

# Human aldose reductase and human small intestine aldose reductase are efficient retinal reductases: consequences for retinoid metabolism

Bernat CROSAS\*<sup>†1</sup>, David J. HYNDMAN<sup>†</sup>, Oriol GALLEGO\*, Sílvia MARTRAS\*, Xavier PARÉS\*<sup>2</sup>, T. Geoffrey FLYNN<sup>†</sup> and Jaume FARRÉS\*

\*Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, E-08193 Bellaterra (Barcelona), Spain, and <sup>†</sup>Department of Biochemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6

Aldo–keto reductases (AKRs) are NAD(P)H-dependent oxidoreductases that catalyse the reduction of a variety of carbonyl compounds, such as carbohydrates, aliphatic and aromatic aldehydes and steroids. We have studied the retinal reductase activity of human aldose reductase (AR), human small-intestine (HSI) AR and pig aldehyde reductase. Human AR and HSI AR were very efficient in the reduction of all-*trans*-, 9-*cis*- and 13-*cis*-retinal ( $k_{\text{cat}}/K_m = 1100\text{--}10\,300\text{ mM}^{-1}\cdot\text{min}^{-1}$ ), constituting the first cytosolic NAD(P)H-dependent retinal reductases described in humans. Aldehyde reductase showed no activity with these retinal isomers. Glucose was a poor inhibitor ( $K_i = 80\text{ mM}$ ) of

retinal reductase activity of human AR, whereas tolrestat, a classical AKR inhibitor used pharmacologically to treat diabetes, inhibited retinal reduction by human AR and HSI AR. All-*trans*-retinoic acid failed to inhibit both enzymes. In this paper we present the AKRs as an emergent superfamily of retinal-active enzymes, putatively involved in the regulation of retinoid biological activity through the assimilation of retinoids from  $\beta$ -carotene and the control of retinal bioavailability.

**Key words:** aldo–keto reductase, enzyme kinetics, retinoic acid, retinol, tolrestat.

## INTRODUCTION

Retinoic acid, the oxidized form of vitamin A (retinol), is essential for tissue development and differentiation, and cellular functions [1]. Retinoic acid exerts its regulatory function bound to nuclear receptors [2]. In recent years, an increasing body of evidence has highlighted the role of retinoic acid signalling in important processes such as endothelium proliferation, nervous-system function and cancer therapy [3,4]. The metabolic pathway of retinoids, which accounts for the regulation of retinoic acid levels, includes several oxidation steps, binding to specific proteins and storage as retinyl esters. Retinol oxidation and retinal reduction are key reactions that define a limiting step for the production of retinoic acid [5,6].

Most vertebrate alcohol dehydrogenase (ADH) enzymes of the medium-chain dehydrogenase/reductase (MDR) family, and several short-chain dehydrogenase/reductases (SDRs), are active with retinoids and may account for the physiological oxidation of retinol and reduction of retinal [5,6]. Recently we have characterized a chicken aldo–keto reductase (AKR), closely related to the aldose reductase (AR) and AR-like enzymes, which is active with ethanol and very efficient in all-*trans*-retinal and 9-*cis*-retinal reduction, suggesting a contribution of this enzyme to the metabolism of retinoids [7]. Moreover, docking simulations, performed in the same study, predicted that the human AR structure could productively bind all-*trans*-retinal, suggesting that this molecule could also be a substrate for AR.

AKR enzymes are monomeric NAD(P)H-dependent oxidoreductases that catalyse the reduction of carbonyl compounds [8]. AKR enzymes are widely distributed in Nature and have been grouped into structural and functional families, which include aldehyde reductase (AKR1A), AR (AKR1B), hydroxysteroid

dehydrogenase (AKR1C), ketosteroid (oxosteroid) reductase (AKR1D) and aflatoxin reductase (AKR7A) families, all of them found in humans. For many of these enzymes, several physiological substrates have been proposed, the enzymes being involved in important biochemical pathways, such as aldehyde detoxification, osmoregulation, fructose synthesis, steroid conversion and catecholamine metabolism [9]. AR is active with glucose and other monosaccharides, lipid peroxidation aldehydes, corticosteroids and the cholesterol derivative 2-methylpentanal ('isocaproaldehyde') [10–13]. This enzyme has been suggested to participate in the development of secondary diabetic complications, owing to its ability to reduce glucose to sorbitol, a hyperosmotic compound [14,15]. The role of AR in an euglycaemic environment is still unclear. In the last decade, a group of enzymes closely related to the ARs, the AR-like subfamily [8,15], has been described. This group contains at least one human member (AKR1B10), henceforth known as human small-intestine (HSI) AR [16,17] and several forms from rodent species (AKR1B7–AKR1B9) [18–20]. These enzymes show low or null activity with aldoses. Aldehyde reductase exhibits a more restricted substrate specificity than that of AR, showing low activity with glucose [21].

In the present study we have searched for retinal reductase activity in three different AKR enzymes: AR, HSI AR and aldehyde reductase. Human AR and HSI AR were found to be highly active with several retinal isomers, whereas aldehyde reductase did not show activity with retinals. The finding that retinal reductase activity is spread throughout the AR family represents the emergence of a novel retinal-active superfamily, the AKRs, and provides new data for understanding the regulation of retinoid metabolism.

Abbreviations used: ADH, alcohol dehydrogenase; AKR, aldo–keto reductase; AR, aldose reductase; ARIs, aldose reductase inhibitors; HSI, human small intestine; MDR, medium-chain dehydrogenase/reductase; SDR, short-chain dehydrogenase/reductase.

<sup>1</sup> Present address: Department of Cell Biology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115, U.S.A.

<sup>2</sup> To whom correspondence should be addressed (e-mail xavier.pares@uab.es).

## EXPERIMENTAL

### Enzyme purification

Human AR, HSI AR and pig aldehyde reductase were recombinantly expressed and purified using the following procedure. Luria–Bertani medium, with [NaCl] decreased to 5 g/l and supplemented with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml), was inoculated with an *Escherichia coli* BL21DE3pLysS strain transformed with the plasmid pET16b containing the cDNA for each enzyme. Liquid culture (500 ml) was incubated at 28 °C with shaking at 250 rev./min until an attenuation ( $D_{600}$ ) of 0.7 was reached. Then T7 RNA polymerase gene expression was induced by the addition of isopropyl β-D-thiogalactoside up to 1 mM, for 4 h. Cells were harvested by centrifugation at 8000 g for 15 min, resuspended in 15 ml of 5 mM imidazole/0.5 M NaCl/20 mM Tris/HCl, pH 7.9, and frozen at –20 °C. Cells were lysed by sonication with five to ten pulses (30 s each) and centrifuged at 12 000 g. The supernatant was filtered through a sterile, low-protein-binding syringe tip filter (Millex-HV; 0.45 µm pore size; Millipore) and applied to a nickel-charged chelating Sepharose column (5 ml; Amersham Biosciences) at a flow rate of 0.5 ml/min. The column was washed with 50 ml of 60 mM imidazole/0.5 M NaCl/20 mM Tris/HCl, pH 7.9, and the enzyme was eluted with 60 ml of a linear gradient (60 mM–1 M imidazole). Purity was assessed by electrophoresis on SDS/polyacrylamide gels and staining with Coomassie Blue.

### Enzyme kinetics

Activity with substrates other than retinoids was determined by monitoring the change in NADPH concentration at 25 °C in a diode-array spectrophotometer (Hewlett–Packard 8452A) by measurements at 340 nm, in 0.1 M sodium phosphate, pH 7.5. One unit of activity corresponds to 1 µmol of coenzyme utilized/min, based on a molar absorption coefficient ( $\epsilon_{340}$ ) of 6220 M<sup>-1</sup>·cm<sup>-1</sup>. Activity during the purification procedures was determined with 5 or 50 mM glyceraldehyde, and 200 µM NADPH. Retinal reduction and retinol oxidation were monitored at 400 nm, in the presence of 0.02 % Tween 80, using reported molar absorption coefficients for the retinoid isomers [22]. A 295 nm UV cut-off filter was used to prevent photodegradation of NADPH and retinoids.

Kinetic parameters were obtained from initial-rate activity measurements, with substrate concentrations that ranged from 0.1 ×  $K_m$  to 10 ×  $K_m$ . Each individual rate measurement was done in duplicate. At least three independent determinations were performed for each kinetic constant. Values were calculated using ENZFITTER (Elsevier Biosoft) or Kinetics Software (Hewlett–Packard HP89512), using a non-linear Marquardt's regression method, and expressed as the mean ± S.D. The concentration of active enzyme was calculated by coenzyme titration in a PerkinElmer fluorimeter according to a published procedure [23]. Tolrestat was a gift from Wyeth–Ayerst Research (Princeton, NJ, U.S.A.). Inhibition-constant ( $K_i$ ) values for glucose and tolrestat were calculated from the secondary plot of slope values from the double-reciprocal plot versus inhibitor concentration. Experimental data points were adjusted with a linear-regression method. Inhibition assays with all-*trans*-retinoic acid (1.5–100 µM) were run in the presence of 30 µM 9-*cis*-retinal and 200 µM NADPH.

### Solid-phase extraction and HPLC analysis of retinoids

Retinoids in the enzymic reaction mixture were analysed by HPLC. Handling of retinoids was done under red light and

**Table 1 Kinetic constants of AKR enzymes with glucose and other aldehydes at pH 7.5**

All determinations were performed in 0.1 M sodium phosphate, pH 7.5, with 0.2 mM NADPH.  $K_m$  values are in mM and  $k_{cat}$  values in min<sup>-1</sup>. Abbreviations: N.S., no saturation up to 1.8 M glucose. ALR, aldehyde reductase.

Substrate	Enzyme ...	Human AR	HSI AR	Pig ALR
Glucose	$K_m$	76 ± 14	N.S.	N.S.
	$k_{cat}$	97 ± 2	N.S.	N.S.
	$k_{cat}/K_m$	1.3 ± 0.2	N.S.	N.S.
Glyceraldehyde	$K_m$	0.044 ± 0.002	5.7 ± 0.6	4.65 ± 0.7
	$k_{cat}$	250 ± 16	640 ± 30	2500 ± 90
	$k_{cat}/K_m$	5700 ± 450	110 ± 10	540 ± 80
Glyceraldehyde (0.02 % Tween 80)	$K_m$	0.043 ± 0.002	6.1 ± 1.6	5.52 ± 0.4
	$k_{cat}$	340 ± 6	550 ± 10	2500 ± 50
	$k_{cat}/K_m$	7900 ± 390	90 ± 20	450 ± 30
Pyridine-3-aldehyde	$K_m$	0.014 ± 0.0014	0.037 ± 0.005	0.7 ± 0.08
	$k_{cat}$	245 ± 12	185 ± 8	11 000 ± 90
	$k_{cat}/K_m$	17 500 ± 1900	5000 ± 700	16 000 ± 1800

in the presence of butylated hydroxytoluene (Sigma) in the reaction buffer. Retinoids were extracted using Oasis<sup>TM</sup> Extraction Cartridges HLB (Waters) and eluted with methanol (HPLC grade). Samples were kept protected from light at –20 °C until use. HPLC analysis was performed on a Waters 600 separation module, 996 photodiode-array detector and a LiChrosorb RP18 column (250 mm × 4.6 mm; 5 µm pore size; Phase Separations). Separation of retinoids was achieved using an isocratic solvent system consisting of acetonitrile/1 % (w/v) ammonium acetate (4:1, v/v) at a constant flow rate of 1.2 ml/min. Retinoids were monitored at a wavelength of 340 nm. Analysis of the chromatograms was performed using Waters Millennium software.

### Docking with retinoids

The interaction of retinoids with human AR and pig aldehyde reductase was studied using the Protein Data Bank co-ordinates [23,24] and the procedure previously described [7]. To prepare the structures for docking simulations, hydrogen atoms were added, methyl groups rotated and the atomic contacts minimized. Retinal in an extended conformation (carbon  $\chi$  angles of single bonds = 180°) was placed into the substrate-binding pocket in the same orientation as the ternary ligand testosterone (1AFS) [25]. Non-rigid docking simulations were based upon a Monte Carlo procedure [26], allowing free movement of the substrate, of its rotatable bonds, and of the  $\chi$  angles of the residues inside a 5 Å (1 Å = 0.1 nm) radius from the docked substrate. Additional distance restraints were imposed: 1.0–2.4 Å between the aldehyde oxygen of retinal and the catalytic hydroxy group of Tyr<sup>48</sup>, and 2.0–2.4 Å between C-15 of retinal and C-4 of NADPH.

## RESULTS

### Characterization of retinal reductase activity of human AR, HSI AR and pig aldehyde reductase

We have expressed and purified human AR, HSI AR and pig aldehyde reductase and kinetically characterized the enzymes at physiological pH (Table 1). The present recombinant forms of

**Table 2** Kinetic constants of AKR enzymes with retinoids at pH 7.5

All determinations were performed in 0.1 M sodium phosphate (pH 7.5)/0.02 % Tween 80, with 0.2 mM NADPH or NADP<sup>+</sup>.  $K_m$  values are in mM and  $k_{cat}$  values in min<sup>-1</sup>. Abbreviations: N.A., no activity. N.D., not determined, since activity was very low, less than 0.001 units/mg of enzyme.

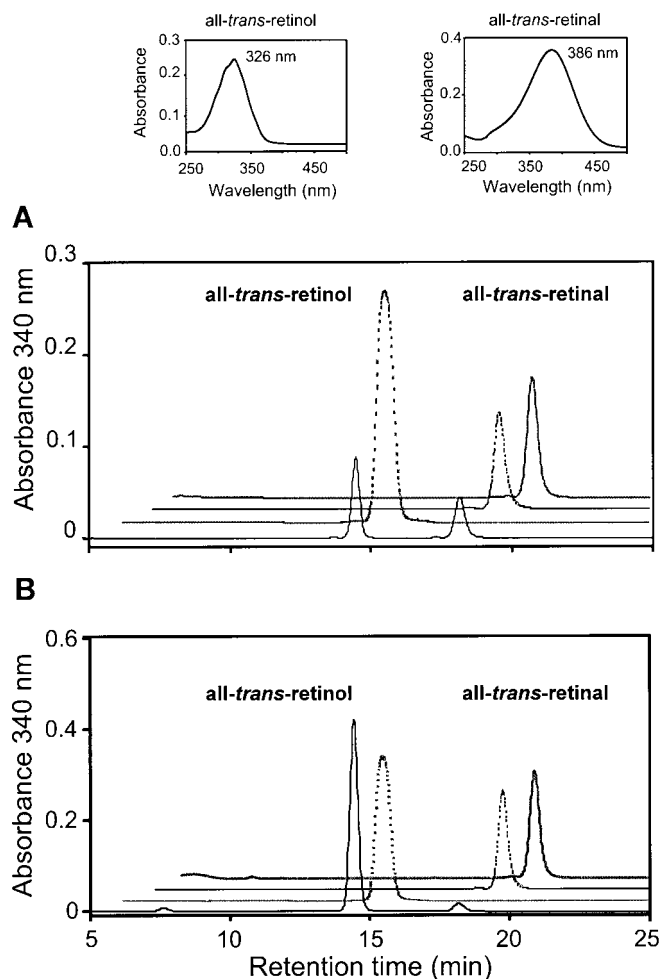
Substrate	Enzyme ...	Human AR	HSI AR	Pig ALR
All- <i>trans</i> -retinal	$K_m$	0.010 ± 0.002	0.019 ± 0.004	N.A.
	$k_{cat}$	15 ± 1	193 ± 4	N.A.
	$k_{cat}/K_m$	1500 ± 320	10 300 ± 2200	N.A.
9- <i>cis</i> -Retinal	$K_m$	0.012 ± 0.003	0.013 ± 0.004	N.A.
	$k_{cat}$	30 ± 1	76 ± 2	N.A.
	$k_{cat}/K_m$	2600 ± 650	5800 ± 1800	N.A.
13- <i>cis</i> -Retinal	$K_m$	0.014 ± 0.002	0.012 ± 0.004	N.A.
	$k_{cat}$	15 ± 1	94 ± 2	N.A.
	$k_{cat}/K_m$	1100 ± 170	7800 ± 2600	N.A.
All- <i>trans</i> -retinol	$K_m$	N.D.	0.010 ± 0.001	N.A.
	$k_{cat}$	N.D.	0.04 ± 0.001	N.A.
	$k_{cat}/K_m$	N.D.	4.0 ± 0.4	N.A.

these three enzymes showed properties with standard substrates similar to those reported previously [10,21,27]. The  $K_m$  value for glyceraldehyde exhibited by human AR was 44  $\mu$ M, a value 100-fold lower than that shown by human HSI AR and pig aldehyde reductase. Human AR had a  $K_m$  value for glucose of 76 mM, whereas HSI AR and aldehyde reductase showed low activity and no saturation with this substrate. Pyridine-3-aldehyde was a good substrate for the three enzymes, showing high catalytic efficiency (Table 1).

The three reductases were also assayed for activity with all-*trans*-, 9-*cis*- and 13-*cis*-retinal (Table 2), using the methodology described by our group for the analysis of MDR ADHs [28]. Both human AR and HSI AR enzymes were active with the three retinal isomers assayed, whereas aldehyde reductase did not show activity with any of them. All-*trans*-retinol was identified as the product of the enzymic reaction when all-*trans*-retinal was used as a substrate (Figure 1). Human AR showed  $K_m$  values of 10–14  $\mu$ M with the retinal isomers, and moderate  $k_{cat}$  values. HSI AR exhibited  $K_m$  values with all-*trans*-, 9-*cis*- and 13-*cis*-retinal similar to those of human AR. However, the  $k_{cat}$  values of HSI AR were higher than those observed for human AR, with all-*trans*-retinal being the best retinoid substrate ( $k_{cat}/K_m = 10300 \text{ mM}^{-1} \cdot \text{min}^{-1}$ ).

Kinetic constants for the reverse reaction were determined with HSI AR, using all-*trans*-retinol and NADP (Table 2). The  $K_m$  value for all-*trans*-retinol was 10  $\mu$ M and the  $k_{cat}$  0.04 min<sup>-1</sup>, a value 4800-fold lower than that determined for the forward reaction. A very low retinol oxidation activity was detected for human AR. The low catalytic efficiency for alcohol oxidation is consistent with the reported high specificity of AKR enzymes for carbonyl reduction [29]. The presence of 0.02 % Tween 80, used to solubilize retinoids [28], did not modify enzyme properties, on the basis of the  $K_m$  and  $k_{cat}$  values exhibited by human AR, HSI AR and pig aldehyde reductase with glyceraldehyde, as compared with the values obtained in a detergent-free buffer (Table 1).

The effect of glucose on the all-*trans*-retinal reductase activity of human AR was evaluated by inhibition assays. Initial-rate measurements of all-*trans*-retinal reductase activity were determined in the presence of 0, 5 (euglycaemia), 20 (hyper-

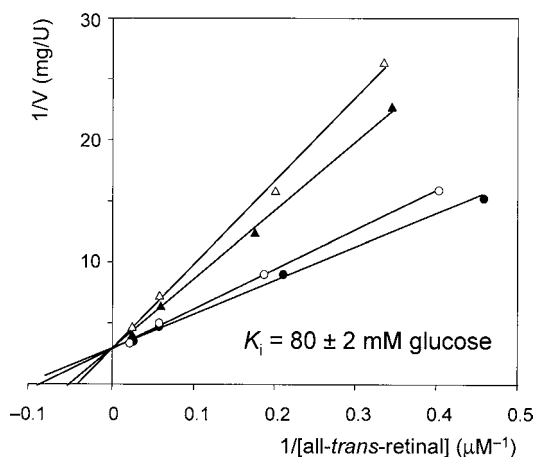


**Figure 1** Identification by HPLC analysis of all-*trans*-retinol as the product of the reaction catalysed by human AR (A) or HSI AR (B), using all-*trans*-retinal as substrate

The enzymic reaction was carried out in 0.1 M sodium phosphate buffer (pH 7.5)/0.2 mM NADPH/30  $\mu$ M all-*trans*-retinal for 20 min at 25 °C. The chromatographic profile of retinoids in the reaction mixture is shown by the black continuous line (bottom curve). As a control, the same procedure was performed in the absence of enzyme (grey continuous line; top curve). Identity of peaks for retinol and retinal was verified by co-elution with standards for retinol (grey broken line; second curve up) and retinal (black broken line; third curve up), and comparison of their spectral properties between 250 and 500 nm by using photodiode-array detection (upper insets).

glycaemia) and 100 mM glucose. Glucose inhibited competitively all-*trans*-retinal reduction, with an apparent  $K_i$  value of  $80 \pm 2$  mM (Figure 2). These results indicate that glucose is a poor inhibitor of the retinal reductase activity of human AR, even at the high glucose concentrations (20 mM) usually found in the diabetic condition.

The inhibition of human AR and HSI AR by tolrestat, a typical AR inhibitor (ARI), was studied using all-*trans*-, 9-*cis*- and 13-*cis*-retinal as substrates (Table 3 and Figure 3). Tolrestat inhibition followed a non-competitive pattern, with  $K_i$  values ranging between 0.09 and 4.2  $\mu$ M. Tolrestat inhibited equally well AR and HSI AR activities with each retinal isomer tested, with the exception of all-*trans*-retinal reduction by AR, which showed the lowest inhibitory effect. All-*trans*-retinoic acid (1.5–100  $\mu$ M) was not effective in inhibiting the retinal reductase activity of either AR or HSI AR.



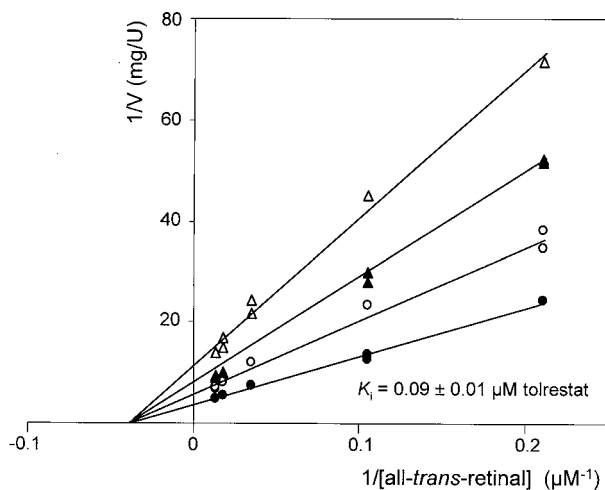
**Figure 2** Inhibitory effect of glucose on the retinal reductase activity of human AR

Double-reciprocal plot from initial-rate measurements of the reduction of all-*trans*-retinal by human AR, at different glucose concentrations (●, 0 mM; ○, 5 mM; ▲, 20 mM; and △, 100 mM). S.E.M. values were less than 10%. Abbreviation: U, units.

**Table 3** Inhibition constants for tolrestat of retinal reductase activity catalysed by AKR enzymes

All determinations were performed in 0.1 M sodium phosphate, pH 7.5, in the presence of 0.2 mM NADPH.  $K_i$  values were calculated from secondary plots by adjusting values from a non-competitive pattern with a linear-regression method.

Substrate	$K_{i, \text{tolrestat}} (\mu\text{M})$	
	Human AR	HSI AR
All- <i>trans</i> -retinal	$4.16 \pm 0.03$	$0.09 \pm 0.01$
9- <i>cis</i> -Retinal	$0.26 \pm 0.03$	$0.17 \pm 0.01$
13- <i>cis</i> -Retinal	$0.82 \pm 0.12$	$0.38 \pm 0.10$



**Figure 3** Inhibition by tolrestat of retinal reductase activity of HSI AR

Double-reciprocal plot from initial-rate measurements of all-*trans*-retinal reduction showing a non-competitive inhibition pattern, at different tolrestat concentrations (●, 0.1  $\mu\text{M}$ ; ○, 0.2  $\mu\text{M}$ ; ▲, 0.33  $\mu\text{M}$ ; and △, 0.5  $\mu\text{M}$ ). S.E.M. values were less than 10%. Abbreviation: U, units.

## Docking with retinoids

The co-ordinates for the three-dimensional structures of human AR [24] and pig aldehyde reductase [23] were used to perform simulations of the binding of all-*trans*- and 9-*cis*-retinal isomers. All-*trans* and 9-*cis*-retinal could bind to human AR (Figure 4A), and defined a retinal-binding site, mainly constituted by residues from loops A, B and C [30]. The carbonyl is placed close to Tyr<sup>48</sup>, His<sup>110</sup> and the nicotinamide of NADPH. The aliphatic chain of retinal is surrounded by Trp<sup>20</sup>, Trp<sup>111</sup>, Phe<sup>122</sup>, Cys<sup>298</sup> and Leu<sup>301</sup>. In the external region of the substrate-binding pocket, Trp<sup>219</sup> and Leu<sup>300</sup> face the  $\beta$ -ionone ring (Figure 4C).

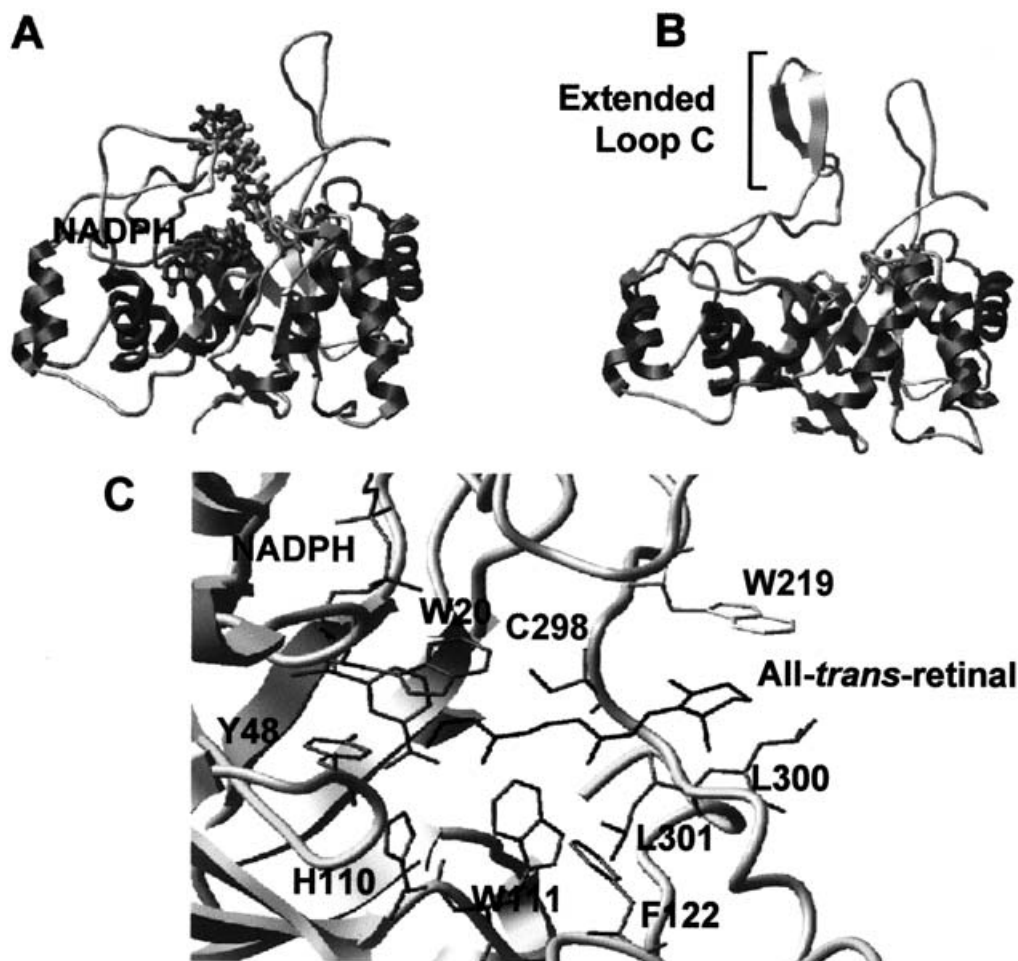
In contrast, pig aldehyde reductase could not accommodate retinal molecules. Docking with all-*trans*- and 9-*cis*-retinal resulted in severe contacts against amino acid side chains from the substrate-binding pocket. An insertion of nine amino acid residues in loop C restricts the access of retinoids to the cavity (Figure 4B, cf. Figure 4A). Similarly to what has been observed with other substrates and inhibitors [27,31,32], the larger loop C could be the determinant for the absence of activity of pig aldehyde reductase with retinoids.

## DISCUSSION

In previous studies [7,33] the search for an ADH in avian tissues resulted in the finding of the first reported AKR enzyme with retinal reductase activity. We therefore speculated on the possibility that other members of the AKR superfamily could also use retinal. In fact, our docking studies suggested that all-*trans*-retinal binds to human AR in a productive manner [7]. In the present work we have studied the activity of different mammalian AKRs with several retinal isomers. We have shown that at least two types of enzyme, AR and AR-like, have high activity with retinoids, suggesting that reduction of retinal is an extended property of the AKR1B family.

Human AR and HSI AR are efficient in reducing all-*trans*-, 9-*cis*- and 13-*cis*-retinal, with  $K_m$  values (10–19  $\mu\text{M}$ ) similar to those described for other substrates of physiological relevance, such as 4-hydroxynonenal and 2-methylpentanal [12,13]. In contrast, pig aldehyde reductase was not active with retinoids. The kinetic constant values of the retinal-active AKR do not differ substantially from those of the MDR and SDR retinal/retinal oxidoreductases [5,6], and are compatible with a physiological utilization of retinoids. In this regard, the catalytic efficiency of human AR and HSI AR with retinal isomers ( $k_{\text{cat}}/K_m = 1100$ – $10300 \text{ mM}^{-1} \cdot \text{min}^{-1}$ ) is comparable with that of human ADH forms ( $k_{\text{cat}}/K_m = 3300 \text{ mM}^{-1} \cdot \text{min}^{-1}$ , for ADH4 with all-*trans*-retinal) [34]. A few microsomal enzymes from the SDR superfamily display NADPH-dependent retinal reductase activity [5,6,35–37]. However, their true catalytic efficiency is often difficult to assess in the available partially purified preparations of these membrane-bound enzymes [38].

Human AR exhibits a wide and hydrophobic substrate-binding pocket defined by amino acid residues close to the active site and by three external loops [24,30]. The presence of aromatic and apolar residues has been correlated with a higher efficiency for hydrophobic substrates [24]. Although the substrate-binding pocket does not appear to be designed for carbohydrate binding, interactions of Trp<sup>111</sup> with the 2'-hydroxy group of monosaccharides have been proposed as a structural basis for sugar binding in ARs [39]. In a previous docking study [7] we suggested that, in addition to good complementarities with the residues of the substrate-binding pocket, the distance between the carbonyl group of all-*trans*-retinal and Tyr<sup>48</sup>, His<sup>110</sup> and C-4 of NADPH was adequate for productive binding. In the present



**Figure 4** Retinoid-binding site of human AR

(A) Ribbon representation of human AR structure with NADPH and docked all-*trans*-retinal (dark grey) and 9-*cis*-retinal (grey). (B) Ribbon representation of pig aldehyde reductase structure. The larger loop C (cf. A) is indicated. (C) All-*trans*-retinal docked to human AR. Residues within a radius of 3 Å from the substrate molecule are indicated.

study, we have performed docking simulations with both all-*trans*- and 9-*cis*-retinal to human AR. In good agreement with previous characterizations of the substrate-binding pocket of human AR [9,24], we now find that aromatic (Trp<sup>20</sup>, Trp<sup>111</sup>, Phe<sup>122</sup> and Trp<sup>219</sup>) and apolar (Leu<sup>300</sup> and Leu<sup>301</sup>) residues face the bound retinal substrate and define a retinal-binding site (Figure 4C).

Recent studies on AR and aldehyde reductase suggest that the C-terminal loop (loop C) is critical for substrate specificity, catalytic efficiency and inhibitor sensitivity [27,31,32]. The most significant structural difference between ARs and aldehyde reductases is the size of loop C, which is nine residues longer in aldehyde reductases. Interestingly, aldehyde reductase did not exhibit activity with retinoids and, consistently, its three-dimensional structure could not accommodate retinal molecules, in part because of steric constraints imposed by loop C (Figure 4B).

#### The retinal reductase function of human AR

AR exhibits retinal reductase activity levels comparable with those of other oxidoreductases of MDR and SDR families [22,35–37,40]. The enzyme is distributed in many human organs and

tissues [17,41], where it may contribute to the regulation of endogenous retinoid concentrations. Retinoic acid is formed by successive oxidation of retinol to retinal, by MDR and SDR enzymes, and of retinal to retinoic acid by retinaldehyde dehydrogenases. While the first step is reversible, the oxidation of retinal is an irreversible process. Therefore retinal is a crucial molecule, and its cellular levels should be strictly controlled. AR is the first cytosolic oxidoreductase described in mammals that can participate exclusively in the reductive direction from retinal to retinol, due to its cofactor specificity and kinetic properties. This could be of functional relevance in the maintenance of suitable levels of retinoic acid.

Remarkably, AR has been shown to be up-regulated in hyperglycaemia [15] and in certain types of cancer [17,42–44]. Induction of AR in these pathologies could have as a consequence an increased reduction of retinal to retinol, thus affecting the production of retinoic acid from retinal, which in turn could alter retinoic acid homeostasis. In fact, retinoic acid has been shown to have an important regulatory function in tumour-cell proliferation [1], as well as in eye [45], kidney [46,47], vascular tissues [48] and the central nervous system [49,50], which are target tissues for diabetic pathology. According to this, the utilization of retinal by AR could have a role in cell proliferation and in secondary

diabetic complications. To check up on this provocative notion, AR activity with retinal will have to be correlated *in vivo* with the status of the retinoic acid signalling pathway. Finally, in the present study, we have shown that tolrestat, pharmacologically used in animal models as a therapy for diabetic pathology, is also a strong inhibitor of the AR activity with retinal. This information provides a new rationale for the use and side effects of ARIs in the treatment of diabetes, and may open novel avenues to their potential application in other clinical disorders such as vascular diseases and cancer.

### The retinal reductase function of HSI AR

The high catalytic efficiency of HSI AR with retinal isomers ( $k_{\text{cat}}/K_m = 5800\text{--}10300 \text{ mM}^{-1} \cdot \text{min}^{-1}$ ) makes this enzyme the most efficient human retinal reductase ever described. HSI AR is expressed mainly in human small intestine and in adrenal gland [16,17]. In small intestine, absorption of  $\beta$ -carotene represents an important source of retinoids.  $\beta$ -Carotene is excised by a dioxygenase, generating all-*trans*-retinal and 13-*cis*-retinal [51], which are then reduced to retinol in the erythrocytes. The kinetic properties and tissue distribution of HSI AR suggest that reduction of carotene-derived retinal may be a physiological function of this enzyme. Regarding the putative role in retinoid assimilation, inhibition of HSI AR by tolrestat is extremely relevant, since this pharmacological compound is normally administered orally.

### Role of dehydrogenases and reductases in the regulation of retinaldehyde levels

The characterization of the retinal reductase activity in members of the AKR superfamily provides novel elements for understanding the homeostasis of retinal and retinoic acid. Retinal plays a central role in the metabolic pathway of retinoids. In fact, the reversible oxidation of retinol to retinal is believed to be the limiting step for the pathway of retinoic acid synthesis [5,6]. To regulate retinal levels, tissues need both retinol dehydrogenases and retinal reductases, and their interplay may provide a fine tuning of retinoic acid production. Hitherto, the enzymes involved in this step were exclusively assigned to MDR and SDR, two superfamilies of oxidoreductases that exhibit the Rossmann fold [52]. Within MDR, the vertebrate ADH family contains several forms active with retinoids [6,22,34,40,53–56]. Moreover, several SDR members define a group of membrane-bound enzymes for which activity with retinoids has also been well proved [5,6]. The inclusion of AR and HSI AR into the metabolism of retinoids defines a new scenario, with a completely different structural scaffold, the  $(\alpha/\beta)_8$  barrel of AKR, that also accounts for retinoid oxidoreduction. The recent description of retinal reductase activity in chicken AKR [7] and in prostaglandin F synthase [57], which are also members of the AKR superfamily, supports the notion that this activity occurs in many AKR enzymes. Control of retinal levels is, therefore, also performed by the widely spread group of cytosolic retinal reductases. This may have profound consequences in the regulation of the retinoic acid synthesis pathway. If ARs were involved in down-regulation of retinoic acid production, it would be advantageous if retinoic acid did not inhibit retinal conversion into retinol. We have shown this to be the case for human AR, while retinoic acid inhibits retinol oxidation by members of the MDR and SDR superfamilies [34,58]. Moreover, the involvement of ARs in tumour-cell proliferation and in the onset of diabetic complications provides an interesting link between retinoic acid regulation and these pathologies.

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