A Vertebrate Aldo-keto Reductase Active with Retinoids and Ethanol*

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Enzymes of the short chain and medium chain dehydrogenase/reductase families have been demonstrated to participate in the oxidoreduction of ethanol and retinoids. Mammals and amphibians contain, in the upper digestive tract mucosa, alcohol dehydrogenases of the medium chain dehydrogenase/reductase family, active with ethanol and retinol. In the present work, we searched for a similar enzyme in an avian species (Gal·lus domesticus). We found that chicken does not contain the homologous enzyme from the medium chain dehydrogenase/reductase family but an oxidoreductase from the aldo-keto reductase family, with retinal reductase and alcohol dehydrogenase activities. The amino acid sequence shows 66–69% residue identity with the aldo reductase and aldose reductase-like enzymes. Chicken aldo-keto reductase is a monomer of M, 36,000 expressed in eye, tongue, and esophagus. The enzyme can oxidize aliphatic alcohols, such as ethanol, and it is very efficient in all-trans- and 9-cis-retinal reduction (kcat/Km = 5,300 and 32,000 mm⁻¹min⁻¹, respectively). This finding represents the inclusion of the aldo-keto reductase family, with the (αβ)m barrel structure, into the scenario of retinoid metabolism and, therefore, of the regulation of vertebrate development and tissue differentiation.

Short chain dehydrogenases/reductases (SDR),¹ medium chain dehydrogenases/reductases (MDR), and aldo-keto reductases (AKR) are three oxidoreductase superfamilies that catalyze the oxidation and reduction of a broad range of alcohol and aldehyde compounds of physiological or pharmacological significance (1–3).

In MDR, the alcohol dehydrogenase (ADH) family constitutes a complex system grouped into several classes (4, 5). ADH1 is the classical enzyme responsible for liver ethanol metabolism, which also exhibits activity with retinoids (6, 7). ADH2 is a liver enzyme that may marginally contribute to ethanol and retinoid metabolisms (8, 9). ADH3 is a glutathione-dependent formaldehyde dehydrogenase (FALDH) inactive with retinoids (6). ADH4 exhibits a unique epithelial distribution and is abundant in the upper gastrointestinal tract mucosa and eye (10–12). It is very efficient with retinoids rather than with ethanol, and it has been proposed to have a role in retinoic acid synthesis (6, 13–15). ADH7 has been described only in chicken embryo, exhibiting activity with retinoids (16). ADH8 is an NADP(H)-dependent form with tissue distribution and substrate specificity similar to those of mammalian ADH4, which has been described in amphibians (17).

The SDR superfamily contains an alcohol dehydrogenase that accounts for ethanol metabolism in Drosophila (18) and several mammalian forms that exhibit activity with retinoids (15, 19, 20). Within the AKR superfamily, several groups of enzymes with related structure and function have been established (3). Among them, the aldo reductase (AR), aldehyde reductase, and hydroxysteroid dehydrogenase (HSD) families have been profoundly characterized in mammals. The AR family has been suggested to be involved in the development of secondary diabetic complications (21) because of its ability to reduce glucose to sorbitol (22), a hyperosmotic compound. Other roles in aldehyde detoxification, osmotic homeostasis, steroid conversion, and catecholamine metabolism have been also proposed for AR (23–26).

Several forms including human ARL1 (aldose reductase-like 1) (27), small intestine reductase (28), mouse vas deferens reductase (29), mouse fibroblast growth-factor-regulated protein (30), and Chinese hamster ovary reductase (31) have been described as a distinct group (3) sharing 70% amino acid identity with AR sequences.

Vitamin A or retinol (ROL) is the precursor of retinal (RAL) and of retinoic acid (RA). RAL is necessary for vision, and RA is essential for growth, development, and cellular functions (32, 33). Biosynthesis of RA from vitamin A implies reversible oxidation of ROL to RAL and irreversible oxidation of RAL to RA. The latter compound, in the all-trans- and 9-cis-isomeric forms, exerts its regulatory function bound to nuclear receptors (34).

In photoreceptor cells, 11-cis-RAL binds to opsins to form rhodopsin. When one photon is absorbed by rhodopsin, 11-cis-RAL is isomerized to all-trans-RAL, which dissociates from opsins. In all-trans-RA and 9-cis-RAL synthesis from ROL, and in rhodopsin regeneration, several oxidation steps occur, which are catalyzed by MDR and/or SDR enzymes, important for the regulation of these processes (15, 20). No activity with retinoids has been even reported in enzymes from any ARK group.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ295030.

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1 The abbreviations used are: SDR, short chain dehydrogenase/reductase; ADH, alcohol dehydrogenase; AKR, aldo-keto reductase; AR, aldo reductase; FALDH, glutathione-dependent formaldehyde dehydrogenase; HSD, hydroxysteroid dehydrogenase; MDR, medium chain dehydrogenase/reductase; RA, retinoic acid; RAL, retinal; ROL, retinol; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; bp, base pair(s).
In this study we have recognized an activity in the upper digestive tract of chicken, similar to that of ADH from the MDR family previously identified in mammals (11, 35) and amphibians (17). However, this avian enzyme was not an ADH of the MDR family, but, surprisingly, an AKR. Thus, we have here characterized the first member of the AKR superfamily with NADP(H)-dependent retinal reductase and ethanol dehydrogenase activities.

**EXPERIMENTAL PROCEDURES**

**Avian Tissues—**Chicken (*Gallus domesticus*) tissues were obtained immediately after death from a local slaughterhouse. The tissues were cut, weighed, homogenized in 10 mM Tris-HCl, 2 mM dithiothreitol, and stored at ~80°C. For enzyme analysis, tissues were thawed, fragmented, and homogenized in 10 mM Tris-HCl, 2 mM dithiothreitol, pH 7.6 (1:1 w/v).

**Enzyme Purification—**Tissue samples (tongue and esophagus, 390 g) were pooled and homogenized in 10 mM sodium phosphate, 2 mM dithiothreitol, pH 7.0. The homogenate was centrifuged (27,000 × g, 30 min, 4°C), filtered through glass wool, ultracentrifuged (85,000 × g, 30 min, 4°C), and filtered again. The supernatant was dialyzed against the initial buffer and applied to a CM-Sepharose (Amersham Pharmacia Biotech) column (2.5 cm × 40 cm) equilibrated with the same buffer. The column was washed with 400 ml of buffer followed by a linear gradient (500 ml) of increasing NaCl concentration (0–250 mM). The fractions exhibiting octanol dehydrogenase activity were pooled, concentrated, and loaded onto an anion exchange column (DiAloa P500) (2 × 20 cm). The preparation was dialyzed against the initial buffer and applied to a 2′,5′-ADP-agarose (Amersham Pharmacia Biotech) column (1 × 20 cm). The column was washed with the same buffer, and the active fractions were pooled, concentrated, dialyzed against 10 mM Tris-Cl, 2 mM dithiothreitol, pH 8.2, and applied to an HPLC system (Waters 600) equipped with a Q column. Elution of the enzyme was performed with a linear gradient of sodium acetate (0–300 mM). The active fractions were dialyzed against 10 mM Tris-Cl, 2 mM dithiothreitol, pH 8.2, and applied to a fast protein liquid chromatography (Amersham Pharmacia Biotech) equipped with a Sephacryl-S200HR column. Protein concentrations were determined by the Bio-Rad protein assay method, using bovine serum albumin as a standard.

**Starch Gel Electrophoresis—**Electrophoresis on starch gel was performed as described (11), except that the gel buffer contained 0.74 mM MOPS, 2.2 M formaldehyde, 1% agarose gel and trans-ferred onto Niylon membranes (Schleicher & Schuell) by means of a TurboBlotter (Schleicher & Schuell). Northern Blot Hybridization—Total RNA was isolated from frozen tissues by the guanidinium thiocyanate method (38). RNA-poly(A) + was isolated from 20-μg aliquots of total RNA with the Quickprep® Micro mRNA Purification Kit (Amersham Pharmacia Biotech). The first strand of the cDNA was synthesized by using the R1R1-(dT)17 primer adapter and Expand High Fidelity DNA polymerase kit (Roche Molecular Biochemicals). The PCR products were directly sequenced or phosphorylated with T4 polynucleotide kinase (Roche Molecular Biochemicals), cloned into the Smal site of pBluescript II SK+ (+), and sequenced (AlfExpress, Amersham Pharmacia Biotech).

**Mass Spectrometry—**Matrix-assisted laser desorption/ionization time-of-flight mass spectra were acquired on a Bruker Biflex spectrometer equipped with a nano-ES ionization inlet.

**Activity—**Activity was determined by monitoring the change in NADPH concentration at 25 °C in a Varian Cary 219 spectrophotometer by measurements at 340 nm, in 0.1 M sodium phosphate, pH 7.5. Glutathione-dependent formaldehyde dehydrogenase activity was measured as reported (39). One unit of activity corresponds to 1 μmol of reduced coenzyme formed or utilized per min, at 25 °C, based on an absorption coefficient of 6260 M −1 cm −1 at 340 nm. Steroids were dissolved in 0.3 M methanol. Methanol was not a substrate nor an inhibitor of chicken AKR under the present conditions (maximum methanol concentration reached in the assay was 45%).

**Kinetic parameters were obtained from activity measurements, with substrate concentrations that ranged from at least 0.1 × Km to 8 × Km. Each individual rate measurement was run in duplicate. Three independent determinations were performed for each kinetic constant. Kinetic constants were calculated using ENSFITTER (Elsevier Biosoft) and expressed as the means ± S.D.**

**Protein Modeling and Retinoid Docking—**A three-dimensional model of chicken AKR was constructed by adopting its amino acid sequence of chicken AKR was constructed by adopting its amino acid sequence into the known fold of human AR (Protein Data Bank entry code 1ADS) of chicken AKR was constructed by adopting its amino acid sequence into the known fold of human AR (Protein Data Bank entry code 1ADS) of chicken AKR was constructed by adopting its amino acid sequence into the known fold of human AR (Protein Data Bank entry code 1ADS) of chicken AKR was constructed by adopting its amino acid sequence into the known fold of human AR (Protein Data Bank entry code 1ADS).

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2 D. Hirschberg, E. Cederlund, A. P. Jonsson, B. Crosas, J. Farrés, X. Fares, T. Bergman, and H. Jornvall, in preparation.
Fig. 1. Starch gel electrophoresis of chicken tissue homogenates. Activity staining using 2-buten-1-ol as a substrate and NADP as cofactor. T, tongue; H, heart; Ey, eye; B, brain; K, kidney; I, small intestine; Es, esophagus; L, liver; P, purified chicken AKR.

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified chicken AKR. Lane 1, enzyme preparation eluted from the HPLC column. Lane 2, purified enzyme after the fast protein liquid chromatography step.

procedure (44), allowing free movement of the substrate, of its rotatable bonds, and of the $\chi$ angles of the residues inside a 5 Å radius from the docked substrate. Additional distance restraints were imposed: 1.0–2.4 Å between the aldehyde oxygen of RAL and the catalytic hydroxyl group of the docked substrate. Additional distance restraints were imposed: 1.0–2.4 Å between the aldehyde oxygen of RAL and the catalytic hydroxyl group of the purified chicken AKR.

Multiple Alignments and Phylogenetic Trees—Protein and DNA sequences were searched in the GenBank, EMBL, PIR, and SwissProt data bases with the FASTA (45) and BLAST (46) algorithms. The Internet AKR Web page was regularly consulted to update information. Sequences were searched in the GenBank, EMBL, PIR, and SwissProt databases with the FASTA (45) and BLAST (46) algorithms. The Internet AKR Web page was regularly consulted to update information.

RESULTS

Enzymatic Forms with ADH Activity in Chicken Tissues—Several ADH-type bands were detected on starch gel electrophoresis (Fig. 1) because of their NADP(H)-dependent activity staining with 2-buten-1-ol. A putative FALDH (or ADH3) appeared as an anodic band with variable intensity in all tissues analyzed. The nature of this band was assessed by glutathione-dependent activity staining with formaldehyde in duplicate gel slices (not shown). Moreover, a cathodic band detected in liver, heart, eye, tongue, and esophagus when 2-buten-1-ol or ethanol was used as a substrate. This strategy only produced DNA fragments corresponding to the octanol dehydrogenase peak. The purified enzyme showed a specific activity of 0.92 unit/mg with octanol (Table I).

With the Sephacryl S200-HR column chromatography, a molecular mass of 36.2 kDa was estimated for the native enzyme using a set of proteins as standards. Moreover, molecular mass values of 36 and 36.6 kDa were determined by SDS-polyacrylamide gel electrophoresis (Fig. 2) and mass spectrometry, respectively. Thus, it can be concluded that the native form is a monomer.

cDNA and Protein Sequences of the Novel Enzymatic Form—Before any data from peptide sequences were available, the first attempt to clone the cDNA was based on degenerated primers deduced from consensus sequences of vertebrate ADH, which were used in the PCR with cDNA from tongue and esophagus. This strategy only produced DNA fragments corresponding to chicken ADH1, which could be marginally expressed in tongue and esophagus, because no active ADH1 was found by starch gel electrophoresis in these tissues (Fig. 1).

These preliminary results suggested that no enzyme homologous to mammalian ADH4 or amphibian ADH8 is expressed in the upper digestive tract from chicken.

We therefore decided to investigate the nature of the novel chicken enzyme by protein analysis. However, attempts at direct sequencer degradation gave no result, suggesting that the N terminus was blocked. Therefore, different sets of the carboxymethylated protein were cleaved with proteolytic enzymes. After fractionation with reverse phase HPLC and subsequent sequencer analysis, scans against data banks showed that the peptides obtained were homologs to fragments of AKR enzymes. Hence, the present enzyme was concluded to be a novel type of AKR, with ADH-like activity. Because of the initial failure to get a cDNA sequence with conventional ADH primers, impurities in the protein preparation, and initial unclarities between protein and cDNA data, the protein analysis was continued until essentially the entire amino acid sequence had been determined by sequencer analysis of 57 peptides from five separate digests (with CNBr, trypsin, N-Asp, Lys-C, or...
Glu-C proteases), covering 311 of 316 residues, i.e. all residues except positions 65–69. Finally, the N terminus was proven to be acetylated by collision-induced dissociation analysis in a quadrupole time-of-flight mass spectrometer of the N-terminal fragment from the Glu-C protease digest, which started with acetyl-Ala, by removal of the initiator Met from the primary translation product.2

Using oligonucleotide primers designed according to the peptide sequences, a DNA fragment of 870 bp was obtained by the PCR. The rapid amplification of cDNA ends method generated three additional DNA fragments corresponding to the 5'9 end (104 and 105 bp) and the 3'9 end (625 bp). The DNA fragments encompassed a 1504-bp full-length cDNA. It included a 44-bp 5'9-flanking region, an open reading frame of 951 bp, encoding a 317-amino acid primary translation product, corresponding to the 316-amino acid acetylated mature form and the initiator Met, and a 509-bp 3'9-untranslated region with a poly(A) tail (Fig. 3). The end results of the protein and cDNA data were in complete agreement.

The amino acid sequence of chicken AKR shows high identity with those of the AR (66–69%) and AR-like (68–69%) families (Table II). The N-terminal region of chicken AKR contains one additional amino acid residue with respect to those of AR and AR-like members. However, the amino acid numbering of AR has been used for the novel enzyme. A phylogenetic tree was built by the bootstrap neighbor-joining method using aldo-keto reductase sequences (Fig. 4). An unrooted representation of the tree emphasized four robust clusters constituted by AR, AR-like, HSD, and aldehyde reductase sequences. The chicken AKR positioned below the split of the AR and AR-like clusters. The novel enzyme contains the catalytic tetrad (Asp43, Tyr48, Lys77, and His110) characteristic of other AKR forms (50), and the residues strictly (Asn 160, Gln 183, and Ser 263) and highly conserved (Ser159, Tyr209, Leu212, Lys262, Arg268, Glu271, and Asn272) involved in NADP(H) binding (3). In contrast to the conserved nature of the coenzyme-binding site, the residues considered important for substrate specificity differ notably between chicken AKR and AR or AR-like sequences (Table II) (see below).

Northern Blot Analysis—Chicken AKR mRNA (1.8 and 4.0 kilobases) was found in the eye and the upper digestive tract (tongue and esophagus) but not in small intestine (Fig. 5). The 1.8-kilobase transcript is likely to represent the mature mRNA. No signal was detected in brain, skeletal muscle, heart, or liver (not shown). These results are consistent with those obtained by starch gel electrophoresis and activity staining (Fig. 1), which also detected active enzyme in eye, tongue, and esophagus, indicating a tissue-specific expression of the chicken AKR gene.

Substrate Specificity of Chicken AKR—Kinetic constants of chicken AKR were determined with several substrates (Tables III and IV). Chicken AKR exhibited a marked NADP(H) preference, suggesting carbonyl reduction as the most likely physiological reaction (Table III). Compounds such as D,L-glyceraldehyde and glyoxal, characteristic substrates for the aldo-keto reductases, were relatively poor substrates for chicken AKR. Moreover, no activity was found with aldose sugars (glu-
cose, galactose, ribose, and xylose) at concentrations up to 150 mM or with steroids (corticosterone, epiandrosterone, dehydroisoandrosterone, 5α-androstane-17β-ol-3-one, and 5-cholesten-3α-ol-7-one) at concentrations up to 150 μM. 3-Nitrobenzaldehyde was a very good substrate, exhibiting the lowest $K_m$ and the highest catalytic efficiency among the compounds tested. Aliphatic aldehydes such as hexanal and 2-trans-hexenal were actively used by chicken AKR, with no significant differences between the two isomers.

An interesting property of chicken AKR is its ADH activity, because no data on aliphatic alcohols as substrates for AKR enzymes are found in the literature. All aliphatic alcohols assayed were substrates that saturated the enzyme. The $K_m$ values decreased steadily as the number of carbons in the aliphatic chain increased, whereas only a small variation was detected in $k_{cat}$ values (Table III).

Among the results obtained with different substrates, the most striking are those concerning retinoids (Table IV), because the AKR superfamily has not been reported before to be active with these physiological compounds. Chicken AKR has the ability to reduce all-trans-, 9-cis-, and 13-cis-RAL isomers and to oxidize all-trans- and 9-cis-ROL. $K_m$ values are similar...

TABLE II
Alignment of amino acid residues constituting the substrate-binding pocket and A, B, and C loops (63) of different AKR enzymes

In bold type and underlined, amino acid residues that correspond to important positions in the substrate-binding pocket. In bold type, amino acid residues identical to those of chicken AKR sequence. In gray, amino acid residues that differ from those of chicken AKR sequence. The amino acid numbering of AR family is used. Protein sequences were taken from data banks, and the alignment was performed using the GCG program PILEUP.

![Fig. 5. Northern blot analysis of chicken AKR.](image)

Fig. 5. Northern blot analysis of chicken AKR. Membrane was prepared with poly(A)⁺ RNA (2 μg/lane). T, tongue; Es, esophagus; I, small intestine; Ey, eye.
for all the RAL isomers, but $k_{cat}$ values differ notably. The best substrate was 9-cis-RAL with a $k_{cat}$ value 5- and 20-fold higher than those for all-trans-RAL and for 13-cis-RAL, respectively, and with an extremely high $k_{cat}/K_m$ ratio (32 000 mM$^{-1}$ min$^{-1}$).

Again, 9-cis-ROL was the best retinoid substrate for the oxidation reaction. No differences were found in the $K_m$ values of all-trans-ROL and 9-cis-ROL with respect to their corresponding aldehydes. In contrast, the $k_{cat}$ values were much lower for the alcohols (Table IV).

**Molecular Model of Chicken AKR**—A molecular model of the chicken AKR tertiary structure was built (Fig. 6). Following amino acid identity (Table II) and phylogenetic criteria (Fig. 4), the structures of the AR and AR-like enzymes were considered the most valid templates. The coordinates of human AR (41), mouse fibroblast growth factor-regulated protein (51), and Chinese hamster ovary reductase (52) three-dimensional structures were used, but no significant differences were found between the three calculated models for chicken AKR (the root mean square deviation was 0.9-1 Å), and thus the one obtained from the human AR template was used as a working object.

The model shows that the residues responsible for the NADPH over NADH preference, i.e. Ly$^6_{9}$ and Arg$^6_{288}$, are conserved in chicken AKR. However, an Asp residue is found at position 264, which is never occupied by residues with acidic side chains in other members of the family. In the crystallographically determined structure, the main chain nitrogen at position 264 faces the 2'-phosphate of NADP(H) and establishes a hydrogen bond with the coenzyme (41). Our model shows that the distance between the charged group of Asp$^{264}$ in chicken AKR and the NADP(H) 2'-phosphate is 4.1 Å and, therefore, a negligible effect on cofactor binding in terms of electrostatic energy should be expected.

The substrate-binding pocket of the chicken AKR model is highly hydrophilic. It includes mostly aromatic (Trp$^{47}$, Tyr$^{6}$, Trp$^{79}$, Tyr$^{111}$, Tyr$^{209}$, and Trp$^{219}$) and aliphatic (Ala$^{45}$, Leu$^{121}$, Leu$^{122}$, and Ile$^{298}$) residues. At the edge of the active site, position 47, typically occupied by apolar aliphatic residues, and position 111, with a conserved Trp in AR, are considered responsible for the sugar and steroid specificity (3, 53).

Significantly, chicken AKR, inactive with pentose or hexose sugars and steroids, contains aromatic and bulky residues at these positions, Phe$^{47}$ and Tyr$^{111}$ (Table II). The absence of Cys residues at positions 298 and 303 could also contribute to the kinetic properties of chicken AKR with glyceraldehyde and aldosugars. Thus, human AR C298S and C303S mutants show a decrease in catalytic efficiency with glucose and xylose, and the C298S mutant shows an increased $K_m$ with glyceraldehyde (54).

The presence of Leu$^{121}$ and Leu$^{122}$, in loop A and Ile$^{298}$, Pro$^{299}$, Val$^{300}$, Pro$^{301}$, Gln$^{302}$, and Ser$^{303}$ in loop C (Table II), in addition to the Phe$^{47}$ and Tyr$^{111}$ substitutions, confer a distinct geometry to the substrate-binding cleft with respect to human AR (Fig. 6, B and C) that could be correlated with the substrate specificity of chicken AKR. The cleft of the chicken enzyme is structured as a funnel shaped cavity that is suitable for binding hydrophobic substrates containing the carbonyl reactive group at the end of an aliphatic chain, such as retinoids, but unsuitable for bulky polycyclic compounds, such as steroids (Fig. 6B). In contrast, the structure of human AR active site (Fig. 6C) is appropriate for steroid binding as well as for retinoids (see below).

**Docking Simulations**—The interaction of RAL with chicken

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**Table III**

Kinetic constants of chicken AKR with aldehydes, alcohols, and cofactors

All determinations were performed in 33 mM sodium phosphate, pH 7.5, with 100 μM NADP or 80 μM NADPH, except for coenzyme constants.

| Substrate | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (μM$^{-1}$ min$^{-1}$) |
|-----------|------------|------------------------|-----------------------------------|
| D,L-glyceraldehyde | 6.1±0.9 | 313±22 | 51 |
| Glyoxal | 103±12 | 950±80 | 9.2 |
| 3-Nitrobenzaldehyde | 0.0013±0.0002 | 1100±66 | 846000 |
| Hexanal | 1.3±0.1 | 870±20 | 670 |
| 2-trans-Hexenal | 0.85±0.02 | 900±56 | 940 |
| Ethanol | 720±90 | 16±0.6 | 0.022 |
| Pentanol | 14±2 | 20±1.2 | 1.4 |
| Hexanol | 1.4±0.1 | 29±0.8 | 20 |
| Octanol | 0.030±0.004 | 32±1 | 1060 |
| NADPH | 0.010±0.001 | 28±3 | 2800 |
| NADPHb | 0.006±0.001 | 935±43 | 155000 |
| NADPHc | 2±0.4 | 20±2 | 10 |
| NADPH | N.A. | | |

a Using 0.25 mM octanol as a substrate.

b Using 10 mM 3-nitrobenzaldehyde as a substrate.

c No saturation up to 1.5 mM NADH.

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**Table IV**

Kinetic constants of chicken AKR with retinoids

Determinations were performed with 100 μM NADP or 80 μM NADPH, in 33 mM sodium phosphate, 0.02% Tween 80, pH 7.5. N.A., no activity.

| Substrate | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (μM$^{-1}$ min$^{-1}$) |
|-----------|------------|------------------------|-----------------------------------|
| all-trans-retinal | 0.032±0.004 | 170±15 | 5300 |
| 9-cis-retinal | 0.027±0.005 | 862±85 | 32000 |
| 13-cis-retinal | 0.040±0.008 | 39±4 | 1000 |

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**An Aldo-keto Reductase Active with Retinoids**

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AKR was studied by docking simulations in our three-dimensional model. The molecule of all-trans-RAL fitted the substrate-binding pocket, and many features suggest that this interaction may correspond to a productive complex. The position of the RAL molecule in the substrate-binding pocket was found to be such that the re face of the aldehyde group is parallel to the nicotinamide ring (23, 53). The RAL aliphatic chain does not clash against any of the voluminous side chains that line the cavity, and the b-ionone ring of the retinoid showed complementarities with residues from loops A and C. The atomic distances between the oxygen of the aldehyde group and the hydroxyl group of Tyr48, and the Ne of His110 (catalytic residues) in the model were 1.8 and 3 Å, respectively. The atomic distance between the C4 of the nicotinamide ring and the C15 of RAL was 2.2 Å (Fig. 6D). The angles drawn by lines through nicotinamide C4-nicotinamide H4R-RAL C15 and the nicotinamide H4R-RAL C15-RAL oxygen were 150° and 100°, respectively. Moreover, 9-cis-RAL also bound to the substrate-binding pocket of chicken AKR with distances to catalytic residues similar to those of the all-trans isomer (not shown). These distances and angles in the model are compatible with those determined for a transition state (50, 55). All-trans-RAL also could be docked to the substrate-binding pocket of the crystal structure of human AR. Although this molecule adopted a different orientation to the one observed in chicken AKR, the distances of the carbonyl group of all-trans-RAL to the catalytic residues and NADPH predict a productive binding (Fig. 6E).

The absence of activity of chicken AKR with steroids was studied by docking simulations with testosterone (not shown). The substrate-binding pocket could harbor the testosterone molecule; however, unfavourable contacts were found with Phe47 (Fig. 6B), the side chain of which was sandwiched between the protein main chain and the steroid. Interestingly, these contacts disappeared in the docking simulation when Phe was substituted by Val, the residue found in human AR at position 47.
An aldohexose dehydrogenase, named ADH4, active with ethanol and retinoids and present in the eye and digestive tract organs has been described in mammals (6, 11, 56, 57). In the stomach of amphibians, an aldohexose dehydrogenase with similar substrate specificity, but a separate origin has been reported (ADH8) (17). Interestingly, ADH8 exhibits a NAD(P) preference over NAD(H), a feature unique to this ADH in the MDR superfamily from animals. We, therefore, searched for a similar enzyme, either NAD(H) or NADP(H)-dependent, in an avian species, G. domesticus. An NADP(H)-dependent enzyme, active with ethanol and retinoids, was found in digestive organs and in the eye of chicken. But, unexpectedly, it does not correspond to an ADH of the MDR superfamily but to a completely different enzyme, a reductase of the AKR superfamily. We have characterized this chicken AKR enzyme and showed that it exhibits distinct structural and functional properties.

Chicken AKR showed a $K_m$ value of 6 mM with D,L-glyceraldehyde, 100–300-fold higher than that reported for human AR with this substrate (20–70 $\mu$M) (22, 54, 58) but similar to the values exhibited by human AR-like and Chinese hamster ovary enzymes (2 and 28 mM, respectively) (27, 31). An important feature of chicken AKR is the absence of activity with glucose, whereas AR enzymes are typically active with this compound (22, 25). In contrast, with the exception of human AR-like enzyme (27), AR-like forms are inefficient (59) or not active at all (25, 31) with glucose.

Chicken AKR is not active with steroids containing hydroxyl or ketone groups in their 3α, 17β, or 20α positions. In contrast, many AKR enzymes, such as HSDs, human AR, Chinese hamster ovary reductases, and mouse vas deferens reductase, catalyze steroid conversion (3, 22, 25, 31, 59), although the human AR-like enzyme is not active with progesterone nor with 17α-hydroxyprogesterone (27). Therefore, in relation to the kinetic properties with both sugars and steroids, chicken AKR exhibits more similarities with the AR-like than with the AR enzymes.

Chicken AKR is active with aliphatic aldehydes and the corresponding alcohols, including ethanol. To our knowledge, activity with ethanol has never been reported for any member of the AKR family. The higher hydrophobicity of the substrate-binding site of chicken AKR, as compared with other enzymes of the family, is probably at the basis of its specific activity. The localization of avian AKR in upper digestive tract tissues is probably at the basis of its distinct specificity. The catalytic efficiency of chicken AKR for all-trans-RAL is similar to that of the most efficient ADHs (7, 13, 14, 17). In conclusion, the present AKR kinetic constants, mostly $k_{cat}$ and $k_{cat}/K_m$, are similar or even higher than those of the SDR and MDR enzymes active with retinoids, further supporting a role of chicken AKR in RAL reduction.

Another unexpected feature is that chicken AKR can transform retinoids with high catalytic efficiency. The $k_{cat}/K_m$ values of chicken AKR with all-trans-RAL and 9-cis-RAL are similar to those for human AR with its best physiological substrates (22, 23) and add a physiological role of chicken AKR in RAL reduction. Chicken AKR is the fastest 9-cis-RAL reductase known. Its $k_{cat}$ (860 min$^{-1}$) is several times higher than those for the best MDR ADHs (7, 13, 14, 17), which are faster enzymes than the SDR RAL reductases (15). The $k_{cat}/K_m$ (catalytic efficiency) is also extremely high for 9-cis-RAL (32000 mM$^{-1}$min$^{-1}$), only comparable with the value of amphibian ADH8 with all-trans-RAL (33750 mM$^{-1}$min$^{-1}$) (17). Moreover, the catalytic efficiency of chicken AKR for all-trans-RAL is similar to that of the most efficient ADHs (7, 13, 14, 17). In conclusion, the present AKR kinetic constants, mostly $k_{cat}$ and $k_{cat}/K_m$, are similar or even higher than those of the SDR and MDR enzymes active with retinoids, further supporting a role of chicken AKR in RAL reduction.

DISCUSSION

In the light of these results, it seems reasonable to propose a new and critical biological function for the AKR superfamily, the metabolism of retinoids. Chicken AKR, like mammalian ADH4, may perform two different roles in regard to retinoid metabolism: 1) regeneration of rhodopsin and involvement in the homeostasis of RAL through the reduction of all-trans-RAL to all-trans-RAL in the eye tissues and 2) conversion of retinoids and the regulation of RA synthesis, essential in tissue differentiation and maintenance in ocular and digestive tract tissues.

The upper digestive tract and the eye of vertebrates need an oxidoreductase capable of metabolizing ROL and RAL. In mammals, an NAD(H)-dependent ADH (ADH4) of the MDR family seems to participate in this function. In amphibians, an NADP(H)-dependent ADH (ADH8), also of the MDR family, accounts for this activity. In the present avian line, a different structure, an NADP(H)-dependent aldo-keto reductase, is used for this function. It is relevant to consider that this specificity toward retinoids may be also present in previously well characterized AKR members from other vertebrate groups, including human. In agreement to this, our docking simulations of the crystallographically determined human AR structure with retinoids suggest a productive binding of these compounds.

Activity-wise, SDR, MDR, and AKR superfamilies have evolved convergently, although they structurally exhibit divergence with distant relationships (3, 60). In SDR and MDR, the convergence has given rise to paradigmatic cases in the metabolisms of ethanol and retinoids. Thus, the widespread MDR ADH is the one responsible for ethanol metabolism in vertebrates, yeast, and bacteria. However, in Drosophila, which lacks an MDR ADH, an SDR ADH exerts that function (61). Moreover, several forms of SDR and MDR are ROH dehydrogenases or RAL reductases, apparently involved in physiological retinoid metabolism (15, 20). We have shown here that AKR has also evolved concomitantly with SDR and MDR in terms of ethanol and ROL metabolism. The (α/β)$_9$ barrel of the AKR tertiary structure, with a conserved core and variable loops in outer domains, responsible for substrate binding, represents a highly adaptable scaffold, able to evolve and develop distinct functions (62) as the retinoid oxidoreduction, here demonstrated. The inclusion of the AKR superfamily in the metabolism of retinoids defines a new scenario where the triad SDR-MDR-AKR would participate in the cellular regulation of the essential signaling function of retinoids.

Note Added in Proof—Chicken AKR has been designed AKR1B12 based on cluster analysis including members of the AKR superfamily, as recommended by Jeż et al. (J. M., Flynn, T. G., and Penning, T. M. (1997) Biochem. Pharmacol. 54, 639–647).
A Vertebrate Aldo-keto Reductase Active with Retinoids and Ethanol
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