HHS Public Access

Author manuscript

Subcell Biochem. Author manuscript; available in PMC 2017 August 10.

Published in final edited form as:

Subcell Biochem. 2016 ; 81: 127–161. doi:10.1007/978-94-024-0945-1_5.

RETINOIC ACID SYNTHESIS AND DEGRADATION

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Abstract

Retinoic acid was identified as the biologically active form of vitamin A almost 70 years ago, but the exact enzymes and control mechanisms that regulate its biosynthesis and degradation are yet to be fully defined. The currently accepted model postulates that RA is produced in two sequential oxidative steps: first, retinol is oxidized reversibly to retinaldehyde, and then retinaldehyde is oxidized irreversibly to RA, which is inactivated by conversion to hydroxylated derivatives. This chapter describes the history, development and recent advances in our understanding of the enzymatic pathways and mechanisms that control the rate of RA production and degradation. Gene knockout studies provided strong evidence that the members of the short chain dehydrogenase reductase superfamily of proteins play indispensable roles in retinoic acid biosynthesis during development. Furthermore, recent finding that two of these proteins regulate the rate of retinoic acid biosynthesis by mutually activating each other provided a novel insight into the mechanism of this regulation. Despite significant progress made since the middle of the 20th century many unanswered questions still remain, and there is much to be learned, especially about trafficking of the hydrophobic retinoid substrates between membrane bound and cytosolic enzymes and the roles of the retinoid binding proteins.

5.1 INTRODUCTION

Humans obtain vitamin A with diet in the form of retinyl esters from animal sources or β-carotene from plant sources. After absorption, these dietary forms of vitamin A are converted enzymatically into the biologically active metabolite, retinoic acid (RA). As too much or too little of RA is equally harmful, the levels of RA in tissues have to be tightly controlled. However, although RA was identified as the biologically active form of vitamin A almost 70 years ago (Arens and Van Dorp 1946; Van Dorp and Arens 1946a; Van Dorp and Arens 1946b), the exact enzymes and control mechanisms that regulate its biosynthesis and degradation are yet to be fully defined. The currently accepted model postulates that RA is produced in two sequential oxidative steps: first, retinol is oxidized reversibly to retinaldehyde, and then retinaldehyde is oxidized irreversibly to RA, which is inactivated by conversion to hydroxylated derivatives (Figure 5.1).

Other chapters focus on the dietary uptake, storage and mobilization of retinol. This chapter will focus on the history, development and recent advances in our understanding of the enzymatic pathways and mechanisms that control the rate of RA production and degradation.

5.2 HISTORY

At first, there were doubts about the natural occurrence of RA as a normal product of retinol metabolism because some of the initial attempts to demonstrate RA formation from retinol or retinaldehyde in vivo have been unsuccessful (Krishnamurthy et al. 1963), and little if any RA could be found in tissues after administering milligram doses of RA (Arens and Van Dorp 1946; Redfearn 1960). Other studies showed that small amounts of RA rapidly appeared in rat tissues (Deshmukh et al. 1965) and bile (Dunagin et al. 1964) after parenteral administration of milligram quantities of retinaldehyde. However, these observations raised a question of whether RA is formed only in systems treated with abnormally large amounts of retinaldehyde. Eventually, it was demonstrated that RA forms in vivo in rat liver and small intestine from microgram quantities of intrajugularly administered ^{14}C-radiolabeled retinol (Emerick et al. 1967). Thus, by the end of the 1960s, it was established that RA is a natural product that is formed in very small quantities from either retinol or retinaldehyde. Using ^{14}C-labeled RA, it was also established that RA and its metabolites are rapidly excreted in the bile of bile-duct cannulated rats (Dunagin et al. 1965; Zachman et al. 1966).

Characterization of the Enzymes That Catalyze the Reversible Oxidation of Retinol to Retinaldehyde

Initially, studies conducted with homogenates of frog and cattle retinas demonstrated that the vitamin A aldehyde (retinene) found in rhodopsin could be reduced to vitamin A alcohol by an enzymatic process (Bliss 1948; Wald 1950) (Figure 1, step 1, reductive conversion of retinaldehyde to retinol), which required reduced diphosphopyridine nucleotide (NADH) as coenzyme (Wald and Hubbard 1949). The reduction was reversible (Figure 1, step 1, oxidation of retinol to retinaldehyde), and because it was also catalyzed by crude rabbit liver extracts, which contain high levels of alcohol dehydrogenases, known to interconvert alcohols and aldehydes, and also by crystalline horse liver alcohol dehydrogenase (ADH) (Bliss 1951), it was suggested that the reduction of retinaldehyde and oxidation of retinol were both catalyzed by an alcohol dehydrogenase (Bliss 1950; Bliss 1947).

This notion was reinforced by the finding that the same enzyme in rat liver appeared to be responsible for both the NADH-dependent retinaldehyde-reductive and NAD-dependent ethanol-oxidizing activities (Zachman and Olson, 1961). Furthermore, ethanol was shown to inhibit the oxidation of retinol by human liver and cattle retina (Mezey and Holt 1971), providing further evidence for potential involvement of ADH in metabolism of retinol.

The possibility that ADH was naturally involved in metabolism of retinol attracted the attention of ADH researchers who were interested in identifying the endogenous physiologically occurring substrates for ADH isoenzymes, which were highly expressed in liver and were best known for their ability to metabolize ethanol. ADHs belong to the medium-chain dehydrogenase/reductase (MDR) superfamily of proteins and are dimeric zinc metalloproteins with ~350-residue subunits. The human and mouse ADH families each contain six classes of cytosolic ADH enzymes (Höög and Östberg 2011; Persson et al. 2008) (Table 5.1). Interest in retinol as the natural substrate for ADH prompted studies from several laboratories which showed that in humans, class IV ADH (ADH4, also known as stomach σσ ADH) was the most catalytically efficient retinol dehydrogenase in vitro,

followed by ADHs of class I (ADH1) and liver-specific class II (ADH2) (Boleda et al. 1993; Saubi et al. 1993; Yang et al. 1994; Han et al. 1998; Crosas et al. 2000). ADH4 protein was proposed to catalyze the oxidation of retinol to retinaldehyde in the epithelia of the stomach, esophagus, skin, and respiratory tract (Haselbeck and Duester 1997; Haselbeck et al. 1997). ADH1 was thought to be the primary retinol dehydrogenase in liver, where it was highly expressed and, in the mouse, constituted 0.9% of liver protein (Algar et al. 1983). The ubiquitously expressed Class III ADH (ADH3) did not exhibit significant catalytic activity towards retinol and functioned primarily as a GSH-dependent formaldehyde dehydrogenase (Molotkov et al. 2002).

While kinetic analyses indicated that ADH4 and ADH1 isoenzymes had significant retinol dehydrogenase activity *in vitro*, the *in vivo* role of ADHs in retinoid metabolism came under scrutiny when Leo and Lieber (1984) described a strain of deermouse that genetically lacked ADH and the cytosolic NAD^+-dependent retinol dehydrogenase activity in its liver and testes without apparent adverse effects. Evidence supporting the existence of non-ADH retinol dehydrogenases also came from earlier studies. Koen and Shaw reported of a retinol dehydrogenase in liver and retina that was distinct from ethanol dehydrogenase (Koen and Shaw 1966). Napoli (1986) showed that 220 mM ethanol did not impair RA production from 10 μM retinol or retinaldehyde by LLC-PK1 cells, an epithelial, non-tumorigenic cell line derived from pig kidney. In addition, the well-known inhibitor of ADH activity, 4-methylpyrazole, did not inhibit RA synthesis significantly at concentrations as high as 10 mM.

These observations prompted a search for an alternative retinol dehydrogenase, unrelated to classical ethanol-oxidizing cytosolic ADH. Leo et al. (1987) reported that liver of ADH-null deermice contained a microsomal retinol dehydrogenase that had a 2.5-fold higher activity with NAD^+ than with $NADP^+$ and was not inhibited by 4-methylpyrazole, the strong inhibitor of the cytosolic retinol dehydrogenase activity. The microsomal enzyme also catalyzed the reverse direction, the reduction of retinaldehyde to retinol. Together, the cytosolic and microsomal fractions accounted for 94% of the total NAD^+-dependent retinol dehydrogenase activity of liver homogenate, with the microsomal activity constituting only about 16% of the total activity.

Lieber's group also showed that liver microsomes from either ADH+ or ADH− deermice did not produce RA from retinol, but the addition of cytosol to the microsomes resulted in the formation of RA (Kim et al., 1992). The authors concluded that retinol was converted to retinaldehyde by the liver microsomes in ADH− deermice, and then retinaldehyde was oxidized to RA by the cytosolic enzymes. The intermediate role of retinaldehyde in the production of RA was further demonstrated by the experiments where the addition of unlabeled retinaldehyde to labeled retinol resulted in isotopic dilution of the RA formed upon incubation with ADH+ deermice cytosol, and also by the finding that tritiated RA was produced from tritiated retinol in the mixture of microsomes and cytosol from ADH− deermice but not by microsomes or cytosol alone.

Another important observation related to the conversion of retinol to RA was made by Napoli who showed that the rate-limiting step in this process was the dehydrogenation of

retinol, as demonstrated using LLC-PK1 cells (Napoli, 1986). The oxidation of retinol to retinaldehyde was consistently 30–60-fold slower than the oxidation of retinaldehyde to RA. Overall, less than 5% of the retinol added to the cells was converted into RA. The synthesis of RA in LLC-PK1 cells was not sensitive to inhibitors of alcohol metabolism, providing further evidence for the existence of retinol dehydrogenases distinct from ethanol dehydrogenases.

Faced with the growing number of enzymes that oxidize retinol *in vitro* and uncertainty with respect to their physiological significance, Napoli and colleagues speculated that if, as had been suggested, intermediates pass through metabolic pathways by direct transfer between proteins (Clegg 1984; Srivastava and Bernhard 1986; Bernhard 1988), rather than by diffusion through the aqueous phase, the first protein in the pathway from retinol to RA would be the ubiquitous cellular retinol binding protein type I (CRBPI), which binds cellular retinol specifically (Ong et al. 1982). Thus, Napoli and colleagues proposed that the physiologically relevant retinol dehydrogenase should be able to recognize the CRBPI-bound retinol (holo-CRBPI) as substrate. To test this idea, Posch et al. (1991) assayed the activity of microsomal fractions from rat liver, kidney, lung, and testes with holo-CRBPI as substrate. The microsomal enzymes appeared to prefer $NADP^+$ over NAD^+ as a cofactor for oxidation of holo-CRBPI to retinaldehyde, because the V_{max} with $NADP^+$ was 3.4-fold higher than that with NAD^+. Notably, the rate for the oxidation of free retinol with $NADP^+$ as cofactor (7.4 pmol/min mg) was about 20-fold higher than that with holo-CRBPI (143 pmol/min × mg). The estimated K_m values for holo-CRBPI and unbound retinol were quite similar, ~1.6 and ~4 μM, respectively. Based on the K_d value of CRBPI for binding of retinol (16 nM), the authors calculated that the amount of free retinol present in holo-CRBPI preparation was not sufficient to support the reaction rate observed using holo-CRBPI as substrate; hence, holo-CRBPI must have been the actual substrate for the microsomal retinol dehydrogenase.

Further work from this group focused on molecular identification of the microsomal $NADP^+$-dependent retinol dehydrogenase (RoDH) and characterization of its interaction with holo-CRBPI. Boerman and Napoli (1995) attempted to extract and purify the microsomal enzyme based on its activity toward 2.5 μM holo-CRBPI. Total purification was not achieved because the RoDH lost activity as purity increased. The final preparation of partially purified RoDH active toward holo-CRBPI contained two major bands, one of 34 kDa and another of 54 kDa. To identify the polypeptide that interacts with CRBPI, microsomes were incubated with CRBPI that was covalently labeled with a UV-activated, cleavable cross-linking reagent. The reagent was radioiodinated such that the iodine would be transferred covalently from the CRBPI to any target protein upon activation and cleavage. A dominant radiolabeled band was observed consistently at 37 kDa but not 54 kDa. Interestingly, this band was not recognized by the antibodies raised against the 34 kDa polypeptide purified using holo-CRBPI activity assay. The authors interpreted this observation as suggesting that the antigenic determinant was contributed by limited epitopes that were altered by crosslinking.

Protein sequencing of four internal polypeptides obtained by a trypsin digest of the 34 kDa band revealed that one internal peptide (L**W**GLV**NNAG**ISVPV) had a sequence highly

conserved in proteins that belong to the superfamily of short chain dehydrogenases/ reductases (SDRs) (Persson, 1991). SDRs have been previously shown to be involved in metabolism of sugars, steroids, and prostaglandins. The identification of the 34 kDa polypeptide as a member of the SDR superfamily suggested that SDRs are also involved in metabolism of retinoid substrates.

By the mid-90s, two major types of enzymes emerged as candidates for the role of retinol dehydrogenases: the cytosolic ADHs and the microsomal SDRs. However, whether both of these enzyme families contribute to RA biosynthesis remained unclear.

Characterization of the Enzymes that Irreversibly Oxidize Retinaldehyde to RA

In contrast to ambiguity surrounding the identification of physiologically relevant retinol dehydrogenases, there was a general consensus early on that the second step in biosynthesis of RA, the oxidation of retinaldehyde to RA, was catalyzed by the soluble enzymes present in cell cytosol (Kim et al. 1992). Studies from several groups provided evidence that the aldehyde form of vitamin A was oxidized irreversibly to vitamin A acid, and that this reaction was catalyzed by aldehyde dehydrogenases (ALDHs) in calf and rat liver (Dmitrovskii 1961; Futterman 1962; Elder and Topper 1962; Mahadevan et al. 1962; Dunagin et al. 1964; Lakshmanan et al. 1964) (Figure 1, step 2). Thus, it was concluded that together with alcohol dehydrogenase, the aldehyde-oxidizing enzymes provided a pathway from the vitamin A alcohol to the acid (Dmitrovsky, 1961; Futterman, 1962; Bamji et al. 1962; Mahadevan et al. 1962), similarly to the metabolism of ethanol to acetaldehyde and further to acetic acid.

To identify the isoenzymes of ALDH family of proteins that were active towards retinaldehyde, Sladek and co-workers examined the activities of several candidate ALDHs (Lee et al. 1990a; Lee et al. 1990b; Lee et al. 1991; Dockham et al. 1992). Their studies showed that only one of chromatographically resolved ALDHs from human liver, ALDH-1, catalyzed the oxidation of retinaldehyde with K_m value of 0.3 μM (Dockham et al. 1992).

Another major contribution was provided by Dräger and coworkers whose work focused on the identification of retinaldehyde-active ALDHs that were essential for RA biosynthesis in mouse embryonic retina during development (McCaffery et al. 1991; McCaffery et al. 1992; McCaffery et al. 1993; McCaffery and Dräger 1994). These researchers used a highly sensitive RA reporter cell line that allowed them to detect low levels of RA produced by ALDHs in different segments of the developing retina and spinal cord. Using a combination of immunohistochemistry, isoelectric focusing, and biochemical assays they were able to identify three separate forms of ALDH that differed in their isoelectric points, catalytic efficiency, sensitivity to inhibitors, and their spatio-temporal expression pattern during development. One of the ALDH activities confined to the dorsal part of the retina was identified as the previously characterized murine AHD2 class I ALDH (RALDH1). In addition to this previously characterized enzyme, McCaffery et al. found two novel aldehyde dehydrogenase activities, named V1 and V2. V2 was of special interest because it was the first aldehyde dehydrogenase detectable in the early mouse embryo.

Taking a different approach, Napoli and colleagues searched for the retinaldehyde-oxidizing ALDH by fractionating rat liver cytosol by anion-exchange chromatography and assaying the catalytic activities of fractions with 2 μM retinaldehyde as substrate and NAD^+ as cofactor (Posch et al. 1992). The purified enzyme (RALDH1) was shown to oxidize retinaldehyde in the presence of CRBPI with an average K_m of 0.13 μM. By isoelectric point (~8.3), the rat retinal dehydrogenase was most similar to human ALDH IV (Goedde and Agarwal 1987) and mouse AHD-2 (Rout and Holmes 1985). The exact molecular identities of the ALDHs responsible for the oxidation of retinaldehyde to RA in rat and mice remained unknown until 1996.

Characterization of the Enzymes Involved in Degradation of RA

In vivo studies using ^{14}C-labeled RA showed that administered RA disappeared quickly from the animal (Redfearn 1960; Van Dorp and Arens 1946). For example, all of the ^{14}C from 16 μg of ^{14}C-labeled RA injected intravenously into rats was recovered within 48 h (DeLuca and Roberts 1969). The products of RA metabolism included retinoyl β-glucuronide (Dunagin et al. 1965; Dunagin et al. 1966; Lippel and Olson 1968); decarboxylated metabolites (DeLuca and Roberts 1969), and polar metabolites (Ito et al. 1974) (Figure 1, step 3). Hanni and co-workers identified 4-oxoretinoic acid as well as the all-*trans* and 9-*cis* isomers of 5′-hydroxy-RA in the feces of rats after a single intraperitoneal dose of 27.2 mg of all-*trans*-RA (Hänni et al. 1976; Hänni and Bigler 1977).

With the advent of high-pressure liquid chromatography (HPLC) and application of this technique to the separation of retinoids, Frolik and co-workers were able to demonstrate that incubation of [^3H]RA in the presence of hamster liver 10,000 × *g* supernatant produced several metabolites that were more polar than the parent compound. Two of these metabolites were identical with synthetic all-*trans*-4-hydroxy-RA and all-*trans*-4-oxo-RA (Frolik et al. 1978; McCormick et al. 1978a; Frolik et al. 1979). Napoli and colleagues identified 5,8-oxy-RA as a metabolite of administered all-*trans*-RA in the intestine of vitamin A deficient rats (Napoli et al. 1978). However, it was pointed out that the in vivo product was most likely the 5,6-epoxide of RA and that 5,8-oxy-RA was derived from 5,6-epoxide under the acidic conditions of the extraction procedure (McCormick et al. 1978b; Napoli et al. 1978).

To determine the characteristics of the enzyme systems involved in metabolism of RA, Roberts et al. (1979a, 1979b) investigated the RA metabolism by subcellular fractions of hamster intestine and liver. They found that RA metabolism was induced in the tissues of vitamin A deficient hamsters by pretreatment of the animals with oral doses of RA. The major classes of metabolites separated by HPLC were more polar than RA and retained the carboxyl carbon atom. The metabolic activity was localized in the 100,000 × g pellet, required NADPH and oxygen, and was strongly inhibited by carbon monoxide and by some cytochrome P-450 inhibitors. The authors concluded that the initial steps in the deactivation of the RA molecule were mediated by a cytochrome P-450-type enzyme system. Cytochrome P-450 enzymes are membrane-bound mono-oxygenases encoded by the *CYP* supergene family that catalyze the oxidative metabolism of both endogenous and exogenous compounds, including carcinogens, drugs, steroids, fatty acids, and prostaglandins (Zanger

et al. 2014). Interestingly, the oxidation of RA was specifically induced by the RA itself. Classic cytochrome P-450 or P-448 inducers phenobarbital or d-methylcholanthrene induced RA catabolism to only a minor extent.

Leo et al. (1984) showed that feeding rats with a diet containing a hundred times the normal amount of vitamin A resulted, within 2 to 3 weeks, in an increase in total hepatic microsomal cytochrome P-450 content. They confirmed that this was associated with an enhanced conversion of all-*trans*-RA to polar metabolites including 4-hydroxy- and 4-oxo-RA by isolated microsomes. They also showed that purified cytochromes P-450f and b promoted conversion of RA to polar metabolites, including 4-hydroxy-RA. In addition, this group showed that purified human P450IIC8 metabolized both retinol and RA to corresponding 4-hydroxy-retinoids and other polar metabolites.

Roberts et al. (1992) examined eight purified rabbit cytochrome P-450 (P-450) isozymes for their activities toward RA. Cytochrome P-450s 2B4 and 1A2 were the most active RA 4-hydroxylases, but the maximum velocities of P-450 2B4 for hydroxylation of retinol and retinal were much greater than that with RA as substrate. None of the isozymes investigated was found to convert the 4-hydroxy derivative to the 4-oxo derivative. Additional evidence for the involvement of CYP enzymes in metabolism of RA came from studies of Van Wauwe et al. (1992), who demonstrated that P-450 inhibitor liarozole potently inhibited the C-4 hydroxylation of RA.

Together these studies established that a major metabolic pathway of RA consisted of the hydroxylation at position C-4 of its cyclohexenyl moiety to form 4-hydroxy-RA, which was then oxidized further to 4-keto-RA and more polar metabolites, with glucuronylation of RA possibly being limited to liver and intestine. Both *in vitro* and *in vivo,* the C-4 hydroxylation of RA was determined to be mediated by a cytochrome P-450-dependent monooxygenase system. In addition, it was proposed that RA bound to cellular retinoic acid binding protein type I (CRABPI) could serve as substrate for RA-metabolizing cytochrome P-450s (Fiorella and Napoli 1994). In mammals, several purified CYPs were shown to be capable of converting RA to more polar metabolites in reconstituted systems, although generally specificity for RA was not high. An open question remained whether or not there could be (RA-inducible) CYP family members specifically dedicated to the hydroxylation of RA.

5.3 DEVELOPMENT OF THE FIELD

Oxidation of Retinol to Retinaldehyde: Re-evaluation of ADH Kinetic Constants

The comparison of the catalytic properties of the cytosolic ADH isoenzymes and the microsomal RoDH toward retinol was complicated by the methodological differences in measurements of their activities. Initially, the assays of the retinol oxidizing activity of ADH isoenzymes were conducted by a continuous spectrophotometric assay (Boleda et al. 1993; Yang et al. 1994), which measured the appearance of retinaldehyde based on its absorbance at 400 nm (ε_{400}=29 500 M^{-1} cm^{-1}), where retinol does not absorb. However, these assays required solubilization of retinoids using detergents such as Tween-80, which acted as an apparent competitive inhibitor of retinol oxidation, increasing the K_m values of ADHs for retinoids by up to 100-fold (Martras et al. 2004). On the other hand, the activity of

microsomal RoDH was measured in a reaction buffer containing 2 mM egg yolk L-α-phosphatidylcholine and retinol bound to CRBPI or added directly to buffer from ethanol stock solution. Reaction products were extracted with hexane and separated by HPLC (Boerman and Napoli 1995).

To exclude the inhibitory effect of Tween-80, ADH kinetics with retinoids were re-measured in a detergent-free system using bovine serum albumin as the “solubilizing” agent for retinol (Gallego et al. 2006), because CRBPI-bound retinol was shown not to serve as substrate for ADH (Kedishvili et al. 1998). These assays produced much lower K_m values of ADH isoenzymes for retinol (0.14–0.3 μM) (Table 5.1) and retinaldehyde (0.29–0.8 μM), which were in the same range or lower than the K_m values of rat RoDH for free or CRBPI-bound retinol. The k_{cat} values of ADH isoenzymes varied from 4 to 190 min^{-1} for the oxidation of retinol and 2.3 to 300 min^{-1} for the reduction of retinaldehyde. In agreement with the previous measurements, the most catalytically efficient enzyme was class IV ADH (ADH4), followed by class I ADH (ADH1B2) and class II ADH (ADH2) (reviewed in Parés et al. 2008). However, the *in vivo* roles of ADH isoenzymes in RA biosynthesis remained unclear.

ADH Gene Knockouts

With the development of transgenic technologies, it became possible to assess the *in vivo* roles of the numerous retinoid-active oxidoreductases by knocking out the gene of interest. An extensive analysis of the contribution of individual ADH isoenzymes to retinoid metabolism *in vivo* was carried out by Duester and colleagues. Gene knockout studies revealed that neither of ADH genes was essential for survival during embryonic development (reviewed in Parés et al. 2008). When maintained on normal chow diet, *Adh4*$^{-/-}$ mice, *Adh1*$^{-/-}$ mice, *Adh3*$^{-/-}$ mice, and *Adh1/4*$^{-/-}$ double knockout mice were all viable and fertile. When subjected to vitamin A-deficient diet during gestation, *Adh1*$^{-/-}$ mice faired no worse than wild-type mice. *Adh4*$^{-/-}$ mice had 34% more stillbirths than did wild-type mice (Deltour et al. 1999), whereas *Adh1/4*$^{-/-}$ offspring displayed more growth and survived longer than *Adh4*$^{-/-}$ mice. The authors speculated that the additional loss of ADH1 moderated the negative effect of a loss of ADH4 during gestational vitamin A deficiency by reducing retinol turnover. Remarkably, *Adh3*$^{-/-}$ adult mice on standard mouse chow were noticed to weigh significantly less (24.7 g vs. 34.3 g at 14 weeks of age) than wild-type mice, suggesting a growth deficiency (Molotkov et al. 2002). Retinol-supplemented diet containing 10-fold higher vitamin A than standard mouse chow increased growth of *Adh3*$^{-/-}$ mice nearly to normal compared with WT. From all these observations, the authors concluded that ADH3 and ADH4 each played essential roles in survival and growth during vitamin A deficiency, but that ADH1 was not essential. Furthermore, Duester and colleagues reasoned that ADH3 would be the best candidate for a retinol dehydrogenase among ADHs, because ADH3 was the most conserved and ancient form that was present in all vertebrates and exhibited ubiquitous tissue expression pattern, in contrast to ADH4, which is absent in the liver. Therefore, they re-measured the activity of mouse ADH3 using a more sensitive HPLC assay and showed that ADH3 oxidized retinol to retinaldehyde at the rate of 105 pmol/min × mg, which was comparable to the activity of the partially purified rat microsomal RoDH1 (115 pmol/min × mg). These results were interpreted to indicate that ADH3 generates RA during vitamin A deficiency. However, others pointed out that the

cause of death of the-null mice was not reported, and that RA was not quantified in serum or tissues (Napoli 2012). Therefore, it could not be excluded that vitamin A deficiency aggravated the problems caused by disruption of other non-retinoid metabolic or detoxifying pathways that would normally involve ADH.

An important insight into the possible roles of ADH isoenzymes in retinol metabolism was provided by dosing ADH knockout mice with retinol. These metabolic assays showed that *Adh1*$^{-/-}$ mice were unable to process a large dose of retinol as efficiently as wild-type mice. Two hours after oral administration of 50 mg/kg of retinol plasma levels of RA in *Adh1*$^{-/-}$ mice were 23-fold lower than in WT mice. In comparison, *Adh3*$^{-/-}$ mice had only a 3.6-fold reduction in plasma RA levels, and *Adh4*$^{-/-}$ mice had RA levels similar to those in WT mice (Molotkov et al. 2002). The authors concluded that ADH1 plays a dominant role in clearance of an acute dose of retinol, while ADH3 also contributes but to a lesser extent and ADH4 plays little or no role in liver retinol metabolism, in agreement with its absence in liver.

Finally, an interesting observation was made using mice lacking both ADH1 and CRBPI. Compared with wild-type mice, *CRBPI*$^{-/-}$ mice had greatly reduced levels of retinyl esters in the liver, while *Adh1*$^{-/-}$ mice exhibited a significant increase in liver retinyl esters (Molotkov et al. 2004). Importantly, relatively normal liver retinyl ester levels were observed in *CRBPI*$^{-/-}$/*Adh1*$^{-/-}$ mice. During vitamin A deficiency, the additional loss of *Adh1* completely prevented the excessive loss of liver retinyl esters observed in *CRBPI*$^{-/-}$ mice for the first 5 weeks of deficiency and greatly minimized this loss for up to 13 weeks. *CRBPI*$^{-/-}$ mice also exhibited increased metabolism of a dose of retinol into RA, and this increased metabolism was not observed in *CRBPI*$^{-/-}$/*Adh1*$^{-/-}$ mice. These findings indicated that CRBPI protected retinol from the oxidation by an ADH in agreement with the previous report (Kedishvili et al. 1998). Others noted that retinol metabolism catalyzed by ADH likely *contributes* to retinoid toxicity by generating high concentrations of RA during exposure to vitamin A doses that saturate CRBPI and defeat the mechanism of innocuous retinoid storage as RE (Napoli 2012). Toxicity from RA generated by ADH1 from high doses of retinol would be especially damaging to fetuses since a relatively low single dose of retinol (3 mg/kg) was shown to induce 71% incidence of cleft palate in mice, and a higher dose (39 mg/kg) induced 76% incidence of neural tube defects (Biesalski 1989). Adult animals would not be expected to suffer similar toxicity since major developmental processes would be completed and excessive RA was shown to be rapidly eliminated (Redfearn 1960; Van Dorp and Arens 1946; DeLuca and Roberts 1969).

In summary, the work by Duester and colleagues demonstrated that different ADH isoenzymes had different capacities for metabolizing retinol unbound to CRBPI. Their findings also indicated that the retinol dehydrogenase activities of ADH isoenzymes, especially of ADH1, would play the primary role in rapid elimination of a massive dose of retinol but that these enzymes were not essential for the tightly controlled production of RA during embryogenesis. This left open the question of which enzyme was responsible for the oxidation of retinol to retinaldehyde for the generation of transcriptionally active RA during embryonic development.

Cloning of the Microsomal SDR Retinol Dehydrogenases

While other researchers focused on the roles of various ADH isoenzymes in retinoid metabolism, Napoli and coworkers continued their investigation of the 34 kDa microsomal SDR that was partially purified from rat liver using holo-CRBPI as substrate (Boerman and Napoli, 1995). A PCR fragment obtained using primers based on the sequences of two internal peptides from this SDR allowed to isolate the full-length cDNA, which encoded a 317-amino acid polypeptide with a calculated molecular mass of 34.9 kDa (Chai et al. 1995). This polypeptide, named RoDH1 (same as RoDH3 (Chai et al. 1996; Belyaeva and Kedishvili 2006)), shared 52% amino acid sequence identity with another member of the SDR superfamily that was cloned earlier in 1995 and found to exhibit 11-*cis*-retinol dehydrogenase activity but not all-*trans*-retinol dehydrogenase activity (Simon et al. 1995). Thus, retinoids were identified as common substrates for a group of related newly identified members of the SDR superfamily of proteins (Table 5.2).

The properties of the recombinant rat RoDH1 were examined by transiently transfecting P19 cells with RoDH1 expression construct (Chai et al. 1995a). These assays showed that the 10,000 × g supernatant of homogenized P19 cells catalyzed the production of retinaldehyde from holo-CRBPI with the average K_m value of 0.9 μM. However, the expression of rat RoDH1 was restricted to liver, whereas RA was produced by many different cell types and tissues, suggesting that additional retinol dehydrogenases must exist. Shortly after cloning of rat RoDH1, Napoli and colleagues reported cloning of a second 317-amino acid rat SDR retinol dehydrogenase, RoDH2 (Chai et al. 1995b). This enzyme shared 82% sequence identity to RoDH1 and was expressed not only in liver, but also in kidney, brain, lung, and testis. Similarly to RoDH1, recombinant RoDH2 expressed in P19 cells produced retinaldehyde from holo-CRBPI with an average K_m value of 2 μM and had a higher activity with $NADP^+$ than with NAD^+.

In 1997, a surprising finding added a new wrinkle to the developing story of microsomal RoDH enzymes. Biswas and Russell (1997) reported that both rat RoDH1 and its newly identified human homolog, which shares 62% sequence identity with rat RoDH1, exhibited a potent 3α-hydroxysteroid dehydrogenase activity, converting weak androgen, 3α-androstanediol, into the potent androgen dihydrotestosterone. However, in the reactions with 3α-hydroxysteroids, these enzymes preferred NAD^+ over $NADP^+$, and the K_m values for androgens were much lower (~ 0.1 μM) than the reported K_m values for free or CRBP1-bound retinol (1–2 μM) (Chai et al. 1995a; Chai et al. 1995b). Interestingly, human RoDH (also known as RoDH-like-hydroxysteroid dehydrogenase or RL-HSD) was also found to possess weak 3β-hydroxysteroid dehydrogenase activity and to act as a 3(α→β)-hydroxysteroid epimerase, converting 3α-hydroxysteroids to 3β-hydroxysteroids (Huang and Luu-The 2000; Chetyrkin et al. 2001; Penning 2011; Bauman et al. 2006). Finally, both the rat and human RoDHs manifested oxidative 17β-hydroxysteroid dehydrogenase activities when presented with appropriate substrates (Biswas and Russell 1997). Thus, RoDH-like enzymes appeared to be multifunctional.

In humans, two more RoDH-like enzymes were identified (Table 5.2). The first enzyme, named RoDH4 (also known as RDH16), exhibited a liver-specific expression pattern, while the second one, named non-hepatic 3α-hydroxysteroid dehydrogenase (also known as

DHRS9), was widely expressed. Both exhibited higher catalytic efficiencies with 3α-hydroxysteroids than with all-*trans*-retinol (Gough et al. 1998; Chetyrkin et al. 2001) but the second one had little or no activity towards all-*trans*-retinol.

In order to assess the *in vivo* contribution of RoDH enzymes to RA biosynthesis, Napoli and colleagues began the cloning and characterization of the murine orthologs of rat and human RoDHs. Due to the gene duplication event, mice had many more RoDH-like genes than rats or humans (Belyaeva and Kedishvili 2006). Importantly, all of the mouse RoDH-like enzymes studied thus far were found to prefer NAD^+ over $NADP^+$ and recognized both 3α-hydroxysteroids and *cis*-retinoids as substrates (reviewed in Napoli 2001). In fact, only one of these mouse enzymes, RDH1, had significant activity towards all-*trans*-retinol (Zhang et al. 2001). Still, the catalytic efficiency of mouse RDH1 towards all-*trans*-retinol (V/K_m of 3 (nmol/min/mg)/μM) was ~10-fold lower than towards 3α-androstanediol (V/K_m of 31 (nmol/min/mg)/μM). Thus, like ADH isoenzymes, the microsomal RoDH/RDH enzymes related to the original rat RoDH1 all-*trans*-retinol dehydrogenase were shown to recognize other substrates besides retinoids. Phylogenetic analysis showed that the rat, human and mouse homologs of rat RoDH1 that exhibited dual retinol/sterol dehydrogenase activity belong to the same branch of phylogenetic tree. This branch of SDRs was designated as SDR9C (Table 5.2). The individual roles of retinol/sterol dehydrogenases in metabolism of retinol remained to be established.

Analysis of RoDH enzymes using in vivo and ex vivo approaches

A targeted knockout of *Rdh1* gene in mice showed that RDH1-null mice were viable (Zhang et al. 2007); hence, the function of RDH1 in retinoid or steroid metabolism was not essential for embryogenesis. However, the adult mice had an interesting phenotype - instead of being smaller when restricted in vitamin A, they grew longer and larger than wild type mice, with increased weight of multiple fat pads, liver, and kidney. There were no detectable changes in RA levels in tissues of RDH1-null mice, possibly due to a downregulation (2.5-fold) of *Cyp26a1* expression, but the amount of retinol on low vitamin A diet (0.6 IU/g) was increased in liver and kidney (1.5–2-fold) relative to wild-type. Thus, RDH1 clearly had an impact on retinoid metabolism in adult mice. Whether this was due to its retinol dehydrogenase activity, and whether the contribution of RDH1 to RA biosynthesis was the only role of RDH1 in mouse metabolism remained to be established. However, it was clear that RDH1 was not the primary enzyme that produced RA during embryonic development.

The search for physiologically relevant retinol dehydrogenases appeared to have hit a roadblock when, unexpectedly, a random mutagenesis screen for novel genes essential for mouse embryogenesis linked mutations in another retinoid-active member of the SDR superfamily, Retinol Dehydrogenase 10 (RDH10), to defects in growth and patterning of the forelimb, frontonasal process, pharyngeal arches, and various organs. RDH10 was originally identified in humans based on its sequence similarity to retina SDR1 (retSDR1) (discussed below) (Haeseleer et al. 1998; Wu et al. 2002). RDH10 and retSDR1 (also known as DHRS3) clustered with a different branch of SDR phylogenetic tree, SDR16C (Figure 5.2). Initial characterization of RDH10 using large amounts (200 μg) of microsomal fraction from COS cells transfected with RDH10 expression construct and 1 μCi [^{3}H] all-*trans* retinol

suggested that RDH10 acted as an $NADP^+$-preferring all-*trans*-retinol – specific dehydrogenase (Haeseleer et al. 1998). However, subsequent studies showed that human RDH10 (SDR16C4) recognized not only all-*trans*-retinol, but also 11-*cis* and 9-*cis* retinols as substrates and strongly preferred NAD^+ as a cofactor (Belyaeva et al. 2008), in agreement with the structural determinants of SDR cofactor specificity (Kallberg et al. 2002). The essential role of RDH10 in development was further confirmed by targeted inactivation of RDH10 function in mice. RDH10-null mice displayed severe malformations and embryonic lethality due to insufficient production of RA (Sandell et al. 2007; Rhinn et al. 2011; Ashique et al. 2012). This phenotype could be rescued by supplementation with RA or all-*trans*-retinaldehyde (Rhinn et al. 2011). Interestingly, when overexpressed in a model of human organotypic skin raft culture, RDH10), but not the SDR9C dehydrogenases, induced a phenotype consistent with overproduction of RA, which was characterized by an increased proliferation and reduced differentiation of keratinocytes (Lee et al. 2011). Thus, at least in human epidermis, RDH10, a member of the SDR16C family appears to contribute to RA biosynthesis, while the SDR9C enzymes do not.

The Reverse Reaction: Reduction of Retinaldehyde to Retinol

Studies from various laboratories showed that a fraction of retinaldehyde added to intact cells in culture was oxidized to RA, but the more abundant product was retinol. This indicated that mammalian cells contained enzymes that reduce retinaldehyde to retinol. Thus the steady-state levels of cellular retinaldehyde available for RA biosynthesis could be controlled by retinaldehyde reductases. Several members of the SDR superfamily of proteins were found to catalyze the interconversion between retinol and retinaldehyde in vitro with NADP(H) as the preferred cofactor (Haeseleer et al. 1998; Kedishvili et al. 2002; Belyaeva and Kedishvili 2002; Belyaeva et al. 2003; Kasus-Jacobi et al. 2003; Belyaeva et al. 2005; Lee et al. 2007; Belyaeva et al. 2008) (Table 5.2, SDR7C and SDR16C family). When expressed in intact cultured cells, these NADP(H)-preferring enzymes unidirectionally converted retinaldehyde to retinol (Lee et al. 2007). Interestingly, one of these SDRs, which was named originally retina SDR1 (retSDR1) and later renamed Dehydrogenase Reductase 3 (DHRS3, SDR16C1), was shown to be inducible by RA in human neuroblastoma cell lines, THP-1 monocytes, and rat liver (Cerignoli et al. 2002; Zolfaghari et al. 2012).

DHRS3 was identified at the level of cDNA in the expressed sequence database (EST) of GenBank as a photoreceptor visual cycle all-*trans*-retinol dehydrogenase (Haeseleer et al. 1998) based on its sequence similarity to previously cloned retinoid-active SDRs (Chai et al. 1995a; Simon et al. 1995). However, the mRNA encoding DHRS3 was found to be expressed in many human tissues, including adult heart, placenta, lung, liver, kidney, pancreas, thyroid, testis, stomach, trachea, and spinal cord, as well as fetal tissues such as kidney, liver, and lung (Haeseleer et al. 1998; Cerignoli et al. 2002). Initial assays of DHRS3 enzymatic activity using recombinant protein overexpressed in Sf9 insect cells suggested that this protein was capable of catalyzing the transfer of ^3H from [^3H]NADPH, but not from NADH, to 10 μM all-*trans*-retinaldehyde (Haeseleer et al. 1998). The exact rate of DHRS3-catalyzed reaction was not reported but the retinaldehyde reductase activity of the enzyme appeared to be much lower than that of other SDRs with NADPH-dependent retinaldehyde reductase activity (Belyaeva et al. 2003; Belyaeva et al. 2005). Furthermore, overexpression

of DHRS3 in SK-N-AS neuroblastoma cells stimulated the accumulation of retinyl esters, but did not result in quantifiable changes in the conversion of retinol to retinaldehyde or RA (Cerignoli et al. 2002). Nevertheless, a knockdown of *dhrs3a* gene expression in zebrafish embryos appeared to increase the expression of *cyp26a1* gene and RARE-GFP reporter construct in the spinal cord (Feng et al. 2010), suggesting that *dhrs3a* regulated RA levels *in vivo*. In mice, targeted knockout of *Dhrs3* resulted in reduction in the levels of retinol and retinyl esters and a slight increase in RA levels, modest changes in the expression of several RA target genes, and embryonic lethality late in gestation (Billings et al. 2013; Adams et al. 2014).

The discrepancy between the severity of the *in vivo* effects of *Dhrs3* gene knockout on retinoid metabolism and embryo survival and the seemingly negligible *in vitro* activity of DHRS3 towards retinaldehyde was puzzling. To resolve this puzzle, Adams et al. (2014) undertook an in-depth study of the properties of DHRS3 as an enzyme. Unexpectedly, these studies revealed that human DHRS3 required the presence of another retinoid-active SDR, human RDH10, in order to display its full enzymatic potential. Furthermore, RDH10 was in turn activated by the presence of DHRS3. The mutual activation of DHRS3 and RDH10 did not depend on the catalytic activity of either protein and occurred equally well when wild-type proteins were substituted with the corresponding active site mutants of the partner proteins. The RDH10-activated DHRS3 was found to act as a high-affinity/high efficiency all-*trans*-retinaldehyde–specific reductase that did not recognize 11-*cis*-retinaldehyde as substrate and preferred NADPH as a cofactor. When co-expressed in HEK293 cells, the two proteins were co-localized in characteristic ring structures within the endoplasmic reticulum (Adams et al. 2014) that were described previously by two other groups (Deisenroth et al. 2011; Jiang and Napoli 2013).

Importantly, the retinol dehydrogenase activity of the membrane fraction isolated from E14.5 DHRS3-null mouse embryos was about 2-fold lower than the activity of wild-type embryos; and mouse embryonic fibroblasts isolated from E14.5 embryos produced 1.7-fold less retinaldehyde from retinol than MEFs from wild-type embryos (Adams et al. 2014). RDH10 was shown to be the major retinol dehydrogenase during embryonic development (Rhinn et al. 2011). Hence, the results obtained with DHRS3-null mouse embryos confirmed that the mutually activating interaction between RDH10 and DHRS3 occurred *in vivo* and suggested that this mechanism was conserved across species.

Oxidation of Retinaldehyde to RA: Cloning and Characterization of Retinaldehyde Dehydrogenase Isoenzymes

Fractionation of rat tissue cytosol by anion-exchange chromatography revealed the existence of at least four retinaldehyde dehydrogenase isoenzymes (Posch et al. 1992). The quantitatively major isoenzyme in liver, kidney, and testis, RALDH1 was purified from rat liver but cloning of the corresponding cDNA from liver was complicated by the abundance of numerous other ALDHs. To avoid abundant hepatic ALDHs, Wang et al. (1996) screened rat testis cDNA library for RALDHI cDNA using PCR primers based on sequences of two highly conserved amino acid-segments in the mammalian ALDHs. As a result of this screening, a cDNA clone encoding a previously unknown ALDH, distinct from RALDH1,

was identified. This isoenzyme was named RALDH2, because it catalyzed the oxidation of free retinaldehyde with the apparent K_m value of 0.7 ± 0.3 μM and the V_{max} value of 105 ± 4 nmol/min × mg of purified protein expressed in *E. coli*. The kinetic constants for the oxidation of retinaldehyde in the presence of a 2-fold molar excess of apo-CRBPI, which binds retinaldehyde with a relatively high K_d of 50–100 nM (Levin et al. 1988; Li et al. 1991), were 0.2 ± 0.06 μM (apparent K_m) and 62 ± 15 nmol/min × mg (V_{max}).

At the same time, Zhao et al. (1996) succeeded in cloning a novel mouse ALDH with activity indistinguishable from that of V2 embryonic retinaldehyde dehydrogenase described in retina from mouse P19 teratocarcinoma cells. Because of the scarcity of the V2 enzyme in *vivo,* these researchers screened candidate cell lines for the presence of the V2 activity using a zymography bioassay. They found that P19 cells induced by treatment with RA expressed a new retinaldehyde dehydrogenase activity that was absent in uninduced cells. To clone the corresponding cDNA, they designed degenerate oligonucleotide primers corresponding to two highly conserved protein motifs in ALDH sequences and employed PCR to screen cDNA synthesized from mRNA of PI9 cells, untreated and treated with retinoic acid, for aldehyde dehydrogenase cDNAs. Restriction digest of the 450-bp PCR product showed that only the RA-treated P19 cells expressed unique PCR-amplified sequence resistant to SalI digestion. The sequence of this product allowed the isolation of the full-length cDNA encoding murine RALDH2. The murine and rat RALDH2 protein sequences turned out to be 100% identical.

Rat RALDH1 was cloned from rat kidney cDNA library (Bhat et al. 1995) and testis cDNA library (Penzes et al. 1997). Rat RALDH1 was found to share ~72% amino acid sequence identity with RALDH2. A third RALDH, RALDH3, was later identified and originally referred to as ALDH6 (Grün et al. 2000). The fourth cloned RALDH, RALDH4, was found to recognize only 9-*cis*-retinaldehyde as substrate and not all-*trans*-retinaldehyde (Lin and Napoli 2000; Lin et al. 2003).

Under the currently accepted nomenclature, all all-*trans*-retinaldehyde-oxidizing ALDHs belong to ALDH1A family: ALDH1A1 (RALDH1), ALDH1A2 (RALDH2), and ALDH1A3 (RALDH3). The *in vivo* roles of individual ALDH isozymes in RA biosynthesis were summarized in several recent review articles (Napoli 2012; Marchitti et al. 2008). In brief, ALDH1A2 was shown to be the primary enzyme responsible for RA biosynthesis at most sites during embryogenesis. *Aldh1a2*$^{-/-}$ mice died early in embryonic development due to defects in heart morphogenesis (Niederreither et al. 1999). ALDH1A3 had a more limited role during development. *Aldh1a3*$^{-/-}$ mouse embryos survived until birth but died shortly thereafter from defects in nasal development (Dupé et al. 2003). In addition, ALDH1A3 was reported to play a major role in protection of adult tissues against carcinogenesis (reviewed in Marchitti et al. 2008). ALDH1A1 was not essential for embryogenesis, but could have a role in RA biosynthesis during adulthood, because the livers of *Aldh1a1*$^{-/-}$ mice displayed reduced RA biosynthesis and increased serum retinaldehyde levels after treatment with retinol (Fan et al. 2003; Molotkov and Duester 2003).

To compare the catalytic properties of the retinaldehyde-active ALDH isoenzymes, Bhat and colleagues performed kinetic analysis of all three murine ALDHs under the same conditions,

allowing for direct comparison of their properties (Table 5.3). ALDH1A3 was the most catalytically efficient enzyme, but it had a relatively high K_m value for all-*trans*-retinaldehyde (Table 4.2) (Sima et al. 2009). ALDH1A1 and ALDH1A2 had similar catalytic efficiency but the K_m value of ALDH1A2 was much lower than that of ALDH1A1 (Table 5.3) (Gagnon et al. 2002; Gagnon et al. 2003). Thus, ALDH1A1 was the least potent retinaldehyde dehydrogenase of the three enzymes. All three ALDHs recognized other substrates in addition to retinaldehyde, including aldehydes derived from lipid peroxidation. In fact, ALDH1A1 was shown to play a crucial role in protection of the mouse eye lens and cornea from lipid peroxidation aldehydes and cataract formation induced by oxidative stress, as demonstrated using various *Aldh1a1*$^{-/-}$ mouse models (Lassen et al. 2007).

RA degradation: Cloning and Characterization of the RA-Inducible RA-Metabolizing CYP Enzymes

It was not until the development of molecular cloning techniques that cytochrome P-450 enzymes specific for RA metabolism were identified. It was shown that in F9 cells, RAR-mediated signaling could play an important role in regulating the enzymes responsible for RA-induced self-degradation (Boylan et al. 1995). White et al. (1996) used mRNA differential display to identify zebrafish genes regulated by RA during exogenous RA exposure. This approach allowed them to isolate a cDNA, P450RAI, encoding a novel member of the cytochrome P450 family. In COS-1 cells transfected with the P450RAI cDNA, all-*trans*-RA was rapidly metabolized to more polar metabolites including 4-oxo-RA and 4-OH-RA.

Subsequently, these investigators cloned the two first mammalian RA-inducible RA-metabolizing cytochromes P450 (hP450RAI and P450RAI-2) of the novel class of cytochromes (CYP26) and demonstrated that they were responsible for generation of several hydroxylated forms of all-*trans*-RA, including 4-OH-RA, 4-oxo-RA, and 18-OH-RA (White et al. 1997; White et al. 2000). Competition experiments with other retinoids suggested that all-*trans*-RA was the preferred substrate. The authors concluded that these enzymes (renamed CYP26A1 and CYP26B1), played a key role in RA metabolism, functioning in a feedback loop where RA levels were controlled in an autoregulatory manner. Later, a third CYP26C1 was identified that also preferred all-*trans*-RA as substrate but could metabolize 9-*cis*-RA and was much less sensitive than the other CYP26 family members to the inhibitory effects of ketoconazole (Taimi et al. 2004).

Targeted gene knockout studies showed that CYP26A1-null mouse fetuses died at mid-late gestation, with multiple organ defects that were consistent with excessive RA signaling (Abu-Abed et al. 2001). CYP26B1-null mice that were born alive died right after birth due to respiratory defects (Yashiro et al. 2004). In human fetus CYP26A1 was found to be the major enzyme in brain while CYP26B1 was found in all other tissues except brain. However, in adult human tissues, the expression patterns of CYP26A1 and CYP26B1 at the level of both mRNA and protein showed a significant overlap (Topletz et al. 2012). With the exception of liver and lung, where CYP26A1 was the predominant form, all other human adult tissues contained higher levels of CYP26B1. This tissue distribution pattern was

supported by another study (Xi and Yang 2008), with minor discrepancies that could be potentially attributed to variability in the quality of tissue samples.

Despite the low sequence identity (43%), CYP26A1 and CYP26B1 were shown to have very similar enzymatic properties, hydroxylating RA to form 4OH-RA and 18OH-RA, and then hydroxylating these primary products further (Topletz et al. 2012). Purified recombinant CYP26A1-His_6 had a lower affinity but a ~10-fold higher rate for formation of 4OH-RA than CYP26B1-His_6 (Table 5.4). CYP26A1 also had a 2–10-fold higher catalytic activity towards hydroxylated forms of RA, making it ~20-fold more efficient than CYP26B1 in the overall depletion of RA. Considering that CYP26A1 expression in liver is very sensitive to RA levels (reviewed in Ross and Zolfaghari 2011), the high catalytic efficiency of this low affinity enzyme would enable CYP26A1 to rapidly bring down the excessive levels of RA.

CYP26C1 was found to share 45% amino acid identity with CYP26A1 and 51% - with CYP26B1, but it exhibited a distinctly different expression pattern and catalytic properties. CYP26C1 was expressed mainly during embryonic development, but appeared to be nonessential since CYP26C1-null mice had no apparent abnormalities (Uehara et al. 2007). In adult tissues, it was detected at low levels in adrenal gland, lung, spleen, testis, brain, adult liver and ovary (Xi and Yang 2008). Unlike the other two enzymes, CYP26C1 prefered 9-*cis*-RA as substrate and exhibited broader substrate specificity in general. Furthermore, CYP26C1 showed a different response to treatment with RA and 9-*cis*-RA, being upregulated in some tissues and cells but downregulated in others (Pennimpede et al. 2010). Of the three CYP26 genes, CYP26C1 is the least conserved and its physiological function remains poorly understood. A frameshift mutation in human CYP26C1 was linked to focal facial dermal dysplasia type IV, a rare syndrome characterized by facial lesions resembling aplasia cutis Slavotinek et al. 2013). A potential role of other members of CYP superfamily of proteins in RA catabolism was discussed in a recent review (Kedishvili 2013).

5.4 CURRENT STATE OF THE FIELD

Enzymes Involved in the Control of Retinol and RA Levels

The currently accepted model of RA biosynthesis postulates that RA is produced in two sequential oxidative steps: first, retinol is oxidized to retinaldehyde, and then retinaldehyde is oxidized to RA (Figure 5.1). The first step is rate-limiting and reversible. The rate of retinaldehyde production is controlled by two types of SDR enzymes: the NAD^+-dependent oxidative SDR(s) that oxidize retinol to retinaldehyde (RDH10 and possibly others), and the NADPH-dependent reductive SDR(s) that reduce retinaldehyde back to retinol (DHRS3 and possibly others) (Figure 5.3). Both types of SDRs are integral membrane proteins. The second step in RA biosynthesis is irreversible and is catalyzed by cytosolic ALDHs: ALDH1A1 (RALDH1), ALDH1A2 (RALDH2), and ALDH1A3 (RALDH3). RA is catabolized by the members of the CYP family of proteins, primarily by CYP26A1, CYP26B1, and CYP26C1.

It must be noted that in addition to these primary enzymes dedicated to retinoid metabolism, there are other enzymes that recognize retinoids as substrates and may play a significant role under certain physiological conditions. For example, ADH isoenzymes may contribute to

elimination of large doses of retinol that exceed the binding capacity of CRBPI and the capacity of the lecithin retinol acyltransferase (LRAT) to convert retinol into the storage forms, retinyl esters. The action of ADH enzymes may lead to short-term toxicity caused by excessive conversion of retinol to RA.

In cancer tissues and precancerous lesions, the interconversion of retinol and retinaldehyde may be influenced by the activities of the cytosolic enzymes that belong to the family of aldo-keto reductases (AKRs) and recognize retinaldehyde as substrate (reviewed in Ruiz et al. 2012). AKRs are enzymes with wide substrate specificity that catalyze the reduction of lipid peroxidation products (e.g., 4-hydroxy-*trans*-2-nonenal), ketosteroids, ketoprostaglandins, and xenobiotic compounds (reviewed in Penning and Drury, 2007). Human AKR1B10 has a very high catalytic efficiency for the conversion of retinaldehyde to retinol. Although normally expressed at very low levels in human tissues, it is known to be overexpressed in certain types of cancers, including hepatocellular carcinoma and lung cancer associated with tobacco smoking (Fukumoto et al. 2005; Jin et al 2006; Diez-Dacal et al. 2011). An upregulation of an enzyme with a potent retinaldehyde reductive activity such as AKR1B10 might adversely affect the levels of RA in cancer cells even though AKR1B10 may not be essential for the maintenance of RA homeostasis under normal conditions (reviewed in Kedishvili 2013).

Besides CYP26 enzymes, several other members of the CYP superfamily of proteins including human CYP3A4, 3A5, 3A7, 1A1, 4A11, 2C8, 2C9, 2C22, 2C39, CYP2S1, and CYP2E1 (Andreola et al. 2004; Leo et al. 1984; Marill et al. 2000; McSorley and Daly, 2000; Nadin and Murray 1999; Chen et al. 2000; Smith et al. 2003; Qian et al. 2010; Wu et al. 2006; Westin et al. 1997; Fan et al. 2004; Ross et al. 2011; Liu et al. 2001; Dan et al. 2005; Thatcher and Isoherranen 2009) were shown to catabolize RA. Most of these CYPs are induced by elevated levels of RA. For example, human CYP2S1 is inducible by RA in skin of a subset of individuals and was shown to produce 4-hydroxy-RA and 5,6-epoxy-RA when expressed in *E. coli* together with NADPH cytochrome P450 reductase (Smith et al. 2003). Human CYP2C8 and CYP2C9 genes are both induced by RA, but the less efficient CYP2C9 may have a greater impact on RA catabolism because it is expressed at about 10-fold higher levels than CYP2C8 and, in addition, it's expression is significantly more responsive to RA treatment (Qian et al. 2010). Rodent orthologs of human CYP2C8 and CYP2C9 (rat CYP2C22 and mouse CYP2C39) also 4-hydroxylate RA (Andreola et al. 2004; Westin et al. 1997; Fan et al. 2004). Interestingly, under normal dietary conditions, the relative level of CYP2C22 mRNA expression in rat liver exceeds that of CYP26A1 by about 100-fold (Ross et al. 2011). Mouse CYP2C39 has a relatively high affinity for RA (K_m of 0.8 μM), but a ~40-fold lower V_{max} value compared to other CYP2C family members (Andreola et al. 2004). Nevertheless, the decreased expression of this enzyme in the liver of mice lacking the aryl hydrocarbon receptor gene has been linked to increased levels of RA, retinol, and retinyl palmitate (Andreola et al. 2004). Ethanol was shown to enhance RA metabolism into polar metabolites through the induction of CYP2E1 in the liver (Liu et al. 2001; Dan et al. 2005). Thus, kinetic characteristics and tissue distribution of various RA-catabolizing enzymes indicate that while CYP26A1 and CYP26B1 may be the primary CYP enzymes for the hepatic clearance of RA due to their high catalytic efficiency (Thatcher et

al. 2010; Thatcher and Isoherranen 2009), other members of CYP superfamily may play an essential role in extrahepatic tissues or in liver under certain conditions.

Regulation of RA Biosynthesis and Degradation by RA Status

Some of the components of the RA biosynthetic and catabolic machinery are regulated by RA *via* a feedback regulation loop (Figure 5.4). RA induces the expression of the retinoid enzymes and binding proteins that promote the synthesis if retinyl esters including CRBPI, LRAT (Ross and Zolfaghari 2004; Zolfaghari and Ross 2000; Matsuura et al. 1997), and DHRS3 (Cerignoli et al. 2002). At the same time, RA induces its own catabolism by up-regulating CYP26 enzymes (Wu and Ross 2010; Bouillet et al. 1997; Ong et al. 1994). STRA6, the plasma membrane receptor for retinol carrier RBP4, is known to be induced by RA in extrahepatic tissues (Wu and Ross 2010; Bouillet et al. 1997) while Retinol Binding Protein Receptor 2 (RBPR2), the recently discovered analogous receptor in rodent liver, is suppressed by RA, presumably to promote the uptake of retinol by extrahepatic tissues under conditions of vitamin A sufficiency (Alapatt et al. 2013).

The regulation of retinol dehydrogenases and retinaldehyde dehydrogenases by RA is less well understood, and it appears that the responses of the genes encoding these enzymes may be species-specific and cell context-dependent. For example, vitamin A-deficient rats have lower levels of *Aldh1a1* (*Raldh1*) mRNA in kidney and liver but elevated levels of *Aldh1a1* in testis (Penzes et al. 1997). Orally administered RA restores *Aldh1a1* expression in kidney, but not in liver (Penzes et al. 1997). In primary keratinocytes, a high concentration of RA (1 μM) appears to upregulate *ALDH1A3* (*RALDH3)*, but has no effect on *ALDH1A1* (*RALDH1*) or *ALDH1A2 (RALDH2)* expression (Koenig et al. 2010). *RALDH3* expression is also induced by RA in organotypic human skin cultures and in an epidermal explant, but is not affected by RA in dermal fibroblasts or HeLa cells. Somewhat more consistent pattern of RA effect on *Aldh* genes is seen in mouse embryos, where treatment with RA results in significant suppression of all three *Aldh* transcripts (Lee et al. 2012). However, chick ALDHs do not respond to either excess or deficiency of RA (Reijntjes et al. 2010).

The effect of RA on the expression of RDH10 in human tissues and cell lines has not yet been fully investigated but there seems to be a difference in the regulation of this gene among different species. For example, in *Xenopus laevis*, rdh10 expression is suppressed by RA (Strate et al. 2009), but in chick, RDH10 is not affected by either the excess or absence of RA (Reijntjes et al. 2010). A more systematic analysis of RA effect on the expression of retinol dehydrogenases and retinaldehyde dehydrogenases is needed to better understand the effect of vitamin A status on the conversion of retinol to retinaldehyde in different species.

5.5. THE FUTURE

Despite significant progress made since the middle of the 20th century many unanswered questions still remain and there is much to be learned. RDH10 is the major retinol dehydrogenase responsible for RA biosynthesis during embryonic development. However, the molecular patterning defects in RDH10-null mice do not reflect a complete state of RA deficiency (Rhinn et al. 2011), suggesting that additional retinol dehydrogenases exist. RDH10 shares the highest sequence similarity with two other members of SDR16C family

of proteins, RDHE2 and RDHE2S) (Table 5.2 and Figure 5.2). In humans, the genes encoding RDHE2 (SDR16C5) and RDHE2S (SDR16C6) are located in close proximity to the RDH10 gene (SDR16C4), and may have originated from a common ancestor as a result of gene duplication. Proteins encoded by the mouse orthologs of human *RDHE2* and *RDHE2S* genes (SDR16C11 and SDR16C12, respectively) function as all-*trans*-retinol dehydrogenases *in vitro* and increase the rate of RA biosynthesis from retinol when expressed in living cells (Lee et al. 2009; Belyaeva et al. 2012). Whether these two enzymes contribute to RA biosynthesis during development or adulthood is not yet known, but studies in frogs indicate that the frog ortholog of these enzymes is essential for frog embryonic development (Belyaeva et al. 2012). Frogs have a single gene, *rdhe2* (*sdr16c90*) in position orthologous to mouse *Rdhe2* (*Sdr16c11*) and *Rdhe2s* (*Sdr16c12*) genes. The enzyme encoded by the frog gene acts as a highly active retinol dehydrogenase that promotes RA biosynthesis in living cells (Belyaeva et al. 2012). Thus, the retinol dehydrogenase activity of RDHE2/RDHE2S enzymes is conserved in lower vertebrates. Further studies are needed in order to elucidate the roles of mammalian RDHE2 and RDHE2S in RA biosynthesis. Finally, in addition to RDH10, RDHE2, and RDHE2S, the SDR16C family includes two relatively weak steroid dehydrogenases, 17β-HSD11 (SDR16C2) (Brereton et al. 2001), and 17β-HSD13 (SDR16C3) (Liu et al. 2007). Whether the latter two enzymes also have activity towards retinoids has not yet been examined.

The recent discovery that RDH10 and DHRS3 mutually activate each other (Adams et al. 2014) raises new questions about the impact of this interaction on the cellular levels of RA. It is not yet known whether and how the two proteins physically interact; whether their interaction is influenced by the metabolic state, RA levels, oxidative stress, etc. It is not yet known whether disruption of RDH10 and DHRS3 interaction may lead to pathophysiological changes in cell metabolism. It is also unclear whether the interaction between RDH10 and DHRS3 is limited to these two proteins or whether they have other partners among SDR proteins.

DHRS3 has now been proven to act as a physiologically relevant retinaldehyde reductase essential for embryonic development (Billings et al. 2013; Adams et al. 2014). However, while the retinol and retinyl ester stores in DHRS3-null mice are significantly reduced (~25% of wild-type), they are not fully depleted. This suggests that DHRS3 is not the only retinaldehyde reductase that functions in the embryo. The molecular identity of additional retinaldehyde reductases that are responsible for the maintenance of the residual retinoid stores remains to be established.

The molecular mechanisms for trafficking of the highly hydrophobic retinoid substrates and products from one enzyme or binding protein to another remain puzzling. For example, it is not clear whether RDH10 obtains retinol directly from CRBPI or from the membranes. *In vitro* data suggest that CRBPI inhibits the activity of RDH10 towards retinol (Lee et al. 2011); however, the effect of CRBPI on RDH10 activity *in vivo* remains unexplored, and thus far, there is no evidence of direct protein-protein interaction between RDH10 and CRBPI.

Since RDH10 and DHRS3 interact, it is possible that DHRS3 obtains retinaldehyde directly from RDH10, but experimental proof of such substrate channeling is still lacking. Furthermore, retinaldehyde is also utilized by ALDH isoenzymes, which are believed to reside in the cytosol. How the cytosolic ALDHs are able to compete for the hydrophobic retinaldehyde with the membrane-bound DHRS3, and whether CRBPI is involved in this process is currently unknown.

A recent study demonstrated that when expressed in COS7 cells (Jiang and Napoli 2013), mouse RDH10 co-localized with mitochondria/mitochondrial associated membranes (MAM), in close proximity to CRBPI. During acyl ester biosynthesis RDH10 partially re-located to lipid droplets ((Jiang and Napoli 2013). DHRS3 was also shown to be associated with lipid droplets (Deisenroth et al. 2011). The co-localization of RDH10 and DHRS3 in ring structures similar to lipid droplets was confirmed by Adams et al. (2014). Whether ALDH isoenzymes localize near the membranes or near CRBPI and whether the subcellular localization of retinoid-metabolizing enzymes and retinoid-binding proteins is affected by the cellular retinoid status or other metabolic factors remains unknown.

ABBREVIATIONS

RA	all-*trans*-retinoic acid
RAR	retinoic acid receptor
ADH	alcohol dehydrogenase
NAD	nicotinamide adenine dinucleotide (diphosphopyridine nucleotide)
NADP	nicotinamide adenine dinucleotide phosphate
MDR	medium chain dehydrogenase/reductase
CRBPI	cellular retinol binding protein type I
CRABPI	cellular retinoic acid binding protein type I
RALDH	retinaldehyde dehydrogenase
RDH or RoDH	retinol dehydrogenase
CYP	cytochrome P450
SDR	short-chain dehydrogenases reductase
AKR	aldo-keto reductase

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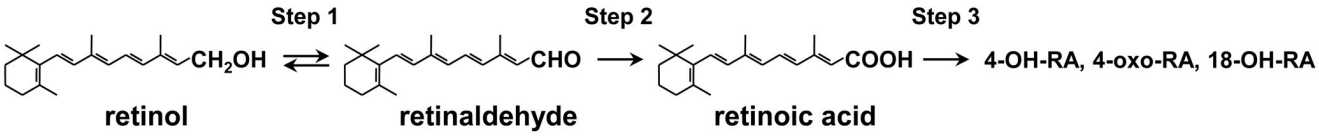

Figure 5.1. Pathway of Retinoic Acid Biosynthesis and Degradation

Retinol is oxidized reversibly to retinaldehyde (Step 1). Retinaldehyde is oxidized irreversibly to retinoic acid (Step 2). Retinoic acid is eliminated by conversion to 4-*hydroxy* (4-OH-RA), 4-*oxo* (4-oxo-RA), and 18-*hydroxy* (18-OH-RA) intermediates (Step 3).

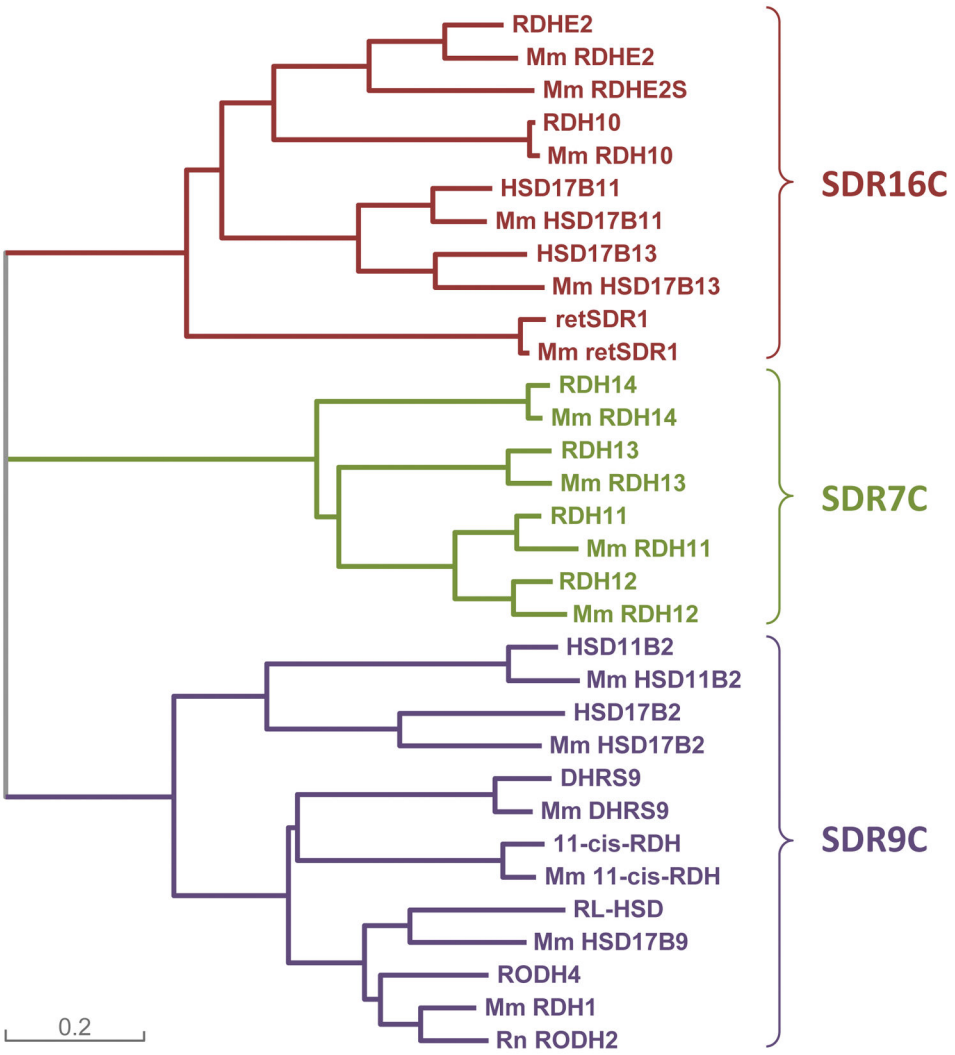

Figure 5.2. Neighbour-Joining Phylogenetic Tree of Retinoid-Active SDRs

Three branches of SDRs that include human and rodent retinoid-active enzymes implicated in retinoic acid biosynthesis are shown. SDR9C group is comprised of enzymes with preference for NAD(H) as cofactor. SDR7C group is comprised of enzymes with preference for NADP(H) as cofactor. SDR16C group includes both NAD(H) and NADP(H) -preferring enzymes. Murine enzymes are prefixed with Mm, rat enzymes are prefixed with Rn. Scale bar, 0.2 amino acid substitutions per site.

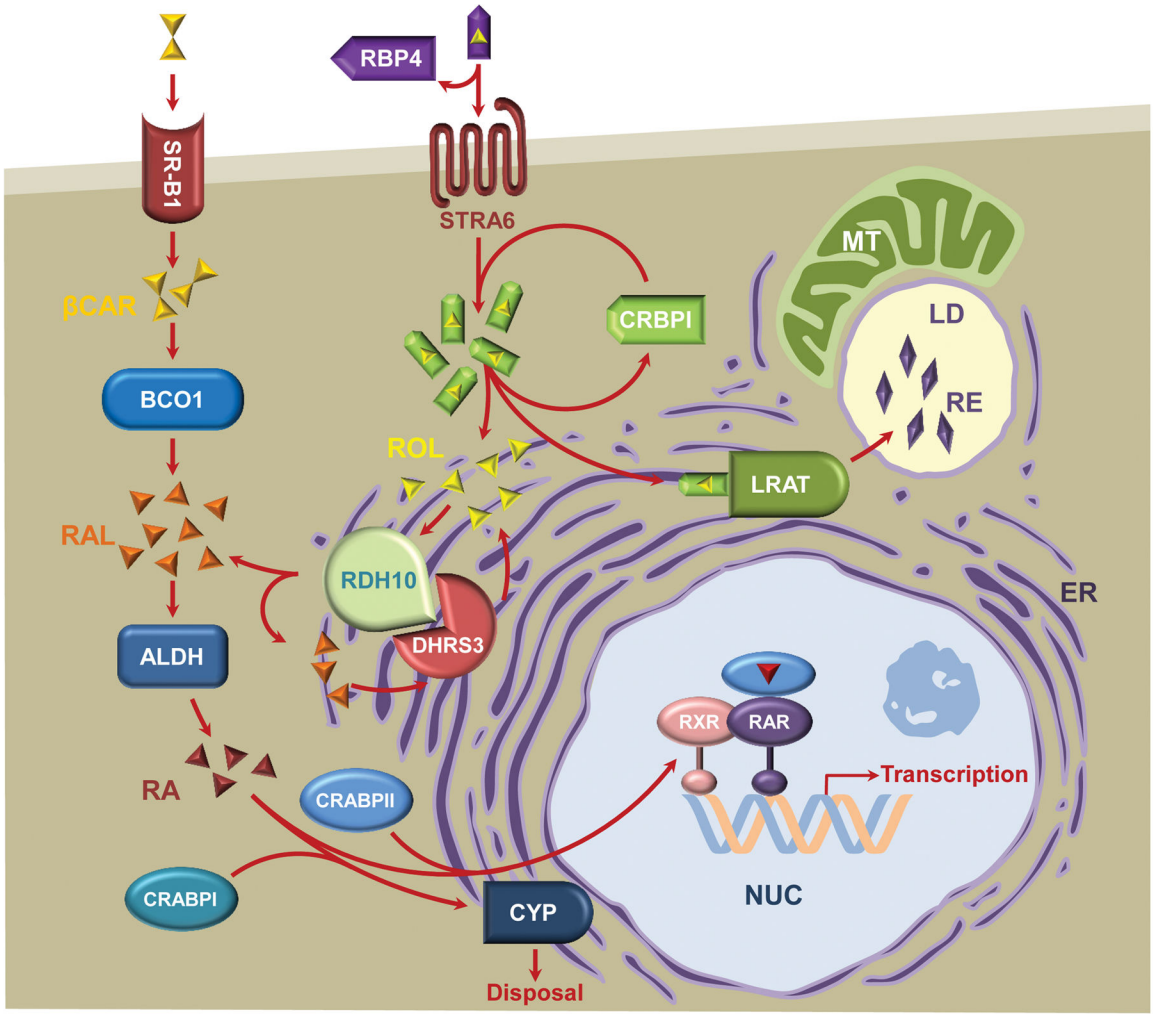

Figure 5.3. Components of Retinoid Metabolic and Signaling System

Retinol (ROL, depicted as yellow pyramids) is delivered to extrahepatic cells bound to plasma Retinol Binding Protein 4 (RBP4). HoloRBP4 binds to RBP4 receptor STRA6. Cellular Retinol Binding Protein type I (CRBPI) accepts retinol from STRA6 in the cytoplasm and delivers retinol to membranes of endoplasmic reticulum (ER), where retinol is either esterified by lecithin retinol acyl transferase (LRAT) to retinyl esters (RE, depicted as purple rhombus) or oxidized by Retinol Dehydrogenase 10 (RDH10) to retinaldehyde (RAL, depicted as orange pyramids). Retinaldehyde is oxidized further to retinoic acid (RA, depicted as brown pyramids) by Aldehyde Dehydrogenase (ALDH) in the cytoplasm or is reduced back to retinol by Retinaldehyde Reductase (DHRS3) in the membranes. Retinoic acid binds to Cellular Retinoic Acid Binding Proteins (CRABPs) type I or type II and is transferred by holoCRABPII to nucleus (NUC) for binding to heterodimers of Retinoic Acid Receptors (RAR and RXR) or delivered to Cytochrome P450 enzymes (CYP) by holoCRABPI for degradation. In addition, β-carotene (βCAR, depicted as duplicate of olive pyramids) is taken up by the cells through scavenger receptor class B, type 1 (SR-B1) and cleaved into two molecules of retinaldehyde by β-carotene oxygenase type 1 (BCO1).

Retinaldehyde derived from β-carotene may be oxidized to retinoic acid or converted to retinol as described above. Other abbreviations: LD, lipid droplet; MT, mitochondria.

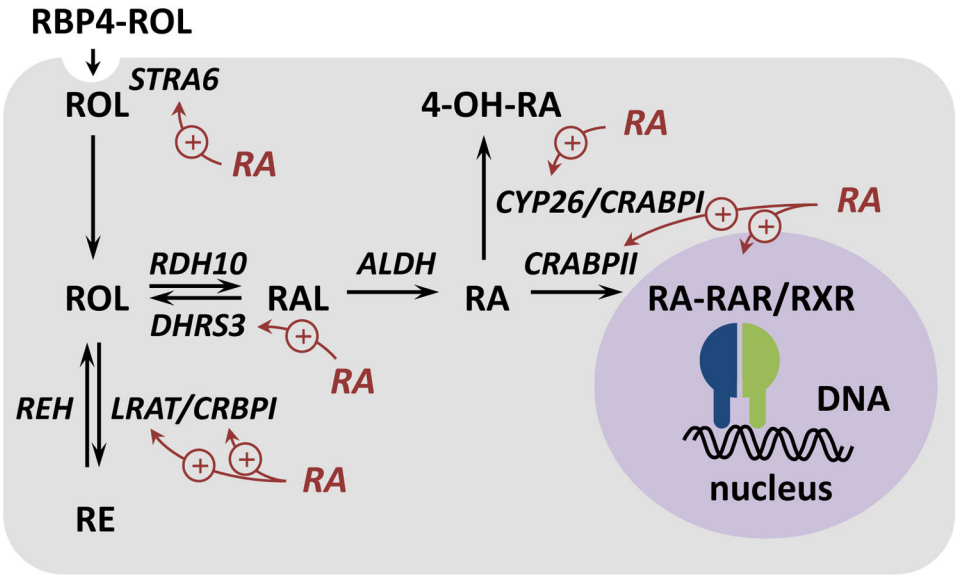

Figure 5.4. Regulation of Retinoid Metabolism by Retinoic Acid Status

Abbreviations are as in Figure 5.3. Retinoic acid (RA) induces the expression of STRA6, LRAT, CRBPI, DHRS3, CYP26, CRABPII, and some forms of RAR.

Table 5.1

Classes of Human ADH and their kinetic constants towards retinol.

Class	Gene name	Retinol as substrate		
		K_m, μM	k_{cat}	k_{cat}/K_m
I	ADH1A	-	-	-
	ADH1B	0.3	21	70,000
	ADH1C	-	-	
II	ADH2	0.14	4	29,000
III	ADH3	-	-	-
IV	ADH4	0.3	190	640,000
V/VI	ADH5/6	-	-	-

ADH5 and ADH6 have been identified at the gene and transcriptional levels only, and their functions are still unknown. Kinetic constants of human ADH3 activity towards retinol have not been reported.

Table 5.2

Nomenclature and Properties of SDR Enzymes.

	Species	Cofactors[a]	Substrates	Nomenclature	Other names
SDR7C					
RDH11	human	NADP(H)	*at*ROL/RAL, *c*ROLs/RALs	SDR7C1	PSDR1, RalR1
	mouse	NADP(H)	*at*ROL/RAL, *c*ROLs/RALs, nonanal, nonenal	SDR7C9	SCALD
RDH12	human	NADP(H)	*at*ROL/RAL, *c*ROLs/RALs, nonanal, nonenal	SDR7C2	
	mouse		ND	SDR7C10	
RDH13	human	NADPH	*at*RAL	SDR7C3	
	mouse		ND	SDR7C11	
RDH14	human	NADP(H)	*at*RAL, 9*c*RAL, *at*ROL	SDR7C4	PAN2
	mouse		ND	SDR7C12	
SDR9C					
RoDH4	human	NAD(H)	*at*ROL/RAL, *c*ROLs/RALs, 3α-HS	SDR9C8	RDH16, RDH-E
RDH1	mouse	NAD^+	*at*ROL, 9*c*ROL, 3α-HS	SDR9C17	
RoDH1	rat	$NADP^+$, NAD(H)[b]	holoCRBPI, 3α-HS	SDR9C29	RDH7
RoDH2	rat	$NADP^+$, NAD(H)[b]	holoCRBPI, 3α-HS	SDR9C28	RDH2
RL-HSD	human	NAD(H)	*at*ROL, *at*RAL; 3α-HS	SDR9C6	HSD17B6, 3α-HSE
	mouse	NAD(H)	*at*ROL/RAL, *c*ROLs/RALs; 3α-HS, 17β-HS	SDR9C13	HSD17B6, 17β-HSD9
DHRS9	human	NAD(H)	3α-HS, may be *at*ROL	SDR9C4	RDHL, retSDR8, RDH-TBE, RoDH-E2, 3α-HSD
	mouse		ND	SDR9C12	
	rat		may be *at*ROL	SDR9C26	eRolDH2
11-*cis*-RDH	human	NAD^+	*c*ROLs; 3α-HS	SDR9C5	RDH5
	mouse	NAD(H)	*c*ROLs/RALs	SDR9C21	
HSD11B2	human	NAD(H)	11β-HS	SDR9C3	Corticosteroid 11β-HSD
	mouse			SDR9C11	
HSD17B2	human	NAD(H)	17β-HS, 20α-HS	SDR9C2	Estradiol 17β-HSD
	mouse			SDR9C10	
SDR16C					

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	Species	Cofactors[a]	Substrates	Nomenclature	Other names
retSDR1	human	NADP(H)	*at*RAL	SDR16C1	DHRS3
	mouse			SDR16C9	
RDH10	human	NAD(H)	*at*ROL/RAL, *c*ROLs/RALs	SDR16C4	
	mouse			SDR16C10	
RDHE2	human	NAD(H)	*at*ROL	SDR16C5	
	mouse	ND	*at*ROL	SDR16C11	
	frog	NAD(H)	*at*ROL	SDR16C84	rdhe2, MGC80593
RDHE2S	mouse	ND	*at*ROL	SDR16C12	
HSD17B11	human	ND	3α-androstanediol	SDR16C2	Pan1b, retSDR2
	mouse			SDR16C7	
HSD17B13	human	$NADP^+$	cortisol	SDR16C3	SCDR10B
	mouse			SDR16C8	

[a]Some of the SDR enzymes can bind both NAD(H) and NADP(H) but their K_m values may differ by an order of magnitude. Only the preferred cofactors are listed.

[b]Rat RoDH1 and RoDH2 were reported to prefer $NADP^+$ with all-*trans*-retinol as substrate [Boerman and Napoli 1995; Chai et al. 1995a; Chai et al. 1995b], but NAD^+ - with 3α-hydroxysteroids [Biswas and Russell 1997; Hardy et al. 2000].

ND, no data. References to these studies are cited in the text. Nomenclature for SDR proteins can be found at http://www.sdr-enzymes.org/. To search the database, choose field “family name” and enter SDR9C or SDR7C or SDR16C to view all currently known members. Human SDR16C6 is not included in the Table because it appears to be a pseudogene.

Table 5.3

Kinetic Properties of Purified Mouse Retinaldehyde Dehydrogenases.

Parameters	**ALDH1A1**[a]	**ALDH1A2**[b]	**ALDH1A3**[c]
K_m, μM	11.6	0.66	3.9
V_{max}, nmol×min^{-1}×mg^{-1}	85.6	4.31	306

[a,b]Mouse ALDH1A1 (RALDH1) and ALDH1A2 (RALDH2) proteins were expressed in *E. coli* as N-terminal fusions to glutathione-S-transferase (GST) and purified using GST-affinity column [Gagnon et al. 2002; Gagnon et al. 2003].

[c]RALDH3 was expressed in *E. coli* as a His-tagged protein and affinity-purified using Ni-NTA column [Sima et al. 2009]. The enzymatic products were analyzed by high pressure liquid chromatography.

Table 5.4

Kinetic Properties of Purified Human CYP26 Enzymes.

Parameters	**CYP26A1[a]**	**CYP26B1[b]**
K_m, nM	50	19
V_{max}, pmol×min^{-1}×pmol P450^{-1}	10	0.8

CYP26A1 and CYP26B1 were expressed as C-terminally His$_6$-tagged proteins using the Baculovirus expression system and purified using cobalt affinity column. The reaction products were analyzed by LC–MS/MS [Topletz et al. 2012].