* BL21p*LysS* strain as a fusion protein, containing an additional six histidine residues and a thrombin cleavage site at the N-terminus. The protein was purified from *E. coli* extracts by affinity chromatography on a nickel agarose column [40].

Purification of rat AFAR

Rat AFAR was purified from soluble extracts of liver by column chromatography with a combination of glutathione–Sepharose, matrex gel Orange A, CM-cellulose and polybuffer exchanger PBE 94. Soluble extract was prepared from the livers of three rats in 20 mM sodium phosphate buffer, pH 7.0 (buffer A), and applied to glutathione–Sepharose (1.6 cm × 15 cm) to remove glutathione *S*-transferases. The flow-through fractions from the glutathione affinity column were combined and loaded onto matrex gel Orange A (1.6 cm × 12.0 cm), which was equilibrated and washed with at least 6 column volumes of buffer A. Once the eluate from this column had an absorbance at 280 nm of less than 0.05 the Orange A was developed, sequentially, by elution first with 5 mM NADP in buffer A, secondly with 600 mM NaCl in buffer A, and thirdly with 2 M NaCl in Buffer A. The majority of rat AFAR was found to be recovered in the fractions eluted by 600 mM NaCl and, after dialysis against 10 mM sodium phosphate buffer (pH 6.7), this material (total volume about 15 ml) was applied to a 1.6 cm × 45.0 cm column of CM-cellulose equilibrated with the same buffer. Continued elution of this column with 10 mM sodium phosphate buffer (pH 6.7) resulted in the resolution of three protein-containing peaks: peak 1 was recovered in the flow-through fractions (40–60 ml), peak 2 was retarded (90–110 ml) and peak 3, which was retained, was eluted by 100 mM NaCl (160–175 ml). Peak 1 from CM-cellulose, which contained rat AFAR, was dialysed against 20 mM Tris/CH_3COOH, pH 8.1, before being subjected to chromatofocusing on PBE 94 in the pH range 8.0–5.0. Rat AFAR was eluted from the chromatofocusing column at approx. pH 6.0. The preparation of AFAR obtained by this method migrated as a single protein band when analysed by SDS/polyacrylamide-gel electrophoresis.

Enzyme and protein assays

Aldehyde- and ketone-reducing activity was routinely measured with a Shimadzu UV-3000 double-beam recording spectrophotometer by following the initial rate of oxidation of NADPH at 340 nm ($\epsilon = 6270\ M^{-1} \cdot cm^{-1}$). The assays were performed at 25 °C in reaction mixtures of 1 ml containing 100 mM sodium phosphate buffer, pH 6.6, and 0.2 mM NADPH (or 0.2 mM NADH). Typically, the final concentration of substrate in the reaction mixture was between 0.1 mM and 10 mM (depending on solubility) with a maximum concentration of 2% (v/v) methanol or 4% (v/v) acetonitrile as carrier; neither methanol nor acetonitrile was found to interfere with the assay or enzyme activity under these conditions. With assays of purified enzyme preparations, approx. 10 μg of protein was added to the cuvette to initiate the reaction and the reaction rate was measured against an identical blank with no enzyme added. Activity was measured for 4 min, over which time a change in absorbance of greater than 0.05 at 340 nm was deemed to be significant. Apparent K_m values for AFAR were determined by measuring the initial reaction rate over a range of substrate or cofactor concentrations (i.e. between $0.5K_m$ and $6K_m$) and were calculated with the Ultrafit curve-fitting software (Biosoft, Cambridge, U.K.) using the Marquardt algorithm. Inhibitors of aldo–keto reductase activity were added to the 4-NBA reaction mixture after the change in absorbance had been measured for a short period (approx. 1 min) so that their effect on the reaction rate could be compared directly with that obtained in the absence of inhibitor.

The catalytic oxidation of alcohols by AFAR was determined at 25 °C with either 1-acenaphthenol or 4-nitrobenzyl alcohol as substrates, using NADP as cofactor, in 100 mM sodium phosphate buffer over the pH range 6.0–9.0.

Protein concentrations were measured with a kit from Bio-Rad Laboratories Ltd. (Hemel Hempstead, Hertfordshire), which employed the method of Bradford [41]. The assay was standardized with BSA.

RESULTS AND DISCUSSION

Characterization of AFAR and rAFAR

We have previously shown [40] that the AFB_1-metabolizing aldehyde reductase purified from rat liver has a similar specific activity towards 4-NBA as the recombinant enzyme (rAFAR). To characterize the enzyme further, the cofactor requirement was determined. Both enzyme preparations can use NADH in addition to NADPH as cofactor, but the affinity for NADH is significantly lower than that observed for NADPH (Table 1). With 4-NBA as substrate, both enzymes have a pH optimum of 6.5 but, as is apparent from Figure 1, they can function over a broad pH range (pH 5–9).

Specificity of rAFAR in reduction of carbonyl substrates

Our previous studies have shown that AFAR catalyses the reduction of both AFB_1-dialdehyde and 4-NBA, but nothing further has been reported about other compounds that serve as

Table 1 Comparison of apparent K_m of recombinant AFAR and rat-liver AFAR for NADPH and NADH

Enzyme activity of recombinant AFAR and rat-liver AFAR was measured as described in Materials and Methods. The K_m values for NADPH and NADH were calculated from the initial reaction rates over a range of cofactor concentrations, with the concentration of 4-NBA at 1 mM.

	Substrate varied			
	NADPH		NADH	
Enzyme	K_m (μM)	V_{max} (μmol · min^{-1} · mg^{-1})	K_m (μM)	V_{max} (μmol · min^{-1} · mg^{-1})
AFAR	2.07 ± 0.57	1.31 ± 0.11	342 ± 32	0.57 ± 0.35
rAFAR	2.58 ± 0.08	1.24 ± 0.85	480 ± 22	0.67 ± 0.19

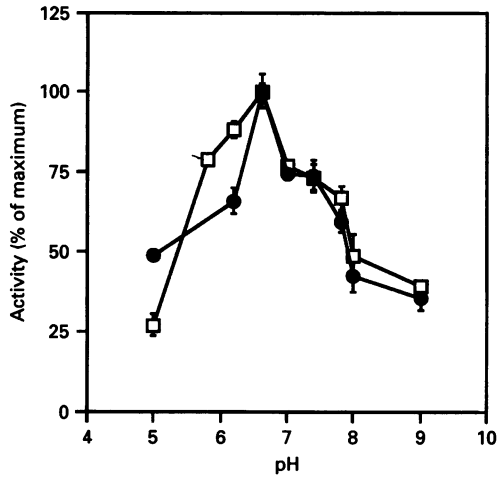

Figure 1 Optimum pH for the reduction of 4-nitrobenzaldehyde by AFAR and rAFAR

The 4-NBA reductase activity was measured between pH 5.0 and pH 9.0 in either 100 mM sodium phosphate (pH 5.0–8.6) or 100 mM glycine/NaOH (pH 9.0) using the manual assay procedure described in the text. The data points for rat-liver AFAR (●) and recombinant rAFAR (□) represent the average of duplicate measurements, with the S.E. represented by error bars.

substrates for this enzyme. To establish the substrate specificity of this enzyme, the ability of rAFAR to reduce a wide spectrum of aldehyde- and ketone-containing compounds has been studied (Tables 2 to 5).The compounds investigated include substrates of other carbonyl-reducing enzymes as well as products of lipid peroxidation and known toxic carbonyl-containing chemicals. It is apparent that, like many aldo–keto reductases, rAFAR has a broad specificity, and is able to reduce both aldehydes and ketones. From a comparison of the initial reaction rates, rAFAR displays the highest activity for 9,10-phenanthrenequinone (9,10-PQ), an aromatic diketone where the two carbonyl groups are on adjacent carbon atoms (Table 3). Other cyclic dicarbonyl compounds such as camphorquinone and acenaphthenequinone are also metabolized effectively by rAFAR and appear to serve as better substrates for rAFAR than straight-chain dicarbonyls such as methylglyoxal and 2,3-butanedione (Table 3). The positioning of carbonyl groups on non-adjacent carbon atoms as in 2,4-pentanedione seems to decrease substantially the ability of the enzyme to reduce one or other of the groups (Table 3). It was found that rAFAR is unable to reduce other diketones such as menadione. The fact that AFAR is unable to metabolize 3-hydroxy-2-butanone, a potential product of 2,3-butanedione (Table 4), suggests that this enzyme does not reduce both carbonyl groups sequentially.

The rAFAR also showed high reductase activity with aromatic aldehydes such as 4-NBA (Table 2) but little activity for aromatic ketones such as 4-nitroacetophenone (Table 4). Assays using substituted benzaldehydes suggest that the activity of rAFAR depends on the reactivity of the C atom in the carbonyl moiety. For example, rAFAR activity is substantially greater with compounds such as 2-NBA and 4-NBA, which contain an electron-withdrawing group on the benzene ring, than with the unsubstituted benzaldehyde. By contrast with 4-NBA and 2-NBA, the activity of rAFAR is significantly lower with compounds containing electron-donating groups, such as 4-hydroxybenzaldehyde or 4-methoxybenzaldehyde (Table 2).

Among saturated aliphatic aldehydes, rAFAR is able to reduce the negatively charged succinic semialdehyde (SSA), but possessed low activity for hexanal and propanal. It is unable to reduce the sugars D-xylose, D-glucuronate, D-galactose and D-glucose but does exhibit low activity towards DL-glyceraldehyde (Table 2).

In addition, rAFAR has low reductase activity with a number of physiologically important α,β-unsaturated carbonyl compounds, such as 4-HNE and acrolein (Table 5), both of which are produced during lipid peroxidation. AFAR seems to be inactive with the base propenals adenine-N^1-propenal and thymine-N^1-propenal (results not shown). The cytotoxic synthetic tripeptide ALLN, a potent inhibitor of cysteine proteases, is also a substrate for rAFAR (Table 2).

Oxidation of alcohol substrates by AFAR

The ability of both recombinant and rat-liver AFAR to oxidize either 1-acenaphthenol or 4-nitrobenzyl alcohol has been investigated over a range of pH values with NADP as cofactor (results not shown). In common with several other aldo–keto reductases [42], rAFAR could not be shown to oxidize any of the alcohols tested.

Comparison of the substrate specificities of AFAR and other aldo–keto reductases

Enzymes that catalyse the reduction of aldehyde-containing or ketone-containing compounds were originally divided on the basis of substrate specificity into three groups, namely aldose reductase, aldehyde reductase and carbonyl reductase [1]. Aldose reductase catalyses the reduction of sugars. Aldehyde reductase is active towards aromatic aldehydes and, with a few exceptions, cannot reduce ketone groups. Carbonyl reductase readily catalyses the reduction of diketones, aromatic ketones and

Table 2 Ability of rAFAR to reduce aldehydes

Enzyme activity was measured as described in Materials and Methods. Activities are expressed as a percentage of 4-nitrobenzaldehyde reductase activity.

Group	Substrate	Concentration (mM)	*V* (%)
Aromatic aldehydes	4-Nitrobenzaldehyde	1	100*
	2-Nitrobenzaldehyde	1	182
	4-Chloro-3-nitrobenzaldehyde	1	133
	3-Nitrobenzaldehyde	1	61
	Benzaldehyde	1	11
	3-Pyridine carboxyaldehyde	1	6.5
	4-Carboxybenzaldehyde	1	4.9
	Phenylacetaldehyde	1	< 0.1
	4-Hydroxybenzaldehyde	1	< 0.1
	3,4-Dimethoxybenzaldehyde	1	< 0.1
Aliphatic aldehydes	Succinic semialdehyde	1	99
	Hexanal	1	3.4
	Propionaldehyde (propanal)	1	1.5
	Formaldehyde	1	< 0.1
Peptide aldehyde	*N*-Acetyl-leucyl-leucyl-norleucinal	1	11
Aldoses and aldohydo acids	DL-Glyceraldehyde	1	8.3
	D-Xylose	10	< 0.1
	D-Glucuronic acid	10	< 0.1
	D-Glucose	10	< 0.1

* The specific activity for 4-nitrobenzaldehyde was 1345 nmol · min^{-1} · mg^{-1} under standard assay conditions

Table 3 Ability of rAFAR to reduce dicarbonyls

Enzyme activity was measured as described in Materials and Methods. Activities are expressed as a percentage of 4-nitrobenzaldehyde reductase activity.

Group	Substrate	Concentration (mM)	*V* (%)
Dicarbonyls	9,10-Phenanthrenequinone	0.05	259
	Acenaphthenequinone	0.1	169
	2,3-Bornanedione (camphorquinone)	1	165
	Phenylglyoxal	1	161
	2,3-Butanedione (diacetyl)	1	29
	Methylglyoxal	1	34
	Glyoxal	1	20
	2,4-Pentanedione (acetyl acetone)	1	4.8
	Acetonyl acetone	1	1.0
	5α-Androstanedione	1	0.5
	Aflatoxin B_1 dialdehyde	0.05	0.4
	Menadione	1	< 0.1

Table 4 Ability of rAFAR to reduce ketones

Enzyme activity was measured as described in Materials and Methods. Activities are expressed as a percentage of 4-nitrobenzaldehyde reductase activity.

Group	Substrate	Concentration (mM)	*V* (%)
Aromatic ketones	Doxorubicin (adriamycin)	0.25	< 0.1
	4-Benzoylpyridine	1	0.5
	Acetophenone	1	< 0.1
	4-Nitroacetophenone	1	< 0.1
	Metyropone	1	< 0.1
Aliphatic ketones	Acetoacetic acid	1	1.1
	Acetone	1	< 0.1
	Methyl ethyl ketone	1	< 0.1
	3-Hydroxy-2-butanone	1	< 0.1

Table 5 Ability of rAFAR to reduce α,β-unsaturated carbonyls

Enzyme activity was measured as described in Materials and Methods. Activities are expressed as a percentage of 4-nitrobenzaldehyde reductase activity.

Group	Substrate	Concentration (mM)	V (%)
α,β-Unsaturated carbonyls	Crotonaldehyde	1	3.6
	2-Furaldehyde	1	4.4
	trans-Cinnamaldehyde (3-phenyl-2-propanal)	1	2.5
	4-Hydroxynonenal	1	1.5
	Acrolein	1	1.3
	trans-2-Hexenal	1	0.6

Table 6 Catalytic properties of AFAR

Apparent K_m and V_{max} values were estimated from the initial velocities measured over a range of substrate concentration, with the NADPH concentration constant at 0.2 mM, as described in Materials and Methods.

	rAFAR			AFAR		
Substrate	K_m (mM)	V_{max} (μmol · min^{-1} · mg^{-1})	V_{max}/K_m (min^{-1} · mg^{-1} · ml)	K_m (mM)	V_{max} (μmol · min^{-1} · mg^{-1})	V_{max}/K_m (min^{-1} · mg^{-1} · ml)
4-Nitrobenzaldehyde	0.72 ± 0.05	2.58 ± 0.08	3.58 ± 1.60	0.70 ± 0.21	2.28 ± 0.35	3.26 ± 1.67
9,10-Phenanthrenequinone	0.03 ± 0.01	8.97 ± 0.96	299.00 ± 96.00	0.02 ± 0.01	8.04 ± 0.03	402.00 ± 3.00
Succinic semialdehyde	0.14 ± 0.02	1.46 ± 0.07	10.43 ± 3.50	0.19 ± 0.08	1.77 ± 0.03	9.31 ± 0.37
2-Nitrobenzaldehyde	0.58 ± 0.02	2.12 ± 0.03	3.65 ± 1.50	0.33 ± 0.05	2.50 ± 0.01	7.57 ± 0.33
Phenylglyoxal	0.46 ± 0.12	3.19 ± 0.21	6.94 ± 1.75	0.57 ± 0.03	3.52 ± 0.01	6.17 ± 0.33
3-Pyridine carboxaldehyde	8.88 ± 3.98	2.49 ± 0.48	0.28 ± 0.12	7.44 ± 2.29	1.78 ± 0.21	0.24 ± 0.09
Diacetyl	19.28 ± 1.40	3.84 ± 0.16	0.20 ± 0.11	28.69 ± 3.53	3.81 ± 0.24	0.13 ± 0.07
Camphorquinone	2.39 ± 0.35	3.36 ± 0.20	1.41 ± 0.57	2.88 ± 1.24	3.50 ± 0.67	1.21 ± 0.54
Methylglyoxal	7.22 ± 1.26	1.13 ± 0.04	0.16 ± 0.03	3.29 ± 1.88	1.12 ± 0.08	0.34 ± 0.04
Glyoxal	45.28 ± 13.80	2.16 ± 0.22	0.05 ± 0.01	46.18 ± 0.29	2.72 ± 0.01	0.06 ± 0.03
DL-Glyceraldehyde	49.28 ± 20.61	1.49 ± 0.41	0.03 ± 0.02	36.78 ± 19.25	0.67 ± 0.20	0.02 ± 0.01

quinones. Other carbonyl-reducing enzymes are now known to exist, and many are named after the substrate used for their original purification, although they were subsequently shown to be capable of metabolizing many different substrates.

The range of substrates that rAFAR can reduce distinguishes it from other enzymes so far characterized. For example, unlike aldose reductase, rAFAR cannot reduce the sugars D-glucose or D-xylose. Although many of the compounds that are among the best substrates for rAFAR are also utilized by 3α-HSD (9,10-phenanthrenequinone, camphorquinone and acenaphthenequinone), rAFAR is unable to reduce 4-nitroacetophenone or catalyse the oxidation of 1-acenaphthenol. This lack of reversibility even at high pH is similar to that found with certain other aldo–keto reductases [42]. Its ability to effectively reduce substrates containing diketones indicates that it has a similar substrate specificity to the structurally unrelated carbonyl reductase. However, unlike human carbonyl reductase and quinone reductase, rAFAR cannot reduce menadione [37,43].

Kinetic properties of recombinant and native rat AFAR

To allow comparisons between the kinetic properties of the AFB_1-metabolizing aldehyde reductase and other carbonyl-reducing enzymes, the apparent Michaelis constants of recombinant and native rat AFAR for some of the model substrates were determined. The values for each substrate tested are comparable between the recombinant enzyme and that purified from rat liver (Table 6). The results show that the enzyme has a relatively high affinity for 9,10-PQ (K_m 20 μM), moderate affinity for SSA (K_m 190 μM), low affinity for 4-NBA (K_m 700 μM) and weak affinity for DL-glyceraldehyde (K_m 37 mM).

Among other carbonyl-reducing enzymes, rat-liver 3α-HSD has a K_m of 1.9 μM for 9,10-PQ and a K_m of 55 μM for 4-NBA [34]. Interestingly, rAFAR has a significantly higher K_m for 4-NBA than other rat aldehyde reductases [44]. A comparison of the K_m values of AFAR and those of other rodent aldo–keto reductases (Table 7) shows that AFAR is distinct from enzymes described previously. AFAR is readily distinguishable from all other rodent aldehyde reductases by its high K_m for 4-NBA and DL-glyceraldehyde.

Inhibition of rAFAR activity

Like other aldo–keto reductases, the activity of rAFAR towards 4-NBA is sensitive to inhibition by various chemicals (Table 8). AFAR displayed a similar, though not identical, level of sensitivity to inhibition by phenobarbital and valproate as succinic semialdehyde reductase (SSR1) from rat brain [45]. However, rAFAR was found to be significantly more susceptible to inhibition by NaCl and Li_2SO_4 than aldehyde reductase from rat kidney [50]. When compared with mouse aldo–keto reductases, rAFAR was found to be inhibited to a similar extent to murine aldehyde reductase I by phenobarbital, but the mouse enzyme is substantially more sensitive to inhibition by Li_2SO_4 than rAFAR [46]. AFAR was also found to be less sensitive to phenobarbital,

Table 7 Comparison of kinetic properties of rat AFAR with other cytosolic carbonyl-metabolizing enzymes from rodents

Abbreviations used: 2-NBA, 2-nitrobenzaldehyde; 3-PC, 3-pyridine carboxaldehyde; GA, glyceraldehyde.

			K_m (μM)					
Enzyme	Reference	Substrate ...	4-NBA	2-NBA	9,10-PQ	SSA	3-PC	GA
AFAR	This study		700	330	20.0	190	7440	36780
Rat 3α-HSD	34		55	—	1.9	—	—	—
Rat high-K_m AR	44		80	—	—	130	—	4500
Rat SSR1	45		—	—	—	140	—	—
Rat SSR2	45		—	—	—	28	—	—
Mouse AR1	46		33	116	—	—	71	2500
Mouse AR2	46		133	769	—	—	250	1671
Mouse DD1	47		24	2	—	—	—	—
Guinea pig AR1	48		160	—	—	—	2200	—
Guinea pig AR2	48		40	—	—	—	903	—
Guinea pig AR3	48		30	—	—	—	880	8260
Gerbil AR1	49		100	50	120	—	111	280

Table 8 Effects of inhibitors on activity of AFAR towards 4-NBA

Activities were measured as described in Materials and Methods and are expressed as a percentage of 4-NBA activity with no inhibitor present.

Inhibitor	Concentration (mM)	Enzyme activity remaining towards 4-NBA (%)
None		100
Quercetin	0.01	34
Ethacrynic acid	0.10	22
Indomethacin	0.10	35
Phenobarbital	1.00	71
Sodium valproate	1.00	59
NaCl	100.00	77
Li_2SO_4	100.00	49

quercetin and ethacrynic acid than the carbonyl-metabolizing enzymes from gerbil (AR1, CR1 and CR2), although AFAR and gerbil AR1 display similar sensitivities to indomethacin [49].

CONCLUSIONS

The present study has demonstrated that AFAR can reduce a wide spectrum of carbonyl-containing compounds. However, the inability of AFAR to reduce D-xylose or D-glucose demonstrates that it does not participate in sugar metabolism. It can reduce several physiological substrates such as SSA, and to some extent glyceraldehyde, and it is possible that in normal rat liver it performs overlapping functions with other enzymes. The ability of AFAR to reduce SSA to 4-hydroxybutyrate may represent an important physiological function of the enzyme because succinic semialdehyde is produced *in vivo* as an intermediate of the 4-aminobutyrate shunt pathway. Reduction of SSA by enzymes in rat brain has been reported [44] and it is thought that 4-hydroxybutyrate may have a neurophysiological role [51] because high-affinity binding sites have been reported for this metabolite in synaptic-membrane preparations [52]. It is less clear whether the reduction of SSA is of significance in tissues other than brain.

The ability of AFAR to reduce a diverse range of compounds suggests that it has the potential to metabolize, and hence protect against, substances other than AFB_1-dialdehyde. Among the compounds examined, AFAR appears to have greatest detoxification capacity for dicarbonyls, several of which (e.g. methylglyoxal) are mutagenic in *Salmonella* strains [17]. In addition to protection against toxic substances, AFAR may also be involved in protecting against the products of oxidative stress. We have found that AFAR can catalyse the reduction of 4-HNE, hexanal and acrolein (Tables 2 and 5). All three of these aldehydes are produced during lipid peroxidation [5] and as they can interact with DNA and protein [53] it is reasonable to suppose that AFAR could prevent genotoxic and cytotoxic damage caused by products of reactive oxygen species.

As AFAR is expressed at only low levels in the livers of rats fed on control diets it might be thought that it plays only a minor role in normal hepatic metabolism. However, the fact that synthetic antioxidants such as ethoxyquin can increase the hepatic levels of AFAR by up to 20-fold [39] may mean that its role is more important under certain dietary conditions.

This paper provides the first description of the catalytic properties of AFAR. Our data show that this enzyme possesses a broad substrate specificity but has highest activity for aromatic aldehydes and aromatic dicarbonyls, some of which are mutagenic. It also exhibits high activity towards SSA, a property shared by relatively few reductases.

We are extremely grateful to Dr. H. Esterbauer for supplying research materials. We thank Kate Cardale for help with initial experiments in this project. This work was supported by the Medical Research Council, Grant G9200034SA.

REFERENCES

1 Wermuth, B. (1985) Prog. Clin. Biol. Res. **174**, 209–230
2 McMahon, R. E. (1982) in Metabolic Basis of Detoxication (Jakoby, W. B., Bend, J. R. and Caldwell, J., eds.) pp. 91–104, Academic Press, New York
3 Hoffman, P. L., Wermuth, B. and von Wartburg, J. P. (1980) J. Neurochem. **35**, 354–366
4 Vogel, K., Bentley, P., Platt, K.-L. and Oesch, F. (1980) J. Biol. Chem. **255**, 9621–9625
5 Esterbauer, H., Cheeseman, K. H., Dianzini, M. U., Poli, G. and Slater, T. F. (1982) Biochem. J. **208**, 129–140
6 Grollman, A. P., Takeshita, M., Pillai, K. M. R. and Johnson, F. (1985) Cancer Res. **45**, 1127–1131
7 Stone, E. J., Hall, R. M. and Kazeniac, S. J. (1975) J. Food Sci. **40**, 1138–1141
8 Felsted, R. L. and Bachur, N. R. (1981) in Enzymatic Basis of Detoxication, vol. 1 (Jakoby, W. B., ed.), pp. 281–293, Academic Press, New York

9 Belinsky, M. and Jaiswal, A. K. (1993) Cancer Metast. Rev. **12**, 103–117
10 Inoue, S., Sharma, R. C., Schimke, R. T. and Simoni, R. D. (1993) J. Biol. Chem. **268**, 5894–5898
11 Parekh, H. K. and Sladek, N. E. (1993) Biochem. Pharmacol. **46**, 1043–1052
12 Tipton, K. F. and Singer, T. P. (1993) J. Neurochem. **61**, 1191–1206
13 Guzelian, P. S. (1982) Annu. Rev. Pharmacol. Toxicol. **22**, 89–113
14 Judah, D. J., Hayes, J. D., Yang, J.-C. et al. (1993) Biochem. J. **292**, 13–18
15 Schauenstein, E., Esterbauer, H. and Zollner, H. (1977) Aldehydes in Biological Systems: Their Natural Occurrence and Biological Activities, Prion, London
16 Kanazu, T., Shinoda, M., Nakayama, T., Deyashiki, Y., Hara, A. and Sawada, H. (1991) Biochem. J. **279**, 903–906
17 Marnett, L. J., Hurd, H. K., Hollstein, M. C., Levin, D. E., Esterbauer, H. and Ames, B. N. (1985) Mutation Res. **148**, 25–34
18 Sladek, N. E., Manthey, C. L., Maki, P. A., Zhang, Z. and Landkamer, G. J. (1989) Drug Met. Rev. **20**, 697–720
19 Lindahl, R. (1992) Crit. Rev. Biochem. Mol. Biol. **27**, 283–335
20 Bosron, W. F. and Li, T.-K. (1981) in Enzymatic Basis of Detoxication, vol. 1 (Jakoby, W. B., ed.), pp. 231–248, Academic Press, New York
21 Riley, R. J. and Workman, P. (1992) Biochem. Pharmacol. **43**, 1657–1669
22 Tomlinson, D. R., Stevens, E. J. and Diemel, L. T. (1994) Trends Pharmacol. Sci. **15**, 293–297
23 Penning, T. M., Smithgall, T. E., Askonas, L. J. and Sharp, R. B. (1986) Steroids **47**, 221–247
24 Smithgall, T. E., Harvey, R. G. and Penning, T. M. (1988) J. Biol. Chem. **263**, 1814–1820
25 Carper, D., Nishimura, C., Shinohara, T. et al. (1987) FEBS Lett. **220**, 209–213
26 Bohren, K. M., Bullock, B., Wermuth, B. and Gabbay, K. H. (1989) J. Biol. Chem. **264**, 9547–9551
27 Pawlowski, J. E., Huizinga, M. and Penning, T. M. (1991) J. Biol. Chem. **266**, 8820–8825
28 Stolz, A., Rahimi-Kiani, M., Ameis, D., Chan, E., Ronk, M. and Shively, J. E. (1991) J. Biol. Chem. **266**, 15253–15257
29 Ciaccio, P. J. and Tew, K. D. (1994) Biochim. Biophys. Acta **1186**, 129–132
30 Deyashiki, Y., Ogasawara, A., Nakayama, T., Nakanishi, M., Miyabe, Y., Sato, K. and Hara, A. (1994) Biochem. J. **299**, 545–552
31 Winters, C. J., Molowa, D. T. and Guzelian, P. S. (1990) Biochemistry **29**, 1080–1087
32 Watanabe, K., Fujii, Y, Nakayama, K. et al. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 11–15
33 Wilson, D. K., Bohren, K. M., Gabbay, K. H. and Quiocho, F. A. (1992) Science **257**, 81–84
34 Pawlowski, J. E. and Penning, T. M. (1994) J. Biol. Chem. **269**, 13502–13510
35 Takahashi, M., Fujii, J., Teshima, T., Suzuki, K., Shiba, T. and Tanaguchi, N. (1993) Gene **127**, 249–253
36 Wermuth, B., Bohren, K. M., Heinemann, G., von Wartburg, J.-P. and Gabbay, K. H. (1988) J. Biol. Chem. **263**, 16185–16188
37 Wallin, R., Gebhardt, O. and Prydz, H. (1978) Biochem. J. **169**, 95–101
38 Williams, J. B., Lu, A. Y. H., Cameron, R. G. and Pickett, C. B. (1986) J. Biol. Chem. **261**, 5524–5528
39 Hayes, J. D., Judah, D. J. and Neal, G. E. (1993) Cancer Res. **53**, 3887–3894
40 Ellis, E. M., Judah, D. J., Neal, G. E. and Hayes, J. D. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 10350–10354
41 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
42 Leinweber, F. J., Greenough, R. C., Schwender, C. R., Kaplan, H. R. and DiCarlo, F. J. (1972) Xenobiotica **2**, 191–202
43 Wermuth, B. (1981) J. Biol. Chem. **256**, 1206–1213
44 Rivett, A. J., Smith, I. L. and Tipton, K. F. (1981) Biochem. J. **197**, 473–481
45 Rumigny, J. F., Maitre, M., Cash, C. and Mandel, P. (1980) FEBS Lett. **117**, 111–116
46 Tulsiani, D. R. P. and Touster, O. (1977) J. Biol. Chem. **252**, 2545–2550
47 Sawada, H., Hara, A., Nakayama, T., Nakagawa, M., Inoue, Y., Hasebe, K. and Zhang, Y.-p. (1988) Biochem. Pharmacol. **37**, 453–458
48 Sawada, H., Hara, A., Kato, F. and Nakayama, T. (1979) J. Biochem. **86**, 871–881
49 Molowa, D. T., Wrighton, S. A. and Guzelian, P. S. (1986) Arch. Biochem. Biophys. **251**, 487–494
50 Ohta, M., Tanimoto, T. and Tanaka, A. (1990) Chem. Pharm. Bull. **38**, 1639–1643
51 Turner, A. J. and Whittle, S. R. (1983) Biochem. J. **209**, 29–41
52 Benavides, J., Rumigny, J. F., Bourguignon, J. J., Cash, C., Wermuth, C. G., Mandel, P., Vincendon, G. and Maitre, M. (1982) Life Sci. **30**, 953–961
53 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

Received 19 April 1995/2 August 1995; accepted 15 August 1995