A novel isoenzyme of aldehyde dehydrogenase specifically involved in the biosynthesis of 9-*cis* and all-*trans* retinoic acid

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The pleiotropic effects of retinoids are mediated by two families of nuclear receptors: RAR (retinoic acid receptors) and RXR (retinoid X receptors). 9-*cis*-Retinoic acid is a specific ligand for RXR receptors, whereas either 9-*cis*- or all-*trans*-retinoic acid activates the RAR receptor family. The existence of RXRs suggests a new role for isomerization in the biology of retinoic acid. We report here the identification of an aldehyde dehydrogenase in the rat kidney that catalysed the oxidation of 9-*cis*- and all-*trans*-retinal to corresponding retinoic acids with high efficiency, 9-*cis*-retinal being 2-fold more active than all-*trans*-retinal. Based on several criteria, such as amino acid sequence, pH optimum, and inhibition by chloral hydrate, this enzyme was found to be a novel isoenzyme of aldehyde dehydrogenase. 9-*cis*-Retinol, the precursor for the biosynthesis of 9-*cis*-retinal was identified in the rat kidney. The occurrence of endogenous 9-*cis*-retinol and the existence of specific dehydrogenase which participates in the catalysis of 9-*cis*-retinal suggest that all-*trans*-retinoi(d) isomerization to 9-*cis*-retinoi(d) occurs at the retinol level, analogous to all-*trans*-retinol isomerization to 11-*cis*-retinol in the visual cycle.

INTRODUCTION

Retinoids (vitamin A and its analogues) are a group of signalling molecules which regulate diverse aspects of cell differentiation, embryonic development, growth and vision [1,2]. The cellular retinoid receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) are members of the steroid receptor superfamily that function as ligand-dependent transcription factors [3–6]. The classification into the RAR and RXR subfamilies is mainly based on sensitivity to retinoid ligands, primary structure and ability to regulate expression of different target genes [7–9]. Since 9-*cis*- or all-*trans*-retinoic acid are ligands for RARs whereas 9-*cis*-retinoic acid is the ligand for RXRs, the identification and regulation of enzymes involved in the biosynthesis of these retinoids are crucial to an understanding of the physiological processes, whereby these compounds regulate cell differentiation and morphogenesis.

In previous studies, we reported high levels of retinoic acid and vitamin A-dependent retinal-oxidizing enzyme activity in the rat kidney [10,11]. We recently purified this enzyme from rat kidney cytosol and investigated its properties [12]. The apparent molecular mass of the native enzyme was 140000 Da. It had a pI of 8.5 and a subunit molecular mass of 53 kDa by SDS/PAGE, exhibiting the lowest K_m for all-*trans*-retinal substrate. In this report, we present the N-terminal and partial amino acid sequence of this enzyme and investigated its specificity towards stereoisomers of retinal.

MATERIALS AND METHODS

9-*Cis*-, 13-*cis*- and all-*trans*-retinal were purchased from the Sigma Chemical Company (St. Louis, MI, U.S.A.), and 11-*cis*-retinal was a generous gift from Dr. Rosalie Crouch of the Medical University of South Carolina (U.S.A.). The retinal isomers were more than 99% pure, as judged by h.p.l.c. Ultrascorb 5 μM octadecyl silane was obtained from Phenomenex. Zorbax CN was purchased from Dupont Canada.

Enzyme assay

The standard incubation mixture, in 100 mM phosphate buffer (pH 7.5) contained: 0.02% Tween-80, 1.61 mM dithiothreitol, 603 μM NAD and 200–300 ng of pure enzyme. Reactions were initiated by addition of the substrates (1.34–16 μM), 9-*cis*-, all-*trans*-, 11-*cis*- and 13-*cis*-retinal in 2.5 μl of dimethylsulphoxide, which was incubated for 60 min at 25 °C. The reaction was terminated by cooling the reaction mixture in ice-cold water. Retinoic acids produced were determined by h.p.l.c. as described previously [11]. From the known molar absorption coefficients of *cis*-isomers of retinoic acid [13], appropriate corrections were made on the all-*trans*-retinoic acid standard curve to quantify the *cis*-isomers of retinoic acid produced in the enzymic reaction.

N-terminal and partial amino-acid sequence

Since the N-terminal of retinal dehydrogenase (RALDH) was blocked, the non-enzymic method for deblocking proteins was used [14]. The enzyme was fragmented for internal sequence by chemical cleavage with cyanogen bromide [15]. The cleaved protein was subjected to SDS/PAGE and transferred on to polyvinylidene difluoride (PVDF, Problott, ABI) membrane [16]. The N-terminal sequence of the deblocked enzyme and fragments on PVDF membrane were obtained by automated Edman degradation performed on a Model 470A gas-phase sequencer equipped with an on-line Model 120A phenylthiohydantoin analyser (Applied Biosystems Inc.) [17].

Extraction and quantification of retinol isomers

Kidneys from Sprague–Dawley rats (Canadian Breeding Farm) maintained on a vitamin A-deficient diet (ICN Nutritional Biochemicals) for 8 weeks and supplemented with 600 μg/week of retinyl palmitate (vitamin A-sufficient rats) were used to extract the endogenous retinol isomers. It was essential to

Abbreviations used: RAR, retinoic acid receptor; RXR, retinoid X receptor; CRBP, cellular retinol-binding protein; PVDF, polyvinylidene difluoride; RALDH, retinal dehydrogenase; AHD-2, mouse liver cytosolic aldehyde dehydrogenase; Pb, phenobarbital-induced cytosolic rat liver aldehyde dehydrogenase; E_1, human liver cytosolic aldehyde dehydrogenase; CRABP, cellular retinoic acid-binding protein.

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maintain the rats on this controlled diet to eliminate the other sources of vitamin A such as β-carotene which is normally present in the stock diet. The kidneys were frozen in liquid nitrogen as soon as they were removed and stored at −80 °C. After homogenization of the whole kidney with 2.0 ml of phosphate-buffered saline, 2 ml of ethanol was added. The retinoids were then extracted three times with 16 ml of petroleum ether (b.p. 30–60 °C). The petroleum ether in the combined extract was dried with anhydrous sodium sulphate and evaporated under nitrogen gas, and the residue was dissolved in 200 μl of 1 % (v/v) 2-octanol in hexane. To minimize the non-specific isomerization, all operations were conducted under yellow light and in ice. Retinol isomers from the extract were separated by h.p.l.c. as described previously [18].

RESULTS AND DISCUSSION

The N-terminal and partial amino-acid sequences of the enzyme RALDH are shown in Figure 1. A total of 125 amino acids in the sequence (approx. 25 % of the entire sequence) was obtained. The partial amino-acid sequence of RALDH showed 95, 93 and 83 % sequence identity with mouse liver cytosolic aldehyde dehydrogenase (AHD-2), phenobarbital-induced cytosolic rat liver aldehyde dehydrogenase (Pb), and human liver cytosolic aldehyde dehydrogenase (E_1) respectively. The distribution of unidentical amino-acid residues seemed not to be uniform between these four aldehyde dehydrogenases. Between RALDH and Pb 100 % sequence identity was observed in amino acids 1–18 and 79–90, while only 63 % similarity was seen in amino acids 105–120. However, there was 100 % identity between RALDH and AHD-2 in the same regions. On the other hand, specific differences between RALDH and AHD-2 in amino-acid residues in sequence were evident at positions 204, 246 and 431.

We found that the pH optimum of purified enzyme for all-*trans*-retinal oxidation was 7.5, which is similar to the earlier observation for retinal-oxidizing enzyme activity found in the kidney cytosol [11]. Pb has been shown to be highly active at pH 9 with propionaldehyde as substrate [19]. It is not known whether Pb catalyses retinal oxidation. Chloral hydrate is a potent inhibitor (91 % inhibition at 50 μM concentration) of RALDH activity, whereas it does not affect Pb activity [19].

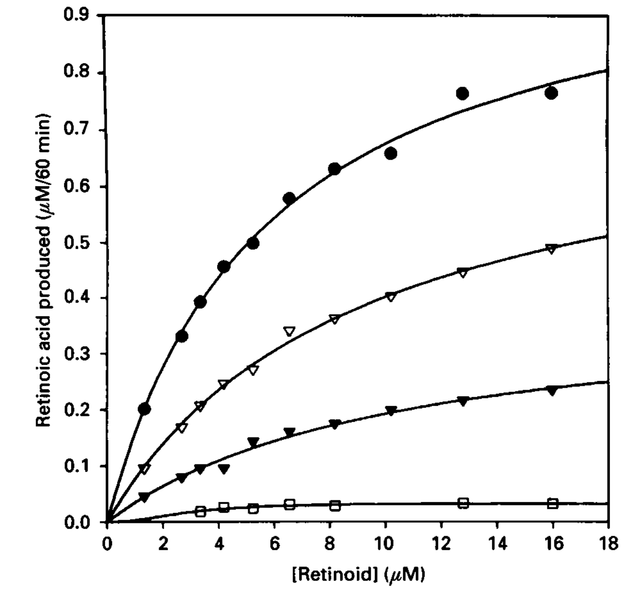

Figure 2 Saturation curves of RALDH with retinal isomers

Incubation conditions are described in the Materials and methods section. 9-*Cis*- (●), all-*trans*- (▽), 11-*cis*- (▼) and 13-*cis*- (□) retinal. Each point in the curve represents the average of four replicates (less than 6% variation between each replicate).

mRNA expression for Pb has been detected by Northern blot in the rat kidney [20]; however, Pb mRNA levels are not inducible by phenobarbital in the kidney, suggesting that aldehyde dehydrogenase expressed in this organ is different from liver Pb. This partial amino-acid sequence of RALDH represents the first report of primary structure of a constitutive cytosolic aldehyde dehydrogenase in the rat.

The high degree of similarity between RALDH and mouse AHD-2, at least in the regions sequenced, indicates that these isoenzymes are equivalent forms in rats and mice. Recently, it has been shown that AHD-2 oxidized all-*trans*-retinal to retinoic acid with a low K_m at pH 8.2 [21]. However, AHD-2 is apparently

	1 – 18	79 – 90	95 – 104	105
RALDH:	S S P A Q P A V P A P L A N L K I Q	M D A S E - G - L L N K	M E R D - L L L A T	M E A - N G G K
Pb:	S S P A Q P A V P A P L A N L K I Q	M D A S E R G C L L N K	M E R D R V L L A T	M E S M N A G K
AHD-2:	S S P A Q P R V P A P L A D L K I Q	M D A S E R G C L L N K	M E R D R L L L A T	M E A L N G G K
E1:	S S S G T P D L P V L L T N L K I Q	M D A S E R G R L L Y K	I E R D R L L L A T	M E S M N G G K

	120	204 – 224	234 – 255
RALDH:	V F A N A Y L S	M A S L I - E - G F P - - V V - - V - G Y	M D V D K V A F T G - T Q V G K L I K E A A
Pb:	I F T H A Y L L	M A S L I K E A G F P P G V V N V V P G Y	M D I D K V S F T G S T E V G K L I K E A A
AHD-2:	V F A N A Y L S	L A S L I K E A G F P P G V V N I V P G Y	M D V D K V A F T G S T G V G K L I K E A A
E1:	L Y S N A Y L N	V A S L I K E A G F P P G V V N I V P G Y	M D I D K V S F T G S T E V G K L I K E A A

	393 – 437
RALDH:	M - I A K E E I F G P V Q Q I M K F K - I D - V I K R A N N T - Y G L A A G V F T K D L D
Pb:	M R I A K E E I F G P V Q Q I M K F K S I D E V I K R A N N T P Y G L A A G V F T K D L D
AHD-2:	M R I A K E E I F G P V Q Q I M K F K S V D D V I K R A N N T T Y G L A A G L F T K D L D
E1:	M R I A K E E I F G P V Q Q I M K F K S L D D V I K R A N N T F Y G L S A G V F T K D I D

Figure 1 Comparison of the N-terminal and partial amino-acid sequences of RALDH with amino-acid sequences of other cytosolic aldehyde dehydrogenases

Phenobarbital-induced cytosolic rat liver dehydrogenase (Pb) [20], mouse liver cytosolic aldehyde dehydrogenase (AHD-2) [38] and human liver cytosolic aldehyde dehydrogenase (E_1) [39] are compared. Amino-acid residues are boxed when they are different from RALDH. Dashes represent unidentified amino acids in RALDH.

Table 1 Specificity of RALDH for retinal isomers

Kinetic constants were obtained by fitting the data from the saturation curves to Michaelis–Menten and Lineweaver–Burk kinetic plots with the computer program Enzfitter [40]. Values are the averages of two independent determinations, where each point in the curve in each determination represents the average of four replicates.

Retinal isomer	K_m (μM)	$V_{max.}$ (μM/60 min)	$V_{max.}/K_m$
9-*Cis*	5.70	1.10	0.19
All-*trans*	9.8	0.77	0.08
11-*Cis*	10.50	0.39	0.04

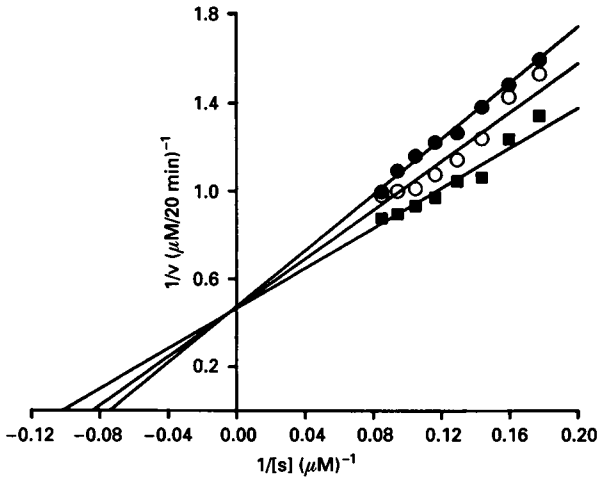

Figure 3 A double-reciprocal plot of inhibition of RALDH activity by all-*trans*-retinol

The incubation conditions are described in the Materials and methods section, except that it was carried out at 37 °C for 20 min. The substrate used was all-*trans*-retinal. All-*trans*-retinol concentrations: ■, none; ○, 2 μM; ●, 3 μM. Each point in the curve represents the average of four replicates (less than 10% variation between each replicate).

not expressed in the mouse kidney [22]. Taken together, these observations suggest that RALDH isolated from the rat kidney is a novel isoenzyme of aldehyde dehydrogenase involved in retinoic acid biosynthesis.

We examined the ability of RALDH to catalyse the oxidation of 9-*cis*-, 11-*cis*- and 13-*cis*-retinal in order to evaluate substrate specificity. Non-specific isomerization of retinal isomers during incubation was minimized (< 0.1 %) by reducing the incubation temperature to 25 °C and by using 0.02 % Tween-80 instead of 0.5 mg of BSA as solubilizing agent. Saturation kinetics with retinal isomers showed that 9-*cis*-retinal had the highest activity (Figure 2). The catalytic efficiency of 9-*cis*-retinal was 2-fold higher than that of all-*trans*-retinal (Table 1). Furthermore, we found that the oxidative products of *cis*-retinal by RALDH were *cis*-retinoic acids [23]. Interestingly, 13-*cis*-retinal catalysis was barely detectable in all concentrations used. These kinetic data clearly demonstrate the specific catalytic selectivity of RALDH towards 9-*cis*-retinal. It is evident from the structures and catalytic activities of retinal stereoisomers that there exist at least two sites in the enzyme molecule where the substrate interacts. First, the retinoi(d) side-chain drives initial binding of the molecule. Secondly, *trans*-orientation of the 13–14 double bond is essential for efficient catalysis. Among the well-known retinal stereoisomers, the bent structure at 9–10 carbon atoms and *trans*-configuration at the terminal aldehyde group (9-*cis*-retinal) favour efficient catalysis. This observation suggests that 9-*cis*-retinal is the preferred structure and could be the physiological substrate for RALDH.

We have reported previously that all-*trans*-retinol is a potent inhibitor of all-*trans*-retinoic acid synthesis by crude cytosolic preparations from the rat kidney [11]. Therefore, we examined the influence of the inhibition by all-*trans*-retinol on RALDH activity. All-*trans*-retinol competitively inhibited all-*trans*-retinal oxidation with an apparent inhibition constant (K_i) of 9.1 μM, which is similar to the K_m for all-*trans*-retinal (9.8 μM) (Figure 3). Similarly, 9-*cis*-retinol competitively inhibited the catalytic oxidation of 9-*cis*-retinal with a K_i of 5.4 μM (similar to the K_m for 9-*cis*-retinal) (results not shown). Since we did not observe the catalysis of either all-*trans*-retinol or 9-*cis*-retinol to the respective isomers of retinal by RALDH, the inhibition caused by these retinol isomers was due to competition for recognition sites in the enzyme molecule with respective stereoisomers of retinal.

In the frog liver, using radioactive all-*trans*-retinol, the formation of 9-*cis*-retinol has been demonstrated [24]. However, the occurrence of endogenous 9-*cis*-retinol in the mammalian tissues has not been reported yet. To determine whether 9-*cis*-retinol occurs *in vivo* in the rat kidney, we extracted retinoids from this organ as described in the Materials and methods section. Figure 4 illustrates a representative h.p.l.c. profile of the endogenous retinol isomers present in kidney extract. We found significant amounts of endogenous 9-*cis*-retinol in the normal (vitamin A-sufficient) rat kidney (peak 5, Figure 4). We estimated from peak areas that rat kidney contained 0.64 nmol of 9-*cis*-retinol and 6.23 nmol of all-*trans*-retinol per whole kidney (average of three independent determinations from three rat kidneys) respectively. To examine whether 9-*cis*-retinol could derive from all-*trans*-retinol during the extraction, kidney tissue was spiked with tritiated all-*trans*-retinol (New England Nuclear, 99.9 % pure), homogenized and extracted. Subsequent h.p.l.c. fractionation of the extract revealed that 3 % of the radioactivity resided in 13-*cis*-retinol and less than 0.8 % of the radioactivity in 9-*cis*-retinol. This indicates that the endogenously present 9-*cis*-retinol is not formed from all-*trans*-retinol during extraction.

Recent studies have shown that cellular retinoid-binding proteins are involved in vitamin A metabolism [25–27]. Rat kidney expresses two cellular retinoid-binding proteins (CRBPs); one is specific for all-*trans*-retinol (CRBP type I) and the other binds specifically all-*trans*-retinoic acid (CRABP type I) [28]. Neither of these proteins bind all-*trans*- or 9-*cis*-retinal *in vitro* [29,30]. Furthermore, all-*trans*-retinol and all-*trans*-retinoic acid have been shown to be the endogenous ligands of CRBP (I) and CRABP (I) respectively [31], suggesting that these binding proteins are unlikely to provide retinal substrates to RALDH *in vivo*. Therefore, CRBP (I) and CRABP (I) may not play direct roles in the enzymic oxidation of retinal to retinoic acid.

In our studies we tested the ability of a purified novel aldehyde dehydrogenase to catalyse the oxidation of various retinal isomers using Tween-80 as solubilizing agent. Although in a number of studies the investigators have used various organic solvents and detergents in retinol and retinal dehydrogenase assays [21,32,33], we chose a 0.02 % concentration of Tween-80 in our incubations mainly because this concentration did not affect the activity of the enzyme. In addition, as reported by Xavier and Julia [34], we also observed the reproducibility of the measurements in the presence of 0.02 % Tween-80 [12].

Several investigators have unequivocally demonstrated that 9-*cis*-retinoic acid is an endogenous ligand for the nuclear RXR

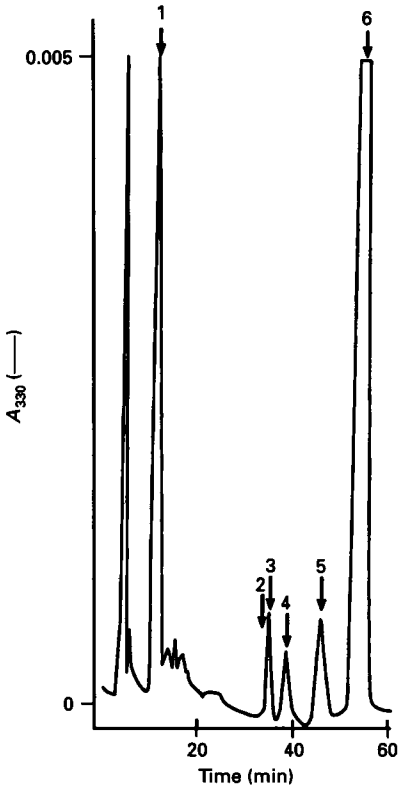

Figure 4 Representative h.p.l.c. chromatogram indicating the presence of 9-*cis*-retinol in the rat kidney

The lipid extract from the kidney was fractionated on Phenomenex–ODS and Zorbax CN columns connected in series and developed with a mobile phase of 1% (v/v) 2-octanol in hexane at a flow rate of 2.0 ml/min [18]. Arrow marks 1, 2, 3, 5 and 6 represent the elution positions of standard retinyl acetate, 11-*cis*-retinol, 13-*cis*-retinol, 9-*cis*-retinol and all-*trans*-retinol respectively. Peak 4 is an unidentified retinol isomer present in the kidney extract. Standard retinol isomers were obtained by reduction of respective retinal isomers with sodium borohydride [18].

[7,8]. Furthermore, the occurrence of 9-*cis*-retinoic acid in the mouse liver and kidney, where RXR is highly expressed, has been reported [8]. Therefore, regulation of all-*trans*-retinoi(d) isomerization to 9-*cis*-retinoi(d) could be a key step in retinoic acid physiology. Although the formation of 9-*cis*-retinoic acid from all-*trans*-retinoic acid has been found *in vitro* in cell incubations, it is not known whether this reaction is catalysed by an enzyme. Since non-enzymic isomerization of all-*trans*-retinoic acid to 9-*cis*-retinoic acid occurs when all-*trans*-retinoic acid is incubated with cells for more than 16 h [35], it is likely that the 9-*cis*-retinoic acid detected in these studies is due to non-specific formation from all-*trans*-retinoic acid. The demonstration of 9-*cis*-retinal as the most preferred substrate for RALDH in the present study and the occurrence of 9-*cis*-retinol in the kidney strongly suggests that isomerization occurs at the retinol-oxidation step. In the process of 9-*cis*-retinoic acid biosynthesis, we propose that all-*trans*-retinol is isomerized to 9-*cis*-retinol, and 9-*cis*-retinol is oxidized by an as yet unidentified dehydrogenase to 9-*cis*-retinal. 9-*cis*-Retinal is further oxidized by RALDH to 9-*cis*-retinoic acid. This proposed pathway leading to the production of 9-*cis*-retinoic acid is further supported by work done on the visual process, where retinal pigment epithelium specifically isomerizes all-*trans*-retinol to 11-*cis*-retinol [36,37].

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