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Enzymes and Binding Proteins Affecting Retinoic Acid Concentrations

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Free retinoids suffer promiscuous metabolism *in vitro*. Diverse enzymes are expressed in several subcellular fractions that are capable of converting free retinol (retinol not sequestered with specific binding proteins) into retinal or retinoic acid. If this were to occur *in vivo*, regulating the temporal–spatial concentrations of functionally-active retinoids, such as RA (retinoic acid), would be enigmatic. *In vivo*, however, retinoids occur bound to high-affinity, high-specificity binding proteins, including cellular retinol-binding protein, type I (CRBP) and cellular retinoic acid-binding protein, type I (CRABP). These binding proteins, members of the superfamily of lipid binding proteins, are expressed in concentrations that exceed those of their ligands. Considerable data favor a model pathway of RA biosynthesis and metabolism consisting of enzymes that recognize CRBP (apo and holo) and holo-CRABP as substrates and/or affecters of activity. This would restrict retinoid access to enzymes that recognize the appropriate binding protein, imparting specificity to RA homeostasis; preventing, e.g. opportunistic RA synthesis by alcohol dehydrogenases with broad substrate tolerances. An NADP-dependent microsomal retinol dehydrogenase (RDH) catalyzes the first reaction in this pathway. RDH recognizes CRBP as substrate by the dual criteria of enzyme kinetics and chemical crosslinking. A cDNA of RDH has been cloned, expressed and characterized as a short-chain alcohol dehydrogenase. Retinal generated in microsomes from holo-CRBP by RDH supports cytosolic RA synthesis by an NAD-dependent retinal dehydrogenase (RalDH). RalDH has been purified, characterized with respect to substrate specificity, and its cDNA has been cloned. CRABP is also important to modulating the steady-state concentrations of RA, through sequestering RA and facilitating its metabolism, because the complex CRABP/RA acts as a low K_m substrate.

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INTRODUCTION

Retinoic acid (RA) modulates gene expression during development and postnatally to control the differentiation state or entry into apoptosis of numerous cell types in many organs [1, 2]. It is reasonable to expect that the multi-loci biosynthesis of RA would be highly regulated and the rate of its turnover would be managed, to carefully orchestrate its temporal–spatial concentrations [3–5]. After all, it is not only the expression of the RA and RX receptors that mediate retinoid function, but also the state of their association with retinoids.

Although it has been accepted for decades that two successive reactions convert retinol into RA, with retinal as intermediate, progress in understanding regulation of RA biosynthesis has been hampered by lack of detailed knowledge of the enzymes involved. A major obstacle has been the proclivity of retinoids to serve as substrates *in vitro* for multiple enzymes, which has obscured focus on those few (?) that are most likely to be physiologically relevant. In liver, for example, multiple cytosolic and microsomal enzymes have the capability of catalyzing the oxidation of free retinol or retinal *in vitro*, especially when non-physiological concentrations of free retinoids are used as substrates [see discussion in ref. 5]. Interpreting such data is problematic, insofar as they do not necessarily identify significant pathways *in vivo*. Enzymes important to RA biosynthesis should recognize the predominant form of retinol *in vivo*.

The discovery of retinoid binding proteins unveiled a new dimension of retinoid biology [6–8], but their function(s) are not yet understood fully. Retinol in liver

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occurs bound in the protected environment of cellular retinol binding-protein (CRBP). CRBP consists of two perpendicular sets of five peptide anti-parallel β strands. The resulting flattened "β-clam" enfolds retinol in a high-affinity ($K_d \sim 0.1$–1 nM) binding pocket and isolates it from the cellular environment, with the hydroxyl group buried deeply in the protein [9, 10]. The concentration of CRBP exceeds that of retinol, ensuring maximum binding of the retinoid. CRBP may confer specificity on RA biosynthesis by restricting retinol access to enzymes capable of recognizing the retinol/retinol binding-protein "cassette". This would prevent opportunistic interaction of retinol with enzymes that do not recognize the protein outer layer of the "cassette", protect retinol from non-enzymatic oxidation, and protect cells from the membrane-disrupting potential of free retinol.

A pathway elucidated recently relies on a microsomal, NADP-dependent RDH that does recognize as substrate the "cassette" of retinol bound to CRBP [5, 11, 12]. The retinal generated in microsomes from holo-CRBP subsequently supports cytosolic RA synthesis by a NAD-dependent retinal dehydrogenase (RalDH); the retinal may be transferred from microsomes to cytosol by CRBP [13]. This pathway is depicted in Fig. 1, in context of a broader model of retinoid homeostasis, in which the ratio apo-CRBP/holo-CRBP determines the proportion of retinol channelled into retinyl esters vs that converted into RA. In this model, CRBP influences the amount of retinol sequestered from circulation [14]. The resulting holo-CRBP supports RA biosynthesis by serving as substrate (quantitatively minor pathway) or acts as substrate for retinol esterification catalyzed by lecithin:retinol acyl transferase, LRAT (quantitatively major relative to RA biosynthesis) [15, 16]. In the event of decreasing plasma retinol deficiency, hydrolysis of retinyl esters by cholate-independent retinyl ester hydrolase, REH (as opposed to the historically-studied, cholate-obligate esterase that hydrolyzes cholesterol and retinol esters and triacylglycerol, summarized in Fig. 2) mobilizes stored retinyl esters to maintain cellular retinol concentrations [17–19]. This occurs through the dual mechanisms of apo-CRBP inhibition of LRAT [20] and stimulation of retinyl ester hydrolysis by cholate-independent REH [21]. Apo-CRBP does not inhibit microsomal RDH, unless present in higher than usual physiological amounts. As a result, as cellular retinol concentrations decrease, the increase in apo-CRBP would promote recharging of CRBP, while sparing holo-CRBP for RA biosynthesis, by preventing its use as substrate for retinol esterification. RA biosynthesis would be sustained at the expense of storing retinol.

Fig. 1. Model of a pathway of RA biosynthesis and metabolism showing the relationships among enzymes and retinoid binding proteins.

Fig. 2. Characteristics of esterases that hydrolyze retinyl esters. The cholate-dependent esterase has a specific requirement for cholate, hydrolyzes cholesteryl esters (CE) and is inhibited by anti-cholesteryl esterase (CEase).

The rate of RA metabolism also affects its concentration available for activating the retinoid receptors. CRABP modulates the effectiveness of RA by sequestering the retinoid and forming a substrate, holo-CRABP, with a lower K_m than free RA [22]. Both actions decrease the concentration of free RA. Binding alone would cause a 50-fold decrease in free RA, i.e. calculating from a K_d of 1 nM with RA at 50 nM and CRABP at 100 nM.

Roles for retinoid-binding proteins in retinoid metabolism are consistent with related observations about their localization during embryogenesis. In the early mouse embryo, limb ectoderm expresses CRBP, whereas the underlying mesenchyma expresses CRABP [23, 24]. Ectoderm is most sensitive to vitamin A deficiency, indicating a requirement for RA, whereas mesenchyma is most sensitive to RA excess, indicating a protective role for CRABP. Thus, RA need and CRBP co-localize, whereas sensitivity to RA and CRABP co-localize, at least in this instance.

RETINOL DEHYDROGENATION

The first step in RA biogenesis entails dehydrogenation of retinol into retinal. CRBP imparts specificity

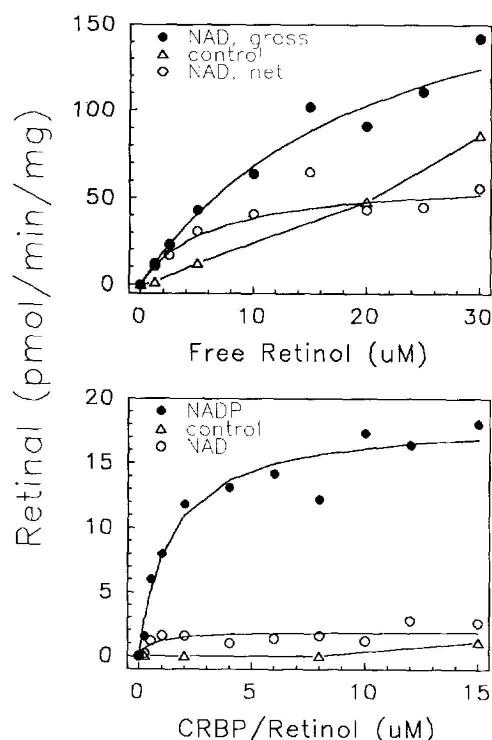

Fig. 3. Comparison of retinal synthesis in microsomes from free retinol vs retinol bound to CRBP. (Top panel) With free retinol as substrate, NAD (●) or NADP (not shown) support retinal synthesis and artifactual oxidation of retinol is high (△). Net or enzyme-catalyzed retinal synthesis (○) can be significantly lower than total synthesis, depending on the substrate concentration. (Bottom panel) With retinol bound to excess CRBP as substrate (1.4-fold molar ratio, to ensure complete binding), major retinal formation occurs only in the presence of NADP (●) with NAD largely restricted from interaction with retinol (○). Artifactual oxidation is essentially non-existent (△).

to this microsomal reaction by decreasing artifactual oxidation of retinol and preventing the NAD-catalyzed reaction, revealing that under physiological conditions, i.e. excess CRBP relative to retinol, an NADP-dependent enzyme catalyzes retinal production (Fig. 2) [11, 12]. This dehydrogenase recognizes only all-*trans*-retinoids and discriminates against 9-*cis*- and 13-*cis*-retinol. Although it recognizes free all-*trans*-retinol and 3,4-didehydroretinol equally well, it does not use CRBP-bound 3,4-didehydroretinol efficiently as substrate, indicating that not all CRBP–retinoid complexes can serve as efficient substrates for metabolism of their

Table 1. Cytosolic retinal dehydrogenases separated by anion-exchange chromatography

Rat tissue	Fraction (% total activity)			
	P1	P2a	P2b	P2c
Liver	67	17	—	16
Kidney	85	3	3	9
Testes	67	—	—	33

ligands. This suggests another potential mechanism whereby CRBP would influence retinoid signalling, *viz.* by influencing the nature of the RA synthesized. For example, in the adult human, conversion of retinol into 3,4-didehydroretinol and the latter into 3,4-didehydroretinoic acid occurs in keratinocytes [25], which express only low levels of CRBP [26]. A similar phenomenon of differential ligand used as substrate has been observed with CRABP and RA metabolites (see below).

To identify the polypeptide associated with the active site of RDH, a partially-purified rat liver microsomal extract was allowed to react with [^{125}I]phenylarsine oxide. This inhibitor of RDH binds with spatially-proximal sulfhydryl groups and forms covalent adducts [27]. Upon SDS–PAGE and autoradiography a single ^{125}I-band was observed at ~34 kDa, despite the presence of at least seven major polypeptides. Embodiment of the active site in this 34 kDa polypeptide was confirmed by allowing microsomes or a glycerol-extract of microsomes to react with CRBP bound to a peptide cross-linking reagent. CRBP was covalently labelled with a UV-activated, cleavable crosslinking reagent, radioiodinated such that the iodine would be transferred from CRBP to any target protein after activation and cleavage with dithiotreitol (Fig. 3). With this reagent, a dominant radiolabeled band was observed consistently in the presence of NADP. In the absence of cofactor this band was not observed and its intensity in the presence of NADPH or NAD was 31 and 25% of that observed with NADP, respectively. The dependence of crosslinking with holo-CRBP on cofactor indicates that holo-CRBP delivered its radiolabel to an enzyme that follows an ordered bisubstrate reaction mechanism, i.e. an NADP-dependent dehydrogenase.

The 34 kDa band was subjected to protein microsequencing and the data were used to design primers for RT-PCR using a rat liver cDNA library. A 1.8 kb RDH clone encoding a ~34 kDa polypeptide was isolated and expressed. The RDH has the predicted amino acid residues of a short-chain alcohol dehydrogenase (SCAD), especially the six amino acid residues conserved in 24 known SCAD [28]. RDH also had 16 of the 19 amino acid ressidues identical in at least 17 of the 24 SCAD. The putative SCAD cofactor binding site, G(X)$_3$GXG, and active site, Y(X)$_3$K, were present in the correct relative positions. The RDH has ~47% amino acid sequence similarity and 23% identity with rat 11β-dehydrogenase [29]. In addition, as with other SCAD such as the steroid 11β-dehydrogenase [30, 31], carbenoxolone, the steroidal aglycone of the licorice-derived glycyrrhizin, inhibits RDH activity in microsomes or expressed from the cDNA, but ethanol up to 860 mM does not.

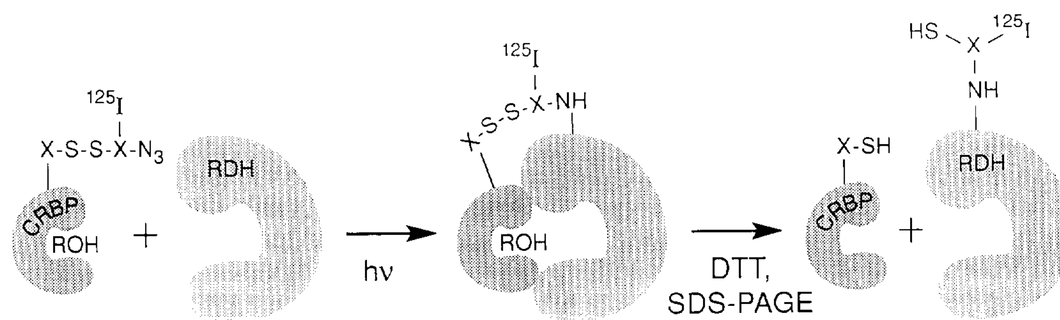

Fig. 4. Strategy to crosslinking RDH with CRBP. CRBP was covalently bound with a cleavable, iodinated, photoactivated protein crosslinker. Upon covalent binding of the derivatized CRBP and cleavage with dithiothreitol, the radioiodine was transferred to the target RDH. Crosslinking occurred only with holo-CRBP (not apo-CRBP) and required a pyridine nucleotide cofactor.

RA SYNTHESIS FROM RETINAL

At least four different cytosolic rat tissue dehydrogenases can be distinguished by anion-exchange chromatography (Table 1). P1, and at least two others P2a and P2c, respond to decreases in vitamin A nutriture with increases in activity. The major isozyme, P1, uses retinal generated in microsomes from holo-CRBP as substrate and seems to recognize retinal as substrate in the presence of excess CRBP [13]. P1 has been purified and its cDNA has been cloned. Its substrate specificity is interesting: in contrast with that of the RDH, which recognizes only all-*trans*-retinoids. P1 recognizes all-*trans*-retinal and 9-*cis*-retinal as substrates with V_{max}/K_m values of 2.1 and 1.5, respectively, but discriminates against 13-*cis*-retinal [32]. This specificity is not unusual. The RA receptors bind all-*trans*-RA and 9-*cis*-RA with equivalent affinity, but discriminate against 13-*cis*-RA [33–35]; this is also true of the epididymal RA binding protein [36].

Recognition of 9-*cis*-retinal may suggest a role for the RalDH in 9-*cis*-RA synthesis (Fig. 4). 9-*cis*-RA can be produced non-enzymatically from all-*trans*-RA, but no evidence supporting an enzyme-catalyzed isomerization has been reported [37]. 9-*cis*-RA could also derive from preformed 9-*cis*-retinoids. 9-*cis*-β-carotene is a natural product that occurs in human foodstuffs [38, 39] and dietary 9-*cis*-β-carotene causes accumulation of 9-*cis*-β-carotene and 9-*cis*-retinol in human and rat tissues [40, 41]. One function of 9-*cis*-retinal dehydrogenase activity could be to convert 9-*cis*-retinal, produced from dietary 9-*cis*-retinol, or from 9-*cis*-β-carotene, into 9-*cis*-RA, the endogenous ligand of RX receptors [34, 42]. 9-*cis*Retinol also may be produced from all-*trans*-retinyl esters by a pathway analogous to the one that converts all-*trans*-retinyl esters into 11-*cis*-retinol [43]. Potential mechanisms of *trans* to 11-*cis*-isomerization are compatible with 9-*cis*-isomer production [44].

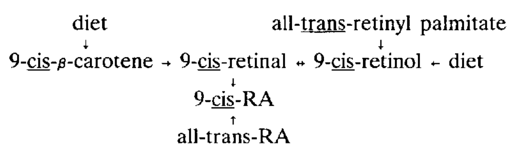

Fig. 5. Possible routes of 9-*cis*-RA biosynthesis.

RA METABOLISM

Metabolism of RA begins with oxidation of the β-ionone ring to produce hydroxy RAs (Fig. 5). The relative contributions of the two pathways depicted depends on the tissue, e.g. testis has the highest rate of 18-hydroxylation, but kidney shows no detectable 18-hydroxylation, and both tissues produce 4-hydroxy-RA and/or metabolites derived from 4-hydroxy-RA [45, 46].

Cloistering RA in CRABP, while permitting metabolism seems to operate throughout CRABP-expressing tissues as a mechanism of controlling the concentrations of free RA. *In vitro* CRABP serves as a low K_m substrate for RA metabolism (Table 2), revealing a mechanism for the relationship among RA potency, the rate of RA metabolism and the concentration of CRABP [22, 46–49]. The effect of CRABP on the rate of metabolism, however, is retinoid specific. 4-Hydroxy-RA also binds to CRABP, but when bound is metabolized slowly, if at all, compared to the elimination $t_{1/2}$ of 40 min of CRABP-bound RA [46]. Both RA and 4-hydroxy-RA, however, are rapidly metabolized in their free forms (elimination $T_{1/2}$ values *in vitro* of ~35–40 min). Arrest of 4-hydroxy-RA metabolism by CRABP shows that not all CRABP–retinoid complexes are recognized as substrates. This phenomenon, similar to that observed with CRBP and 3,4-didehydroretinol,

Table 2. CRABP influence on RA concentrations and function

1.	↑ elimination $t_{1/2}$ of RA, ↑ RA potency (↓ ED_{50})
2.	↑ CRABP expression, ↓ RA potency (↑ ED_{50})
3.	holi-CRABP is a low K_m substrate for RA metabolism (2 nm for holo-CRABP, with 7-fold ↑ V_m/K_m, vs 50 nM for free RA)
4.	↑ CRABP expression, ↑ RA metabolism

See text for references.

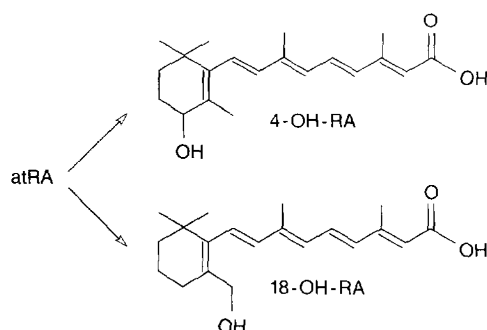

Fig. 6. Initial steps in RA metabolism. Tissue-dependent reactions convert RA into 4-hydroxy-RA or 18-hydroxy-RA, plus other metabolites. 4-Hydroxy-RA undergoes conversion into 4-oxo-RA; other metabolites also probably are derived from 4-hydroxy- and 18-hydroxy-RA.

is consistent with ligands affecting the function of retinoid-binding proteins by modifying the conformations of the binding proteins. Thus, retinoids other than retinol and RA may have their metabolism altered by the nature of their interactions with retinoid binding proteins. This may be important in determining which activated retinoids exert influence in specific loci.

CONCLUDING SUMMARY

The use of retinoid binding proteins as substrates in RA biosynthesis and metabolism prompted focus on relatively few enzymes that show promise as the most physiologically significant of the many possibilities presupposed by *in vitro* experiments with free retinoids (Table 3). The insight generated has supported a detailed, molecular approach to determining how RA homeostasis is maintained. Nevertheless much remains to be revealed about these pathways. Only preliminary studies have been made into regulation, e.g. [50].

Other related problems demand scrutiny, as well. For example, a cytosolic dehydrogenase has been reported that recognizes holo-CRBP as substrate, but is very sensitive to inhibition by apo-CRBP [5, 11, 51]. Seemingly, such an enzyme would function optimally only when CRBP and retinol concentrations are equivalent—a situation that does not seem to occur under normal circumstances. What would be the role of such a dehydrogenase in RA biosynthesis? The biosynthesis of 9-*cis*-RA also remains poorly understood. Does it derive solely from all-*trans*-RA or, as the substrate specificity of the RalDH implies, does it also derive from 9-*cis*-retinal, potentially available via several sources?

Table 3. Retinoic acid homeostasis

Synthesis	Catabolism
Holo-CRBP as substrate	Holo-CRABP as substrate
Microsomal, NADP-dependent RDH	Microsomal, NADPH-dependent
Cytosolic, NAD-dependent RALDH	*P*450s
Many tissues and cell lines	Many tissues and cell lines
NOT feedback regulated by physiological [RA], [apoCRABP], [holoCRABP]	Multiple, tissue-dependent pathways
RalDH induced by RA deficiency	Induced by RA excess
Depressed by PGE and TPA	Induced by xenobiotics (TCCD)

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