Retinoid processing in vivo - characterization and structure-function analysis of retinol dehydrogenases

Kristian Tryggvason

Stockholm 2003
Anything is possible!

This thesis was written without a single drop of coffee.
Abstract

Retinoids (vitamin A derivatives) are vital nutrients that regulate a variety of physiological processes, including embryonic development, postnatal growth, reproduction, cell differentiation as well as maintenance of the immune system and vision. Most of the physiological functions of retinoids are believed to be controlled by retinoic acid (RA) altered gene expression via two classes of nuclear hormone receptors, the RARs and RXRs. However, in the eye, 11-cis retinal acts as the visual chromophore that can be activated by light leading to a visual response.

The active retinoids, RA and 11-cis retinal, are generated from a common precursor, retinol. RA is synthesized from retinol in a two-step process, first by a retinol dehydrogenase (RDH) to retinal, and further by a retinal dehydrogenase to RA. To generate 11-cis retinal, retinol is first isomerized to 11-cis retinol and then oxidized by RDHs to 11-cis retinal. To date 20 different RDHs have been identified. In this study we focused on the characterization and structure-function analysis of two RDHs to expand the knowledge of the cellular pathways generating active retinoids.

The murine RDH4 oxidizes cis-retinols into corresponding aldehydes. We revealed that RDH4 was in fact the murine homologue of the human RDH5, based on the amino acid similarity, substrate specificity and protein expression patterns. These enzymes are thought to have a role in chromophore generation in the visual cycle. Genetical evidence for the role of RDH5 in 11-cis retinal synthesis was provided from mutations found in the RDH5 gene that caused fundus albipunctatus, a stationary form of night blindness. Intriguing was that both RDH4 and RDH5 enzyme domains faced the endoplasmic reticulum (ER) lumen. Later we showed that CRAD1, an enzyme closely related to RDH5, had a similar membrane topology. Considering the similarity of the microsomal RDHs, this suggests a lumenal orientation for most if not all of the RDHs.

Analysis of the two RDHs, CRAD1 and RDH5, revealed that they had short cytosolic tails of 6-7 amino acids. To examine the significance of these tails, we generated mutants with deleted tails. A reporter assay was developed to measure the enzyme activities of RDHs in transfected cells. Strikingly the tail deletion mutants lacked enzymatic activity in the cellular reporter assay, while they retained their activity in vitro. This led us to examine the significance of the cytosolic tails for subcellular localization. Indeed, the deletion mutants had abnormal cellular localizations, indicating that the reporter assay detects intracellular alterations. To map the essential amino acid residues, further mutations were generated in the cytosolic tail. Results generated with the reporter assay from a deletion series, alanine scan and chimeric proteins suggested that a conserved proline residue is important for enzymatic activity in vivo. The abolished activity of the CRAD1 tail deletion mutant could not be rescued by relocating the enzyme to the ER with a well-characterized double lysine ER retention signal. Therefore, the CRAD1 tail might associate with other proteins necessary for enzyme activity in vivo.
# Table of contents

Abstract .......................................................................................................................... 5  
Primary research articles ............................................................................................... 7  
Abbreviations .................................................................................................................. 8  
Introduction ..................................................................................................................... 9  
  The overall picture ......................................................................................................... 9  
  Effects of retinoids ......................................................................................................... 11  
    Perception of light ......................................................................................................... 11  
    Visual cycle ................................................................................................................ 13  
  Regulation of gene transcription .................................................................................. 14  
  Complications associated with retinoid excess or deficiency ................................. 15  
  Retinoids during embryogenesis .................................................................................. 17  
  Defects in the visual cycle ............................................................................................. 17  
  Retinoid intake and storage .......................................................................................... 18  
  From storage to target cells .......................................................................................... 19  
  Cellular retinol binding proteins .................................................................................. 21  
  Biosynthesis of active retinoids .................................................................................... 23  
    The first oxidizing step – retinol dehydrogenases .................................................. 23  
      Alcohol dehydrogenases .......................................................................................... 24  
      Microsomal retinol dehydrogenases ......................................................................... 25  
    Second oxidizing step – retinal dehydrogenases ..................................................... 28  
  Concluding remarks ..................................................................................................... 30  
Aims of this project ......................................................................................................... 33  
Materials and Methods ................................................................................................. 33  
Results and Discussion ................................................................................................... 34  
  Paper I: Analysis of the gene structure, expression profile, intracellular  
    localization and membrane topology of murine RDH5 ........................................... 34  
  Paper II: Development and practical implications for a method capable  
    of analyzing retinol dehydrogenase activity in vivo .............................................. 35  
  Paper III: A Study of the biochemical effects of mutations in the RDH5  
    gene causing fundus albipunctatus ............................................................................ 37  
  Paper IV: Further analysis of the significance of CRAD1 and its C-  
    terminal tail in retinol metabolism ............................................................................ 38  
General Discussion ......................................................................................................... 40  
Acknowledgements ........................................................................................................ 42  
References ...................................................................................................................... 44
Primary research articles


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>9c, 11c</td>
<td>9-cis, 11-cis</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>at</td>
<td>all-trans</td>
</tr>
<tr>
<td>CRAD1</td>
<td>cis-retinol/androgen dehydrogenase 1</td>
</tr>
<tr>
<td>CRBP</td>
<td>cellular retinol binding protein</td>
</tr>
<tr>
<td>CRABP</td>
<td>cellular retinoic acid binding protein</td>
</tr>
<tr>
<td>CRALBP</td>
<td>cellular retinal binding protein</td>
</tr>
<tr>
<td>CYP450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>IRBP</td>
<td>interphotoreceptor retinoid binding protein</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LRAT</td>
<td>lecithin:retinol acyltransferase</td>
</tr>
<tr>
<td>MDR</td>
<td>medium-chain alcohol dehydrogenase/reductase</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RAL</td>
<td>retinal</td>
</tr>
<tr>
<td>Raldh</td>
<td>retinal dehydrogenase</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>RARE</td>
<td>retinoic acid response element</td>
</tr>
<tr>
<td>RBP</td>
<td>retinol binding protein</td>
</tr>
<tr>
<td>RDH</td>
<td>retinol dehydrogenase</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigment epithelium</td>
</tr>
<tr>
<td>ROL</td>
<td>retinol</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>RXRE</td>
<td>retinoid X response element</td>
</tr>
<tr>
<td>SDR</td>
<td>short-chain dehydrogenase/reductase</td>
</tr>
<tr>
<td>TTR</td>
<td>transthyretin</td>
</tr>
<tr>
<td>VAD</td>
<td>vitamin A deficiency</td>
</tr>
</tbody>
</table>
Introduction

The overall picture

The word retinoid was first introduced by M.B. Sporn in 1976 to group all natural and synthetic analogs of vitamin A under the same category in the way carotenoids and steroids had been gathered some time earlier (Sporn et al., 1976). The Oxford English Dictionary defines retinoids as any substances that display vitamin A activity. In the scientific literature, authors have used the word retinoids in a number of different ways. In this thesis, all vitamin A derivatives and synthetic molecules having characteristics of vitamin A will be called retinoids. Fig. 1 illustrates some of the naturally occurring retinoids discussed in this thesis.

Retinoids are small hydrophobic molecules that are involved in a wide range of physiological processes, including vision, embryogenesis, reproduction, bone and skin maintenance as well as lymphocyte activation (Moore, 1957; Wolf, 1996) Humans are not able to produce retinoids and therefore they have to be obtained from the diet. Retinoids are stored in the liver as retinyl esters and released to the blood generally in the form of all-trans retinol (vitamin A). All-trans retinol is a prehormone that has to be metabolized into its active forms, 11-cis retinal, all-trans retinoic acid (RA) and 9-cis RA (Fig. 2).
Figure 2: The cellular metabolic pathways of retinoids. The active metabolites are marked in bold.

In the eye, all-trans retinol is first isomerized to 11-cis retinol and then further oxidized by retinol dehydrogenases (RDH) to 11-cis retinal. When bound in a complex with proteins called opsins, 11-cis retinal can be activated by light, triggering the isomerization of 11-cis retinal to all-trans retinal, which eventually generates a visual response. All-trans retinal is then reduced back to all-trans retinol to complete the process, the so called visual cycle (Saari, 1994). It has also been suggested that retinoids act in non-visual light sensing in the eye, thus regulating the circadian clock that controls our daily 24-hour rhythm (Lucas et al., 2003).

In non-ocular tissues, all-trans retinol is oxidized to RA in a two-step manner. First, retinol is oxidized by a RDH to retinal and then further by a retinal dehydrogenase (Raldh) to RA (Fig. 1). RA is the active metabolite that controls the activation or inhibition of gene transcription via the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). The activation of the RARs and RXRs mediate most of the physiological effects of retinoids, including development, reproduction, and maintenance of bone and skin.
**Effects of retinoids**

The effects of retinoids are manifested in two independent ways. Excluding opsin mediated light detection, all known physiological functions of retinoids are mediated via RAR or RXR that either activate or repress gene transcription (Hart, 2002). However, in the eye, 11-cis retinal acts as the visual chromophore, that can be activated by light to trigger a visual response. (Palczewski and Saari, 1997).

**Perception of light**

Mammalian light perception occurs in the retina of the eye. To be able to understand the complex visual cycle, or visual cycles if there in fact are two ways of producing the visual chromophores, it is important to understand the structure of the eye (Fig. 3). The retina is composed of two parts, the neuroretina and the retinal pigment epithelium (RPE, Fig. 3A). The neuroretina is a highly specialized part of the central nervous system specialized in detecting light, while one of the roles of RPE is to support the neuroretina.

The retina can be divided into four cell layers (Fig. 3B). The role of the RPE, apart from the biochemical part that will be discussed below, is to support and nourish the photoreceptor cells. In addition, the black pigments in RPE cells prevent light from scattering. The photoreceptor cells are specialized in detecting light and they also participate in the biochemical processing of retinoids. The photoreceptor cells mediate their signal to bipolar cells that are interconnected by horizontal and amacrine cells in the inner nuclear layer. Bipolar cells are connected to the brain via ganglion cells. Müller cells give structural support and have a possible role in the cone visual cycle (see below) (Saari, 1999).

There are two different photoreceptor cell types, the rods and cones. Cones function during daylight and are able to detect the wavelength and intensity of light, while rods function during scarce light and can only detect differences in the intensity of light. The cones and
rods are distributed unevenly in the human eye, with cones mostly present in the fovea of the eye, while rods are located in the peripheral retina. A single cone can connect to a single ganglion cell, while up to 1000 rods can be coupled to a single ganglion cell. This secures high accuracy of vision during daylight, and increased light sensitivity during scarce light.

Visual cycle

Already in the 19th century, investigators experimented with extracted retinas and demonstrated that the color of the dark-adapted frog retinas changed from purple to yellow, when exposed to light (Ewald, 1877). It took another 50 years for investigators to show that vitamin A was involved in the process. George Wald was first to predict the visual cycle, and to show that retinoids are an important part of it (Wald, 1934). The visual cycle he proposed resembles that illustrated in Fig. 3C. He was later rewarded a Nobel Prize for his pioneering work on vision.

It has been suggested that rods and cones have different retinoid regeneration cycles. Recent experiments with cone dominant animals have provided strong evidence for this (Mata et al., 2002). The cone visual cycle resembles the rod visual cycle except for that the regeneration cycle occurs between the cones and the Müller cells. In this thesis only the rod visual cycle will be discussed.

In the rod visual cycle, retinoids are taken up from the circulation by RPE cells in the form of all-trans retinol (Fig. 3C). All-trans retinol is either isomerized directly to 11-cis retinol, or first esterified by LRAT to all-trans RE, and then isomerohydrolyzed to 11-cis retinol. This step is still debated, since the enzymes capable of isomerizing all-trans retinol have not been identified. Formed 11-cis retinol is subsequently oxidized mainly by RDH5 to 11-cis retinal, which is transferred from the RPE to the photoreceptor cells, by an unknown mechanism. One candidate carrier protein between the two cell types is the interphotoreceptor retinoid binding protein (IRBP) that binds to 11-cis retinal, and is expressed between the RPE and photoreceptor cells. In the photoreceptor cells, 11-cis retinal is bound covalently to opsin to form rhodopsin. Opsin bound 11-cis retinal can be activated by light to trigger a visual response. Activation leads to the isomerization of 11-cis retinal to all-trans retinal, and the release of retinal from opsin (Saari, 1999). Finally, all-trans retinal is reduced to all-trans retinol by several possible RDHs, to finish the visual cycle (Haeseleer et al., 1998; Haeseleer et al., 2002; Kedishvili et al., 2002; Rattner et al., 2000).
An alternative pathway for rod chromophore regeneration has been suggested by Fong and colleagues. They have isolated an opsin-like RPE-specific retinal G protein coupled receptor (RGR) that is able to bind all-trans retinal and isomerize it with light to 11-cis retinal (Jiang et al., 1993). This regeneration mechanism is similar to a mechanism existing in invertebrates, where opsin bound 11-cis retinal is isomerized by one photon to all-trans retinal followed by regeneration to 11-cis retinal by absorption of another photon. (Hardie, 1986). Chen et al. studied this regeneration pathway by generating RGR null mutant mice. No visual defects were detected in retinoid regeneration in dark-adapted mice, however in constant illumination their ability to respond to light was impaired (Chen et al., 2001). A possible role for the RGR cycle is to regenerate the rod chromophores during illumination. The RGR cycle is light dependent and therefore cannot function in scarce light.

**Regulation of gene transcription**

The non-visual functions of retinoids were discovered later by Wolbach and Howe, when they found keratinization of epithelia in various organs resulting from retinoid deficiency (Wohlbach and Howe, 1925). However, the receptor for RA mediated regulation of transcription, RAR, was identified and cloned much later (Giguere et al., 1987; Petkovich et al., 1987). Soon afterwards, RXR, another RA receptor was identified (Heyman et al., 1992). The receptors have different ligand preferences, the natural high affinity ligands for RAR are all-trans RA and 9-cis RA, while only 9-cis RA can activate RXRs. Actually, RARs and RXRs are groups of receptors that can be divided into three subgroups (RARα, RARβ, RARγ and RXRα, RXRβ, RXRγ), that all have multiple isoforms produced by alternative splicing, differential promoter usage or both (For a thorough review, see Mangelsdorf et al., 1994.

RAR and RXR regulate gene transcription by binding DNA at sites called retinoid response elements (RARE and RXRE), and recruiting the transcriptional machinery. RAR is only able to bind DNA if it is heterodimerized with RXR. In functional RAR/RXR heterodimers, RXR
serves as a silent ligand independent partner. RXR binds DNA also as a homodimer, which can be activated by 9-cis RA (Zhang et al., 1992). In addition, RXR acts as a universal heterodimerization partner for various nuclear hormone receptors, including vitamin D receptors (VDR), liver X receptors (LXR) and peroxisome proliferator activated receptors (PPAR). In functional heterodimers, RXR can act as a silent ligand independent partner (RAR, VDR) or as an active ligand dependent partner (LXR, PPARγ) (Piedrafita, 1999).

Retinoid response elements have been identified in the promoter region of many genes, including RARs, cellular retinol binding proteins (CRBPs), cellular retinoic acid binding proteins (CRABPs), and the Hox genes. Interestingly no response elements have been found in the studied promoter regions of CRAD1 or Raldh2, two retinoid metabolizing enzymes, although it has been shown that mRNA levels of Raldh2 can vary according to retinoid status (Chai et al., 2001; Niederreither K et al., 1997; Wang et al., 2001b). For a more comprehensive list of genes regulated by RA see Balmer and Blomhoff, 2002.

To understand the individual roles of the retinoid receptors, mice depleted of single RARs and RXRs, as well as several double and triple knockout mice strains have been created. The detailed phenotypes of these knockout studies are described elsewhere (Clagett-Dame and DeLuca, 2002). Interestingly, only one of the single knockouts, the depletion of RXRα, was embryonic lethal, indicating redundancy between the receptors. Note however that RARβ and RARγ null mutant mice strains exhibited extensive postnatal lethality. The double knockout mice exhibited more severe symptoms than the single knockouts, but they all resembled the symptoms typical for retinoid deficiency.

**Complications associated with retinoid excess or deficiency**

The significance of retinoids in development was discovered in 1933, when vitamin A deficient piglets were born without eyes (Hale, 1933). Later, the teratogenic effects of excess retinoids were discovered, when 100 mated
rats were fed excess vitamin A during gestation. Only 10 rats carried to term, with 54% of the offspring displaying severe malformations (Cohlan, 1953). Interestingly retinoid excess in rodents had many of the same defects as vitamin A deficiency (VAD) (Nau, 1994). If the physiological vitamin A levels are not met during pregnancy, developmental defects arise, and the severity of the defects are correlated with the retinoid levels within the embryo (Fig. 4) (Morriss-Kay and Ward, 1999).

![Figure 4: An illustration showing the relationship between retinoid levels and embryonic malformations during pregnancy. Abbreviations VAD – vitamin A deficiency. (Freely adapted from Morriss-Kay and Ward, 1999)](image)

In adults, retinoid excess is not common, and usually occurs only during cancer (APL), or acne treatment with retinoids (Collins and Mao, 1999). VAD in adults, causes blindness, immune deficiency, keratinization of the epidermis and eventually death (Moore, 1957). VAD is not a problem of the Western world, but there are more than 100 countries, mostly in Africa and South-East Asia, where VAD is regarded as a public health problem. The most vulnerable groups for being affected by VAD are children and pregnant women. Within these countries, there are between 100-140 million children suffering from VAD. An estimated 250 000 - 500 000 of these children become blind every year and half of them die within 12 months after losing their vision (WHO, 2003).
Retinoids during embryogenesis

Ever since the significance of retinoids in embryonic development was discovered, thorough studies have been conducted in several species to understand its effect during embryogenesis. Studies in several species including pig, mouse, rat and quail suggested that VAD induced abnormalities include, malformations in the eye, liver, thymus, forelimb, heart and cranio-facial area, umbilical hernia, edema, and abnormal digit numbers (Morriss-Kay and Sokolova, 1996). The impact of retinoids in the development of specific organs have been studied extensively, but will not be discussed here further (Clagett-Dame and DeLuca, 2002; Hofmann and Eichele, 1994; Morriss-Kay and Sokolova, 1996).

Once the primary retinoid target tissues became identified, the search for the molecular players started. The roles of proteins known to be involved in retinoid metabolism have been studied by localizing them during embryogenesis and generating mouse strains depleted of these genes. The results of several of these studies will be discussed later. Surprisingly, many of the knock out mouse strains display only small alterations in their retinoid metabolism.

Defects in the visual cycle

A variety of genetic diseases affecting vision have been associated with genes encoding known proteins in the visual cycle. The disease categories are partly heterogeneous, but often include the decrease or loss of either rod or cone vision and degeneration of the retina.

Mutations in the RGR and rhodopsin encoding genes have been associated with retinitis pigmentosa, while mutations in LRAT have been associated with retinal dystrophy. (Dryja et al., 1990; Morimura et al., 1999; Thompson et al., 2001). Several mutations have been found in the RDH5 gene, which are associated with fundus albipunctatus, an autosomal recessive eye disease characterized by white spots in the retina and stationary night blindness. Mutated RDH5 proteins exhibited lower protein
expression levels and decreased enzymatic activity (Lidén et al., 2001; Yamamoto et al., 1999).

Cellular retinal binding protein (CRALBP) binds both 11-cis retinol and 11-cis retinal and is expressed exclusively in the eye. The precise function is unclear, but it has been suggested that the holo-form acts as substrate for both esterification and oxidation of 11-cis retinol. Mutations in CRALBP have been associated with several different forms of retinal diseases and mice lacking the CRALBP gene show an impaired isomerization of all-trans retinol to 11-cis retinol suggesting a role as a 11-cis retinol receptor after isomerization (Saari, 2000; Saari et al., 2001). Mice lacking IRBP showed degeneration of the photoreceptors (Palczewski et al., 1999). A protein highly expressed in the RPE cells, RPE65, has been proposed to function as a holo-RBP receptor in the RPE membrane (Båvik et al., 1993). Even though the role of RPE65 in retinoid metabolism in the eye is still unclear, mutations in the gene RPE65 have been associated with retinal dystrophy and Leber’s congenital amaurosis (Gu et al., 1997; Marlhens et al., 1997).

**Retinoid intake and storage**

Retinoids are obtained from the diet in two forms, as β-carotene from plants or as retinyl esters from animal sources (Goodman and Blaner, 1984). The two sources of retinoids have different uptake mechanisms in the gut.

β-carotene is directly taken up by the small intestine brush border membrane cells, called enterocytes. It was proposed as early as 1965 that an enzymatic activity within the enterocytes cleaves β-carotene symmetrically into two retinal molecules (Goodman et al., 1966; Olson and Hayaishi, 1965). The enzyme β-carotene 15,15’-dioxygenase was finally cloned from *Drosophila* in 2001 and shortly thereafter from man and mouse (Paik et al., 2001; Redmond et al., 2001; vonLintig et al., 2001; Yan et al., 2001). After the cleavage of β-carotene into two molecules of retinal, the formed retinal is reduced to retinol by
microsomal retinal reductases. The retinyl esters are hydrolyzed to retinol before they enter the gut (Rigtrup et al., 1994).

The two pools of retinoids are combined in the enterocytes as retinol, which is then re-esterified by lecithin:retinol acyltransferase (LRAT). The formed retinyl esters are packed into triglyceride-rich chylomicrons and secreted into the lymph, and eventually taken up by endocytosis, mostly in the liver using specific receptors for ApoE within the chylomicrons (Mortimer et al., 1995). Around 25% of the chylomicron remnants are taken up by extrahepatic tissues like, lung, skeletal muscle and bone marrow (Goodman et al., 1965).

A small amount of RA that is not incorporated into chylomicrons, is secreted to the portal vein by enterocytes. A recent study excluded the possibility that retinol or retinal formed from β-carotene or retinyl esters could be processed to RA within the enterocytes. Part of the portal vein RA derives from nutritional RA, but Kiefer et al. provided another explanation. They cloned an enzyme from the enterocytes, which cleaved β-carotene asymmetrically (Kiefer et al., 2001). One end product of the reaction can easily be processed to RA, which can also be secreted from the enterocytes to the portal vein.

After retinyl esters are taken up by liver hepatocytes, they are hydrolyzed to retinol, and either re-esterified to retinyl esters, or secreted into the circulation as a complex with retinol binding protein (RBP). Some retinoids are stored in the hepatocytes, but the main storage sites for retinoids are the lipid droplets of the specialized liver cells called stellate cells. It is not known how the retinoid transport homeostasis between the hepatocytes, stellate cells and circulation is controlled (Vogel et al., 1999).

**From storage to target cells**

During fasting state, the main retinoid in the circulation is retinol (2-3 µM), but some RA can also be found (4-14 nM) (De Leenheer et al., 1982; Goodman, 1984). Retinol does not circulate free in plasma, but is instead bound in a complex with retinol binding protein (RBP) and transthyretin
(TTR) in a 1:1:1 ratio. The role of RBP is to bind and protect retinol from the aqueous environment, while TTR protects RBP from being filtered through the kidney glomeruli (Mw of RBP is 21 kDa).

RBP and TTR are synthesized primarily in the hepatocytes, although at least RBP is synthesized in some other extrahepatic tissues, including kidney, lungs, heart, skeletal muscle, and eyes (Soprano and Blaner, 1994). In hepatocytes, the ROL-RBP-TTR complex is probably formed already in the endoplasmic reticulum (ER), and is secreted into the circulation. Mice lacking TTR exhibited 60% higher liver RBP levels than wild type mice, and cell cultures released RBP more efficiently when TTR was present (Melhus et al., 1991; Wei et al., 1995). These data suggest that TTR also has a role in releasing RBP-ROL from the hepatocytes.

The release of the main retinoid reservoir from hepatic cells is still unclear, since RBP is not expressed in stellate cells. Several options are possible. It has been suggested that stellate cells release free retinol, which is then bound by apo-RBP (Vogel et al., 1999). Alternatively cells could release ROL bound to endocytosed RBP found in stellate cells after endocytosis (Senoo et al., 1990). These options are not likely since apo-RBP is not secreted to the blood, and although vigorous attempts have been made to localize RBP in stellate cells using different microscopy techniques, all of the efforts have failed (Muto et al., 1971; Suhara et al., 1990).

RBP-bound retinol cannot be the sole source of retinoids in target tissues, as was seen from studies using RBP deficient mice. Newborn RBP deficient mice exhibited impaired vision, which was eventually restored with time. Additionally, thirteen month old mice had higher hepatic retinoid levels and lower plasma levels (12.5% of wild type), than the wild type, but there was no difference in many of the retinoid target tissues including kidney, spleen, testis and ovary (Quadro et al., 1999). When RBP-deficient mice were subjected to retinoid deficient diet, they were unable to release the retinoid stores from the liver. The low plasma retinoid levels were shown to derive from the diet and not the liver. These experiments suggested that RBP is needed for the release of retinoids from the liver, and therefore has an important role in regulation of the plasma retinol levels.
Free RA in the circulation represents another source of retinoids for targets cells, although this source of retinoids is not available for all tissues such as the eye, testis and B-cells (Kurlandsky et al., 1995). RA is the active retinoid metabolite in testis, but it cannot cross the blood-testis barrier. This suggests that retinoids in the testis of RBP deficient, but fertile, mice are obtained in another form. RBP deficient and wild type mice exhibited retinyl esters in the circulation, when held on a retinoid sufficient diet. These retinyl esters are derived from the diet, since the removal of retinoids from the diet exhausted the retinyl esters, and the mice became slowly VAD symptomatic (Quadro et al., 1999). This suggests that the retinoid target tissues are able to obtain retinoids also from circulating retinyl esters.

Two different pathways have been suggested for retinol uptake by target cells. Noy and Xu have suggested that retinol is released from RBP to the target cell membrane, where it is then ‘flip-flopped’ to the other side and ultimately bound to CRBPI (Noy and Xu, 1990). Other groups have suggested the existence of cell specific RBP receptors. Several of these have been identified (Båvik et al., 1991; Senoo et al., 1990; Sivaprasadaraao and Findlay, 1988), but so far only one proposed RPE receptor, p63, has been cloned (Båvik et al., 1993). This protein, also known as RPE65, has been knocked out in mice by gene targeting. The phenotype did not show defects in retinoid uptake, but instead revealed an overaccumulation of all-trans retinyl esters in the RPE, which resulted in re-speculation of the possible role of RPE65 in the isomerization of all-trans retinol to 11-cis retinol (Redmond et al., 1998). These data do, however, not exclude the role of RPE65 as a RBP receptor. The RPE65 deficient mice have the possibility to directly take up retinoids as retinyl esters as seen in RBP knockout mice.

**Cellular retinol binding proteins**

The two first identified intracellular retinol binding proteins were CRBPI and CRBPII. Recently two novel members, termed CRBPIII and CRBPIV, have been added to the family (Folli et al., 2001; Folli et al., 2002; Vogel
et al., 2001). There are also two cellular retinoic acid binding proteins, CRABPI and CRABPII, and an 11-cis retinoid binding protein, CRALBP. The role of retinoid binding proteins is believed to be to solubilize the hydrophobic retinoids in the aqueous environment. They have also been suggested to have functions in the regulation of transport, metabolism and action of the retinoids that they bind (Noy, 2000). CRBPI and CRBPII, bind retinol more efficiently than retinal, while CRABPI and CRABPII bind RA but not retinol, or retinal (Dong et al., 1999; MacDonald and Ong, 1987). The binding proteins show differential expression patterns indicating distinct roles.

In adults, CRBPI has the highest expression levels in liver and kidney (Noy 2000, and references therein). Knock out studies showed that CRBPI was not essential during normal physiological vitamin A conditions, even though the liver retinyl ester stores were decreased with more than 50%. Once retinoid sufficient, mice retinoid stores cannot be depleted by a retinoid deficient diet for its remaining lifespan. However, when the CRBPI knockout mice were subjected to a retinoid deficient diet, the retinoid stores were depleted within 5 months. Therefore, it was suggested that CRBPI has a role in serving as the substrate, in complex with retinol, for LRAT esterification in the liver (Ghyselinck et al., 1999).

CRBPII is almost exclusively expressed in the small intestine in adults. It is the most highly expressed protein in the intestine comprising up to 1% of the total protein. When bound to retinal in the enterocytes, it stimulates the reduction of retinal to retinol (Boerman and Napoli, 1996). CRBPII bound retinol is also an excellent substrate for LRAT esterification (Ong et al., 1994). Therefore, the role of CRBPII has been suggested to be to reduce retinal and esterify retinol in the enterocytes. During pregnancy, CRBPII can also be detected in the placenta. Recently, CRBPII null mutant mice were generated and analyzed (E et al., 2002). Surprisingly the mice were alive and fertile during normal vitamin A diet administration, and there was no significant difference in the reduction of retinal or LRAT enzyme activity between the wild type and CRBPII null mice. However, when subjecting pregnant CRBPII deficient mice to marginal vitamin A diet from gestation day 10 onwards, all of the pups died within 24 hours after birth. This suggested a role for CRBPII in the
maternal placenta in ensuring the developing fetus with adequate levels of retinoids (E et al., 2002).

The roles of the two recently cloned CRBPs are still unclear. CRBPIII has been reported to have similar biochemical properties as CRBPI and CRBPII, but CRBPIV (mouse CRBPIII), has ten-fold lower retinol binding affinity, and does not bind retinal (Folli et al., 2001; Folli et al., 2002; Vogel et al., 2001).

In embryos, both CRABPI and CRABPII are widely expressed, while in adults, CRABPI is expressed almost ubiquitously, and CRABPII is expressed only in skin, uterus, ovary and the choroids plexus (Noy, 2000). The RA-binding proteins have been proposed to have distinct functions. CRABPI is mostly localized in the cytoplasm, and protects the nucleus from acquiring excess retinoids, by directing RA for breakdown. CRABPI-bound RA is a known substrate to RA metabolizing enzymes such as the CYP450s (Fiorella and Napoli, 1991; Fujii et al., 1997). CRABPII has the opposite effect, and is instead believed to sensitize cells for RA. Both binding proteins have been knocked out in mice separately and together. Even the double CRABPI/CRABPII knockout mice were live and fertile, indicating that the CRABPs are dispensable in retinoid homeostasis (Lampron et al., 1995).

**Biosynthesis of active retinoids**

Studies regarding RA biosynthesis have been extensive since the RARs were first identified in 1987 (Giguere et al., 1987; Petkovich et al., 1987). Several different classes of enzymes have been implied to oxidize retinoids from retinol to RA. These protein families are briefly summarized below. Fig. 1 illustrates the reactions these enzymes catalyze.

**The first oxidizing step – retinol dehydrogenases**

Members of several different enzyme families have been suggested to oxidize retinol (an alcohol) to retinal (an aldehyde). The two most studied enzyme families are the classical alcohol dehydrogenases (ADHs) and
Retinol dehydrogenases belonging to the family of short-chain dehydrogenase/reductases (SDRs). There are some reports showing that CYP450s and aldo-keto reductases could also oxidize retinol, but their physiological relevance are yet to be elucidated (Chen et al., 2000; Crosas et al., 2001).

**Alcohol dehydrogenases**

So far five different classes of ADHs have been identified in human and mouse (Duester et al., 2003). ADHs are zinc-dependent enzymes of about 40 kDa that utilize NAD for the oxidation of various primary, secondary and cyclic alcohols (Jörnvall et al., 1999). The ADHs have a wide substrate specificity, including ethanol, S-nitrosoglutathione, hydroxyfatty acids, aldehydes, steroids and retinols, and have been described as a general detoxifying enzyme family for alcohols and aldehydes (Höög et al., 2001; Jörnvall et al., 2000).

Of the ADHs, ADH4 oxidizes retinol, most efficiently followed by ADH2>ADH1>ADH3. The retinol-oxidizing capabilities of ADH5 have not yet been analyzed. ADH4 is partly localized in retinoid target tissues during embryogenesis, while ADH1 is mainly localized in the liver, and ADH3 ubiquitously in all tissues (Duester et al., 2003). While the ADH1, ADH2 and ADH4 have high efficiency on retinol oxidation, they have relatively high $K_m$ values. ADH1 and ADH4 have $K_m$ values of approximately 30 µM, while ADH2 has 7,3 µM. (Han et al., 1998). The $K_m$ value of ADH3 was not published with the rest of the retinoid kinetic data (Molotkov et al., 2002). The high $K_m$ values of ADH4 could have contributed to the fact that it was metabolically inactive in a cellular reporter assay using 1 µM 9-cis retinol as the substrate, while two microsomal RDHs efficiently oxidized retinol (Tryggvason et al., 2001).

To study the relevance of ADHs in retinol oxidation ADH null mutant mice were generated. ADH3 and ADH4 deficient mutant strains exhibited higher death rates than the wild types when subjected to a retinoid deficient diet for two generations. (Molotkov et al., 2002). Additionally, lower levels of RA were detected from the blood of ADH1 and ADH3 deficient mice after an oral retinol dose.
Developmental studies in retinoid target tissues, like the nervous system, suggest that RA synthesis is controlled in a spatio-temporal manner in retinoid target tissues (McCaffery and Dräger, 2000). Considering the high binding efficiencies of CRBPs \((K_d<10 \text{ nM})\), little free retinol is available in retinoid target tissues (Napoli, 1999a). The fact that ADHs are not able to oxidize holo-CRBP combined with their high \(K_m\) values and expression patterns, it is unlikely that ADHs oxidize retinol during normal retinoid conditions. However, they can have a role in metabolizing retinol during retinoid detoxification.

**Microsomal retinol dehydrogenases**

The microsomal retinol dehydrogenases belong to a family of alcohol dehydrogenases called short-chain dehydrogenase/reductases (SDRs). There are more than 1000 enzymes belonging to the SDR family, that cover a wide range of substrate specificities, including steroids, alcohols, prostaglandins, and retinols. Most members of this group are comprised of approximately 250 residues, with two conserved sequences, including the active site Y-X-X-X-K, and the cofactor binding site G-X-X-X-G-X-G, (Jörnvall et al., 1999; Napoli, 1999b). In addition, the 19 identified microsomal RDHs have both N- and C-terminal extensions, which most likely are used for membrane anchoring. (See Table I, for a list of the enzymes including substrate specificities and expression profile.)

The first retinol dehydrogenase belonging to the SDR protein family, 11-cis RDH, was cloned in 1995 (Simon et al., 1995). RDH5, as it was later renamed, was proposed to oxidize the last oxidation step in the chromophore generation in the eye, as suggested by its 11-cis retinoid substrate specificity and high expression in the RPE. This was later verified when mutations in the RDH5 gene, that were associated with fundus albipunctatus, were shown to have lower enzymatic efficiencies (Lidén et al., 2001; Yamamoto et al., 1999).
RDH5 was later found to be expressed in other tissues. Moreover, it was equally as efficient in oxidizing 9-cis retinol, suggesting that RDH5 has dual roles. In the eye, RDH5 functions as 11-cis RDH contributing to the visual cycle, and in non-ocular tissues it oxidizes 9-cis retinol contributing to the 9-cis RA biosynthesis pathway (Driessen et al., 1998; Romert et al., 1998; Romert et al., 2000; Wang et al., 1999). However no defects were observed outside the eye in the RDH5 patients, indicating no essential role for RDH5 outside the eye, or presence of redundant RDHs (Driessen et al., 2000; Shang et al., 2002; Yamamoto et al., 1999). RDH5 deficient mice had a less severe visual phenotype than humans with mutations in the RDH5 gene. The RDH5 deficient mice had only problems with dark adaptation, while human patients were night blind. Possibly, animals that are active at night have a more redundant rod visual cycle than humans.

Of all the RDHs, RDH5 is the most well characterized enzyme, and its association with a visual disease makes it a prototypic RDH. RDH5 is a typical microsomal RDH belonging to the SDR family. The enzyme has a molecular weight of about 32 kDa, has N- and C-terminal membrane anchoring domains that associate the protein with an ER membrane, a

Table I: RDHs, their substrate specificities and expression patterns

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Species</th>
<th>Substrate</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>RoDHI</td>
<td>R</td>
<td>atROL</td>
<td>Liver, lung, testis, brain, kidney</td>
</tr>
<tr>
<td>RoDHII</td>
<td>R</td>
<td>atROL</td>
<td>Liver, kidney, brain, lung, testis</td>
</tr>
<tr>
<td>RoDHIII</td>
<td>R</td>
<td>unknown</td>
<td>Liver</td>
</tr>
<tr>
<td>RDH5</td>
<td>H, M, B</td>
<td>9cROL, 11cROL</td>
<td>Eye, liver, kidney, mammary gland</td>
</tr>
<tr>
<td>CRAD1</td>
<td>M</td>
<td>9cROL, 11cROL</td>
<td>Liver, kidney, intestine</td>
</tr>
<tr>
<td>CRAD2</td>
<td>M</td>
<td>11cROL, atROL</td>
<td>Liver, lung, eye, kidney</td>
</tr>
<tr>
<td>CRAD3</td>
<td>M</td>
<td>9cROL, 11cROL</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>CRADL</td>
<td>M</td>
<td>steroids</td>
<td>Kidney, liver</td>
</tr>
<tr>
<td>RDH1</td>
<td>M</td>
<td>atROL&gt;9cROL</td>
<td>Liver, testis, kidney, heart, brain</td>
</tr>
<tr>
<td>RoDH4/RDH-E</td>
<td>H</td>
<td>atROL</td>
<td>Liver, epidermis</td>
</tr>
<tr>
<td>eRolDH</td>
<td>R</td>
<td>atROL</td>
<td>Uterine epithelia</td>
</tr>
<tr>
<td>RDH-E2</td>
<td>H</td>
<td>atROL</td>
<td>epidermis</td>
</tr>
<tr>
<td>RDH-TBE</td>
<td>H</td>
<td>atROL</td>
<td>trachea, colon, tongue, esophagus</td>
</tr>
<tr>
<td>RDH10</td>
<td>H, B, M</td>
<td>atROL</td>
<td>RPE</td>
</tr>
<tr>
<td>RDH11</td>
<td>H</td>
<td>9cROL, atROL</td>
<td>Eye</td>
</tr>
<tr>
<td>RDH12</td>
<td>H</td>
<td>atRAL</td>
<td>Eye</td>
</tr>
<tr>
<td>RDH14</td>
<td>H</td>
<td>9cROL, atROL</td>
<td>Eye</td>
</tr>
<tr>
<td>prRDH</td>
<td>H, B</td>
<td>atRAL</td>
<td>Eye</td>
</tr>
<tr>
<td>RalR1</td>
<td>H</td>
<td>atRAL</td>
<td>Prostate</td>
</tr>
<tr>
<td>retSDR1</td>
<td>H</td>
<td>atRAL</td>
<td>Eye, heart, liver, pancreas</td>
</tr>
</tbody>
</table>
SDR catalytic domain, and a 7 amino acid C-terminal tail (See Fig. 5A). The amino acid similarity between RDH5 and CRAD1 is 60%, and the hydropathy plots of RDH5 and CRAD1 show striking similarities.

Interestingly, studies on the membrane topology of RDH5 revealed that the catalytical SDR domain faced the ER lumen in both the human and mouse enzymes (Romert et al., 2000; Simon et al., 1999). This was intriguing, since it had been widely accepted that microsomal RDHs use retinol bound to cytosolic cellular retinol binding proteins as substrates, suggesting a cytosolic orientation of the catalytic domain (Napoli, 1999a). CRAD1, a close relative of RDH5, was also subjected to membrane topology studies, and was also shown to have a lumenal orientation (Tryggvason et al., 2001). There are two reports suggesting that RoDH1 and RoDH4 catalytic domains would reside in the cytosol, but the experimental data is not convincing (Lapshina et al., 2003; Wang et al., 2001a). Therefore we suggest that most, if not all, microsomal RDHs have a lumenal orientation of the catalytic domain. (Fig. 5B)

![Figure 5: Characteristic features of microsomal retinol dehydrogenases. A. A hydropathy plot containing three retinol dehydrogenases and a schematic domain structure. Black – mRDH5, red – hRDH5 and blue – CRAD1. B. A proposed domain structure for microsomal retinol dehydrogenases.](image)

Although evidence for RDH5 involvement in the processing of the visual chromophore is undisputable, the role of RDH5 outside the eye has not been clarified. It has been proposed that RDH5 acts as a 9-cis RDH in non-ocular tissues, but it is also able to oxidize some steroids (3α-adiol
and androsterone) (Huang and Luu-The, 2001; Wang et al., 1999). In fact, many of the microsomal RDHs, including CRAD1, can also oxidize hydroxysteroids (Napoli, 2001). Furthermore, CRAD1 is more efficient in catalyzing 3α-adiol and androsterone to their metabolites than retinol (Chai et al., 1997). Therefore, it has been suggested that RDHs can function in both retinol and hydroxysteroid oxidation depending on the substrate availability (Chai et al., 1997; Huang and Luu-The, 2001; Su et al., 1998).

It has been suggested in many primary and review articles, that CRBPs have a major role in retinol oxidation, serving as the substrate when bound to retinol. The fact that cytosolic ADHs cannot oxidize CRBPI bound retinol has been used as a strong argument against ADH involvement in retinol oxidation in vivo (Lapshina et al., 2003; Napoli, 1999a).

Several facts support a role for CRBPI-mediated retinol oxidation. First, both RoDH1 and RoDH4 were able to be crosslinked with holo-CRBP, but not apo-CRBP (Lapshina et al., 2003; Wang et al., 2001a). Second, it has been shown that the efficiency of retinol oxidation is higher when using holo-CRBP than with free retinol (Boerman and Napoli, 1995). However, knockout studies of CRBPI and CRBPII have provided opposing evidence. CRBPI null mutant mice had no obvious phenotypes in retinol oxidation or in vision. Instead, decreased LRAT activity in liver was detected, implying a role for CRBPI in esterification (Ghyselinck et al., 1999; Saari et al., 2001). Additionally, there was no difference in retinal reductase activity in the gut of CRBPII null mutant mice (E et al., 2002).

**Second oxidizing step – retinal dehydrogenases**

Retinal dehydrogenases (Raldhs) belong to a family of enzymes called aldehyde dehydrogenases. In fact, the first Raldh were identified in 1991 by Lee et al. when they explored the retinal oxidizing abilities of all 13 aldehyde dehydrogenase activities known at that time (Lee et al., 1991). Three of the aldehyde dehydrogenases were able to oxidize retinal to RA and they were all cytosolic proteins using NAD as a cofactor. The first
Raldh purified from rat kidney, had a molecular weight of 53 kDa and was NAD dependent in the oxidation of both all-trans retinal and 9-cis retinal to their respective acids (Labrecque et al., 1993). The enzyme was finally cloned in 1995, and was found to have high amino acid similarity to other cloned cytosolic aldehyde dehydrogenases (Labrecque et al., 1995).

Four different Raldhs have been identified and cloned from various species so far (Grun et al., 2000; Lee et al., 1991; Lin and Napoli, 2000; Wang et al., 1996). They all catalyze the irreversible oxidation of retinal to RA. The four enzymes have different substrate specificities and efficiencies that are summarized in Table II. All the Raldhs are evolutionally closely related. Raldh1, Raldh2 and Raldh3 share amino acid similarities of about 75%, while Raldh4 is slightly more distant and shares about 50% amino acid similarity with the other Raldhs (Lin et al., 2003).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Hugo</th>
<th>Other names</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raldh1</td>
<td>ALDH1A1</td>
<td>ALDH1, AHD-2, RalDH(I), RALDH</td>
<td>atRAL = 9cRAL</td>
</tr>
<tr>
<td>Raldh2</td>
<td>ALDH1A2</td>
<td>ALDH11, RALDH2, RalDH(II), V2</td>
<td>atRAL &gt; 9cRAL</td>
</tr>
<tr>
<td>Raldh3</td>
<td>ALDH1A3</td>
<td>ALDH6, V1</td>
<td>at-RAL</td>
</tr>
<tr>
<td>Raldh4</td>
<td>none</td>
<td>similar to ALDH8A1, hALDH12</td>
<td>9cRAL &gt;&gt; atRAL</td>
</tr>
</tbody>
</table>

To address the role of Raldh2 in embryogenesis, Raldh2 null mutant mice were generated. These mice died at midgestation (before E10,5) with several typical VAD defects including a single enlarged heart tube, shortened antero-posterior axis, shortened or non-existing limbs, truncated fronto-nasal region and webbed digits (Niederreither et al., 1999). The embryo was totally RA deficient at the analyzed time points E7,5 and E8,5, except in the eye as was seen from RA activated reporter gene expression in whole mount embryos. This is not surprising, since other Raldhs are not expressed at this stage except for Raldh1, which is expressed in the eye at E8,5. Raldh3 is detected first at E8,75, and Raldh4 after E11,5 (Lin et al., 2003; Mic et al., 2000; Ulven et al., 2000).

A rescue attempt was made for the Raldh2 null mutant mice, by providing the mothers RA between E6,75-E8.25. These embryos performed axial rotation and showed a milder VAD phenotype at E10,5, but still exhibited symptoms related to VAD. Using similar RARE-lacZ reporter-strain mice, RA was detected in a variety of tissues, indicating that the other Raldhs
had started to synthesize RA (Mic et al., 2002). These data indicate that Raldh2 is a major RA synthesizing enzyme during early development, and it cannot be totally compensated for by external RA, as the rescued Raldh2 null mice exhibited typical defects associated with abnormal retinoid levels.

Interestingly, Raldh4 is the first retinal dehydrogenase, which has a marked specificity for 9-cis retinal as a substrate (Lin et al., 2003). There has been a debate if 9-cis retinoids exist in mammals, since they have been difficult to detect. Using conventional methods, several laboratories have detected 9-cis retinoids in adult and embryonic tissues (Arnhold et al., 1996; Heyman et al., 1992; Labrecque et al., 1993), but many others have failed (Horton and Maden, 1995; Ulven et al., 2000). The existence of enzymes specifically oxidizing 9-cis retinoids provides some evidence for a 9-cis RA generating biosynthesis pathway.

Concluding remarks

Research on retinoid metabolism has increased the last decades, ever since it was shown that retinoids regulate gene transcription through nuclear hormone receptors. RA-mediated activation of nuclear hormone receptors indicated that plasma retinol has to be oxidized intracellularly in a two-step manner to RA. However, the field is young and new proteins associated with retinoid processing are still identified on a regular basis.

A need for tight regulation on the processing of the intracellular retinoid levels is shown from various knockout, as well as retinoid excess and sufficiency studies. If there were no tight control, plasma retinoids would diffuse into cells and the oxidized metabolites would probably result in a constitutive activation of the retinoid receptors, considering that 500-1000 times higher retinol are present in the blood compared to the levels of RA needed for the activation of the nuclear hormone receptors. However, the mechanisms underlying the regulation of retinoid metabolism are still largely unknown.

In the target tissues, retinoid metabolism can be regulated on many different levels (Fig. 6). These include the regulation of the cellular
and nuclear uptake of retinoids as well as compartmentalization of retinoids as retinyl esters or as holo-CRBP or holo-CRABP. Other possible regulatory sites are the two oxidation steps and the degradation of RA to inactive oxo-metabolites that are not able to bind the nuclear hormone receptors. (Niederreither et al., 2002a).

![Diagram of retinoid processing pathway](image)

**Figure 6**: The retinoid processing pathway with possible retinoid regulatory sites marked with arrows.

Mice strains that are deficient of genes known to be involved in retinoid processing have mild phenotypes compared to retinoid deficient mice (cellular retinoid binding proteins, RARs, RXRs, RDHs). An exception is the Raldh2 knockout mice, where no RA synthesis occured during early embryogenesis (Niederreither et al., 1999). In addition, some of the phenotypes were more severe when mice were subjected for abnormal retinoid intake (CRBPI, CRBPII). These data indicates that there is redundancy between the regulatory pathways.

That many different genes encoding the machinery required for cellular uptake, metabolism and possibly compartmentalization are co-expressed suggests that RA biosynthesis may be controlled in a spatio-temporal manner. Many of the proteins involved in retinoid processing are widely expressed. However, several RDHs have tissue-specific expression patterns, opening up for a possibility for tissue specific regulation of retinoid processing.

Within the target cells, the crucial regulatory step is most likely the oxidation of retinol to retinal by RDHs. Retinol can be stored in two inactive storage forms, as RE or bound to CRBPs, that can be recruited when needed. Retinal, on the other hand, is a chemically highly active molecule that can not be stored in most cells. That CRABPI and CRABPII double knockout mice were almost normal argues against major
regulation at the RA stage. (Lampron et al., 1995). Although during extreme retinoid conditions many of the reserve regulatory mechanisms are probably needed to preserve physiological retinoid concentrations.
Aims of this project

Despite rapid progress in research on retinoid metabolism in recent years, the fundamental mechanisms of retinol intake to target cells and the regulation of its oxidation to the active metabolites are still largely unclear. In this study we aimed to obtain more information about the retinoid processing pathways, by mainly focusing on the characterization of retinol dehydrogenases CRAD1 and RDH5. The major objectives were:

I. To analyze the gene structure, expression profile, intracellular localization and membrane topology of murine RDH5.

II. To develop a method for analyzing retinol dehydrogenase activity in vivo in transfected cells, and to use this method for structure-function analysis of CRAD1 and RDH5

III. To study the biochemical effects of mutations of the RDH5 gene that causes fundus albipunctatus.

IV. To study the C-terminal tail of CRAD1 in detail in order to understand its importance for localization and enzymatic activity.

Materials and Methods

The complete list of all the material and methods are presented in the individual papers (I-IV).
Results and Discussion

The results of which this thesis is based on are thoroughly presented and discussed in the original papers (I-IV) and here I will only give a brief summary and comments on each paper.

**Paper I: Analysis of the gene structure, expression profile, intracellular localization and membrane topology of murine RDH5**

Our group has previously described the first retinol dehydrogenase (RDH5) and a murine retinol dehydrogenase 5 (RDH4). In this paper we further characterized RDH4, by studying its gene structure, expression pattern and membrane topology to gain more knowledge of its role in retinoid metabolism.

We showed that the RDH4 gene is composed of 5 exons, including the first untranslated exon. The tissue distribution of RDH4 was analyzed by a dot-blot hybridization, which indicated that the highest expression of RDH4 mRNA is found in the eye, liver and kidney. Based on RT-PCR analysis, RDH4 is expressed as three different splice variants that are all expressed in the three tissues with the highest RDH4 expression, although some variation in their expression levels occurs. A dot-blot analysis also revealed that RDH4 is expressed already at E7. This is in accordance with previous studies (Ulven et al., 2000).

Since RDH4 had the highest expression levels in the adult eye, kidney and liver, immunohistochemistry was performed in these organs. RDH4 was first detected at E18 in the developing eye, and a typical adult expression pattern was seen in 16-day-old mice (P16). In the liver, expression was detected in the centrilocular area of the hepatocytes. while in the kidney, RDH4 was expressed in the distal tubules and the transitional epithelium in the renal pelvis.
Human RDH5 has been shown to have typical ER localization with the catalytic domain facing the ER lumen (Simon et al., 1999). This suggested a similar ER membrane orientation for RDH4, considering the similarity of the proteins. Indeed, in an immunofluorescence study, RDH4 was shown to co-localize with concavalin A, a known ER marker. When an ectopic glycosylation site (N-I-S) was introduced to RDH4 (amino acids 71-73), and the protein was expressed in vitro in the presence of canine microsomes, partial glycosylation occurred. N-linked glycosylation of proteins only occurs in the lumen of ER, providing strong evidence for a lumenal orientation of RDH4 (Parodi, 2000).

The fact that RDH4 shares 84% amino acid identity with RDH5, has similar expression patterns, substrate specificity and membrane topology, made us conclude that RDH4 is the mouse homologue of human RDH5.

**Paper II : Development and practical implications for a method capable of analyzing retinol dehydrogenase activity in vivo**

Retinoic acid is generated from retinol in a intracellular two-step process. Two classes of proteins have been shown to oxidize retinol to retinal, the medium chain alcohol dehydrogenases and short chain dehydrogenase/reductases. In this paper we described a novel method for measuring the RDH enzyme activity in vivo in transfected cells.

The assay is based on the fact that the RXR ligand-binding domain can be fused to the yeast transcription factor GAL4. The receptor fusion protein can be activated by RXR ligands to activate the transcription of co-transfected reporter luciferase that is preceded by a thymidine kinase (TK) minimal promoter and four GAL4 binding sites. The luciferase levels can be quantified with a luminometer (de Urquiza et al., 2000).

We showed that the biosynthesis of 9-cis RA from 9-cis retinol requires the concerted action of both a RDH and a Raldh. The limiting factor of this assay is also the target for the activity measurement. We titrated different retinoids, and showed that all-trans retinoids do not
activate the reporter system, indicating an inability of the cells to isomerize all-trans retinoids to 9-cis retinoids. The highest activation was achieved using ~1 µM 9-cis retinol as substrate. Using these conditions, the RDH activity is limiting and therefore allowed us to study its enzymatic activity in vivo.

With this reporter assay, we showed that only CRAD1 and RDH5 oxidized 9-cis retinol. CRAD2 and CRADL showed no 9-cis oxidizing activity, which is in accordance with previous data (Su et al., 1998). However, ADH4, which has been shown to have the highest oxidation activity in vitro, had only little or none enzymatic activity in the in vivo reporter assay.

We further characterized CRAD1 to analyze if its cell biological properties were similar to the related enzyme, RDH5. Immunofluorescence analysis showed that like RDH5, also CRAD1 was localized to the ER. Additionally the catalytic domain of CRAD1 faced the lumen, as was seen from the proteinase protection assays.

Results from studies with other SDRs and hydropathy plot of CRAD1 suggest, that membrane bound retinol RDHs, have two membrane anchoring domains, a luminal catalytic domain and cytosolic tail consisting of 6-7 amino acids (Napoli, 1999b; Romert et al., 2000; Simon et al., 1999; Tryggvason et al., 2001). Since the catalytic domain is on the opposite side of the membrane, it is surprising that this 6/7 amino acid tail is so highly conserved. We constructed mutants lacking both CRAD1 and RDH5 cytosolic tails, to analyze their roles for enzymatic activity. Surprisingly, the tail deletion mutants lacked enzymatic activity in vivo with the reporter assay while they retained enzymatic activity in vitro. These results were very interesting, since this suggested that the cytosolic tails had a role in vivo, but are not necessary for enzymatic activity in vitro. One possible reason for enzymatic inactivity can be cellular mislocalization and inability to recruit partners needed for activity, possibly co-factors. The mutant proteins were analyzed by immunofluorescence, to verify their subcellular localization. Both of the mutants were shown to have abnormal cellular patterning. This suggested that the cytosolic tails of CRAD1 and RDH5 are necessary for subcellular localization, which in turn is crucial for enzymatic activity in vivo.


**Paper III : A Study of the biochemical effects of mutations in the RDH5 gene causing fundus albipunctatus**

Previous studies have shown that mutations in the gene encoding RDH5 are associated with fundus albipunctatus, an autosomal recessive eye disease that causes a form of stationary night blindness. We obtained information of the mutated nucleotides from clinical publications, and Dr. T. Dryja. Mutant constructs were generated by single-stranded mutagenesis, and analyzed for protein expression levels and enzymatic activity. Mutations associated with fundus albipunctatus were shown to occur in both the lumenal ectodomain and the carboxyterminal transmembrane domain.

All mutants analyzed were expressed at lower levels than the wild type enzyme, and had decreased enzymatic activity. That A294P was active in enzyme assays was surprising since the patient with the A294P mutation was compound heterozygous with a second inactive R280H mutant. As fundus albipunctatus is a recessive disease, this combination should have not have produced a disease phenotype, considering the one functioning A294P allele. This led us to examine the possibility that R280H acts in a dominant-negative way to the A294P mutant. The reporter assay was used to analyze this hypothesis. The results showed that A294P was repressed by all the examined mutant RDH5 proteins. This may in part be due to the lower expression levels of the mutant A294P compared to the wild type.

One way to explain how R280H can act in a dominant-negative way towards A294P is if RDH5 is found as dimers *in vivo*, and that functional dimers have to be generated for proper enzymatic activity. This was proven true with two separate cross-linking experiments. Therefore we conclude that RDH5 and possibly other RDHs acts as functional dimers.
Paper IV: Further analysis of the significance of CRAD1 and its C-terminal tail in retinol metabolism

It was reported previously by us, that the CRAD1 and RDH5 catalytic domains faced the ER lumen, although there has remained some dispute about these facts. In addition, the cytosolic tails of CRAD1 and RDH5 have been shown to be necessary for enzymatic activity in vivo. We conducted studies to verify the luminal orientation of the catalytic domain of CRAD1. In addition we analyzed the biochemical properties of CRAD1, which had been subjected to structural changes in the cytoplasmic tail, and analyzed the possible role for CRAD1 in 9-cis RA synthesis in vivo.

The membrane binding properties of CRAD1 and CRAD1 tail deletion mutant (CRAD1-D6) were analyzed to verify that the phenotype seen in CRAD1-D6 mutants do not arise from inadequate membrane anchoring. Both the wild type and CRAD1-D6 were verified to be integral membrane proteins.

To provide additional evidence for the luminal orientation of the CRAD1 catalytic domain, immunocytochemistry combined with selective membrane permeabilization, was used. CRAD1 was detected when cellular membranes were permeabilized with Triton X-100, while no CRAD1 was seen when only the plasma membrane was permeabilized with streptolysin O (SLO). This indicated that the ER membrane had to be permeabilized to allow the antibody to detect the protein. Since the antibody against CRAD1 is raised against the catalytic domain of the protein, this provides additional evidence that the catalytic domain of CRAD1 is located in the lumen of the ER. However, when CYP2E1, an ER resident protein facing the cytosolic side of the ER membrane, was used as a positive control for SLO permeabilization, the protein was detected.

We further characterized the CRAD1 carboxyterminal tail by analyzing a series of deletion mutants, alanine substitutions, addition of amino acids, and several chimeric enzymes. Analysis of the tail deletion mutants revealed a partial correlation between decreased enzymatic activity and abnormal localization. However, normal ER localization per se is not sufficient for enzymatic activity as was seen when the CRAD1 C-
terminal tail was replaced with a well-characterized double lysine ER retention signal. A proline residue was the only single amino acid that showed decreased enzyme activity when single amino acids were mutated to alanine. An addition of an arginine residue close to the ER membrane diminished enzyme activity suggesting the importance of membrane proximity. A FLAG-tag was attached to the wild type CRAD1 and the cytosolic tail was substituted with corresponding tails of CRAD2 or RDH5. None of these mutants showed decreased enzymatic activity. All these data suggest an important role for the invariant proline residue for the enzymatic activity in vivo.

The cytosolic tail can be important for the RDHs in several ways. The tail is a possible target for protein-protein interactions, which might have some regulatory roles for enzyme activity or stability. It is important to remember that at least CRAD1 does not have retinoid response elements in its promoter region, suggesting other post-transcriptional regulatory mechanisms for CRAD1 to respond to cellular retinoid needs. In addition the invariant conserved proline residue in the cytosolic tail is a possible site for covalent modifications e.g. hydroxylation, and therefore a possible regulation mechanism. We explored possible interacting partners for CRAD1 and RDH5, but could not verify any with the techniques we used.

To gain more knowledge of the physiological role of CRAD1, we analyzed the embryonic expression pattern of midgestation mouse embryos by immunohistochemistry. CRAD1 was detected in several RA target tissues including, heart, adrenal glands, lung, craniofacial ectoderm and mesoderm as well as the respiratory tract. The heart showed the most intensive expression. In addition CRAD1 is co-localized with Raldhs in many tissues (Niederreither et al., 2002b). Furthermore, in the heart, CRAD1 is co-localized with both Raldh2 and RXRα. This is interesting, since both RXRα and Raldh2 knockout mice exhibited serious malformations in the heart. (Kastner et al., 1994; Niederreither et al., 2001). It is possible that CRAD1 has a role in 9-cis RA biosynthesis in tissues, when it is co-expressed with Raldhs and RXRs.
General Discussion

The lumenal orientation of the catalytic domain of microsomal RDHs has been a controversy in the retinoid field. The catalytic domains of two RDHs have been shown to have a lumenal ER orientation using conventional experimental methods (Romert et al., 2000; Simon et al., 1999; Tryggvason et al., 2001). Two other laboratories have provided evidence for an opposing model, suggesting that the catalytic domain faces the cytosol. One of the arguments used for suggesting a cytosolic orientation of the catalytic domain of two RDHs, RoDH1 and RoDH4, was the lack of glycosylation (Lapshina et al., 2003; Wang et al., 2001a). A lack of glycosylation does not indicate a cytosolic orientation, since most, but not all, of the proteins located in the ER are glycosylated (Parodi, 2000). In fact, wild type RDH5 is not glycosylated, but after an addition of an ectopic glycosylation site to the protein, RDH5 was partially glycosylated (Romert et al., 2000; Simon et al., 1999). Lapshina et al. also argue that RoDH4 cannot be degraded by proteinase K. However, the little degradation observed in their report requires the presence of both Triton X-100 and proteinase K, suggesting a lumenal catalytic orientation (Lapshina et al., 2003).

There has been no convincing evidence for a cytosolic orientation of the RDHs. Therefore the identification of two microsomal RDHs with a catalytic domain located in the lumen of ER suggests that most if not all RDHs have a lumenal orientation. Given that these proteins probably are lumenal, the function of the cellular binding proteins might have to be re-evaluated, since the lumenal catalytic domain of RDHs are not available for the cytosolic holo-CRBP (or holo-CRALBP).

The development of the reporter assay has been a breakthrough in the development of analytical methods for studying RDHs. The reporter assay has been crucial for our experiments, and this thesis, because many of the experiments mentioned in Papers II, III and IV would have been impossible or difficult to conduct without the reporter assay. It is so far the only efficient analytical method that allows structure-functional analysis of RDHs in vivo. For example, the deletion of the C-terminal tails of CRAD1 and RDH5 had no effects on their enzyme activities in vitro, but
both mutants depleted of the cytoplasmic tails lacked activity in vivo (Paper II). In addition, a replacement of the CRAD1 cytosolic tail to a known ER retention signal, rescued the ER localization but not the enzymatic activity (Paper IV), suggesting an additional cellular role for the CRAD1 tail. These data suggest an in vivo role for the CRAD1 and RDH5 cytosolic tails that could not have been detected with traditional biochemical methods. These findings set a new perspective for RDH research. The experiments showed that in vivo enzyme activity does not need to correlate with in vitro data. Therefore more in vivo data needs to be gathered of the players involved in retinoid processing to eventually be able to understand the entire pathway including the regulatory mechanisms. The reporter system is probably not the only solution to these problems, even though it is not only restricted for RDH analysis. The reporter assay can also be used to screen for possible enzymes with all-trans retinol isomerizing activities, and after the isomerase activity has been identified, the uptake of retinoids can also be studied.
Acknowledgements

This book is a summary of the scientific part of my life for the past few years, a time that has been very interesting and fun in many other aspects as well. During this time, I have matured as a person and as a scientist. There are several people I would like to thank being involved in this special time of my life.

Professor Ulf Eriksson, my supervisor. I would like to express my deep gratitude to you for opening the doors of the Ludwig Institute for a young innocent student like me. I’ve been lucky to have a supervisor like you. You always had the time for us, even when you were busy, and your knowledge of science and everyday life astonishes me. I only hope that someday I will obtain such knowledge.

I would like to thank Professor Ralf Petterson, for providing such excellent facilities, and for creating and maintaining such a first-rate scientific atmosphere here at the Ludwig Institute.

It would have been boring to work all alone. I’ve had the pleasure of working with excellent retinoid researchers like Andras, Anna and Martin. Thank you Andras and Anna for sharing all your knowledge and helping me to a jump-start to retinoid biology.

But there are more people in the group, though they are usually more interested in growth factors than vitamins. Thanks to you, I’ve gained a lot of knowledge of growth factors and angiogenesis. Annica, Barbara, Carina, Christina, Erika, Hong, Karin, Linda, Xuri. You all made our group very special, and I’ve really liked working with all of you. We’ve had a ball, haven’t we LAB4?

We, the Ludwigians, have always had a strong positive WE feeling, always willing to help each other with scientific or non-scientific problems. Not forgetting the Ludwig parties, which have already become legendary. Thank you all the party people, Jonas, Fredrik, Jhansi, Etienne, Hanna, Elisabeth R. (+ others that are mentioned elsewhere... you know who you are). Ludwig is such a well-organized place to work thanks to Birgitta, Charlotta, Erika, Inger and Mats.

I would especially like to thank Diogo, Alex and Linda. Your friendship has meant a lot to me, both at work and elsewhere.

Stijn, I would like to express myself with more than words, but since this is a book, you’ll have to do with words. These five years have been an excellent time of my life, and I am glad I was able to share them with you. All those discussions about nothing and everything over lunches, beers, holidays. Tranan, Fasching, MF... One word dude, Stabil-Aii-z!
Sandra. thanks for lending your boyfriend for all those nights out, and for being such a lovely person. I am glad I’ve got to know you.

Richard, Shahab and Rutger. I’ve learned to know you a bit later, but thank god we did. We’ve had such a good time. I still think The Red Baps should have gotten a record contract. Let the good times continue!

The Gatheringboys + others in Finland, Andreas, Jake, Jani, Jukka A, Jukka P, Kipsi, Kaitsu, Matti, Olli and Taneli. Even if I’ve been here on the wrong side of the Baltic Sea you never abandoned me but instead always greeted me with open arms, when I’ve come back to visit my roots. The Gatherings at strange places are always amazing, and will be remembered. Too bad we can’t meet more often.

One big part of my post-graduate student period has been the involvement with The CMB Pub. Thanks to all of you in The Pub Crew. Thanks especially to MR, for dragging me along. Alistair, Maddis and all the rest of you for keeping the spirit up. I would also like to thank all of you who gathered there behind the bar for making the pubs lively and fun.

Thanks to the rest of the people I’ve interacted with. I like interacting. The Ericson lab has shared their time at various fun parties plus some rare serious occasions. I also have the pleasure to know many fun and interesting people at different institutes including CMB, CGB, MTC and MBB. Thanks for all the good times.

My Polish sofa and Taiwanese computer. Without you two this thesis would have never been finished. Go Mac!

I would like to thank my family for their constant support for everything I’ve done. I would also like to thank Mummu, Vaari and Amma, for always remembering me and giving me a place to stay whenever I pop by.

One especially amazing thing happened to me during my time as a post-graduate student. I met Ulrika. A lot of things have changed since then, but all for the better. Thanks for being there to be found. I cherish you with all my heart.

[Signature]
References


