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Medium-chain and short-chain dehydrogenases/reductases in retinoid metabolism

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Abstract

Retinoic acid (RA), the most active retinoid, is synthesized in two steps from retinol. The first step, oxidation of retinol to retinaldehyde, is catalyzed by cytosolic alcohol dehydrogenases (ADHs) of the medium-chain dehydrogenase/reductase (MDR) superfamily and microsomal retinol dehydrogenases (RDHs) of the short-chain dehydrogenase/reductase (SDR) superfamily. The second step, oxidation of retinaldehyde to RA, is catalyzed by several aldehyde dehydrogenases. ADH1 and ADH2 are the major MDR enzymes in liver retinol detoxification, while ADH3 (less active) and ADH4 (most active) participate in RA generation in tissues. Several NAD^+- and $NADP^+$-dependent SDRs are retinoid active. Their *in vivo* contribution has been demonstrated in the visual cycle (RDH5, RDH12), adult retinoid homeostasis (RDH1) and embryogenesis (RDH10). K_m values for most retinoid-active ADHs and RDHs are close to 1 μM or lower, suggesting that they participate physiologically in retinol/retinaldehyde interconversion. Probably none of these enzymes uses retinoids bound to cellular retinol-binding protein, but only free retinoids. The large number of enzymes involved in the two directions of this step, also including aldoketo reductases, suggests that retinaldehyde levels are strictly regulated.

Keywords

Alcohol dehydrogenase; retinol dehydrogenase; retinol; retinoic acid; retinoid metabolism

Retinoids: biological activity and metabolism

Retinoids comprise a family of polyisoprenoid lipids including vitamin A (retinol) and its natural and synthetic derivatives. Retinoids regulate a wide variety of essential biological processes, such as vertebrate embryonic morphogenesis and organogenesis, cell growth arrest, differentiation and apoptosis, and homeostasis [1,2]. All-*trans*-retinoic acid (all-*trans*-RA), the most potent biologically active metabolite of vitamin A, can both prevent and rescue the main defects caused by vitamin A deficiency in adult animals. In addition, 11-*cis*-retinaldehyde plays a key role in the visual function. Recent evidence suggests that all-*trans*-retinaldehyde is a regulator of adipogenesis, independent of its conversion to RA [3]. Retinoids are being used in the treatment and prevention of particular cancer types [4].

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RA exerts its pleiotropic effects through binding to RA receptors (RARs), which bind to DNA as heterodimers with retinoid X receptors (RXRs). Both receptor types are members of the nuclear hormone receptor superfamily. As RXR/RAR heterodimers, these receptors control the transcription of RA target genes through binding to RA-response elements. Although RARs bind both all-*trans*-RA and 9-*cis*-RA, and 9-*cis*-RA binds to RXRs, it is unnecessary for RXRs to bind a retinoid ligand for RA signaling [5]. Also, because 9-*cis*-RA has not been consistently detected in mammalian cells, the consideration of 9-*cis*-RA as a natural bioactive retinoid remains controversial.

Retinoid transfer from liver, which is the major organ for the storage and metabolism of retinoids, to target tissues occurs mainly in the form of retinol bound to a specific plasma protein, retinol-binding protein (RBP), which delivers retinol to the target cells. In the cell, retinol has two metabolic alternatives: storage and oxidative metabolism. One form of storage is through binding to cellular retinol binding proteins (CRBPI and II). CRBPI shows wide tissue expression, while CRBPII is expressed in the small intestine. They are members of a family of small (~15 kDa) proteins that bind hydrophobic ligands such as long-chain fatty acids and retinoids [6]. Their K_d for retinol is in the low nM range. Therefore most retinol in the cell is bound to CRBP. The protein also binds retinaldehyde but with a higher K_d. Retinol is also stored in the form of retinyl esters. Free retinol and CRBP-bound retinol are substrates for esterification by lecithin:retinol acyltransferase (LRAT), while retinyl ester hydrolase reverts this process.

Of great importance is the oxidative pathway of retinol metabolism, which generates RA. The first step is the reversible oxidation of retinol to retinaldehyde, catalyzed by different forms of alcohol dehydrogenase (ADH) from the MDR superfamily, and a variety of retinol dehydrogenases (RDHs) from the SDR superfamily. Recently retinaldehyde reductase activity has been detected in members of the aldo-keto reductase (AKR) superfamily [7]. Retinaldehyde is irreversibly oxidized to RA by various retinaldehyde dehydrogenases (RALDH1, 2 and 3; i.e. ALDH1A1, 1A2, and 1A3), demonstrated by gene knockout studies in mice to be essential for embryonic development [8,9]. RA can bind to cellular RA binding protein (CRABP), migrate to the nucleus for receptor binding, or be transformed to oxidized and mostly inactive compounds by CYP26, a cytochrome P450 enzyme [10].

This review summarizes the *in vitro* and *in vivo* evidence for the role of ADHs and RDHs in the first step of RA biosynthesis.

In vitro studies of ADHs and retinoids

It has long been known that the relatively nonspecific liver ADH can catalyze the oxidation of retinol to retinaldehyde [11,12]. Moreover, it was recognized that extrahepatic ADH can also perform the reaction [12,13]. The search for an endogenous substrate for ADH, and the identification of the oxidative pathway from retinol to RA as essential in the generation of the most active vitamin A compound, attracted the interest of ADH researchers to retinoid metabolism.

The ADH enzymatic assay

It was early recognized that retinol oxidation catalyzed by ADH is a fast reaction that can be easily followed by a continuous spectrophotometric assay, taking advantage of the absorbance change at 400 nm, where retinol does not absorb and where retinaldehyde exhibits a high absorbance (ε_{400} = 29 500 $M^{-1} \cdot cm^{-1}$). However the low solubility and instability of retinoids in aqueous buffer caused difficulties with the aqueous assay. Following early procedures [14], 0.02% Tween 80 has frequently been added to the activity assay buffer as a retinoid solubilizer [15]. In addition to increased solubility, Tween 80 stabilized the retinoid, providing

a reliable and reproducible assay [16]. However Tween 80 behaves as an apparent competitive inhibitor and, at the concentration used in the assay, it increases the retinoid K_m by up to 100-fold, without affecting k_{cat} [16]. Although this methodology may still serve to compare kinetics of different ADHs, it is not useful when ADHs are compared with other redox enzymes, such as SDRs, the activities of which have been determined using detergent-free buffer. Recently, ADH kinetics with retinoids was measured in a detergent-free system [17] developed by one of us (NK), as described below.

Kinetics of human ADH enzymes with retinoids

Human ADHs, including classes I, II and IV, obey simple Michaelis-Menten kinetics with retinoid substrates, both in the oxidative and reductive directions [18]. Table 1 lists the k_{cat} values of the human ADHs obtained in the presence of Tween 80 in the assay buffer. As indicated above, this methodology gives good estimates for k_{cat}.

Activity with all-trans retinoids

ADH1—Human ADH1 isozymes exhibit similar activity, except for ADH1B1, which is significantly less active. Although K_m constants obtained in the presence of Tween 80 cannot be considered as absolute values, they can be used for comparison between ADHs. ADH1 isozymes show similar K_m values, ranging from 30 to 96 μM [18-21]. They are highly expressed in the liver where they have an established role in detoxification of excess retinol (see below). In addition, low expression is detected in lungs, the gastrointestinal tract, blood vessels, adrenal glands and kidneys, and in small amounts in other organs [22-26], where ADH1 could contribute to the generation of RA during both adulthood and fetal development.

ADH2—Human ADH2 has k_{cat} values similar to those of the best ADH1 isozymes (Table 1), while K_m values are consistently low [18,19]. This was further confirmed by the detergent-free buffer assay, which supports a role of ADH2 in retinol oxidation. ADH2, essentially a liver enzyme, would participate, along with ADH1 in the elimination of the retinol excess.

ADH3—A very small rate or lack of activity was reported for ADH3 with retinoids [18,19, 27]. However, genetic evidence supports the involvement of ADH3 in the generation of RA *in vivo*. Thus, *Adh3* null-mutant mice exhibited reduced RA generation, growth deficiency that could be rescued by retinol supplementation, and postnatal lethality during vitamin A deficiency. The small amount of the ubiquitous [22] ADH3 activity (105 pmol.min^{-1} ·mg protein^{-1} for the mouse enzyme) [28] may be sufficient to account for the low RA levels required for biological functions in tissues lacking other retinol dehydrogenases.

ADH4—This is the most active ADH in both the oxidation of retinol and the reduction of retinaldehyde. It is also the most efficient ADH in terms of k_{cat}/K_m [18-21]. Structural and modeling studies show a wide entrance to the substrate-binding pocket where the cyclohexene ring of the retinoid can fit. An extra space is also provided by a deletion at position 117 (Gly in class I). This allows binding of the retinoid in a more extended conformation than in other ADH classes, which may account for the higher catalytic efficiency of ADH4 [20,29].

Although ADH4 k_{cat} with retinoids is the highest among ADHs, it is still more than 10-fold lower than the k_{cat} for ethanol. This prompted the investigation of the kinetic mechanism of ADH4 with retinoids. In contrast to the well-accepted symmetric ordered sequential mechanism of ADH1 with ethanol, ADH4, when functioning as a retinol dehydrogenase, conforms to an asymmetric mechanism, random for the oxidation of all-*trans*-retinol and ordered for the reduction of all-*trans*-retinaldehyde, under rapid equilibrium in binding the substrate and coenzyme for both reactions [30].

ADH4 is expressed in many epithelial tissues of the body, such as skin, blood vessels, gastrointestinal mucosa and cornea [22-26]. Its high retinol dehydrogenase activity suggests that it has a major role in retinol oxidation where it is expressed.

Activity with cis-retinoids and ring-oxidized retinoids

Kinetic data have been reported for the 7-, 9-, 11- and 13-*cis* isomers of retinol and retinaldehyde [18,20,21] (Table 1), using the Tween 80 method. The 7- and 9-*cis* retinoids were the most active substrates for ADH4. In contrast, ADH1 exhibited more activity toward the all-*trans* isomers. The high activity of ADH4 with 11-*cis*-retinol (k_{cat} = 95 min^{-1}) and its presence in several cellular layers of the retina support a contribution of this enzyme in the redox reactions of the visual cycle [21].

Ring-oxidized derivatives of retinoids are of interest since they are biologically active compounds in several mammalian and lower vertebrate tissues [16]. ADH1 and ADH4 are active with 3,4-didehydroretinol and 3,4-didehydroretinaldehyde, with kinetic constants similar to those of retinol and retinaldehyde. The substrates 4-hydroxyretinol and 4-oxoretinaldehyde showed the highest rate with any ADH among all the retinoids previously tested, with 3-40-fold increases in k_{cat} when compared with the parent retinol and retinaldehyde compounds [16].

Ethanol inhibition of ADH activity with retinoids

Ethanol acts as a competitive inhibitor against retinol in the enzymes of the human ADH family. The magnitude of the apparent K_i values tends to correlate with the K_m for ethanol of the different ADH forms. A small effect should be predicted for ADH3 because of its weak binding of ethanol. The low-K_m ADH1 forms, mainly responsible for alcohol elimination, exhibit K_i values in the 0.04-4 mM range, while constants for ADH2 and ADH4 are 7 and 11 mM, respectively [19,31]. The relatively low K_i values suggest inhibition of retinol oxidation at the moderate ethanol concentration found in blood after social drinking. This has been proposed as a cause of fetal abnormalities due to maternal drinking [32]. The spatiotemporal expression of ADH and other studies in mouse models support this notion. It has been speculated that other alcohol-related diseases, like testicular atrophy and aspermatogenesis, skin diseases, and oral and gastrointestinal cancer, may proceed through inhibition of the retinol dehydrogenase activity of ADH by ethanol and consequent decrease in RA synthesis [19].

ADHs and retinoid metabolism in other mammals and lower vertebrates

Kinetics with retinoids have been studied with the rodent ADH1 and ADH4 forms [20,21,27, 33]. In general, the results are similar to those for the human enzymes. A unique feature is the very different K_m of ADH4 for ethanol, about 2000 mM for the rodent enzymes and 40 mM for the human ADH4. In contrast, the K_m for retinol is very similar for the ADH4 of all the species. Site-directed mutagenesis has demonstrated that a single substitution at position 294, Val in human and Ala in the rodents, is mostly responsible for the kinetic differences. It was concluded that mammalian ADH4 exhibits a conserved function in retinoid metabolism but not in ethanol oxidation, further reinforcing the role of ADH4 as a retinol dehydrogenase in mammals [20].

All vertebrate groups from bony fish to the human contain ADH1 and ADH3, enzymes that can oxidize retinol. Moreover, additional retinoid-active ADHs have been demonstrated in lower vertebrates. Fetal and adult chicken tissues contain an ADH (ADH-F) active with retinol (k_{cat} = 1.5 min^{-1}) and steroids, which was proposed to represent a separate ADH class [34].

Amphibia express an ADH (ADH8) that is unique because it shows a strong preference for $NADP^+$, instead of NAD^+ as the rest of the known vertebrate ADHs. In addition, ADH8

exhibits the largest k_{cat}/K_m ratio of all known vertebrate ADHs with all-*trans* retinaldehyde. Both features support a retinaldehyde reductase function of ADH8 [35].

ADHs in retinoid metabolism during development and adulthood

Several members of the mammalian ADH enzyme family participate in embryonic and adult RA synthesis. In mid-gestation mouse embryos, *ADH1* mRNA is limited to the mesonephric ducts (primitive kidneys), *ADH4* mRNA is limited to craniofacial and posterior mesoderm, but *ADH3* mRNA is found ubiquitously at high levels in all tissues [36]. ADH3 expression remains ubiquitous in late-gestation mouse embryos and adults, whereas ADH1 and ADH4 are expressed in a limited set of tissues that for the most part do not overlap with each other but do overlap with ADH3 [24]. These studies suggest that oxidation of retinol to retinaldehyde is ubiquitous in mammalian embryos (particularly due to ADH3 activity), and that tissue-specific regulation of RA synthesis may occur primarily at the next step by RALDH. However, as discussed below, the recent discovery that the SDR enzyme RDH10 is required for normal embryonic RA synthesis indicates that retinol oxidation can be limiting in particular cells [37].

In vivo studies on the role of ADH in retinoid metabolism

Five classes of ADH exist in both mice and humans, and all these genes in both species are localized to a single tandem cluster with the same gene order and transcriptional orientation [38]. This conservation suggests that ADHs have conserved functions in mice and humans. Gene knockout studies on mouse *Adh1, Adh3* and *Adh4* have provided evidence for functions in retinol metabolism *in vivo* as summarized below (Fig. 1).

Prevention of retinol toxicity by class I ADH (*Adh1*)

Adh1-/- mice do not exhibit defects in growth or survival when maintained on a vitamin A-sufficient diet [28]. However, compared to wild-type mice, *Adh1*-/-mice administered a dose of retinol exhibit a sharp reduction in metabolism of the dose of retinol to RA, which results in a significant increase in vitamin A toxicity [39]. These findings indicate that in order to avoid retinol toxicity it is more beneficial to metabolize retinol to RA through a pathway linked to ADH1 than to allow retinol to accumulate. This may be important not only pharmacologically but also physiologically, as some sources of food (such as liver, milk, and eggs) and vitamin pills contain significant amounts of retinol or retinyl esters which can be toxic if consumed in high enough quantities.

The physiological findings on vitamin A toxicity obtained in *Adh1*-/- mice correlate with the observations that ADH1 (exhibiting high retinol activity) is found at much higher levels in adult liver, the major site for retinol detoxification, than ADH3 (exhibiting low retinol activity), and that ADH4 (the most active form) is absent from liver.

Physiological roles of class III ADH (*Adh3*) during vitamin A deficiency and toxicity

In contrast to *Adh1*-/- mice, *Adh3*-/- mice display an easily noticeable developmental phenotype. *Adh3*-/- mice exhibit 15% postnatal lethality and a growth deficiency, resulting in adult body weights approximately 30% lower than normal [28]. *Adh3*-/- growth deficiency can be rescued by retinol supplementation during postnatal life, suggesting that the defect involves an inability to efficiently utilize retinol when supplied in food at normal levels. This rescue implies that other enzymes can replace the ADH3 function when retinol is present at high levels. The other enzymes may include ADH1 and ADH4, which exhibit *in vitro* retinol oxidation activities about 1000 times higher than that of ADH3 [28].

A role for ADH3 in mammalian development has also been supported by studies on mice subjected to vitamin A deficiency during gestation and postnatal development [28]. *Adh3*-/- mice and *Adh4*-/- mice exhibit 100% postnatal lethality and a severe growth deficiency during vitamin A deficiency, whereas *Adh1*-/- mice behave similar to wild-type (about 40% postnatal lethality and mild growth deficiency). These studies demonstrate that ADH3 and ADH4 both play roles in RA synthesis for postnatal development.

Adh3-/- mice also display a reduction in metabolism of a dose of retinol to RA, suggesting that ADH3 participates in prevention of retinol toxicity [28].

Function of class IV ADH (*Adh4*) during vitamin A deficiency

Adh4-/- mice do not exhibit defects in growth or survival when maintained on a vitamin A-sufficient diet [28,39]. However, as mentioned above, studies on *Adh4*-/- mice have demonstrated that they, like *Adh3*-/- mice, exhibit severely reduced postnatal growth and survival when placed on a vitamin A-deficient diet [28,40]. *Adh1*-/- mice subjected to a vitamin A-deficient diet do not show a reduction in growth or survival compared to wild-type [28], and *Adh1*-/-; *Adh4*-/- double mutants fare no worse than *Adh4*-/- mice during vitamin A deficiency [39]. Thus, ADH3 and ADH4 (but not ADH1) play overlapping roles in supplying retinaldehyde for RA synthesis during developmental RA signaling. The high retinol activity of ADH4 is concluded to be useful in ensuring metabolism of retinol to retinaldehyde to provide RA synthesis for RA signaling when retinol is limiting.

Several experiments using the *Adh4*-/- and *Adh1*-/- single mutants and the *Adh1*-/-; *Adh4*-/- double mutants have shown that ADH4 does not play a significant role in elimination of excess retinol to prevent retinol toxicity [39]. Overall, these studies indicate that ADH4 promotes survival during vitamin A deficiency, whereas ADH1 provides considerable protection against vitamin A toxicity, thus demonstrating largely non-overlapping physiological functions for these enzymes in retinol metabolism.

ADH gene family members have redundant functions in retinol metabolism

Overall, mouse genetic studies have demonstrated that ADH1 function overlaps that of ADH3 in providing protection against vitamin A toxicity, whereas ADH4 function overlaps that of ADH3 in providing protection against vitamin A deficiency. ADH3 can be thought of as a general-purpose retinol dehydrogenase (low activity, ubiquitous), quite sufficient when vitamin A is sufficient, whereas ADH1 and ADH4 are specialized retinol dehydrogenases (high activity, tissue-specific) whose retinoid functions are only indispensable in specific tissues during extreme vitamin A conditions, i.e. retinoid excess (ADH1) or retinoid deficiency (ADH4). Since retinoic acid signaling is found in chordate development [41], additional evidence that ADH3 may be a general-purpose retinol dehydrogenase comes from studies showing that ADH3 is the only ADH detected in primitive chordates [42] and early vertebrates [43]. A redundant function for retinol oxidation shared by several ADHs and SDRs (see below) may account for the lack of an embryonic lethal defect in single *ADH* gene knockouts.

SDRs and retinoids

Historical background

The existence of retinoid-active enzymes other than cytosolic ADHs has been suggested by several studies, which showed that retinoid-metabolizing activities are present in the membrane fractions of many cells [44]. Initial attempts to purify these enzymes have not been successful because they lost most of their activity upon detergent solubilization and extraction from membranes [45]. The only membrane-bound enzyme that was purified from animal tissues

following its activity toward retinol remains rat retinol dehydrogenase 1 (RoDH1). RoDH1 was purified from rat liver microsomes using the CRBPI-bound form of retinol (*holo*CRBPI) as substrate because *holo*CRBPI was thought to represent the physiologically relevant form of retinol and the *holo*CRBPI dehydrogenase activity appeared to be enriched in the microsomal fraction of rat liver [46,47]. The cDNA encoding RoDH1 was cloned shortly thereafter [46, 47], and the primary structure of the deduced protein (317 amino acids) indicated that it belongs to the SDR superfamily.

At about the same time (1995), another membrane-bound retinoid-active SDR was isolated from detergent-solubilized bovine retinal pigment epithelium (RPE) membranes as a protein co-purifying with a major RPE protein, p63 [48]. This SDR was shown to oxidize 11-*cis*-retinol to 11-*cis*-retinaldehyde but showed no activity toward all-*trans*-retinol [48]. Based on this substrate specificity and localization in the RPE, this enzyme was proposed to represent the 11-*cis*-retinol dehydrogenase (11-*cis*-RDH) required for the production of the visual chromophore 11-*cis*-retinaldehyde in the eye. The amino acid sequences of these two enzymes, bovine 11-*cis*-RDH and rat RoDH1, were found to be similar (~50% sequence identity), suggesting common evolutionary origins [49].

Cloning of these first retinoid oxidoreductases prompted identification of numerous additional retinoid-active SDRs. To date, at least 17 different SDR proteins in humans, rats, and mice are known to be active with retinoids (Table 2).

The SDR enzymatic assay

Early on it became clear that the detergent-based retinoid activity assays developed for the MDR-ADH enzymes could not be used for SDRs, because detergents inactivated the SDR enzymes. Initially proposed conditions [46,47] resulted in poorly reproducible data; therefore, a new assay was developed [50]. In this assay, bovine serum albumin (BSA) is used to stabilize and solubilize retinoids in aqueous solutions. Although BSA binds retinoids, it shows a much higherK_d value (2 μM) compared to CRBP [51-53]; and thus it does not interfere significantly with enzymatic activity [17]. Briefly, the substrate stock solution is first prepared by adding the concentrated retinoid in ethanol or DMSO to an aqueous buffer containing an equimolar concentration of BSA. The suspensions are sonicated for 10 min to disperse retinol. The concentration of the solubilized substrate is determined by measurement of the absorbance of the solution at 330 nm for retinol ($\varepsilon = 39000\ M^{-1} \cdot cm^{-1}$) or 386 nm for all-*trans*-retinaldehyde ($\varepsilon = 29500\ M^{-1} \cdot cm^{-1}$). Aliquots of the BSA-solubilized substrate stock solution are then added to assay samples to desired concentration. The final concentration of ethanol or DMSO in the assay does not exceed 1%. The reactions are carried out in siliconized glass tubes and terminated by the addition of cold methanol. Retinoids are extracted with hexane and separated using an HPLC method, which makes it possible to separate and detect *trans* and *cis* retinoid isomers. Reaction rates are determined based on the percent of substrate conversion [54]. This method has been recently applied to ADH kinetic analysis, resulting in K_m values much lower than those determined in the presence of detergent [17].

Human SDR retinoid oxidoreductases

At least 10 human SDR proteins recognize retinoids as substrates. Although each of these proteins is unique in its substrate and cofactor specificity, they can be assigned to two major groups based on their cofactor preference: one group comprises the enzymes with high affinities for NAD^+/NADH, the other those with high affinities for $NADP^+$/NADPH (Table 2).

***NAD+-dependent SDRs*—**The human NAD+-dependent retinoid-active SDRs consist of four enzymes: 11-*cis*-RDH, retinol dehydrogenase 4 (RoDH4), RoDH-like 3α-hydroxysteroid dehydrogenase (RL-HSD) and RDH-like SDR (RDHL, also known as DHRS9) [49].

Human 11-*cis*-RDH recognizes 11-*cis*-retinol, 9-*cis*-retinol and 13-*cis*-retinol as substrates but has little activity toward all-*trans*-retinol (Table 2) [55]. This enzyme exhibits similar kinetic parameters for the oxidation of 11-*cis*-retinol free or bound to cellular retinaldehyde binding protein (CRALBP) [56], suggesting that 11-*cis*-RDH recognizes the CRALBP-bound form of 11-*cis*-retinol (*holo*CRALBP) as a substrate. However, direct binding of *holo*CRALBP to 11-*cis*-RDH has not yet been demonstrated.

Genetic evidence in humans and knockout mice suggests that 11-*cis*-RDH contributes to the oxidation of 11-*cis*-retinol to 11-*cis*-retinaldehyde in retinal pigment epithelium during the retinoid visual cycle [57,58]. Mutations in the gene encoding human 11-*cis*-RDH (*RDH5*) appear to be linked to *fundus albipunctatus*, a rare form of stationary night blindness characterized by a delay in the regeneration of cone and rod photopigments [57].

Human RoDH4 exhibits wide retinoid isomer specificity, accepting all-*trans*-retinol, 13-*cis*-retinol, and 9-*cis*-retinol as substrates (Table 2) [50]. RL-HSD is more stereospecific and is active toward all-*trans*-retinol but not 13-*cis*-retinol [59] (Table 2). RDHL (DHRS9) appears to have a negligible all-*trans*-or *cis*-retinol dehydrogenase activity compared to RoDH4 or RL-HSD *in vitro* [60], but contributes to RA production in transiently transfected cells [61,62].

The four enzymes are catalytically unstable after extraction from microsomal membranes. Therefore, most of their kinetic parameters were obtained using partially purified microsomal fractions containing the recombinant enzymes. However, a successful purification protocol has been developed for RoDH4 [54]. Kinetic characterization of the purified enzyme confirmed the substrate and cofactor specificity established for partially purified RoDH4 (Table 2) [54]. Interestingly, microsomal membranes were found to competitively inhibit RoDH4 activity toward retinol [63], significantly raising the apparent K_m value of RoDH4 for all-*trans*-retinol. All three human SDRs active towards all-*trans*-retinol recognize only the unbound form of this substrate [17].

The human NAD+-dependent retinoid-active SDRs have higher catalytic efficiencies for the oxidation of 3α-hydroxysterols than of retinols (Table 2) [50,59,60].

NADP+-dependent SDRs—At least six human microsomal SDRs possess an NADP+-dependent retinoid activity. Among them, three-RDH11, RDH12 and RDH14-appear closely related [49], while three others - retinal SDR1 (retSDR1), photoreceptor RDH (prRDH), and RDH10 - share little similarity with each other or with the RDH11-14 cluster [49]. With the exception of human RDH12, which appears to have a weak dihydrotestosterone reductase activity [64], the NADP+-dependent retinoid-active SDRs lack significant steroid oxidoreductive activity.

Only two of the NADP+-dependent SDR retinoid oxidoreductases, RDH11 and RDH12, have been purified and kinetically characterized (Table 2) [65,66]. Both enzymes recognize all-*trans* as well as *cis*-retinoids with the apparent K_m values in the low micromolar range. With all-*trans*-retinaldehyde, RDH12 has a twice greater V_{max} value than RDH11 and remains the most catalytically efficient retinaldehyde reductase known. Mutations in RDH12 have been genetically linked to severe early-onset autosomal recessive retinal dystrophy [67,68]. A recent study suggests that RDH12 protects photoreceptors against retinaldehyde-induced toxicity and overproduction of retinoic acid [69].

RDH14 (originally known as PAN2) exhibits a retinaldehyde reductase activity when expressed in insect cells [70,71]. RDH13 was identified as a member of the RDH11-14 group based on sequence similarity [71], but the enzymatic activity has not yet been demonstrated.

In addition to retinoids, some of the RDH11-14 SDRs are quite active toward short-chain and medium-chain aldehydes. However, this property is not always conserved between the human and mouse orthologs. For example, human RDH12 and mouse RDH11 both exhibit high catalytic rates with nonanal, *cis*-6-nonenal, and *trans*-2-nonenal [66,72], but the human ortholog of RDH11 has a very low activity toward these substrates [66].

Information regarding kinetic characteristics of other $NADP^+$-dependent retinoid-active SDRs is very limited. RetSDR1, expressed in membranes of insect cells, reduces all-*trans*-retinaldehyde but not 11-*cis*-retinaldehyde in the presence of NADPH [73]. For human prRDH retinoid activity has not yet been demonstrated but its closely related bovine ortholog reduces all-*trans*-retinaldehyde [74].

Human RDH10 was reported to prefer $NADP^+$ as cofactor. However, unlike retSDR1 or prRDH, this enzyme has little activity in the reductive direction when expressed in COS cells but is active in the oxidative direction [75]. RDH10 does not oxidize 11-*cis* retinol, 9-*cis* retinol, or 13-*cis* retinol in the presence of NAD^+ or $NADP^+$, but it is specific for all-*trans*-retinol. Recently, a search for mouse genes responsible for regulation of growth and pattern formation has identified a new midgestation lethal mouse mutant, called *trex*, which displays craniofacial, limb, and organ abnormalities [37]. The *trex* mutation has been mapped to *Rdh10*. The $RDH10^{trex}$ mutant protein lacks the ability to oxidize retinol, resulting in insufficient production of RA. Supplementation with RA rescued the abnormalities seen in *trex* mutant embryos, suggesting that RDH10 is essential for RA biosynthesis during embryogenesis [37]. RDH10 is the only retinoid-active dehydrogenase from either the SDR or MDR superfamily which has been shown to be indispensable for embryonic development in mice under normal vitamin A supply.

Rodent SDR retinoid oxidoreductases

Most rodent retinoid oxidoreductases have been studied using cell lysates or microsomal preparations of recombinant enzymes transiently expressed in eukaryotic cells. Three closely related enzymes have been identified in the rat: RoDH1, RoDH2 and RoDH3. Partially purified RoDH1 appeared to recognize *holo*CRBPI as substrate, although the activity was severalfold higher with free retinol [46]. As an evidence for the recognition of *holo*CRBPI, CRBPI was shown to cross-link covalently with RoDH1 [76]. RoDH1 prefers $NADP^+$ as cofactor [76], and exhibits high activity with 3α-hydroxysteroids [77]. Properties of RoDH2 are similar to those of RoDH1, while RoDH3 has not yet been characterized. A rat ortholog of human RDHL, named eRolDH (also known as rat Dhrs9 [49]), functions as an all-*trans*-retinol dehydrogenase [78].

At least eight mouse SDRs oxidize retinols *in vitro* (Table 2). Most NAD^+-preferring murine SDRs (CRAD1, CRAD2, CRAD3 and 17β-HSD9) [79-82] have a mixed *cis*-retinoid/3α-hydroxysteroid substrate specificity, and their 3α-HSD activity greatly exceeds the retinol dehydrogenase activity. Mice null for CRAD3 show 2- to 4-fold increases in mRNAs of enzymes that catalyze xenobiotic and steroid metabolism, suggesting a role for CRAD3 in steroid and/or retinoid metabolism [83].

The only identified NAD^+-dependent murine enzyme that uses all-*trans*-retinol is RDH1 [84], although its V_{max} value with retinol is severalfold lower than with 3α-hydroxysteroids (Table 2). *Rdh1* knockout mice have increased liver and kidney retinoid stores, suggesting that RDH1 metabolizes retinoids *in vivo* [85].

In contrast to some of the previous enzymes for which human orthologs are difficult to ascertain, the $NADP^+$-dependent all-*trans*-retinol dehydrogenase RDH10 is highly conserved among species, with 98.6% protein sequence identity between mouse and human [75], consistent with its critical role in embryogenesis (see above).

Finally, a retinaldehyde reductase (RRD, also known as human 2,4-dienoyl-CoA reductase), has been described in peroxisomes [86] (Table 2).

In summary, many retinoid-active SDRs have been identified in human and animal tissues, but much remains to be learned about their catalytic properties and physiological roles. Most of these enzymes exhibit specific tissue distribution patterns and recognize multiple compounds as substrates, suggesting that they may contribute to steroid and medium-chain aldehyde metabolism in addition to metabolism of retinoids. The catalytic properties and substrate specificity of orthologous enzymes in humans and rodents may be quite different. Furthermore, phylogenetic analysis shows that the total number of retinoid-active SDRs varies in different species. Together, these factors may explain the lack of expected phenotype for some of the SDR knockout mouse models.

Comparative activity analysis of ADHs, SDRs and AKRs with retinoids: Effect of CRBP

Estimates of the relative contribution of enzymes of each superfamily (MDR, SDR, AKR) to retinoid metabolism is difficult since kinetics have been performed with different methodologies. SDRs would supposedly play a major role because of their low K_m and their activity with retinol bound to CRBPI [92], in contrast to the lack of activity of MDR-ADHs with CRBPI-retinol [52]. However, recently the kinetic analysis of representative enzymes of each superfamily has been performed, using an HPLC method, with BSA as a solubilizer in a detergent-free buffer [17,93,94] (Table 3). Remarkably, all enzymes exhibit low and similar K_m values, 1 μM or lower, while they differ in their k_{cat} values. The ADH K_m values obtained with this methodology were about 100-fold lower than those obtained in the presence of Tween 80, while k_{cat} values were similar with the two methodologies (Tables 1 and 3). In the oxidative direction, expected for the NAD^+-dependent enzymes, ADHs are much more active retinol dehydrogenases than RoDH4, an NAD^+-dependent SDR. For the $NADP^+$-dependent enzymes (RDH11, RDH12 and the AKRs), expected to function in the reductive direction, RDH12 exhibits the highest catalytic efficiency (900 000 $mM^{-1} \cdot min^{-1}$ [66]).

The activity of the three enzymatic types was also checked in the presence of CRBPI [17,66]. None of the enzymes used retinol (or retinaldehyde) bound to CRBPI but only free retinoids, both in the oxidative and reductive directions. The small activity found with *holo*CRBPI could be fully supported by the amount of retinoid being dissociated from the complex. The activity previously reported for SDR enzymes with all-*trans*-retinol bound to CRBPI probably results from the small amount of the compound that isomerizes to 9-*cis*-retinol in the presence of light and CRBPI [66]. The 9-*cis*-retinol does not bind to CRBPI and could therefore be oxidized. The present methodology, which allows the separation of all *cis/trans*-isomers, conclusively establishes that none of the studied oxidoreductases uses *holo*CRBPI.

In vivo evidence on the ADH1/CRBPI relationship in liver retinoid metabolism

Genetic studies have shown that CRBPI stimulates retinol storage as retinyl esters [95] (Fig. 1). The dominant pathway of degradative retinol turnover in the liver proceeds through oxidation of retinol to retinaldehyde by ADH1 [39], followed by irreversible oxidation of retinaldehyde to RA by RALDH1 [96]. The high retinoid activity of these enzymes may affect the ability of CRBPI to sequester retinol for retinyl ester synthesis. This may also be true for

ADH2, as its main site of expression is the liver. Compared to wild-type animals, *Crbp1*-/- mice exhibit a 3.5-fold decrease in liver retinyl ester stores, whereas *Adh1*-/- mice exhibit a 1.6-fold increase in liver retinyl ester stores [97]. Importantly, *Crbp1*-/-; *Adh1*-/- double mutant mice exhibit liver retinyl ester stores that are not significantly different from wild-type [97]. Thus, the loss of liver retinyl ester stores observed in *Crbp1*-/- mice is due to retinol oxidation catalyzed by ADH1. Further studies have demonstrated that ADH1 is involved in a continuous oxidative turnover of retinol and that CRBPI limits this turnover [97].

These findings have led to the hypothesis that a major physiological function of liver CRBPI is to protect free retinol from ADH1, consistent with *in vitro* studies showing that CRBPI inhibits ADH retinol activity. CRBPI provides a pool of retinol for retinyl ester synthesis that is mostly protected from ADH1 activity. The opposing actions of CRBPI and ADH1 on liver retinol metabolism would control the balance between retinol storage (favored by CRBPI) and retinol turnover (favored by ADH1).

Role of redox enzymes and CRBPI in retinoid metabolism of target tissues and its regulation

Finally, we propose a model for retinoid pre-receptor metabolism and regulation in target tissues (Fig. 2). According to this model, CRBPI would not directly participate in the biosynthesis of RA, and its role would be limited to vitamin A storage. Thus, CRBPI would favor transfer of retinol to membranes, where LRAT would esterify it into retinyl esters. On the other hand, CRBPI would prevent retinol from being oxidized, and only dissociated free retinol would be available to retinol dehydrogenases. CRBPI severely restricts the activity of the dehydrogenases because of its extremely low binding constant (K_d for retinol = 0.1 nM). Retinaldehyde reductases only use free retinoid as well. In contrast, they can still function in the presence of CRBPI because of a higher constant (K_d for retinaldehyde = 50 nM), which results in a significant fraction of free retinoid in solution. A physiological implication of this observation is that the presence of CRBPI appears to favor reduction of retinaldehyde over retinol oxidation. Moreover, in some cases, i.e. in AKR overexpression found in some cancer types [98], retinaldehyde reduction would be further favored.

Members from the three enzyme superfamilies (MDR, SDR, AKR) could participate in retinol and retinaldehyde oxidoreduction. NAD^+-dependent enzymes seem to be best suited for retinol oxidation, with ADH4 being the most efficient. $NADP^+$-dependent enzymes are better adapted for retinaldehyde reduction, RDH12, RDH11 and AKR1B10 being the most efficient [17,66, 94] (Table 3). This *in vitro* evidence is supported by results with cell lines expressing enzymes from each type [17,94]. Relative amounts of oxidizing and reducing enzymes, and of CRBPI, along with their tissue expression pattern and subcellular localization, would constitute important factors in the control of the retinoid metabolites required for biological functions. A prevalent idea is that retinol oxidation is catalyzed by several enzymes in a redundant manner, and that this assures a background level of retinaldehyde in most tissues, while the presence of RALDH would finally control the RA production. However, systems involved are complex, show heterogeneous spatiotemporal distribution, and their expressions change under different physiological and pathological conditions. Therefore other factors can be limiting in a given situation, including the reversible retinol-retinaldehyde interconversion, as recently demonstrated [37]. The multiplicity of the enzymes involved in this step shows that levels of retinaldehyde are critical and strictly controlled.

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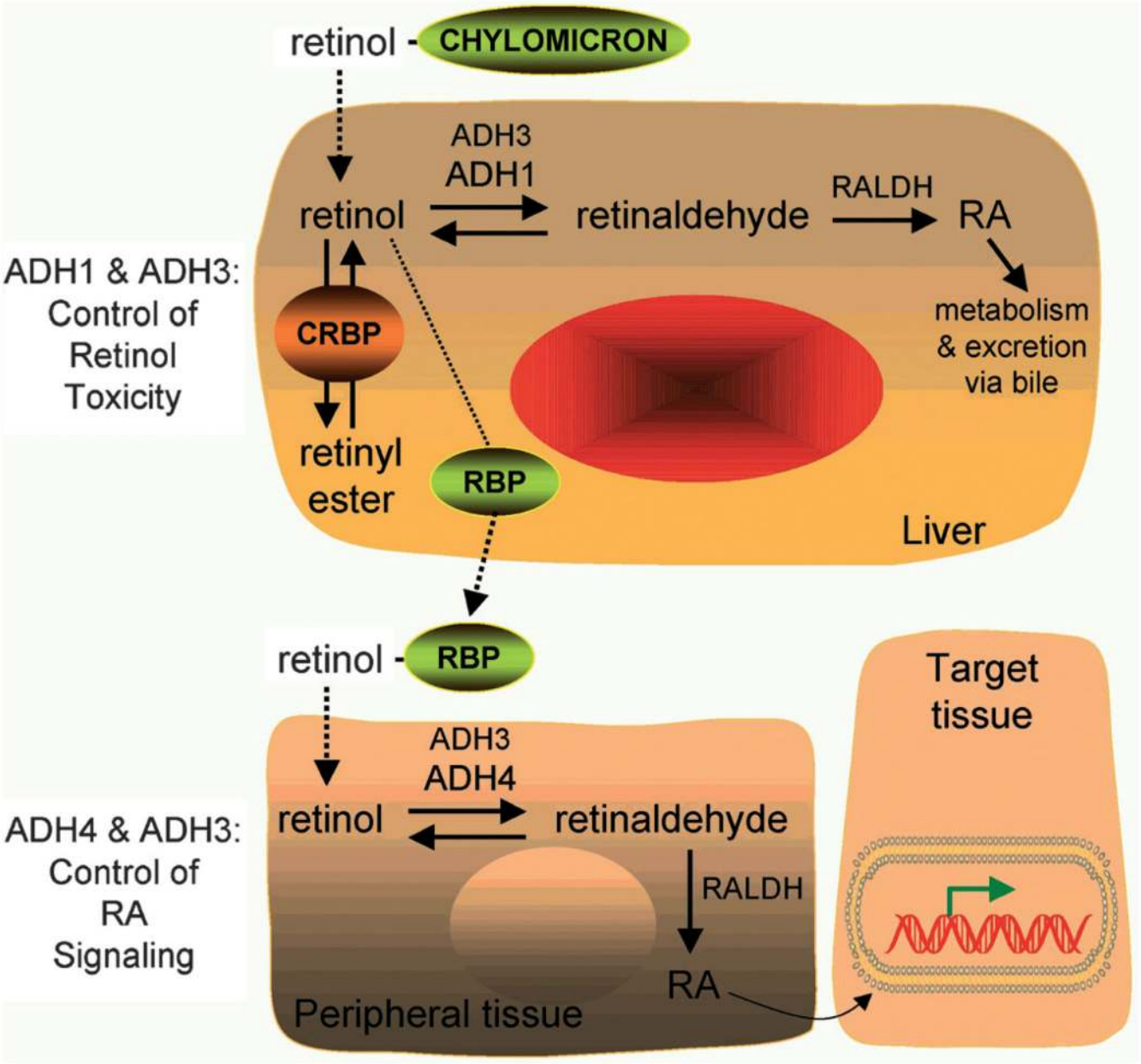

Figure 1.

ADH function during prevention of retinol toxicity and RA signaling. *ADH* gene knockout studies have revealed two important physiological functions for ADH in retinol metabolism. The top portion depicts the retinol oxidation function of ADH1 and ADH3 in liver to stimulate retinol turnover, which prevents retinol toxicity. The bottom portion depicts the retinol oxidation function of ADH4 and ADH3 in peripheral tissues to generate RA for signaling to nearby target cells. Retinol is taken up by liver primarily through retinol bound to chylomicrons and is carried in serum by retinol-binding protein (RBP) secreted from the liver. Cellular retinol-binding protein (CRBP) facilitates conversion of retinol to retinyl esters for storage. RBP transports retinol to peripheral tissues where it is metabolized to RA by ADH and retinaldehyde dehydrogenase (RALDH).

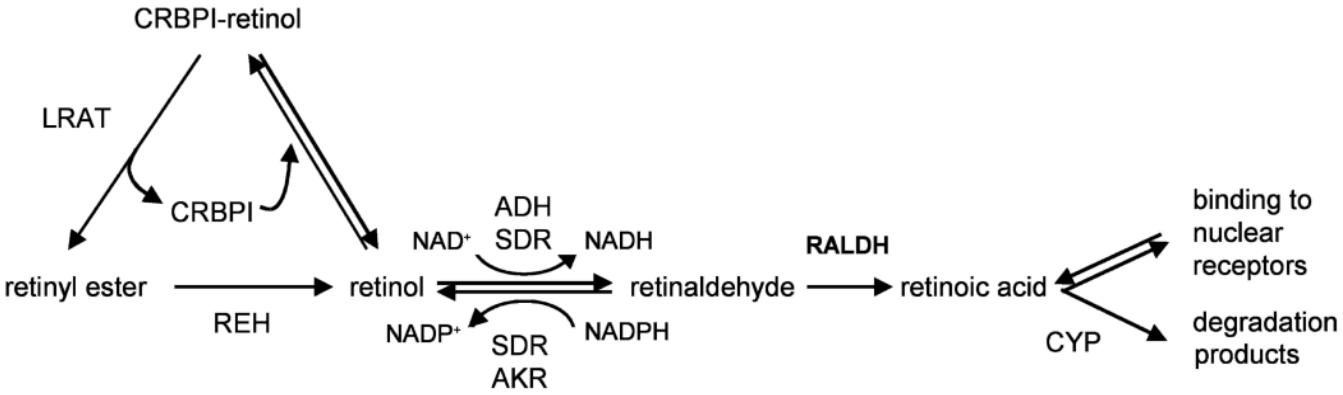

Figure 2.
Schematic diagram of cellular retinoid metabolism. Levels of CRBPI, lecithin:retinol acyltransferase (LRAT) and oxidoreductases influence the retinoid flow either toward the storage pathway or the retinoic acid synthesis [17]. REH, retinyl ester hydrolase; RALDH, retinaldehyde dehydrogenase; CYP, cytochrome P450.

Table 1

Catalytic constants (k_{cat}, min^{-1}) of human ADHs with retinoids in Tween 80.[a]

Substrate	ADH1A	ADH1B1	ADH1B2	ADH1C1	ADH2	ADH4	Ref.
all-*trans*-retinol	5.2	0.9	0.5	5.5	9.1	59	[18]
	11	1.2	17	7.2	3.3	120	[19]
		0.9	7.5			34	[21]
9-*cis*-retinol		0.4	1.8			238	[21]
11-*cis*-retinol		0.4	4.8			95	[21]
all-*trans*-retinaldehyde	3.5	0.3	2.1	0.5	5.5	52	[18]
		0.6	17			55	[21]
9-*cis*-retinaldehyde	2.6[b]	0.9	1.4	1.1[b]	7[b]	95	[21]
11-*cis*-retinaldehyde		0.16	1.7			9	[21]

[a] Determined in 0.1 M sodium phosphate, pH 7.5, 0.02% Tween 80, using 2.4 mM NAD^+ for retinol oxidation, and 0.77-1 mM NADH for retinaldehyde reduction, at 25°C. Values are calculated per subunit (40 kDa).

[b] Taken from [18].

Table 2

Properties of Retinoid-Active SDRs

Enzyme	Substrate								Preferred cofactor	Tissue distribution	Ref.
	All-*trans*-Rol (All-*trans*-Ral)		11-*cis*-Rol (11-*cis*-Ral)		9-*cis*-Rol (9-*cis*-Ral)		3α-adiol				
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}			
Human											
11-*cis*-RDH	Not significant		7.5[1] 2.5[1,2]	117[1] 143[1,2]	6.3	21	6.4	8.7	NAD^+	Widespread	[55,56]
RoDH4	0.3[1,3]	35[1,3]	ND		ND		0.22	6.9	NAD^+	Liver>>>fetal lung>>brain	[50,54,63]
RL-HSD	3.2	1.2	ND		ND		0.13	16.5	NAD^+	Liver>>>lung>placenta, brain, testis, prostate, spleen	[59]
RDHL (DHRS9)	Not significant		ND		ND		7.5	14	NAD^+	Trachea>>>other tissues	[60,61]
retSDR1	(active)		NA		ND		NA		NADPH	Pancreas>liver>heart>kidney>retina>other	[73]
prRDH	(active)		(low activity)		ND		ND		NADPH	Rod and cone photoreceptors	[75]
RDH10	active		NA		NA		ND		$NADP^+$	RPE>>> retina, kidney, pancreas, liver, lung, skeletal muscle, brain	[37,75,87,88]
RDH11	0.6[1] (0.12)[1]	300[1] (500)[1]	ND		0.19	1.6	NA		$NADP^+$/NADPH	Widespread	[65,89]
RDH12	0.4[1] (0.04)[1]	760[1] (1000)[1]	0.16[1] (0.1)[1]	200[1] (1260)[1]	0.16[1] (0.14)[1]	200[1] (390)[1]	NA		$NADP^+$/NADPH	Retina>>>kidney>pancreas>>other	[66]
Rat											
RoDH1	0.6[1,4]	0.1[1,4]	ND		ND	0.1[5]	0.5[5]		$NADP^+$ NAD^+[5]	Liver	[76]
RoDH2	2[4]	ND	ND		ND	3.2[5]	1.3[5]		$NADP^+$ NAD^+[5]	Liver>>kidney>>brain>lung>testis	[90]
Mouse											
CRAD1	ND	0.2	ND	13	5.4	9.9; 55	0.2	27	NAD^+	Liver≅kidney>>>heart>brain	[79]
CRAD2	6.9	0.04	5	0.2	>28	ND	2.2	3.4	NAD^+	Liver>>>lung>eye>kidney>brain	[80]
CRAD3	ND		2.3	40	3.6	35	1.5	19	NAD^+;$NADP^+$	Liver >>> kidney>>testis, lung, small intestine, heart, and brain	[81]
17β-HSD9	3.2	0.4	active		active		1.4	2.8	NAD^+	Liver	[82]
RDH1	2.6	6.7	ND		2.2	3.3	3.1	97	NAD^+	Widespread	[91]
RRD	(2.3)	(40)	ND		ND		ND		NADPH	Liver≅kidney≅heart> testis>>>other	[86]

All activities were measured using crude supernatans of transiently transfected cells unless indicated otherwise. In parenthesis are values for retinaldehydes. K_m, μM; V_{max}, nmol/min mg; ND, not determined; NA, not active; Rol, retinol; Ral, retinaldehyde; adiol, androstanediol

[1] for purified enzymes

[2] in the presence of CRALBP

[3] determined for purified RoDH4 after correction for membrane inhibition [63]

[4] for *holo*CRBPI

[5] measured with NAD^+.

Table 3

Kinetic constants of human MDR, SDR and AKR enzymes with retinoids in BSA

Substrate			ADH1B2[a]	ADH2[b]	ADH4[a]	RDH11[a]	RoDH4[c]	AKR1B1[a]	AKR1B10[a]
all-*trans*-retinol	K_m	(μM)	0.3	0.14	0.3	0.6	0.29		0.4
	k_{cat}	(min^{-1})	21	4	190	11	1.20	NA	7.2
	k_{cat}/K_m	($mM^{-1}·min^{-1}$)	70000	29000	640000	18000	4100		18000
all-*trans*-retinaldehyde	K_m	(μM)	0.40	0.29	0.8	0.12	0.34	1.1	0.6
	k_{cat}	(min^{-1})	5.0	2.3	300	18.0	ND	0.35	27
	k_{cat}/K_m	($mM^{-1}·min^{-1}$)	13000	7900	378000	150000	ND	320	45000

Activities with retinoids were determined in equimolar BSA, 90 mM potassium dihydrogen phosphate, 40 mM potassium chloride, pH 7.4, at 37 °C. Reaction products analysed by HPLC. Data from [a] [17], [b] [93] and [c] [63]. ND, not determined. NA, not active.