Control of Substrate Flow at a Branch in the Visual Cycle†

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ABSTRACT: Photoisomerization of rhodopsin's chromophore, 11-*cis*-retinaldehyde, and subsequent regeneration of the 11-*cis* configuration are accomplished in vertebrates by a series of reactions known as the visual cycle. At one point in the cycle, 11-*cis*-retinol can either be enzymatically oxidized to 11-*cis*-retinaldehyde and exported for visual pigment regeneration or be enzymatically esterified and stored. Partition of substrate at this branch was examined in this study and found to be influenced by cellular retinaldehyde-binding protein (CRALBP), a retinoid-binding protein found in retina. Esterification was reduced to about 10% and oxidation stimulated 2–3-fold in the presence of this protein. Other experiments confirmed that "free" 11-*cis*-retinol was esterified more rapidly than 11-*cis*-retinol complexed with CRALBP and that CRALBP·11-*cis*-retinol was not an inhibitor of the esterification. Following oxidation of CRALBP·11-*cis*-retinol, the reaction product, 11-*cis*-retinaldehyde, was found associated with the binding protein. 11-*cis*-Retinaldehyde is not available for reaction with carbonyl reagents when the retinoid is bound to CRALBP. However, enzymatic oxidation of CRALBP·11-*cis*-retinol in the presence of *O*-ethylhydroxylamine produced *ca.* 30% retinaldehyde *O*-ethyloxime and 70% free 11-*cis*-retinaldehyde, suggesting that about one-third of the retinol oxidized had dissociated from the binding protein. Neither oxidation nor esterification of CRALBP·11-*cis*-retinol was inhibited by including CRALBP·11-*cis*-retinaldehyde in the reaction mixture. The results indicate that CRALBP influences the competition for substrate between two important visual cycle enzymes, and suggest that CRALBP may act as a substrate-routing protein *in vivo*. The mechanism for the opposite effect of CRALBP on oxidation and esterification is not understood.

Absorption of a photon by rhodopsin photoisomerizes 11-*cis*-retinaldehyde to *all-trans*-retinaldehyde and initiates the phototransduction cascade. Bleached visual pigment is regenerated in vertebrates in a series of reactions known as the visual cycle (Wald, 1968). *all-trans*-Retinaldehyde is reduced to *all-trans*-retinol in photoreceptor cells, and moves to the retinal pigment epithelium (RPE) where subsequent enzymatic processing takes place. In RPE, esterification produces *all-trans*-retinyl palmitate, which is converted to 11-*cis*-retinol and palmitate in a reaction involving concerted isomerization and ester hydrolysis (Bernstein et al., 1987; Deigner et al., 1989). 11-*cis*-Retinol is at a branch in the visual cycle as it can either be oxidized to 11-*cis*-retinaldehyde and used for visual pigment synthesis in photoreceptors or be esterified and stored as 11-*cis*-retinyl ester in RPE [see Rando (1991) and Saari (1990) for recent reviews of the visual cycle].

Enzymatic activities at branch points in metabolic pathways are frequently subject to various controls as the partition of substrate to the two (or more) pathways is often precisely controlled and linked to functional parameters of the pathway. In the visual cycle, 11-*cis*-retinyl ester is known to build up in the dark in RPE (Bridges, 1976; Krinsky, 1958), implying that the esterification branch dominates. However, 11-*cis*-retinyl ester can be hydrolyzed and used to produce 11-*cis*-retinaldehyde for visual pigment regeneration during bleaching episodes, suggesting that the dehydrogenase branch has been activated.

The two enzymes of the mammalian visual cycle that process 11-*cis*-retinol have not been extensively characterized. 11-*cis*-Retinol dehydrogenase activity was described several years ago (Lion et al., 1975), and recently partially purified (Suzuki et al., 1993); however, little is known of the structure of the enzyme or its kinetic properties. LRAT, the enzyme responsible for esterification from bovine RPE microsomes, has been studied (Saari & Bredberg, 1988a, 1989; Barry et al., 1989; Shi et al., 1993; Saari et al., 1993a), but, again, no indications of control have been identified.

Cellular retinaldehyde-binding protein (CRALBP) is found in bovine RPE (Bunt-Milam & Saari, 1983) and is known to bind either 11-*cis*-retinol or 11-*cis*-retinaldehyde (Saari et al., 1982). Since retinoids *in vivo* are usually protein-bound (retinyl esters are an exception), we questioned whether presentation of 11-*cis*-retinol complexed with CRALBP would affect the amount of oxidation or esterification. The results of the present study demonstrate that CRALBP results in diminished esterification and enhanced oxidation of 11-*cis*-retinol, relative to the control. The mechanisms for these opposite effects are not known.

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[1] Abbreviations: [^{3}H]-11-*cis*-retinol, [C-15-^{3}H]-11-*cis*-retinol; [^{3}H]-11-*cis*-retinaldehyde, [C-15-^{3}H]-11-*cis*-retinaldehyde; BSA, bovine serum albumin; BSA·11-*cis*-retinol, 11-*cis*-retinol complexed with BSA; CRALBP, cellular retinaldehyde-binding protein; CRALBP·11-*cis*-retinaldehyde or CRALBP·11-*cis*-retinol, CRALBP complexed with either retinoid; DTT, dithiothreitol; LRAT, lecithin:retinol acyltransferase; RPE, retinal pigment epithelium.

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EXPERIMENTAL PROCEDURES

Materials. Materials were purchased or obtained from the following suppliers: [C-15-^{3}H]*all-trans*-retinol and NaB^3H_4, DuPont New England Nuclear; Superose 12 gel filtration column, Pharmacia Fine Chemicals; fatty acid-poor BSA, Sigma Chemical Co. 11-*cis*-Retinaldehyde was obtained through the generosity of the National Eye Institute. [^{3}H]-11-*cis*-Retinol was prepared by reduction of 11-*cis*-retinaldehyde with NaB^3H_4 as described previously (Stubbs et al., 1979). CRALBP was purified from bovine retina and complexed with [^{3}H]-11-*cis*-retinol as previously described (Saari & Bredberg, 1988b). The specific radioactivity was approximately 30 000 dpm/nmol. Retinoids were purified by HPLC when necessary (Saari et al., 1982).

RPE microsomes, the source of both LRAT and 11-*cis*-retinol dehydrogenase activities, were prepared as previously described (Saari & Bredberg, 1990).

Assay of Retinol Esterification. LRAT activity at 37 °C was determined as previously described (Saari & Bredberg, 1990). The reaction mixture included BSA (20–60 μM) to which [^{3}H]-11-*cis*-retinol was added dissolved in ethanol or complexed with CRALBP. The final concentration of ethanol was less than 0.1% in all studies.

Assay of 11-cis-Retinol Dehydrogenase. The assay employed depends on the transfer of ^{3}H from carbon-15 of [^{3}H]-11-*cis*-retinol to NAD during enzymatic oxidation. The appearance of ^{3}H in the aqueous-organic (upper) phase of a CH_2Cl_2–methanol partition was followed. Results obtained with this assay are comparable to those obtained by HPLC analysis of the products of the reaction (Saari et al., 1993b).

Reaction conditions included a 200 μL total volume, 37 °C, of 50 mM Tris buffer, pH 7.2, 1 mM DTT, 30 μM BSA (2 mg/mL), 60 μM NAD, and 10 μM [^{3}H]-11-*cis*-retinol. The latter was added as a solution in ethanol (final ethanol concentration less than 0.1%) or as a complex with CRALBP. Reactions were run in conical 1.5-mL snap-cap tubes and started with the addition of a few microliters of RPE microsomes. Time courses were run in a larger volume, and 200 μL of reaction was removed to conical tubes at desired times for the extraction. The reaction was stopped with the addition of 400 μL of cold methanol and 20 μL of neutralized 1 M NH_2OH followed by incubation for 5 min at 37 °C. Following addition of 180 μL of 1 M NaCl, the tubes were placed on ice, and 400 μL of CH_2Cl_2 was added with vortexing. A brief centrifugation (5 min at 5000 rpm) was used to separate the phases. After removal of the lower phase with a Hamilton syringe, the upper phase was extracted twice more with 400 μL of CH_2Cl_2. Finally, 400 μL of the upper phase was counted in a liquid scintillation spectrometer. Controls were run without addition of NAD or with addition of microsomes to the reaction mixture after addition of methanol.

Gel Filtration. Reaction mixtures containing CRALBP·11-*cis*-retinol, NAD, and RPE microsomes were prepared as described above and incubated at 37 °C. At varying times, samples were withdrawn, chilled to 0 °C, and centrifuged at 200000*g* to remove microsomes and stop the reaction. Portions of the supernatant were applied to a Superose-12 10/30 column (Pharmacia) equilibrated in 50 mM MOPS, pH 7.0, containing 0.2 M sodium acetate. Elution was accomplished with the same buffer at a flow rate of 24 mL/h. The oxidation product CRALBP·11-*cis*-retinaldehyde was detected at 425 nm.

HPLC Separation of Retinoids. Chilled methanol was added to enzymatic reaction mixtures, and the retinoids were extracted with hexane as described (Saari & Bredberg, 1990). HPLC was employed to obtain retinol, retinaldehyde, retinal oximes, and retinyl esters (Saari et al., 1982). Retinoid specific radioactivities were determined by dividing the radioactivity of an isolated retinoid (liquid scintillation counting) by the concentration of the retinoid (spectral analysis).

Trapping of Reaction Intermediates with O-Ethylhydroxylamine. We had previously demonstrated that 11-*cis*-retinaldehyde bound to CRALBP was protected from reaction with carbonyl reagents such as NH_2OH (Stubbs et al., 1979). We further demonstrated in this study that the dehydrogenase reaction was not inhibited by inclusion of up to 40 mM *O*-ethylhydroxylamine (results not shown), indicating that this reagent could be used to trap free aldehydic intermediates in the oxidation reaction. Reaction conditions included buffered RPE microsomes, 33 mM *O*-ethylhydroxylamine, and either 13 μM CRALBP·11-*cis*-retinol or 13 μM BSA·11-*cis*-retinol. As a control, CRALBP·11-*cis*-retinaldehyde was incubated with the reagent but without microsomes. Following a 2-min reaction at 37 °C, the mixtures were chilled and dialyzed at 5 °C to remove hydroxylamine. Retinoids were then extracted as described (Saari & Bredberg, 1990). *O*-ethyloximes of 11-*cis*-retinaldehyde were well resolved from 11-*cis*-retinol by HPLC using the solvent system described by Van Kuijk et al. (1985).

Effect of CRALBP·11-cis-Retinaldehyde on Oxidation and Esterification. CRALBP·11-*cis*-retinaldehyde was added to buffered microsomes and CRALBP·[^{3}H]-11-*cis*-retinol in molar ratios (aldehyde to alcohol) up to 10:1. The effect of these additions on esterification or oxidation was measured using the assays described for these reactions.

RESULTS

Effect of CRALBP on Oxidation and Esterification of 11-cis-Retinol. A previous communication provided evidence for reduction of CRALBP·11-*cis*-retinaldehyde by 11-*cis*-retinol dehydrogenase of RPE (Saari & Bredberg, 1982). In this study, we have expanded our efforts to include the effects of CRALBP on esterification and on oxidation of 11-*cis*-retinol, the direction of the oxidation/reduction reaction believed to be physiologically relevant. The question of whether CRALBP could influence the partition of 11-*cis*-retinol between oxidation and esterification was of particular interest. Both 11-*cis*-retinol dehydrogenase and LRAT are present in bovine RPE microsomes (Saari & Bredberg, 1988, 1989; Zimmerman, 1976), which were used as the source of enzymes in this study.

The experiments described were performed in the presence of a constant amount of BSA, referred to here as a BSA background. BSA is known to bind retinol and retinaldehyde (Futterman & Heller, 1972) with relatively low affinity (Noy & Xu, 1990) and has been used extensively to solubilize retinoids (Blaner & Churchich, 1980; Saari & Bredberg, 1988, 1989; Bernstein et al., 1987). We assume that the low-affinity binding sites of BSA simply buffer the concentration of 11-*cis*-retinol in solution and consider that this represents "free" retinol. Variations in BSA concentration above 20 μM did not affect the rate of oxidation or esterification of 11-*cis*-retinol (data not shown). Inclusion of BSA in reaction mixtures resulted in higher recoveries of extracted retinoids and improved reproducibility.

We compared the amount of esterification and oxidation obtained when microsomes in buffered BSA (30 μM) were mixed with 10 μM 11-*cis*-retinol either complexed with CRALBP or added in a small volume of ethanol (final concentration <0.1% ethanol). Reaction progress curves are

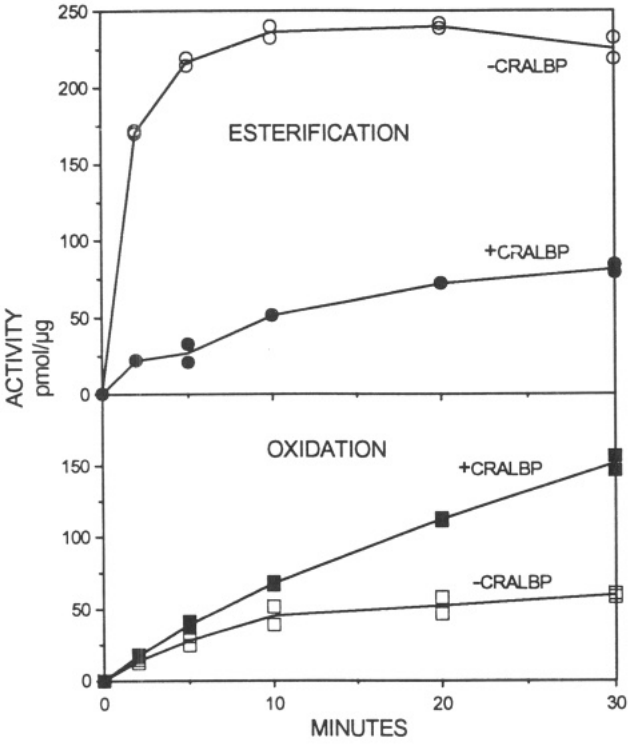

FIGURE 1: Time course of the esterification (upper panel) and oxidation (lower panel) of 11-*cis*-retinol (10 μM) complexed with CRALBP or BSA. BSA was present in both reaction mixtures at 30 μM. Samples were removed for analysis at the times shown. The results of duplicate determinations are shown. Esterification of CRALBP·11-*cis*-retinol is reduced, relative to BSA·11-*cis*-retinol, at all times examined, whereas oxidation of CRALBP·11-*cis*-retinol is stimulated.

shown in Figure 1. Esterification of CRALBP·11-*cis*-retinol was reduced (relative to BSA·11-*cis*-retinol) at all reaction times whereas significant stimulation of CRALBP·11-*cis*-retinol oxidation was evident after 5 min of reaction. For example, after 5 min of reaction, esterification was decreased to 23% whereas oxidation was stimulated to 160%, relative to the BSA control. Thus, esterification dominated with only BSA present, whereas oxidation was favored if the substrate was bound to CRALBP. This experiment has been repeated 3 times with the same qualitative results.

The apparent stimulation of oxidation observed with CRALBP·11-*cis*-retinol could result simply from the increased substrate available for oxidation when the esterification reaction is retarded. However, an examination of the amounts of substrate remaining during the time course indicates that stimulation of the dehydrogenase did not result from this trivial explanation. For instance, at the earliest reaction time measured (2 min), 47% of BSA·11-*cis*-retinol has been processed by LRAT and only 4% by 11-*cis*-retinol dehydrogenase. However, the calculated remaining concentration of BSA·11-*cis*-retinol (5 μM) was above the K_m for the dehydrogenase (approximately 2 μM), indicating that substrate depletion by LRAT is not responsible for the apparently weak 11-*cis*-retinol dehydrogenase activity observed with BSA·11-*cis*-retinol.

An additional experiment also indicated that the ratio of oxidation to esterification is different for BSA·11-*cis*-retinol compared to CRALBP·11-*cis*-retinol; 5 μM BSA·[^{3}H]-11-*cis*-retinol was added to buffered microsomes and NAD with either nonradioactive 5 μM BSA·11-*cis*-retinol (treatment A) or 5 μM CRALBP·11-*cis*-retinol (treatment B). A BSA background of 30 μM was included in both treatments. After

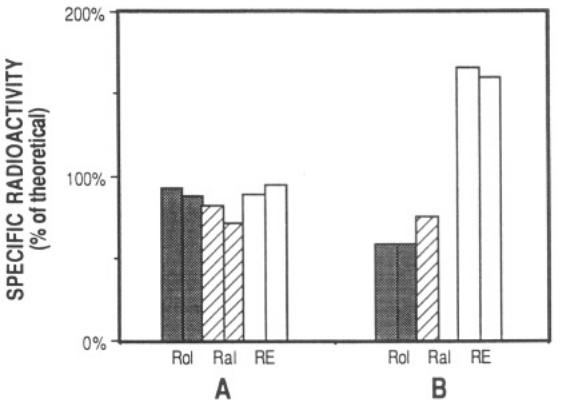

FIGURE 2: Metabolism of BSA·11-*cis*-retinol or CRALBP·11-*cis*-retinol by bovine RPE microsomes. Both treatments include [^{3}H]-11-*cis*-retinol added to a reaction mixture of 50 mM Tris, pH 7.2, 1 mM DTT, 30 μM BSA, and 60 μM NAD. In treatment A, 5 μM 11-*cis*-retinol was added in a small volume of ethanol. In treatment B, 5 μM 11-*cis*-retinol complexed with CRALBP was added. One minute after addition of bovine RPE microsomes, the reaction was stopped, retinoids were extracted, and specific radioactivities were determined. Results of duplicate determinations are shown except for Ral, treatment B, where a sample was lost. The value of 100% refers to the specific radioactivity of 11-*cis*-retinol predicted for complete equilibration of added radioactive and nonradioactive 11-*cis*-retinol. The results of treatment B indicate that BSA·11-*cis*-retinol is esterified more rapidly than CRALBP·11-*cis*-retinol. (Rol = 11-*cis*-retinol, Ral = 11-*cis*-retinaldehyde, RE = retinyl ester.)

1 min, the reaction was stopped with the addition of cold methanol, and retinoids were extracted and separated by HPLC and their specific radioactivities determined. The results, shown in Figure 2, indicate that the specific radioactivities of retinol, retinaldehyde, and retinyl ester, produced by a mixture of BSA·[^{3}H]-11-*cis*-retinol and BSA·11-*cis*-retinol, were approximately the same, and within 90% of that predicted from the mixing of radiolabeled with cold retinol (treatment A). The specific radioactivities of retinoids produced by a mixture of BSA·[^{3}H]-11-*cis*-retinol and CRALBP·11-*cis*-retinol (treatment B, Figure 2) differed considerably. The specific radioactivity of retinyl ester was 180% of the predicted value whereas that of residual retinol was about 65% of the predicted value, indicating that BSA·11-*cis*-retinol was esterified more rapidly than CRALBP·11-*cis*-retinol. There was no difference in the specific radioactivities of retinaldehyde isolated from the two reaction mixtures, consistent with the small stimulation of oxidation observed after 1 min of reaction (Figure 2). In three repeats of this experiment, the specific activity of retinyl ester in treatment B consistently exceeded that of treatment A. Duplicate values are shown in Figure 2 to provide an indication of the precision of the measurements. The half-time of exchange of CRALBP·[^{3}H]-11-*cis*-retinol with 11-*cis*-retinol ($t_{1/2}$ = 2 min), measured in other studies (Saari and Bredberg, unpublished results), suggests that <25% of the bound retinol would have exchanged with the free during the 1-min reaction. It should be noted that oxidation of [C-15-^{3}H]-11-*cis*-retinol to 11-*cis*-retinaldehyde removes a tritium atom from carbon-15, resulting in a product with half the specific radioactivity of the reactant. These results demonstrate that free 11-*cis*-retinol is esterified more rapidly than that bound to CRALBP.

Stimulation of the Rate of Oxidation. Stimulation of oxidation could be explained by a mechanism in which the binding protein–retinoid complex is a better substrate or by an activating effect of the binding protein. The experiments outlined below were designed to explore these possibilities.

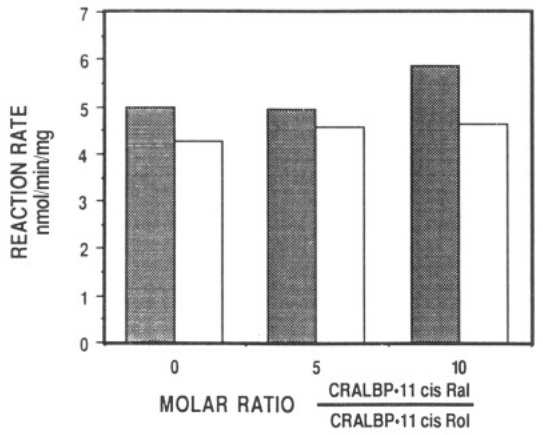

FIGURE 3: Oxidation of CRALBP·[^{3}H]-11-*cis*-retinol in the presence of CRALBP·11-*cis*-retinaldehyde. Reaction mixtures containing CRALBP·[^{3}H]-11-*cis*-retinol (1.5 μM), NAD, microsomes, and varying concentrations of CRALBP·11-*cis*-retinaldehyde were stopped after 1 min, and the amount of retinyl ester (light bars) or 11-*cis*-retinaldehyde (dark bars) was determined. No inhibition of oxidation or esterification was observed. (Ral = retinaldehyde; Rol = retinol.)

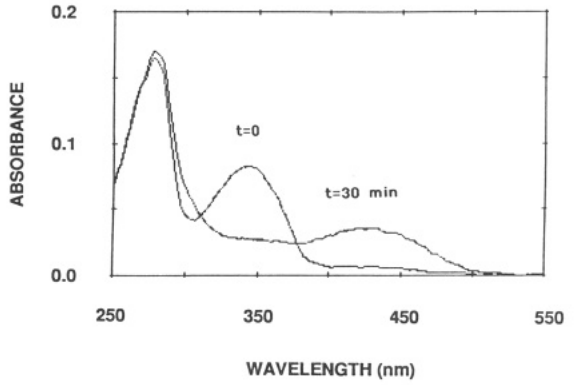

FIGURE 4: 11-*cis*-Retinaldehyde remains bound to CRALBP: spectral evidence. The trace marked "t = 0" illustrates the absorption spectrum of CRALBP·11-*cis*-retinol. The peak absorbance at 330 nm is due to the bound 11-*cis*-retinol chromophore. The trace marked "t = 30 min" illustrates the absorption spectrum isolated from a reaction mixture containing buffered RPE microsomes and NAD at 37 °C for 30 min. The 330-nm peak has largely been replaced by a peak at 425 nm, characteristic of CRALB·11-*cis*-retinaldehyde. The results demonstrate that the product of the reaction is found in association with the binding protein.

Retinaldehyde bound to CRALBP is inert to reaction with hydroxylamine (Stubbs et al., 1978), demonstrating that the aldehyde functional group is not accessible to the water-soluble reagent in this complex. This feature was employed to obtain more information about the mechanism of the oxidation of CRALBP·11-*cis*-retinol. *O*-Ethylhydroxylamine was used since retinaldehyde *O*-ethyloximes derived from this reagent are well resolved from retinols by HPLC (Van Kuijk et al., 1985). Initial experiments determined that the rate of 11-*cis*-retinol oxidation was not affected by *O*-ethylhydroxylamine concentrations up to 40 mM (results not shown). We included 33 mM *O*-ethylhydroxylamine during the enzymatic oxidation of CRALBP·11-*cis*-retinol to determine whether "free" 11-*cis*-retinaldehyde could be detected during the reaction. As a control, CRALBP·11-*cis*-retinaldehyde was incubated with *O*-ethylhydroxylamine for the same reaction time. The following products were found after 2 min of reaction: oxidation of BSA·11-*cis*-retinol, 100% *O*-ethyloxime; oxidation of CRALBP·11-*cis*-retinol, 68% 11-*cis*-retinaldehyde, 32% *O*-ethyloxime; incubation of CRALBP·11-*cis*-retinaldehyde, 100% 11-*cis*-retinaldehyde. This experiment has been repeated with comparable results. The results suggest that about one-third of the substrate is oxidized via a mechanism in which the product, 11-*cis*-retinaldehyde, is accessible to *O*-ethylhydroxylamine. The remaining two-thirds of the retinaldehyde was either never accessible to *O*-ethylhydroxylamine or failed to react with the reagent within the time of the experiment.

If oxidation of CRALBP·11-*cis*-retinol occurs by direct interaction of the enzyme and binding protein, increasing concentrations of CRALBP·11-*cis*-retinaldehyde should inhibit the initial rate of oxidation of CRALBP·11-*cis*-retinol since the conformations of the binding protein complexed with these ligands are likely to be similar. However, no inhibition of CRALBP·[^{3}H]-11-*cis*-retinol oxidation or esterification was observed with up to a 10-fold molar excess of CRALBP·11-*cis*-retinaldehyde over CRALBP·11-*cis*-retinol (Figure 3).

Absorption spectra of CRALBP·11-*cis*-retinol before and after incubation with microsomes and NAD are shown in Figure 4. There is a pronounced loss of absorbance at 340 nm and a gain in absorbance at 425 nm. The former absorbance is due to bound 11-*cis*-retinol whereas the latter is due to bound 11-*cis*-retinaldehyde whose absorption maximum is shifted from 390 to 425 nm on binding to CRALBP (Stubbs et al., 1978). When adjusted for the difference in molar extinction of the bound retinoids and the retinol esterified, the amount of 11-*cis*-retinaldehyde generated equaled 95% of the amount of 11-*cis*-retinol lost. The appearance of 11-*cis*-retinaldehyde bound to CRALBP was also followed using gel filtration chromatography. CRALBP·11-*cis*-retinol was incubated with RPE microsomes and NAD, and samples were taken at varying times, centrifuged at 5 °C to remove the microsomes and stop the reaction, and analyzed by gel filtration. 11-*cis*-Retinol bound to CRALBP absorbs at 340 nm whereas 11-*cis*-retinaldehyde complexed with CRALBP absorbs at 425 nm (Stubbs et al., 1978), as mentioned above. Thus, the generation of a CRALBP·11-*cis*-retinaldehyde complex could be monitored by measuring the absorption at 425 nm. The area of the 425-nm-absorbing component steadily increased with reaction time, indicating that the 11-*cis*-retinaldehyde generated is found associated with CRALBP (Figure 5).

Retardation of the Rate of Esterification. The esterification velocity as a function of substrate concentration was examined in more detail since different slopes might be apparent at high and low concentrations of CRALBP·11-*cis*-retinol where dissociation to "free" retinoid would be favored or diminished. The results of such an experiment are shown in Figure 6, as a double-reciprocal plot. Esterification of BSA·11-*cis*-retinol yielded a single slope with kinetic parameters K_m = 0.63 μM and V_{max} = 102 nmol min^{-1} mg^{-1} (Figure 6B), whereas esterification of CRALBP·11-*cis*-retinol showed evidence of two slopes (Figure 6A). The inset of Figure 6A illustrates an expanded plot of the velocities at higher substrate levels. Two phases of the reaction were clearly evident. A K_m of 1.3 μM and a relatively low V_{max} of 17 nmol min^{-1} mg^{-1} were observed at substrate concentrations below 2.5 μM (inset). At higher concentrations of CRALBP·11-*cis*-retinol (Figure 6A, inset), the reaction showed little signs of saturation and yielded K_m and V_{max} values much larger than those observed with "free" retinoid (42 μM and 248 nmol min^{-1} mg^{-1}, respectively), which probably reflect the contribution of the higher absolute concentrations of 11-*cis*-retinol found at higher CRALBP·11-*cis*-retinol concentrations. The kinetic study is consistent with the notion that "free" 11-*cis*-retinol is esterified more rapidly than CRALBP-bound 11-*cis*-retinol. However, we cannot determine from these data whether 11-*cis*-retinol complexed with CRALBP is directly processed.

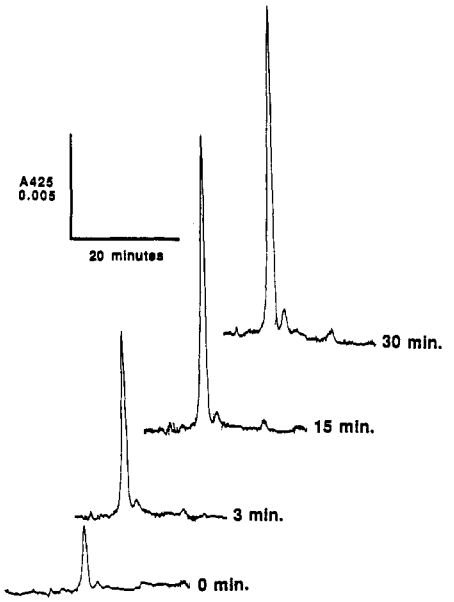

FIGURE 5: Time course for the conversion of CRALBP·11-*cis*-retinol to CRALB·11-*cis*-retinaldehyde. A reaction mixture containing CRALBP·11-*cis*-retinol was incubated with buffered RPE microsomes and NAD at 37 °C. At the times indicated, microsomes were removed by centrifugation, and a portion of the supernatant was applied to a Superose-12 gel filtration column. The traces indicate the column profile obtained as monitored by the absorption at 425 nm, the maximum absorption of 11-*cis*-retinaldehyde bound to CRALBP.

DISCUSSION

Retinoids, with the exception of retinyl esters, are usually associated with specific proteins in nature, and characteristics of the resulting complex affect their metabolism or action. Our earlier comparison of the rates of reduction of CRALBP·11-*cis*-retinaldehyde by various chemical reducing agents and alcohol dehydrogenases led to the hypothesis that the binding protein·retinoid complex was a substrate for 11-*cis*-retinol dehydrogenase *in vitro* (Saari & Bredberg, 1982). However, this earlier study was carried out with the reaction run in the direction of reduction at low pH (5.5), and we wished to extend our studies to more physiological conditions and with the presumed physiological direction of the reaction. We were particularly intrigued by the possibility that a binding protein in RPE could affect the partition of 11-*cis*-retinol between two competing enzymatic reactions: esterification by LRAT (Barry et al., 1989; Saari & Bredberg, 1988, 1989) and oxidation by 11-*cis*-retinol dehydrogenase (Lion et al., 1975). The results indicate that CRALBP dramatically affected the competition of LRAT and 11-*cis*-retinol dehydrogenase for 11-*cis*-retinol. Oxidation was stimulated and esterification retarded, relative to the BSA control.

Several interesting questions are posed by these results. Of general interest is whether the substrate for these enzymes is the dissociated retinoid or the CRALBP·retinoid complex. A second question relates to the mechanism of the opposite effects of CRALBP on the two enzymatic rates, *i.e.*, stimulation of oxidation and retardation of esterification rates. Finally, is the observed *in vitro* effect on metabolic routing likely to play a physiological role in the visual cycle?

Nature of the Substrate. It is clear from a number of studies that both the dehydrogenase and LRAT will process "free" retinol. In view of our results, it is of interest whether either of the two enzymes can directly process 11-*cis*-retinol bound to CRALBP. Experiments presented here clearly indicate that a component of the oxidation of CRALBP·11-*cis*-retinol

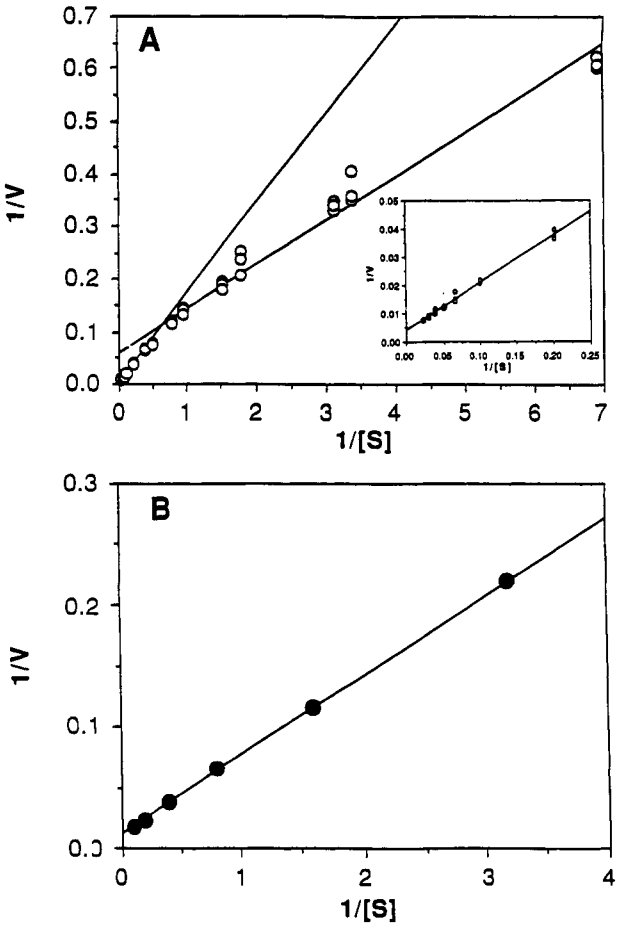

FIGURE 6: Effect of varying CRALBP·11-*cis*-retinol or BSA·11-*cis*-retinol concentration on the velocity of esterification. (A) CRALBP·11-*cis*-retinol. Two slopes are evident for the double-reciprocal plot of the data. The kinetic parameters associated with the reaction at low concentrations of substrate are K_m = 1.3 μM and V_{max} = 17 nmol min^{-1} mg^{-1}. The inset presents the data at a higher substrate concentration on an expanded scale (K_m = 42 μM). (B) BSA·11-*cis*-retinol. A single slope is observed.

involves dissociated retinol but leave open the question of whether protein-bound retinol is a substrate. First, assay of the dehydrogenase reaction in the presence of *O*-ethylhydroxylamine indicated that about one-third of the retinaldehyde generated during the reaction was accessible to the water-soluble reagent during the course of the oxidation. The presence of some 11-*cis*-retinaldehyde in this reaction (68%) could be explained if the rate of reassociation of the product were similar to the rate of oxime formation under the experimental conditions. It could also mean that a fraction of the oxidation occurred without exposing retinaldehyde to the carbonyl reagent. Further experiments are needed to resolve this issue. Second, if the substrate for the dehydrogenase is CRALBP·11-*cis*-retinol, inhibiton of the initial rates of oxidation should have been observed in the presence of CRALBP·11-*cis*-retinaldehyde as identical chromatographic and limited proteolysis profiles suggest that the conformations of CRALBP complexed with these two ligands are very similar. However, there was no effect of CRALBP·11-*cis*-retinaldehyde on the oxidation or esterification rate.

Evidence relating to the reduction of CRALBP·11-*cis*-retinaldehyde by RPE microsomes suggested that the retinoid-binding protein complex is the substrate for the reaction (Saari & Bredberg, 1982). 11-*cis*-Retinaldehyde bound to CRALBP was not reduced by $NaBH_4$, $NaCNBH_3$, borane dimethylamine, or yeast alcohol dehydrogenase and NADH.

However, addition of RPE microsomes and NADH resulted in facile reduction. Thus, although no "free" 11-*cis*-retinaldehyde could be detected with chemical reagents, the dehydrogenase was able to reduce the substrate effectively. While other mechanisms are possible, this study suggested direct interaction of the dehydrogenase and the protein-bound substrate. Determination of the physical parameters associated with retinoid–CRALBP interaction may contribute to a resolution of this apparent contradiction.

The evidence presented here suggests that dissociated 11-*cis*-retinol is the substrate for LRAT. The kinetics of esterification of CRALBP·11-*cis*-retinol show evidence of a biphasic process (Figure 6), which could be interpreted as esterification of bound and free retinoid. However, there is no inhibition of the rate of esterification by added CRALBP·11-*cis*-retinaldehyde (Figure 3). In addition, it is unlikely that the bulky palmitate group could be introduced to 11-*cis*-retinol while it remains bound to CRALBP since the reaction product, retinyl palmitate, has no affinity for the binding protein.

Mechanism of the Opposite Effects on Oxidation and Esterification Rates. If both enzymatic esterification and oxidation occur only with dissociated 11-*cis*-retinol, what mechanisms could account for the stimulation of oxidation and retardation of esterification? We have obtained little evidence that bears directly on this question. One would anticipate that obligatory processing of dissociated retinoid would generally reduce the rate of both reactions since the concentration of "free" retinoid in equilibrium with bound would be low. In addition, since both enzymes are associated with the microsomal membrane fraction from RPE, it is likely that the dissociated retinoid must accumulate to substrate levels in the membranes before appreciable enzymatic processing will occur. The final concentration in the membranes will be a function of the relative affinities of membranes and binding protein for the retinoid (Noy & Xu, 1990). This may be the mechanism responsible for the retardation of the rate of esterification since the evidence presented in Figure 3 suggests that CRALBP is not an inhibitor of LRAT.

Whereas obligatory processing of dissociated retinoid could explain retardation of the rate of esterification observed with CRALBP·11-*cis*-retinol, the oxidation reaction is enhanced (or unaffected at early reaction times), indicating that an additional process is involved. It is possible that CRALBP is an activator of the dehydrogenase. This hypothesis could be tested with apo-CRALBP. We have not been able to prepare regenerable apo-CRALBP in amounts suitable for the analysis. An additional factor may involve the enhanced affinity of CRALBP for the product of the oxidation reaction (11-*cis*-retinaldehyde) (Saari & Bredberg, 1987) and its lack of affinity for the product of the esterification reaction (11-*cis*-retinyl palmitate).

Competition for Substrate in the Visual Cycle. 11-*cis*-Retinol dehydrogenase and LRAT of RPE microsomes compete for 11-*cis*-retinol *in vitro*. The subcellular localization of these enzymes is not known, but it is likely that they compete for 11-*cis*-retinol *in vivo* as well. CRALBP favors oxidation of 11-*cis*-retinol relative to esterification, and it also binds the product of the oxidation reaction with higher affinity than the reactant. The combined action of these two effects could well play a role in directing the metabolism of 11-*cis*-retinol at this critical branch in its metabolism. Recently Okajima et al. (1993) reported that esterification of 11-*cis*-retinol was minimal compared to oxidation in toad eye cup preparations. This suggests that the metabolism of 11-*cis*-retinol is regulated *in vivo* and is consistent with our results, which show diminished esterification of 11-*cis*-retinol in the presence of CRALBP.

Retinoids bound to cellular retinol-binding protein (CRBP) in other tissues have been suggested to be substrates for enzymatic processing. CRBP·*all-trans*-retinol was esterified by LRAT of rat liver microsomes (Ong et al., 1988) or oxidized to retinaldehyde (Posch et al., 1991) in reactions apparently involving direct interaction of enzymes and CRBP. CRBP(II)·*all-trans*-retinaldehyde served as a substrate for a microsomal alcohol dehydrogenase from rat small intestinal mucosa (Kakkad & Ong, 1988), and CRBP(II)·*all-trans*-retinol (Ong et al., 1987) was readily esterified by microsomal LRAT from the same rat tissue. Thus, a physiologic role for CRALBP as a substrate-routing protein is consistent with our earlier study (Saari & Bredberg, 1982) and with studies of other retinoid-binding proteins from other laboratories. Its differing effects on esterification and oxidation are further intriguing.

In summary, enzymatic oxidation of 11-*cis*-retinol bound to CRALBP is favored over enzymatic esterification, whereas the situation is reversed with BSA complexes of the retinoid. The enhanced rate of oxidation could result from activation of the dehydrogenase by CRALBP whereas the reduced rate of esterification could result from obligatory processing of dissociated retinoid. CRALBP may play a role in determining whether 11-*cis*-retinol in the eye is stored as the ester or oxidized to 11-*cis*-retinaldehyde for visual pigment regeneration.

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