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Characterization of the microsomal and partially purified retinal reductase of rat small intestine

Sarah A. Wardlaw and David E. Ong*

Department of Biochemistry, Vanderbilt University, Nashville, Tennessee 37232-0146

A retinal reductase activity found in rat small intestinal mucosa was characterized as a partially purified preparation and as present in a preparation of microsomes. Stereochemical studies revealed that hydrogen was added to the re face of the aldehyde group during reduction of retinal by the microsomal activity, matching the stereochemistry of reduction by isolated intestinal segments. Retinal reduction by this enzyme was determined to be a sequential reaction, most likely with reduced nicotinamide adenine dinucleotide (NADH) binding first. The enzyme was specific for retinal and did not oxidize retinol to a measurable extent in the presence of cellular retinol-binding protein, type II (CRBP II). The presence of greater than stoichiometric amounts of CRBP II did not substantially alter the rate of retinal reduction, however. The Michaelis constants for both the bound and unbound forms of the substrate indicated that in the presence of binding protein the enzyme used bound retinal as the source of substrate, rather than the small amount of free retinal in equilibrium with the binding protein, indicating direct interaction between the enzyme and CRBP II-retinal. Competition experiments indicated increased recognition of the binding protein by the enzyme when the protein was in the "holo" form, with a preference for CRBP II over cellular retinol-binding protein (CRBP), a very similar binding protein found in other cell types. The properties determined strongly suggest this is the enzyme involved in the processing of β-carotene-derived retinal after uptake by the absorptive cell. (J. Nutr. Biochem. 7:222–229, 1996.)

Keywords: CRBP; CRBP II; reductase; retinaldehyde; retinol; vitamin A

Introduction

The retinoid family of compounds serve vital roles within the body where 11-*cis*-retinal is the chromophore of the visual pigments and all-*trans*- and 9-*cis*-retinoic acid function as potent regulators of gene transcription. Other retinoids may also play important roles. All such retinoids are derived from dietary sources, either the long chain acyl esters of retinol ("vitamin A") found in some animal-derived foods or provitamin A carotenes such as β-carotene present in many plants. Consequently, there is interest in the mechanism by which this essential nutrient is obtained from the diet and processed by the intestinal absorptive cell before delivery throughout the body.

The overall steps of the process have been well defined.[1] Dietary retinyl esters are hydrolyzed in the lumen of the intestine by either pancreatic lipases or an intrinsic brush border lipase.[2] The liberated retinol is then internalized by a specific transporter of the absorptive cell.[3] β-Carotene enters the cell as such, with no evidence as yet for a specific uptake mechanism. The β-carotene is then oxidatively cleaved to produce all-*trans*-retinal in a reaction or series of reactions that still require clarification.[4,5] The retinal is then reduced to retinol. Retinol from either source is esterified with long chain fatty acids and incorporated into chylomicrons for release from the cell. Although these steps are clear, the enzymes responsible for the necessary metabolism described above are, in some cases, not yet definitively determined or characterized.

The discovery of cellular retinal-binding protein, type II (CRBP II), expressed primarily, if not solely, in the intestinal absorptive cell of the adult animal,[6] prompted reexamination of several of the metabolic steps. CRBP II is an abundant protein, comprising approximately 1% of the cytosolic protein of intestinal mucosa. This protein binds both retinol and retinal with high affinity ($K_d \approx 10$ nM). As a

Address correspondence to Dr. David Ong, Professor of Biochemistry, School of Medicine, Vanderbilt University, Nashville, TN 37232-0146
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consequence the vast majority of the retinol and retinal present in the absorptive cell would be expected to be bound by this protein. A microsomal acyl-CoA-dependent enzyme activity (ARAT) thought to be responsible for retinol esterification in the absorptive cell[7] was found to be unable to esterify retinol when provided bound to CRBP II.[8] This led to the discovery of a microsomal lecithin-retinol acyltransferase (LRAT) activity in the intestinal mucosa, as well as other tissues, that would effectively esterify retinol provided bound to either CRBP II or the other intracellular retinol binding protein, CRBP.[8,9] Work with cultured cells, the Caco-2 cell as a model for the absorptive cell and the Sertoli cell of the testis, has shown that inhibition of LRAT in the intact cell by phenylmethylsulfonyl fluoride blocks retinol esterification, yet ARAT is not inactivated by this compound.[10,11] This, coupled with the sequestering of retinol from ARAT but not LRAT by CRBP II, has strongly supported LRAT as the physiologically important enzyme for retinol esterification.

Similarly, the enzyme activity originally thought to be responsible for the reduction of β-carotene-derived retinal[12] was found to be ineffective at reducing retinal when the retinal was provided bound to CRBP II.[13] However, a microsomal activity was discovered that would carry out the reduction of retinal in the presence of CRBP II.[13] Here we have examined this enzyme more extensively, both as initially detected in the microsomes and as a solubilized, partially purified preparation. All properties are consistent with it being the sole enzyme responsible for retinal reduction in the absorptive cell.

Methods and materials

Preparation of microsomes and partially purified enzyme

Male, Sprague-Dawley rats purchased from Sasco, Inc. and weighing approximately 300 g were killed by CO_2 inhalation. The proximal ⅔ of the small intestine was removed and all traces of mesentery tissue stripped away. The lumen of the intestine was flushed with 50 ml of cold saline. All subsequent steps were carried out at 4°C. The intestine was cut open longitudinally and washed in saline containing 1 mM DFP for approximately 10 sec. The intestine was then rinsed free of the DFP in fresh saline. The mucosa was scraped from the muscle using a glass microscope slide and placed in a 4× volume (w/v) of saline containing 0.1 mM PMSF and stirred vigorously. The mucosal cells were pelleted by centrifugation at 100 × g for 15 min. The mucosal cells were resuspended in 50 mM potassium phosphate, pH 7.2, containing 1 mM DTT, 0.1 mM EDTA, 0.1 mM NADH, 0.1 mM PMSF (Buffer A) to the original volume and homogenized with a Brinkmann Polytron PT 3000 homogenizer set at 12,000 rpm and operated for 30 sec. Large cellular components were pelleted by centrifugation at 20,000 × g for 15 min and the supernatant liquid reserved. The pellet was resuspended in Buffer A, homogenized as before, and centrifuged again at 20,000 × g. The supernatant liquids from both centrifugations were pooled and poured through three layers of cheesecloth to remove traces of fat and then centrifuged at 100,000 × g for 1 h to pellet the microsomes. The pellets were washed gently with Buffer A and then suspended in a small volume (approximately 1 ml buffer/rat) of Buffer A.

The microsomes were diluted with Buffer A and Triton X-100 (Pierce, protein grade, purchased as a 10% aqueous solution) such that the protein concentration was 5 mg/ml and the detergent concentration was 1%. The solution was stirred gently for 1 hour at 4°C and then centrifuged at 100,000 × g for 1 h. The supernatant liquid was decanted and glycerol was added to a final concentration of 20% (v/v).

A 1.6 cm diameter column was packed with phenyl Sepharose CL-4B media (Pharmacia) to a volume 1.5 times the volume of the solubilized microsomal protein solution. The column was equilibrated with Buffer A that had been diluted with glycerol to a final concentration of 20% (Buffer B). A concentrated solution of CHAPS (Pierce) was added drop-wise to the solubilized microsomes, while stirring, to a final concentration of 0.4% (w/v). Stirring was continued at 4°C for at least 15 min before loading the material onto the column. After loading, the column was washed with one column volume of Buffer B. Bound proteins were then eluted with approximately 8 column volumes of Buffer B containing 0.5% Triton. The flow-rate throughout was 1.0 ml/min.

Preparation of binding proteins and ^{3}H-labeled retinoids

Recombinant CRBP was purified as previously described.[14] Recombinant CRBP II was purified after expression in *Escherichia coli* using similar methods.[15]

[^{3}H]-Retinal was prepared by MnO_2 oxidation of [^{3}H]-retinol[16] labeled at the C-15 position. The resulting [^{3}H]-retinal was separated from any residual [^{3}H]-retinol on an aluminium oxide column as described below (see "Assay of Retinal Reduction") and stored in hexane with 0.1 mg BHT/ml hexane at −70°C.

[^{3}H]-Retinol was prepared by the reduction of unlabeled retinal (Sigma Chemical Company) with NaB[^{3}H]$_4$ (American Radiolabeled Chemicals, Inc.)[17], resulting in [^{3}H]-retinol labeled at the C-15 position. Residual retinal was reduced with an excess of unlabeled $NaBH_4$. The [^{3}H]-retinol was stored in the same manner as [^{3}H]-retinal.

Assay of retinal reduction

The enzyme reaction was carried out in a 13 × 100 mm borosilicate culture tube in 0.1 M imidazole acetate, pH 6.0. Approximately 10 μg of microsomal protein were used in a total reaction volume of 0.1 ml. NADH or NADPH was added to a final concentration of 60 μM. The reaction was begun with the addition of [^{3}H]-retinal such that the final concentration was 3 μM. Free [^{3}H]-retinal was dissolved in DMSO before its addition to the reaction mixture. Bound [^{3}H]-retinal was prepared by adding [^{3}H]-retinal (in DMSO) to a 20% molar excess of CRBP or CRBP II. All retinoid-containing solutions were handled under yellow light. The reaction mixture was incubated in a 37°C water bath for 10 min. The reaction was stopped by the addition of 0.4 ml of cold ethanol containing 0.1 mg BHT/ml ethanol. The addition of 0.5 ml of water and 2 ml of hexane/BHT was followed by approximately 15 sec of vortex mixing. A portion of the hexane layer (1 ml) was then applied to a glass column containing approximately 1 g of aluminum oxide deactivated with 0.1 ml water/g of alumina. Unreacted [^{3}H]-retinal was eluted with 3 × 2 ml of 15% (v/v) diethyl ether in hexane. [^{3}H]-Retinol was then eluted with 3 × 2 ml of 50% diethyl ether in hexane, and the eluant was collected directly into a scintillation vial. After evaporation of the ether/hexane under a stream of nitrogen gas, scintillation fluid was added and the [^{3}H]-retinol was quantitated by means of a scintillation counter.

Assay of retinol oxidation

The assay was carried out as described for the reduction of retinal except the enzyme reaction was carried out in 0.1 M HEPES, pH 8.0 in the presence of 750 μM NAD^+. The reaction was begun

with the addition of [^{3}H]-retinol to a final concentration of 25 μM. Approximately 1 μg of partially purified protein was used in the assay. After application to an aluminium oxide column, the [^{3}H]-retinal was eluted and the radioactivity quantitated.

Alcohol dehydrogenase oxidation/reduction reactions

Equine liver alcohol dehydrogenase (Sigma Chemical Co., 1.5 units/mg) was used to reduce retinal by incubating 50 μg of enzyme/ml reaction volume with 100 μM NADH and 6 μM [^{3}H]-retinol for 10 min at 37°C in pH 6 buffer. The reaction was stopped and the retinal isolated as described above for the assay of retinal reduction. The 50% diethyl ether eluant was dried down under nitrogen, and the retinol-containing residue was dissolved in DMSO and stored at 4°C until needed.

Alcohol dehydrogenase was also used for the oxidation of retinol. 50 μg enzyme/ml reaction volume was incubated with 200 μM NAD^+ and varying amounts of [^{3}H]-retinol for 10 minutes at 37°C in pH 8 buffer. The resulting [^{3}H]-retinal was isolated and counted as described for the assay of retinal reduction.

Retinal reduction by segments of rat small intestine

Adult, male Sprague-Dawley rats were killed by CO_2 inhalation, and the upper half of the small intestine removed and flushed with saline. The upper intestine was cut into four equal segments that were slit open and rinsed vigorously in saline. Each segment was then divided into four more segments that were incubated in Krebs-Ringer phosphate buffer containing 0.5% (w/v) taurocholate and 0.22 μM [^{3}H]-retinal for 10 min at 37°C while shaking at 120 oscillations/min. The uptake of retinal was stopped by removal of the segments from the incubation buffer, followed by rinsing in cold buffer lacking retinal. The segments were stored on ice until homogenized and the lipophilic compounds extracted into hexane as described by Pappas et al.[18] Diethyl ether was added to the hexane extract to a final concentration of 2% (v/v). At this concentration of ether, retinyl esters flowed through an aluminum oxide column as described (see "Assay of Retinal Reduction"). This ester-containing flow-through was collected and taken to dryness under nitrogen. The esters were then taken up in pH 8 buffer containing 4% (w/v) cholate. Cholesteryl ester hydrolase was added to a final concentration of 10 mg/ml and a final detergent concentration of 3%. This mixture was incubated for 20 min at 37°C. The retinoids were then extracted into hexane and the retinol separated on an aluminium oxide column as described for the assay of retinal reduction. The stereochemistry of the retinol was then determined by oxidizing the retinol with alcohol dehydrogenase as described above.

Results and discussion

Solubilization and partial purification of the microsomal retinal reductase

Whether the properties of an integral membrane enzyme are best determined using a preparation of microsomes or after solubilization and purification is a complex issue, particularly when one wishes to relate *in vitro* results to physiological function *in vivo*. In this instance a test of 11 different detergents produced only one (Triton X-100), which reproducibly solubilized the majority of the reductase activity associated with the microsomes. The number of detergents that only partially solubilized the activity indicated that the reductase is an integral membrane protein dependent on its surrounding lipid bilayer for proper function. The fact that the enzyme was found to be much less stable after solubilization suggested that the environment of the reductase would be important for correctly determining its physiological properties.

Enzyme activity was stable after the fractionation of the intestinal mucosal cells and the isolation of microsomes for at least 4 months when stored at −70°C. The activity of the solubilized enzyme was found to be equally stable upon storage under the same conditions, but quite labile when purification procedures where attempted. The addition of a second detergent, and the consequent creation of "mixed" micelles, which might more closely resemble the native membrane environment of the enzyme, did not increase enzyme stability. In fact, the addition of octyl glucoside, SDS, or deoxycholate decreased enzyme activity significantly (data not shown). Enzyme stability was increased, however, by the presence of substrate. The loss of activity brought about by incubation of microsomes at 37°C for 5 min was reduced by 40% with the addition of retinaldehyde. More significantly, incubation in the presence of NADH resulted in no loss of activity over this time period. Such stabilization by the cofactor is most likely responsible for the maintenance of a constant reaction velocity over time during routine assays of the solubilized activity, which were generally carried out for 10 min at 37°C. NADPH and NAD^+ were not as effective as NADH at maintaining activity (see *Figure 1*).

The solubilized, partially-purified material used in these studies was generated by chromatography on phenyl Sepharose media. When passed over a phenyl Sepharose column in the presence of an ionic detergent, greater than 50% of the solubilized proteins were not retained on this support, while all reductase activity bound. The reductase activity could only be eluted by saturating the column with nonionic detergent, which effectively cleared the column of all bound protein. The specific activity of the phenyl Sepharose eluant was approximately the same as that of the microsomes themselves (approx. 300 pmoles retinol produced/min/mg protein). However, SDS-PAGE analysis of the bound and

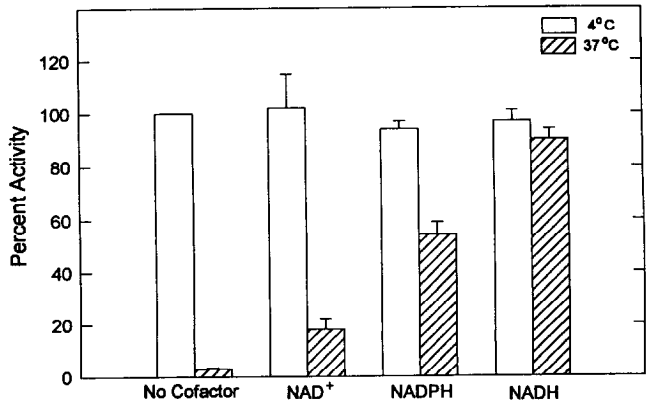

Figure 1 Enhanced enzyme stability in the presence of cofactor. Microsomes were incubated for 10 min at 4°C or 37°C in the presence or absence of cofactor; cofactor concentration = 100 μM. Substrate was then added and the assay carried out as described under "Results and Discussion." The activity is expressed as a percentage of the activity after incubation at 4°C without cofactor (mean of two data points ± range).

nonretained material showed the separation of a significant number of proteins from the reductase, although the remaining protein bands were numerous (data not shown). This solubilized, partially purified activity proved to be unstable to further purification attempts, but was useful for subsequent comparison to activity as expressed in its normal membrane environment. Qualitatively, all important properties of the enzyme activity were retained by this partially purified preparation.

Substrate specificity of the reductase

The specificity of the enzyme for both retinal and reduced nucleotide was determined by retinal reduction in the presence of various analogs of both substrates (see *Table 1*). The resulting activities, expressed as a percentage of activity in the absence of competitor, indicated that the reductase was quite specific for retinal. The compounds tested as inhibitors shared certain structural properties with retinal. β-Ionone contains the same ring structure as retinal, although with a short side chain having a methyl ketone end-group rather than an aldehyde end-group. 3-Methyl-2-butenal, decanal, and tetradecanal are aldehydes of increasing chain length, with tetradecanal being of a length similar to that of retinal. Anhydroretinol contains a methylene end group with a configuration similar to that of an aldehyde group. None of these compounds significantly decreased reduction of retinal by the enzyme, even when present at an approximately 10-fold concentration over that of retinal. These compounds also did not compete with retinal for binding to CRBP II, so were not tested with retinal-CRBP II as substrate. Two forms of retinoic acid, as well as a synthetic retinoid, N-(4-hydroxyphenyl)retinamide, were also tested at similar and higher levels and also found not to compete with retinaldehyde for reduction.[15]

Reductase activity was decreased by several of the cofactor-analogs tested. The most effective compounds were the dyes Cibacron Blue and Procion Red, which are known to interact with the cofactor binding sites of enzymes that use NADH or NADPH. This competition for binding also resulted in the inactivation of the enzyme, because activity could not be recovered by the subsequent addition of a 1,000-fold excess of NADH. (Inactivation required prolonged exposure; significant inactivation was seen only after 30 min of incubation.) This inactivation explained the loss of enzyme activity observed during the testing of these dyes as possible affinity ligands for the purification of the enzyme (data not shown). AMP and ADP also had the ability to compete for cofactor binding, particularly when that cofactor was NADPH, but these too were not found to be effective as affinity matrices. Interestingly, NAD^+ was an effective competitor of NADH binding, yet the other reaction product, retinol, had absolutely no affect on retinal reduction (see *Table 3*), a property that may be due to the order in which the substrates bind.

Order of substrate binding

Retinol production by the microsomal reductase activity was measured over a range of increasing, but nonsaturating, concentrations of retinal, at several constant (also nonsaturating) concentrations of NADH (*Figure 2A*). When both variables were plotted as their inverse, the intersection of the resulting lines (rather than their being parallel to one another) indicated that retinal reduction by this enzyme is a sequential reaction, rather than a nonsequential reaction, i.e. both retinal and NADH bind to the enzyme to form a ternary complex before the formation of either product occurs.

Retinol production was then measured over a range of increasing concentrations of NADH (*Figure 2B*). The concentration of retinal remained constant throughout, but several concentrations of a competitor of NADH binding were used. When both variables were plotted as their inverse, the intersection of the resulting lines at the *Y*-axis (rather than to the left of the *Y*-axis) indicated a purely competitive type of inhibition, rather than a "mixed" inhibition. Under these conditions competitive inhibition could be the result of a reaction sequence requiring the initial binding of NADH before binding of retinal can occur. Competitive inhibition could also result from a reaction sequence in which no

Table 1 The effect of various substrates and substrate analogs on reductase activity

Competitor	Substrate	Source	Molar ratio of competitor to substrate	Percent activity
β-ionone	[^{3}H]Retinal	M[a]	9	109 ± 2[b]
3-methyl-2-butenal	"	P	12	94 ± 4
Decanal	"	P	10	79 ± 2
Tetradecanal	"	P	10	84 ± 10
Anhydroretinol	"	P	10	99 ± 7
NAD^+	NADH	M	3	41 ± 5
Cibacron blue	NADH	M	2.2	5.4 ± 0.02
	NADPH			0
Procion red	"	M	2.2	6.0 ± 2.1
				0
Adenosine 5′monophosphate-	"	M	3.3	98 ± 7
				71 ± 4
Adenosine 2′,5′-diphosphate	"	M	3.3	101 ± 6
				39 ± 1

[a] *P* = partially purified activity; M = microsomal activity.
[b] The activity is expressed as a percentage of the activity in the absence of competitor ± the standard deviation (n = 2).

Table 2 Michaelis constants for free and bound retinal with NADH and NADPH as cofactor

Variable substrate	Saturating substrate	K_M (μM) Microsomal activity	K_M (μM) Partially purified activity
Retinal	NADH	1.18 ± 0.25[a] (5)	0.53 ± 0.01 (2)
Retinal	NADPH	0.96 ± 0.14 (3)	0.42 ± 0.01 (2)
CRBP-retinal	NADH	0.56 ± 0.01 (2)	0.44 ± 0.04 (2)
CRBP-retinal	NADPH	0.68 ± 0.07 (2)	0.61 ± 0.10 (2)
CRBP II-retinal	NADH	0.42 ± 0.02 (2)	0.38 ± 0.05 (3)
CRBP II-retinal	NADPH	1.83 ± 0.08 (2)	0.91 ± 0.16 (4)

[a]±SEM; (n).

particular order of substrate binding is necessary. Differentiating between these two mechanisms requires the use of a competitor of binding of the other substrate, in this case retinal. As mentioned previously no compounds tested were effective competitors for retinal binding, preventing further analysis. However, the failure of retinol to inhibit reduction, in contrast to the inhibition seen with the other product NAD^+, suggested product release may be sequential, with retinol leaving first.

Stereochemistry of retinal reduction by the microsomal reductase and by intestinal gut sheets

Evidence supporting the microsomal reductase as the true physiological enzyme would be obtained if the stereochemistry of reduction by the microsomal activity was shown to be identical to the stereochemistry of retinal reduction in the intact cell. Consequently, the stereochemistry was determined by comparing product retinol obtained from different sources. Advantage was taken of the ability of alcohol dehydrogenase to reduce retinal and oxidize retinol in a stereospecific manner. Because alcohol dehydrogenase adds a hydrogen to the *re* face of an aldehyde group during reduction, retinal with a tritiated aldehyde-hydrogen, when used as a substrate for alcohol dehydrogenase, was reduced to retinol labeled solely at the pro-S position. As a control

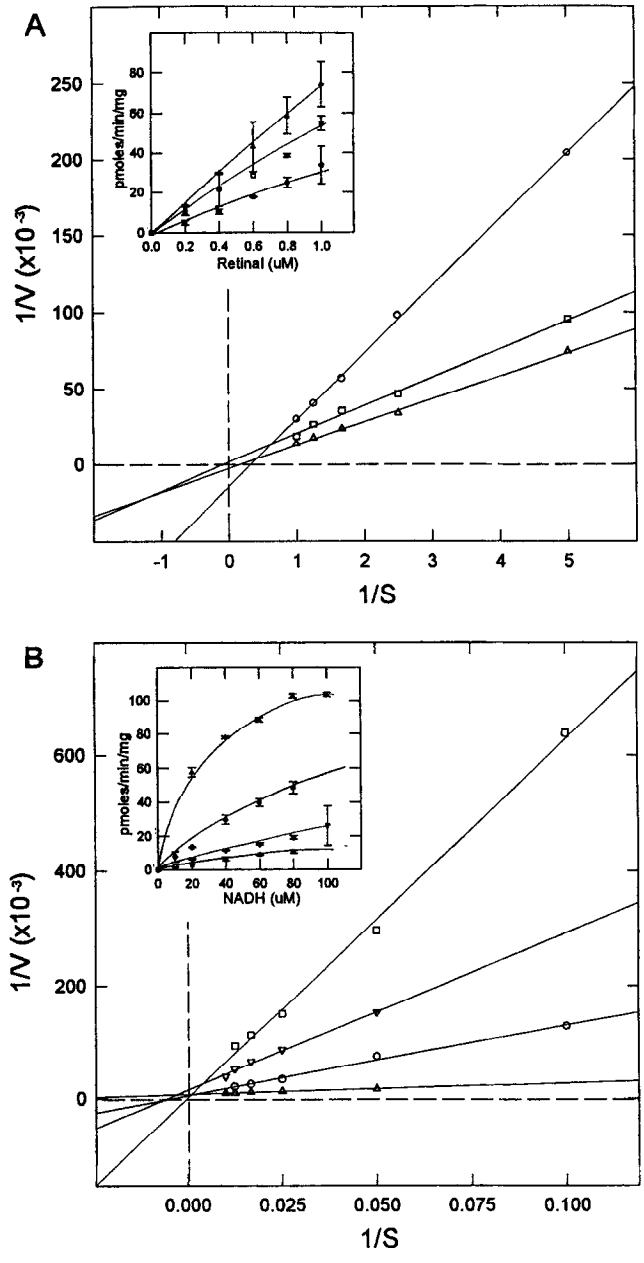

Figure 2 Retinal reduction is a sequential reaction; random-ordered or ordered with NADH binding first. (A) Retinol production as a function of retinal concentration in the presence of ○5, □15, △25 μM NADH (mean of duplicate data points ± range) (inset graph). The intersection of the lines when plotted as a double reciprocal plot indicates a sequential reaction. (B) Retinol production as a function of NADH concentration in the presence of △25, ○50, ▽75, □100 μM Cibacron Blue (mean of duplicate data points ± range); retinal concentration = 1 μM (inset graph). The intersection of the lines at the *Y*-axis when plotted as a double-reciprocal plot indicates a random-ordered or ordered reaction with NADH binding first.

Table 3 The effect of binding proteins and retinol on the reduction of CRBP II-[^{3}H]retinal

Competitor[a]	Microsomal activity (%)	Partially purified activity (%)
apo-CRBP	96 ± 2[b] (4)	78 ± 5 (3)
apo-CRBP II	83 ± 4 (4)	54 ± 2 (3)
Retinol	102 ± 2 (3)	102 ± 11 (7)
CRBP-retinol	101 ± 3 (3)	93 ± 5 (7)
CRBP II-retinol	63 ± 6 (3)	44 ± 4 (3)

[a]Present in a 6:1 ratio over substrate; NADH used as cofactor.
[b]The activity is expressed as a percentage of the activity in the absence of competitor ± the SEM; (*n*).

experiment this retinol was then oxidized to retinal by the alcohol dehydrogenase. As expected, labeled retinal was obtained once again, along with unlabeled NADH (*see Figure 3*).

A second control experiment was conducted by using retinol prepared by the chemical reduction of retinal with $NaB[^3H]_4$ and, therefore, labeled at both the pro-S and pro-R positions. When this retinol was oxidized by alcohol dehydrogenase, both the retinal and the NADH were labeled with tritium, as would be expected with a substrate in which the tritium was not in a stereospecific position.

The stereochemistry of retinal reduction by the microsomal reductase activity was then determined. As above, retinal with a tritiated aldehyde-hydrogen was reduced to retinol by the microsomal reductase. This retinol was then isolated and used as a substrate for oxidation by alcohol dehydrogenase. Labeled retinal was obtained, along with unlabeled NADH, indicating that the reduction of retinal by the microsomal reductase involves the addition of a hydrogen to the *re* face of the aldehyde group in the same manner as that of alcohol dehydrogenase.

This stereochemistry was compared to that of retinal reduction by everted intestinal segments. Small sheets of small intestine from adult rats were incubated at 37°C in an isotonic buffer containing [^{3}H]-retinal and a bile salt for 10 min. Most of the radioactivity recovered from the tissue was retinyl ester, indicating not only that reduction had occurred but that there was a relatively rapid shuttling of the labeled retinol from the reductase to LRAT for esterification. The isolated radioactive retinyl esters were hydrolysed to retinol by cholesteryl ester hydrolase and the recovered labeled retinol used as a substrate for alcohol dehydrogenase. The same distribution of tritium between the reaction products was obtained as had been observed for the retinol produced by the microsomal reductase activity, supporting the proposed role of this enzyme *in vivo* in metabolizing vitamin A after its uptake by the absorptive cell.

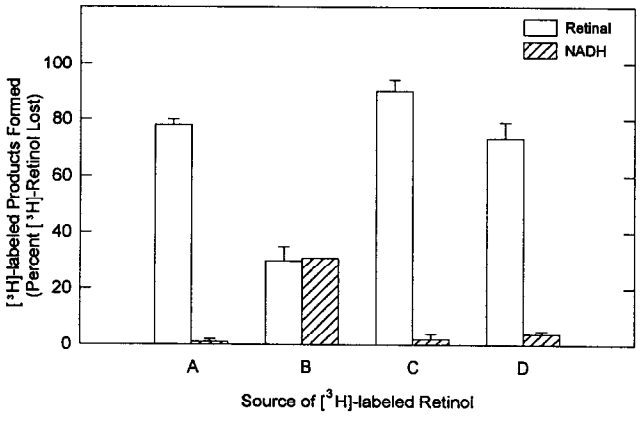

Figure 3 The stereochemistry of retinal reduction by the microsomal activity is the same as that of inverted intestinal segments. The location of the radiolabel after oxidation of [^{3}H]-retinol by liver alcohol dehydrogenase is expressed as a percentage of labeled retinol lost (mean of duplicate data points ± range). The source of the [^{3}H]-retinol: (A) reduction of [^{3}H]-retinal by alcohol dehydrogenase (B) reduction of retinal with $NaB[^3H]_4$ C) reduction of [^{3}H]-retinal by the microsomal reductase activity D) reduction of [^{3}H]-retinal by inverted intestinal segments. Retinal reduction and retinol oxidation were carried out as described under "Methods and materials."

Interaction of retinal reductase with substrates in the presence/absence of cellular retinol-binding proteins

Due to the abundance of CRBP II in the absorptive cell of the small intestine, and its high affinity for both retinol and retinal, the nature of the initial interaction of substrate with the reductase was investigated. The reductase is able to reduce retinal in the presence or absence of binding protein. However, in the presence of binding protein, it is possible that the only interaction is with the small amount of free retinal in equilibrium with that bound to the binding protein. The function of the binding protein, in this case, might simply be to maintain a low level of free retinal in the cell, thereby directing reduction to the microsomal reductase activity with its apparent K_M of 1 μM, rather than to other reductase activities such as the cytosolic activity described by Fidge and Goodman in 1968[12] with much higher K_M values for retinal.

To clarify the reaction mechanism reductase activity was measured in the presence of an increasing concentration of binding protein (see *Figure 4A*). As the amount of binding protein increased, the concentration of free retinal would be expected to decrease, until at a 4:1 ratio of binding protein over retinal, the concentration of free retinal would be calculated to be only 0.2% of starting levels. If the enzyme uses only the free retinal as substrate, its activity would have been expected to decline dramatically as the level of free retinal decreased. This is exactly what was seen for the relatively nonspecific cytosolic retinal reductase activity described by Fidge and Goodman[12] and thought to be responsible for retinal reduction in the absorptive cell (*Figure 4A*). Clearly, this activity was unable to obtain the bound retinal for use as a substrate and almost certainly would not contribute to retinal reduction *in vivo,* unless the level of retinal exceeded the binding capacity of the CRBP II present. The microsomal activity, however, maintained a high rate of reduction in the presence of increasing amounts of binding protein. In fact, this activity increased slightly upon initial introduction of the binding protein, perhaps reflecting better recognition of the complex than the free retinal, with a subsequent transfer of the retinal to the enzyme active site.

The apparent K_Ms determined for the free and bound forms of retinal also do not support the exclusive use of free retinal as substrate. As shown in *Table 2,* when NADH was the cofactor, the apparent K_M for bound retinal was lower than that for free. The K_d for the binding of retinal by CRBP II is approximately 10 nM. At the apparent K_M concentration of 0.42 μM for microsomes, the concentration of free retinal would be approximately 0.06 μM. However, the apparent K_M for free retinal was not 0.06 μM, but was, in fact, 20 times greater, suggesting the direct and preferred use of CRBP II-retinal as a source of substrate. Similar numbers were obtained for CRBP, a protein apparently not present in the absorptive cell, but of interest due to its structural similarity to CRBP II and its similar affinity for retinal. The similar K_Ms for CRBP-retinal and CRBP II-retinal indicated a similar recognition by the enzyme of these two complexes.

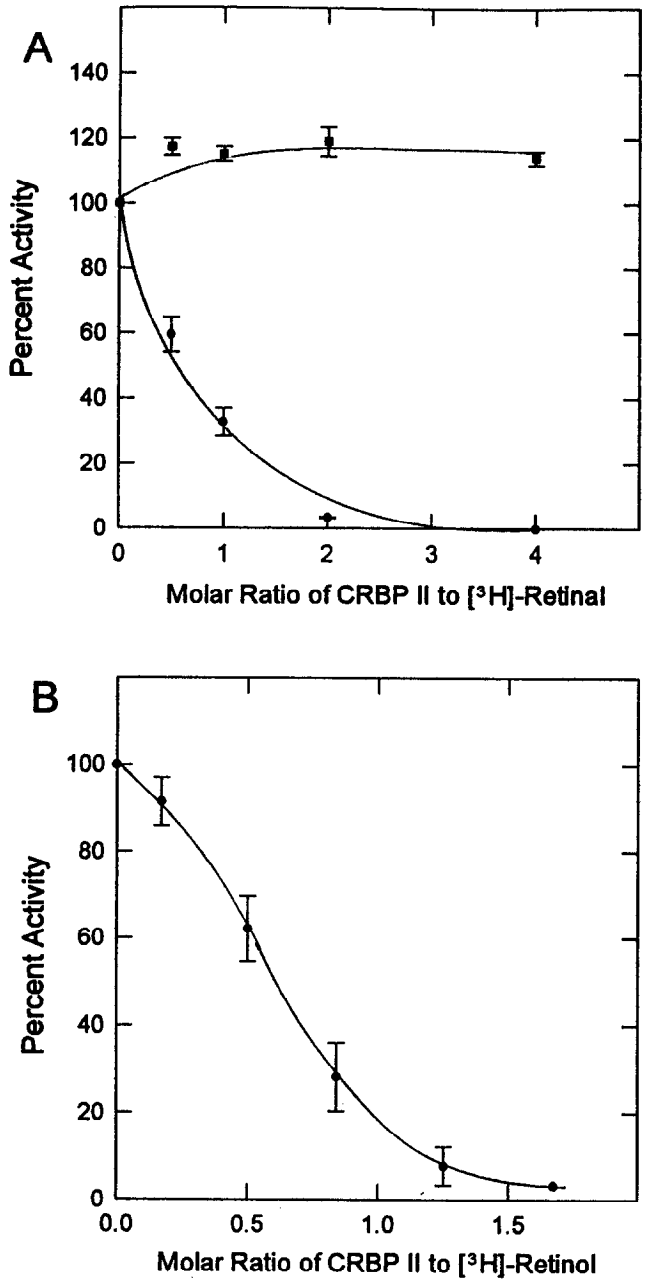

Figure 4 (A) The effect of an increasing concentration of CRBP II on the reduction of [^{3}H]-retinal by the cytosolic and microsomal activities. The activity is expressed as a percentage of activity in the absence of binding protein (mean of two experiments ± range; duplicate data points for each experiment). Equivalent amounts of cytosolic (●) and microsomal (■) protein were assayed. Cytosol was obtained as a by-product of microsome preparation. (B) The effect of an increasing concentration of CRBP II on the oxidation of [^{3}H]-retinol by the partially purified reductase activity. The activity is expressed as a percentage of oxidation in the absence of binding protein ± the standard deviation (n = 3). Oxidation was measured as described under "Methods and materials."

However, with CRBP II there was a statistically significant difference in apparent K_M depending on cofactor used, with a marked decrease in K_M for CRBP II-retinal when NADH was the cofactor. It would appear that the enzyme's interaction with CRBP II-retinal was altered by NADPH in a way that does not occur with CRBP, perhaps as a result of an alteration in the conformational change induced by cofactor binding like that thought to increase the affinity of alcohol dehydrogenase and other enzymes of this type for their second substrate.[19,20] The same pattern of change in apparent K_M values occurred with partially purified material, although all K_M values were lower than with microsomes, perhaps due to the change in the enzyme's lipid environment after solubilization.

Although CRBP II was shown previously not to inhibit reduction of retinal by the microsomal activity (*Figure 4A*), higher levels of binding protein than were tested previously did have some effect (see *Table 3*). At these higher levels, CRBP continued to have no effect on reductase activity, either alone or when binding retinol, although as demonstrated in *Table 2* the reductase appeared to recognize CRBP- and CRBP II-retinal equally well. Retinol alone, when present at a 6-fold higher concentration than that of CRBP II-retinal, also had no affect on reduction. High levels of CRBP II, however, did decrease the rate of reaction, particularly when binding retinol. Such a decrease is perhaps due to a conformational change in the binding protein upon binding of retinol, since retinol alone has no affect on reduction. Structural differences between the apo- and holoforms of the protein in solution have been observed.[14,21] The reductase not only appeared to recognize holo-CRBP II better than apo-CRBP II, but also differentially recognized CRBP and CRBP II. Rat CRBP and CRBP II are the products of two separate, but closely linked genes on the same chromosome. They have a 57% sequence identity and structurally are even more similar. Critical differences in one or more amino acids within their binding sites are thought to be responsible for their different affinities for retinol. Differential recognition of these two proteins by the reductase suggests critical differences on their surfaces as well.

Retinol oxidation

Enzymes of this type (e.g. alcohol dehydrogenase, ocular retinol dehydrogenases), although functioning predominantly in one direction *in vivo,* will often efficiently carry out the reverse reaction *in vitro.* An inability by the intestinal retinal reductase to oxidize retinol would set it apart from these enzymes, and yet would be consistent with a specialized role in the one-way conversion of β-carotene to retinyl esters in the absorptive cell.

When partially purified protein was tested for the ability to oxidize retinol, retinal production was detected using a similar version of the radioactive assay used to measure retinal reduction. The K_M for this retinol oxidation was determined to be approximately 25 μm, considerably higher than the 1 μm K_M for the reduction of retinal. Because most of the retinol in the absorptive cell is thought to be bound to CRBP II, the effect of increasing amounts of CRBP II on retinol oxidation was determined. When a 1:1.5 ratio was reached between retinol and binding protein, retinol oxidation could no longer be detected, indicating that CRBP II-retinol is not a source of substrate for the retinol oxidase activity detected in this material (*Figure 4B*).

Summary

Perhaps the most compelling evidence for the microsomal activity under study here carrying out the reduction of retinal in the absorptive cell is the specificity of the enzyme for its substrate. Not only is the enzyme specific for retinal, but it appears to be the only enzyme activity in the intestinal absorptive cell capable of making use of retinal bound to CRBP II as a substrate. Because of the abundance of CRBP II in these cells and its high affinity for retinal, free retinal is probably at a vanishingly low level within the cell, because the only source is from carotene cleavage. Also supportive of the physiological importance of this enzyme is a reaction stereo-chemistry that matched that of retinal reduction by isolated intestinal segments. In addition, the partially purified activity was found to be ineffective at the oxidation of retinol and blocked completely from oxidizing retinol when retinol was sequestered within a binding protein, unusual for an enzyme of this type. Alcohol dehydrogenase, as well as the two retinol dehydrogenases in the eye, are capable of acting in both directions under the appropriate conditions. Both retinol dehydrogenase activities are described as ones that ''interconvert'' retinol and retinal due to the ease with which they carry out both reactions. Unlike the intestinal reductase, the dehydrogenase of the retinal pigment epithelium (RPE) has been shown to reduce retinal and oxidize retinol when these retinoids were bound to CRALBP, the retinol and retinal binding protein found in the RPE.[22] This quality may be of benefit in the eye where retinoid processing involves the continuous cycling of retinoids between the RPE and rod outer segments, as opposed to the absorptive cell that carries out a one-way uptake, metabolism, and delivery of vitamin A out of the cell. Such reversibility would serve no apparent purpose in the absorptive cell, where retinal reduction is one step in the synthesis of retinyl esters, with the subsequent transport of the esters out of the cell. The inability of the reductase to oxidize retinol bound to CRBP II suggests an enzyme well-suited to its role in the metabolic conversion of β-carotene to retinyl esters after uptake of vitamin A.

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References

1 Ong, D.E. (1994). Absorption of Vitamin A. In *Vitamin A in Health and Disease* (R. Blomhoff, ed.), p. 37–72, Marcel Dekker, Inc., New York, NY USA

2 Rigtrup, K. M., Kakkad, B., and Ong, D. E. (1994). Purification and partial characterization of a retinyl ester hydrolase from the brush border of rat small intestine mucosa: probable identity with brush border phospholipase B. *Biochemistry* **33,** 2661–2666

3 Dew, S. E. and Ong, D. E. (1994). Specificity of the retinol transporter of the rat small intestine brush border. *Biochemistry* **33,** 12340–12345

4 Olson, J. A. (1989). Provitamin A function of carotenoids: the conversion of β-carotene into vitamin A. *J. Nutr.* **119,** 105–108

5 Wang, X.-D., Tang, G.-W., Fox, J. G., Krinsky, N. I., and Russell, R. M. (1991). Enzymatic conversion of β-carotene into β-apocarotenals and retinoids by human, monkey, ferret, and rat tissues. *Arch. Biochem. Biophys.* **285,** 8–16

6 Ong, D. E. (1984). A novel retinol-binding protein from rat. *J. Biol. Chem.* **259,** 1476–1482

7 Helgerud, P., Petersen, L. B., and Norum, K. R. (1982). Acyl CoA: retinol acyltransferase in rat small intestine: its activity and some properties of the enzymic reaction. *J. Lipid Res.* **23,** 609–618

8 Ong, D. E., Kakkad, B., and MacDonald, P. N. (1987). Acyl-CoA-independent esterification of retinol bound to cellular retinol-binding protein (type II) by microsomes from rat small intestine. *J. Biol. Chem.* **262,** 2729–2736

9 MacDonald, P. N. and Ong, D. E. (1988). Evidence for a lecithin-retinol acyltransferase activity in the rat small intestine. *J. Biol. Chem.* **263,** 12478–12484

10 Quick, T. C. and Ong, D. E. (1990). Vitamin A metabolism in the human intestinal Caco-2 cell line. *Biochemistry* **29,** 11116–11123

11 Shingleton, J. L., Skinner, M. K., and Ong, D. E. (1989). Characteristics of retinol accumulation from serum retinol-binding protein by cultured sertoli cells. *Biochemistry* **28,** 9647–9653

12 Fidge, N. H. and Goodman, D. S. (1968). The enzymatic reduction of retinal to retinol in the rat intestine. *J. Biol. Chem.* **243,** 4372–4379

13 Kakkad, B. P. and Ong, D. E. (1988). Reduction of retinaldehyde bound to cellular retinol-binding protein (type II) by microsomes from rat small intestine. *J. Biol. Chem.* **263,** 12916–12919

14 Herr, F. M. and Ong, D. E. (1992). Differential interaction of lecithin-retinol acyltransferase with cellular retinol binding proteins. *Biochemistry* **31,** 6748–6755

15 Dew, S. E., Wardlaw, S. A., and Ong, D. E. (1993). Effects of pharmacological retinoids on several vitamin A-metabolizing enzymes. *Cancer Research* **53,** 2965–2969

16 Bridges, M. M. and Alvarez, R. A. (1982). Measurement of the vitamin A cycle. *Methods Enzymol.* **81,** 463–485

17 Liau, G., Ong, D. E., and Chytil F. (1981). Interaction of the retinol/cellular retinol-binding protein complex with isolated nuclei and nuclear components. *J. Cell Biol.* **91,** 63–68

18 Pappas, R. S., Newcomer, M. E., and Ong, D. E. (1993). Endogenous retinoids in rat epididymal tissue and rat and human spermatozoa. *Biol. Reprod.* **48,** 235–247

19 Eklund, H., Samama, J.-P., Wallen, L., Brändén, C.-I., Åkerson, Å., and Jones, T. A. (1981). Structure of a triclinic ternary complex of horse liver alcohol dehydrogenase at 2.9 Å resolution. *J. Mol. Biol.* **146,** 561–567

20 Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A., Rossmann, M. G., Smiley, I. E., Allison, W. S., Everse, J., Kaplan, N. O., and Taylor, S. S. (1973). Structure-function relationship in lactate dehydrogenase. *Proc. Nat. Acad. Sci.* **70,** 1968–1972

21 Jamison, R. S., Newcomer, M. E., and Ong, D. E. (1994). Cellular retinoid-binding proteins: limited proteolysis reveals a conformational change upon ligand binding. *Biochemistry* **33,** 2873–2879

22 Saari, J. C. and Bredberg, L. (1982). Enzymatic reduction of 11-cis-retinal bound to cellular retinal-binding protein. *Biochim. Biophys. Acta* **716,** 266–272