Reduction of Retinaldehyde Bound to Cellular Retinol-binding Protein (Type II) by Microsomes from Rat Small Intestine*

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Cellular retinol-binding protein, type II (CRBP(II)), an abundant protein of the rat small intestine, has recently been shown to be able to bind retinaldehyde in addition to retinol (MacDonald, P. N., and Ong, D. E. (1987) J. Biol. Chem. 262, 10550–10556). Retinaldehyde is produced in the intestine by oxidative cleavage of β-carotene. The next step in the intestinal metabolism of vitamin A is the reduction of retinaldehyde to retinol which is then esterified for incorporation into chylomicrons. In the present study retinaldehyde bound to CRBP(II) was found to be available for reduction by microsomal preparations from rat small intestinal mucosa. The microsomal activity was about 8 times greater than the activity observed for an equal amount of cytosolic protein. Retinaldehyde reduction utilized either NADH or NADPH as cofactor, with NADH being slightly more effective. The apparent K_m for retinaldehyde-CRBP(II) was 0.5 μM, and the V_max was approximately 300 pmol/min/mg protein, a rate more than sufficient for the needs of the animal. The product retinol remained complexed to CRBP(II). The microsomal enzyme activity reduced free and bound retinaldehyde to approximately the same extent, although the aldehyde function of retinaldehyde bound to CRBP(II) was less accessible to chemical reducing agents than that of free retinaldehyde. Retinol bound to CRBP(II) could not be oxidized by the microsomal activity in the presence of NAD+, while free retinol or retinol bound to bovine serum albumin was oxidized to retinol. The more favorable reduction versus oxidation of retinol bound to CRBP(II) consequently favored the reaction known to be required for the ultimate conversion of β-carotene to retinyl esters for export from the gut.

When β-carotene is the source of vitamin A, the first step in intestinal metabolism is the oxidative cleavage of the carotene to form retinaldehyde (1). In vivo studies have established that the retinaldehyde is then reduced to retinol, esterified with long chain fatty acids, and the resulting retinyl esters incorporated into chylomicrons that are released to the lymph (2–4). In the rat, little carotene passes the gut unmethylated; most is cleaved to form vitamin A (2). The cleavage enzyme is found in the soluble extract of the mucosa of the small intestine. The intestinal mucosa has also been shown to have a soluble enzyme activity that will reduce free retinol to retinol (5). A microsomal activity was not observed under the conditions employed in that study.

Cellular retinol-binding protein, type II (CRBP(II)) is present in high levels in the mature absorptive cells of the small intestine (6). We have shown that this protein presents retinol to the appropriate enzyme for the esterification that occurs prior to incorporation of vitamin A into the chylomicrons (7). In addition to binding retinol, CRBP(II) can also bind retinaldehyde (8). This raised the possibility that retinaldehyde-CRBP(II), rather than free retinaldehyde, might be the physiological substrate for reduction. Consequently we have investigated the reduction of retinaldehyde bound to CRBP(II) by extracts of intestinal mucosa. We report here a previously undescribed intestinal microsomal activity that reduced retinaldehyde bound to CRBP(II). Its properties suggest it may be a physiologically important activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—All-trans-retinaldehyde, all-trans-retinol, butylated hydroxytoluene, and NaBH₄ were obtained from Sigma. Dimethyl sulfoxide, borane dimethylamine complex, and NaCNBH₃ were obtained from Aldrich. NADH, NADPH, and NAD⁺ were products of Pharmacia LKB Biotechnology Inc. All solvents were HPLC grade and were obtained from Burdick and Jackson Laboratories Inc. Normal chow-fed rats were obtained from Sasco. Rat CRBP(II), both CRBP(II)-A and CRBP(II)-B, were purified from 1-day-old rat pups as previously described (6) or by a similar method from adult rat intestine. The two forms appear to be identical except that the α-amino group of peak B is blocked (6). All data shown in this paper were from studies with CRBP(II)-A. However, CRBP(II)-B was examined and found to behave similarly.

**Preparation of Intestinal Microsomes and Cytosol**—Intestinal microsomes were isolated as previously described (7). The microsomes were stored at −70 °C without dithiothreitol. The 100,000 × g supernatant liquid from the microsome preparation was submitted to further centrifugation at 115,000 × g for 150 min to remove any residual microsomes and stored at −70 °C. Protein content was determined using a modified Lowry procedure (9).

**Reduction Assay**—All incubations were carried out in duplicate, under subdued light, in disposable borosilicate culture tubes in a shaking water bath at 37 °C. The retinaldehyde-CRBP(II) complex was added as such or was prefractioned in the reaction vessel by the addition of retinaldehyde in dimethyl sulfoxide to a 20% excess of apo-CRBP(II). Retinaldehyde-CRBP(II) (3 μM) was incubated for 11 min with 80 μM NADH or NADPH and 100 μg/ml microsomal protein in 0.1 M imidazole acetate buffer, pH 6.0, unless otherwise specified. The incubation volume was 250 or 500 μl. The reaction was stopped by the addition of 4 volumes of ice-cold ethanol containing 100 μg/ml butylated hydroxytoluene, transferred to extraction tubes using glass Pasteur pipettes, and the lipids extracted into hexane as previously described (8).

**High Performance Liquid Chromatography**—All HPLC procedures used a Spectra Physics SP8700 solvent delivery system and a Spectra

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Physique pump fitted with a Rhodine injection valve and a 100-µl sample loop. Elution was monitored at 550 nm with a V4 variable wavelength filter detector connected to a Spectra Physic integrator. The stationary phase was a Whatman Partisil 5 silica column (4.6 mm x 25 cm) coupled to a Supelcosil silica guard column (2 cm). The mobile phase was hexane/1,4-dioxane (9:1) at a flow rate of 2 ml/min. Under these conditions, retinaldehyde and retinol eluted at 3.6 and 10 min, respectively. All-trans- and 13-cis-retinol were quantitated by comparing their integrated peak areas to a calibration curve of the amount of pure retinol injected onto the column versus the resulting integrated peak areas.

**Discussion**

**Reduction of Retinaldehyde with Chemical Agents**—Reductions with NaCNBH₃ and borane dimethylamine were in 0.1 M sodium acetate buffer at pH 5.5. Reductions with NaBH₄ were carried out in 0.1 M Tris-HCl at pH 7.5. Unbound retinaldehyde or retinoldehyde-CRBP(II) (1 µM) was incubated with 300 mM NaCNBH₃, 300 µM borane dimethylamine, or 50 mM NaBH₄ for 20 min at 37 ℃. The incubation volume was 1.0 ml.

**RESULTS**

**Enzyme-catalyzed Retinaldehyde Reduction**—Initial experiments examined both microsomal and cytosolic protein prepared from the scraped mucosa of rat small intestine for an activity that would reduce retinaldehyde bound to CRBP(II). Retinaldehyde-CRBP(II) reduction was followed by extracting the reaction mixtures into organic solvent and analyzing the retinoids by HPLC. As can be seen in Fig. 1, microsomal protein contained an activity capable of reducing all-trans-retinaldehyde bound to CRBP(II). The major product of the reduction was all-trans-retinol. Variable but small amounts of 13-cis-retinol were recovered as well which may have arisen from all-trans-retinol during the extraction process. The reaction was quantitated by summing the areas of both 13-cis-retinol and all-trans-retinol peaks. No retinol was produced when NADH was omitted (Fig. 1, lower tracing).

When an equivalent amount of soluble protein was compared to microsomal protein in this system, considerably less reduction of retinaldehyde-CRBP(II) was observed (Fig. 2). Generally 8-10-fold lower activity was observed for cytosolic protein compared to microsomal protein. The cytosolic activity was considerably more active with free retinaldehyde as substrate, however. The activity toward free retinaldehyde was somewhat variable from experiment to experiment but always was at least 3-fold greater than with retinaldehyde-CRBP(II). This cytosolic activity has previously been described (5).

Because a microsomal activity in the gut had not been described previously, efforts were concentrated on its characterization. The reaction was proportional to microsomal protein added up to about 100 µg/500 µl of reaction mixture and was linear for about 20 min. No retinol production was observed in the absence of microsomal protein or with heat-inactivated microsomes, indicating that the enzyme activity was not a property of the carrier protein, CRBP(II). The reaction was optimal between pH 5.0 and 6.0 and completely absent at pH 8.0.

**Rate of Reduction Versus Substrate Concentration**—The rate of retinol formation was saturable with increasing concentrations of retinaldehyde-CRBP(II) (Fig. 3). The apparent $K_{m}$ value for retinol formation, derived from a Woolf plot (Fig. 3, inset), was 0.46 ± 0.04 µM. The value for $V_{max}$ varied between 235 and 360 pmol/min/mg protein with different
preparations of microsomes. The microsomal activity would also reduce unbound retinaldehyde. Unbound retinaldehyde yielded a $K_m$ of 0.78 ± 0.20 μM and a $V_{max}$ similar to that observed for retinaldehyde-CRBP(II). Both NADH and NADPH were effective cofactors for the reduction of retinaldehyde-CRBP(II), with NADH preferred somewhat (Fig. 4). The apparent $K_m$ values were 10 μM for NADH and 18 μM for NADPH.

When reaction mixtures were passed over a small Sephadex G-25 column, product retinol was recovered bound to CRBP(II), determined by observing the characteristic fluorescence excitation spectrum of retinol-CRBP(II) (6). Further, if the fraction containing CRBP(II) was then extracted and analyzed by HPLC, a retinaldehyde peak was obtained in addition to the retinol peak. This suggested that both retinaldehyde and the product retinol remained bound to CRBP(II) under the reaction conditions employed. It was possible that CRBP(II) might have transferred retinaldehyde to the active site of the microsomal enzyme and then retrieved the product retinol. To explore this possibility we added increasing amounts of apo-CRBP(II) to the incubation mixture to see if the overall rate of reaction might be increased by having increased amounts of acceptor for the product. No change in rate was observed in the presence of apo-CRBP(II). Reaction of Unbound Retinaldehyde and Retinaldehyde-CRBP(II) with Chemical Reducing Agents—Because the microsomal enzymic reaction showed only a slight preference for retinaldehyde-CRBP(II) compared to free retinaldehyde, the accessibility of the aldehyde groups of free and bound retinaldehyde for chemical reduction by borane dimethylamine was examined. Because extraction of the reaction mixtures releases retinaldehyde from CRBP(II), borane dimethylamine was removed from the retinoid-CRBP(II) complexes by gel filtration on Sephadex G-25 prior to extraction to prevent reduction from occurring during the extraction process. To compare reduction of free retinaldehyde under equivalent conditions, after incubation sufficient apo-CRBP(II) was added to bind all retinaldehyde and retinol present in the reaction mixture. The reducing agent was then separated from the retinoid-CRBP(II) complexes by Sephadex G-25 chromatography before extraction. Little reduction of retinaldehyde-CRBP(II) by borane dimethylamine was observed compared to the reduction accomplished by the microsomal enzyme activity (Table I). However, a 5-fold greater reduction of free retinaldehyde than of retinaldehyde-CRBP(II) was observed. Similar results were obtained with NaCNBH$_3$ and NaBH$_4$.

**Oxidation of Retinol Bound to CRBP(II)—**When retinol-CRBP(II) was incubated with 200 μg of microsomal protein/ml and 60 μM NAD$^+$ at pH 7.5 or 8.5 for 20 min at 37°C, little or no oxidation of retinol could be detected (estimated to be less than 0.3 pmol/min/mg protein). However, under similar conditions, free retinol was oxidized to retinaldehyde at a rate of 15 pmol/min/mg protein. Retinol bound to bovine serum albumin was oxidized at a rate similar to that obtained with free retinol. The rate of reduction obtained under optimal conditions with the same microsomal preparation was 320 and 290 pmol/min/mg protein for retinaldehyde-CRBP(II) and unbound retinaldehyde, respectively. Thus, the ratio of the rate of reduction versus oxidation was over 1000 for retinoid bound to CRBP(II) but only about 20 for unbound retinoid.

**DISCUSSION**

An important step in the intestinal metabolism of the provitamin β-carotene to vitamin A is reduction of all-trans-retinaldehyde that arises from the oxidative cleavage of the carotene. Previous work demonstrated a cytosolic enzyme activity from small intestine of rat that reduces free retinaldehyde to retinol (5). However, the considerable amount of CRBP(II) present in the small intestine (about 1% of the total soluble protein), all of which is confined to the mature absorptive cell, suggests that any retinaldehyde in those cells would be bound to CRBP(II). Retinaldehyde bound to CRBP(II) was found here to be considerably restricted from the cytosolic enzyme activity, compared to unbound retinaldehyde, but was freely reduced by a microsomal activity. The microsomal protein recovered from the proximal two-thirds of the small intestine of one rat (about 20 mg) had sufficient reductase activity to reduce 1.2 mg of retinaldehyde/day operating at half-maximal velocity ($V_{max}$ = 330 pmol/min/mg), about 200 times the daily vitamin A requirement of the rat (10).

The apparent inability to oxidize retinol-CRBP(II) contrasted with the facile reduction of retinaldehyde-CRBP(II) may be explained by the observation that retinol appears to fit into the binding site of CRBP(II) somewhat differently than does retinaldehyde (8). Retinol is bound so that the five conjugated double bonds appear to be in a fully planar configuration, revealed by the appearance of fine structure in the absorption spectrum of the bound retinol. However, no fine
structure appears in the spectrum of retinaldehyde when it binds to CRBP(II), suggesting that the double bond of the ring has not been brought into the same plane as the four double bonds of the side chain. Perhaps the different fits in the binding site of CRBP(I1) change the degree of exposure of the aldehyde/alcohol function at the end of the side chain so that the alcohol is less accessible to the enzyme.

Although free and bound aldehyde were reduced at almost identical rates by the microsomal enzyme, it appeared that access to the aldehyde function of retinaldehyde when bound to CRBP(II) was somewhat restricted because it was reduced by chemical reducing agents much more slowly than was free retinaldehyde. Perhaps interaction of retinaldehyde-CRBP(II) with the enzyme caused a change in exposure of the aldehyde function, permitting reduction equal or greater than that obtained with free retinaldehyde. However, we found no evidence that the retinaldehyde actually was transferred from CRBP(II) to a binding site on the enzyme.

Other particulate enzyme activities that reduce or oxidize retinoids have been described. Membrane preparations from visual cell outer segments can reduce all-trans-retinaldehyde to retinol using either NADPH or NADH as cofactor (11). That activity will also oxidize retinol to retinaldehyde; no preference for reduction over oxidation could be demonstrated (12). In addition, NADPH appears to be the preferred cofactor, in contrast to the reaction under study here. The pigment epithelium of the eye also contains a retinaldehyde reductase, specific for 11-cis-retinaldehyde with little or no activity for all-trans-retinaldehyde. The activity will also oxidize 11-cis-retinol (13). Interestingly this enzyme will also utilize retinaldehyde bound to cellular retinaldehyde-binding protein (14), an abundant protein of the pigment epithelium (15). Thus that situation is similar to what we observed here, with the enzyme able to utilize either bound or free aldehyde. However, using bound retinoid as a substrate for the pigment epithelium activity did not appear to change the relative rates of oxidation/reduction, in contrast to the intestinal microsomal reductase activity. Liver also contains a microsomal activity that will oxidize retinol or reduce retinaldehyde (16). The effect of retinoid-binding proteins on the reaction was not examined.

The ability of CRBP(II) to reduce retinaldehyde reduction by the cytosolic activity and favor reduction by the microsomal activity is similar to the effect of CRBP(II) on the esterification of retinol. Free retinol can be esterified by an acyl-CoA-dependent microsomal enzyme (17). However, when bound to CRBP(II), retinol is restricted from that reaction and is esterified by a different microsomal enzyme that utilizes lecithin as acyl donor (7, 18). Consequently when retinaldehyde and retinol are bound to CRBP(II), intestinal metabolism may be carried out by two previously undescribed microsomal enzymes. That both activities are microsomal opens the possibility that the enzymes may be physically close together on the endoplasmic reticulum, permitting an efficient and directed production of retinyl esters from the retinaldehyde produced by carotene cleavage. Further work will be directed to exploring this possibility.

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