Retinal Tubulin Binds Macular Carotenoids

Paul S. Bernstein,† Nikita A. Balashov,* Edward D. Tsong,† and Robert R. Rando†*

Purpose. To investigate the biochemical mechanisms responsible for the specific uptake, concentration, and stabilization of the carotenoids lutein and zeaxanthin in the macula.

Methods. Soluble extracts of bovine retina mixed with radioactive carotenoids were purified by hydrophobic interaction, ion exchange, and gel filtration chromatography. Carotenoid-associated proteins in these purified preparations were identified through photoaffinity labeling and protein microsequencing. Similar purifications on human macular tissue without the addition of exogenous carotenoids also were performed.

Results. Experiments on bovine retinal tissue demonstrated that tubulin is the major soluble carotenoid-binding protein. When soluble extracts of human macular protein were examined, the endogenous carotenoids lutein and zeaxanthin were found to copurify with tubulin.

Conclusions. Tubulin is found in abundance in the receptor axon layer of the fovea, where it can serve as a locus for the deposition of the high concentrations of macular carotenoids found there. The binding interaction of carotenoids and tubulin in the Henle's fiber layer could play an important role in the photoprotective effects of the macular carotenoids against the progression of age-related macular degeneration. The association of carotenoids with tubulin, a protein that can form highly ordered linear arrays, may provide an explanation for the dichroic phenomenon of Haidinger's brushes. Invest Ophthalmol Vis Sci. 1997;38:167–175.

The human macula, that specialized region of the retina responsible for high-resolution visual acuity, selectively accumulates two xanthophyll carotenoids derived from the diet, lutein and zeaxanthin.[1–4] A recent epidemiologic study has demonstrated a strong inverse correlation between dietary intake of lutein and zeaxanthin and the risk of progression of age-related macular degeneration (AMD), the leading cause of blindness among the elderly in the United States.[5] It is thought that the macular carotenoids protect against light-induced damage to the retina by filtering out damaging wavelengths of light or by acting as antioxidants.[6]

Extensive work has shown that lutein and zeaxanthin are concentrated specifically in the foveal receptor axon layer (Henle's fibers) and the inner plexiform layer, with zeaxanthin more concentrated in the foveal center and lutein spread more diffusely throughout the retina.[1–4] This type of distribution implies that there must be a system specifically to take up, concentrate, and store the macular carotenoid pigments, especially because lutein and zeaxanthin are relatively minor constituents of the total carotenoid pool in the bloodstream. Moreover, because approximately 50% of the zeaxanthin in the retina is present as the *meso*–diastereomer, a form not found in the human diet and undetectable in human serum, it has been suggested that the retina may be a site for the enzymatic interconversion of lutein to *meso*-zeaxanthin.[7]

Abnormalities of the uptake, concentration, and metabolic systems for carotenoids could have profound effects on macular function and could be involved in the pathogenesis of AMD. One study in monkeys has shown that a carotenoid-free diet results in the absence of the macular yellow pigment, as well as in changes consistent with early AMD, such as increased drusen.[8] The concept that some human tissues selectively accumulate certain carotenoids is not

*From the *John A. Moran Eye Center, University of Utah School of Medicine, Salt Lake City, Utah, and the †Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts.*
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Reprint requests: Paul S. Bernstein, John A. Moran Eye Center, University of Utah School of Medicine, 50 North Medical Drive, Salt Lake City, Utah 84132.

unique; the corpus luteum of the ovary, under the influence of luteinizing hormone, concentrates high levels of a carotenoid tentatively identified as β-carotene.[9,10] The biochemical basis for this carotenoid uptake system has not yet been defined either.

The structure and function of carotenoproteins, proteins that bind carotenoids, have been examined in detail in numerous invertebrate systems. The best studied carotenoid binding protein is crustacyanin, the astaxanthin-binding protein responsible for the color of lobster shells. This multimeric protein, whose subunit sequences are related to retinoid-binding proteins, has been sequenced completely and crystallized.[11,12] Numerous other carotenoid-binding proteins from plants and microorganisms have been purified, characterized, and cloned.[13,14] Many of these seem to be involved in photoprotection. By contrast, little is known about carotenoproteins in vertebrates beyond the knowledge that low-density lipoprotein (LDL), transthyretin, β-lactoglobulin, and albumin can carry carotenoids in the bloodstream.[15–18] There also has been a preliminary report of a β-carotene-binding protein from rat liver.[19] It also is thought that astaxanthin, a carotenoid colorant used in commercial fish food, binds to muscle actin in salmon.[18] In light of the extraordinary concentrations of lutein and zeaxanthin found specifically in the primate macula, it is hypothesized that macular carotenoid-binding proteins may be responsible for this specificity.

Besides lutein and zeaxanthin, one other carotenoid is known to be taken up by the human retina. Canthaxanthin is a relatively rare di-keto carotenoid in the human diet, and it is normally undetectable in the blood or in the eye. When ingested in large quantities for a prolonged period for tanning purposes, it causes a crystalline retinopathy.[20] Surprisingly, despite the massive deposition of crystals in the retina, patients are usually asymptomatic. Other vertebrates, such as birds, reptiles, and amphibians, accumulate a variety of carotenoids in cone oil droplets, which may act as optical filters for individual cones.[21] Cone oil droplets are not found in placental mammals.

In the studies reported here, protein–carotenoid interactions were investigated by incubating soluble bovine retinal extracts with radioactive carotenoids. Protein-bound carotenoids were purified by hydrophobic interaction, anion exchange, and gel filtration chromatography. Tubulin was identified as the major carotenoid-binding protein in these purified mixtures of proteins by photoaffinity labeling and protein microsequencing. Similar studies performed on tissue derived from human macula using endogenous carotenoids were consistent with the finding that retinal tubulin binds macular carotenoids.

The binding of carotenoids by tubulin in the eye has not been reported before. This binding interaction may play an important role in the physiology of the macula and of other biologic systems.

MATERIALS AND METHODS

Unless otherwise noted, all procedures were performed under subdued light, with cooling to 4°C whenever possible to prevent carotenoid photooxidation. Lutein was obtained from Kemin Industries (Des Moines, IA). Zeaxanthin was purchased from Indofine Chemical Company (Somerville, NJ). Radioactive carotenoids, [7,7′,8,8′-^{14}C]-(*3R,3R′*)-zeaxanthin (specific activity, 126.9 μCi/mg) and [6,6′,7,7′-^{14}C]-canthaxanthin (specific activity, 105.5 μCi/mg) were generous gifts from Hoffmann–LaRoche (Basel, Switzerland). Both carotenoids had >90% radiochemical purity by high-performance liquid chromatography (HPLC). Protein purification columns were purchased from Pharmacia Biotech (Piscataway, NJ), and HPLC columns were purchased from Rainin (Woburn, MA). Laboratory chemicals and immunochemicals were purchased from Sigma (St. Louis, MO). Protein assays were performed using the Pierce (Rockford, IL) bicinchonic acid method.

Tissue Preparation

Soluble bovine retinal proteins (approximately 5 mg/ml) were prepared as previously described in a study of retinoic acid-binding proteins.[22] Briefly, this involved homogenization and sonication of fresh or frozen retinas in 20 mM Tris buffer (pH 7.5), which was followed by centrifugation at 100,000*g* at 4°C for one hour. The supernatant was stored at −80°C until later use.

Human retinal tissue processing was based on previous studies that characterized the macular carotenoids.[4] Donor eyes usually were collected within 12 hours of death and were processed immediately to remove the cornea for transplantation. The remaining portion of the globe was opened at the equator, and the anterior portion was dissected. Using the dissecting instruments, the vitreous was removed as much as possible. A trephine was used to punch out a 4- to 5-mm circle of tissue centered on the fovea, and the circular piece of retinal tissue was transferred with a forceps to a microcentrifuge tube. Alternatively, the retina could be detached and its connections to the optic nerve severed. The retina was flatmounted onto a piece of plastic, and the macular area was dissected with a trephine as described above.

Collected human maculae from one to six eyes were combined in a microcentrifuge tube, and 0.5 to 1.0 ml of Tris buffer was added. Homogenization was achieved with a microtip probe of an ultrasonic cell disrupter for several seconds on ice. Typically, one human macula yields approximately 0.5 mg of total protein and 0.2 to 0.3 mg of soluble protein. All re-

search performed on the donated postmortem human material adhered to the tenets of the Declaration of Helsinki.

Ion Exchange Chromatography of Bovine Retinal Carotenoid-Binding Proteins

Five milligrams of soluble bovine retinal protein in 1 ml of 20 mM Tris buffer (pH 7.5) was mixed with 10 l of a stock solution of 1 μCi of 2 mM ^{14}C-canthaxanthin (CTX) or ^{14}C-zeaxanthin (ZX) in tetrahydrofuran for a final concentration of 20 μM/l CTX or ZX. After a 1-hour incubation in the dark at room temperature, the material was passed through a small hydrophobic interaction column (hydroxyalkoxypropyl dextran, type IV, substituted 10% by weight with alkyl chains of C_{15}-C_{18}) to remove unbound carotenoid from the solution. The sample was loaded onto a HiTrap Q anion exchange column (Pharmacia) eluted with a gradient from 0 to 1 M NaCl at 1 ml/minute. One-milliliter fractions were collected. Eluted protein was monitored at 280 nm, and 0.5-ml portions of each fraction were counted for radioactivity in Hydrofluor (National Diagnostics, Somerville, NJ).

Photoaffinity Labeling of Carotenoid-Binding Proteins

Peak fractions of radioactivity from the ion-exchange step using ^{14}C-CTX as the ligand were combined and exposed to an intense visible light source from a photochemical reactor fitted with a uranium filter (Ace Glass, Vineland, NJ) at 4°C for 1 hour. Portions of the photolabeled material were extracted with chloroform–methanol to remove unbound radioactivity using previously published methods,[22] and they were concentrated before sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) were performed.

Photolabeled material was concentrated and further purified by gel filtration chromatography on a Superose 12 column (Pharmacia). One quarter of each fraction was counted for radioactivity, and the remainder of the fractions containing the peak of radioactivity was processed for SDS–PAGE and autoradiography.

Peak fractions were combined, concentrated, and transferred to Pro–Blott polyvinylidene difluoride membranes (Applied Biosystems, Foster City, CA) after SDS–PAGE. After staining with Ponceau S, the major protein band identified by autoradiography was excised and sent for protease digestion and peptide sequencing.

Purification of Carotenoid-Binding Proteins From Human Macula

Soluble proteins from one to six human maculae were separated by ion-exchange chromatography on 1 ml HiTrap Q or Resource Q columns (Pharmacia) without the addition of exogenous carotenoids using a BioLogic protein purification system (Bio-Rad, Hercules, CA). Absorbance measurements at 280 nm (protein) and 436 nm (carotenoid) were determined simultaneously. Portions of fractions with significant absorbance at 436 nm were extracted into hexane and injected onto an HPLC system (Waters, Milford, MA) fitted with a Rainin Microsorb-MV cyano column (5 μm, 4.6 mm × 25 cm) for carotenoid analysis. The isocratic elution solvent was 20% methylene chloride, 0.25% methanol, 0.1% diisopropylethylamine, 80% hexane at 1 ml/minute with detection at 450 nm, a system that allows baseline separation of lutein and zeaxanthin.[23] Fractions with significant amounts of carotenoid associated with protein were analyzed further by SDS–PAGE and western blotting.

Western Blots

Protein samples were concentrated in Microcon-10 centrifugal concentrators (Amicon, Beverly, MA) and dissolved in SDS–PAGE sample buffer containing β-mercaptoethanol. After SDS–PAGE, separated proteins were transblotted to nitrocellulose using a Bio-Rad semidry apparatus. After blocking with 5% nonfat milk, the blots were probed with a 1:1000 dilution of the primary mouse monoclonal antibody to α-tubulin (clone B-5-1-2). The secondary antibody was goat anti-mouse IgG(F_c) linked to alkaline phosphatase. Visualization was by 5-bromo-4-chloro-3-indoloyl phosphate–nitro blue tetrazolium reagent.

RESULTS

Purification of the Major Carotenoid-Binding Protein From Bovine Retina

Initial studies to identify possible ocular carotenoid-binding proteins used bovine retina as the tissue source to conserve scarce human and primate macular tissue until suitable purification procedures and conditions had been established more firmly. Bovine ocular material is easily available in large quantities. No macula was present, however, so the focus of these preliminary experiments had to be on general mechanisms of carotenoid accumulation by the retina. Lutein and zeaxanthin are present in the bovine retina in small quantities, comparable to the amounts found in human peripheral retina. Thus, we found it advantageous to add exogenous radioactive carotenoids during the purification process to enhance detection sensitivity.

A radioactive carotenoid ligand, either ^{14}C-ZX or ^{14}C-CTX, was mixed with soluble bovine retinal proteins and incubated in the dark for 1 hour at room temperature to allow for carotenoid binding. After

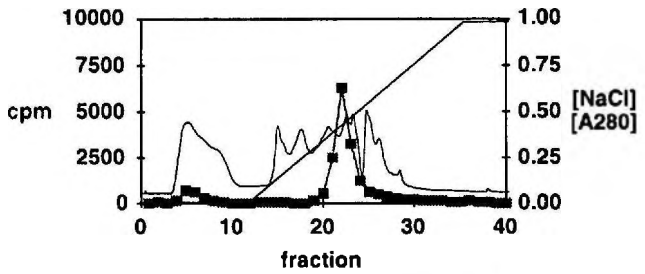

FIGURE 1. Ion exchange chromatography of ^{14}C-canthaxanthin (CTX) bound to bovine retinal protein. Soluble protein from bovine retina (5 mg) was incubated with 20 μM canthaxanthin labeled with ^{14}C (1 μCi). After passing through a hydrophobic interaction column, the sample was loaded onto a HiTrap Q anion exchange column; this was followed by elution with a gradient from 0 to 1 M NaCl. (*line*) Protein (A280). (*boxes*) Radioactive counts.

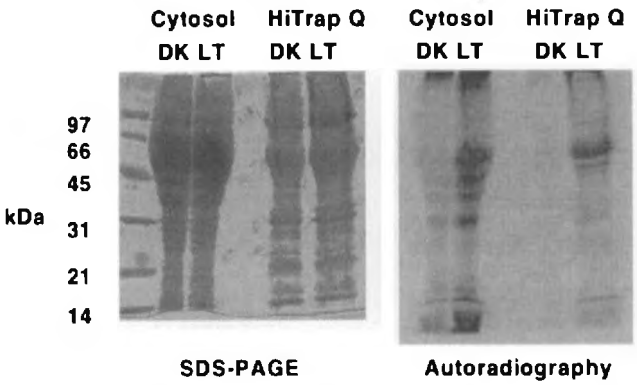

FIGURE 2. Photoaffinity labeling of carotenoid-binding proteins. Two samples of bovine retina cytosol (approximately 500 μg) were incubated with 0.5 μCi of ^{14}C-canthaxanthin (CTX) for 1 hour in the dark (20 μM). One sample was exposed to intense visible light for 1 hour (LT), whereas the other was kept in the dark (DK). Portions of fractions 21 to 23 from the experiment in Figure 1 were treated in a similar manner, except that no supplementary radioactive CTX was added. All samples were extracted with $CHCl_3$–MeOH and were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis with Coomassie staining and autoradiography. Several bands of protein were photoaffinity labeled in bovine cytosol, and the purified radioactive protein peak from the HiTrap Q column showed predominant labeling of a protein band at 55 kDa.

passage through a small hydrophobic interaction column to remove excess unbound carotenoid, the samples were loaded onto an anion exchange column under low salt conditions. Elution with a linear gradient from 0 to 1 M NaCl yielded a single discrete peak of radioactivity that eluted at approximately 0.5 M NaCl (Fig. 1). High-performance liquid chromatography analysis of organic extracts of the peak radioactive fractions revealed that the added radioactive carotenoids indeed were responsible for the radioactivity in these fractions.

Coomassie and silver staining of SDS–PAGE gels of the peak radioactive fractions after ion exchange chromatography revealed a complex mixture of proteins. Photoaffinity labeling was performed to identify the proteins in the mixture responsible for carotenoid binding. The peak fractions carrying ^{14}C-CTX were combined and exposed to intense visible light in a photochemical reactor apparatus to photolabel the binding proteins. Similar photoaffinity labeling experiments using bovine retina cytosol also were carried out. Extraction with chloroform–methanol, followed by SDS–PAGE and autoradiography, demonstrated four bands of radioactive labeling in the 25- to 60-kDa range in bovine retina cytosol, whereas a single major band of radioactivity corresponding to 55 kDa, as well as several minor bands, were present in the ion-exchange purified fractions (Fig. 2).

Further purification by gel filtration of the photoaffinity-labeled peak fractions from the experiment in Figure 2 demonstrated a single peak of radioactivity that eluted just after the void volume on a Superose 12 column (Pharmacia) (Fig. 3). Extraction with chloroform–methanol, followed by SDS–PAGE and autoradiography, showed that the photoaffinity-labeled 55-kDa protein eluted in the peak fractions of radioactivity (Fig. 4).

A sufficient amount of this labeled protein was purified and transblotted to polyvinylidene difluoride and sent for protein sequencing. Protease digestion and peptide sequencing demonstrated a mixture of α- and β-tubulin [TIGGGDDSFNTFFSETGAG (α-tubulin 41-59), LAVNMVPF(P)R (β-tubulin 253-262), and IREEYPDR (β-tubulin 155-162)]. All three sequences were 100% matches to highly conserved regions of tubulin. Purified bovine brain tubulin (Sigma) could

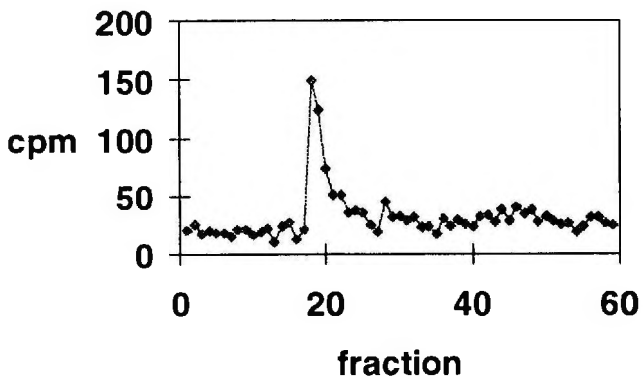

FIGURE 3. Gel filtration of bovine retinal carotenoid-binding protein. Photoaffinity-labeled protein derived from fractions 21 to 23 of the experiments in Figures 1 and 2 were concentrated and loaded onto a Superose 12 gel filtration column eluted with 0.2 M NaCl and 20 mM Tris buffer (pH 7.5) at 0.45 ml/minute. One quarter of each 1-minute fraction was counted for radioactivity. A single major peak of radioactivity eluted just after the void volume.

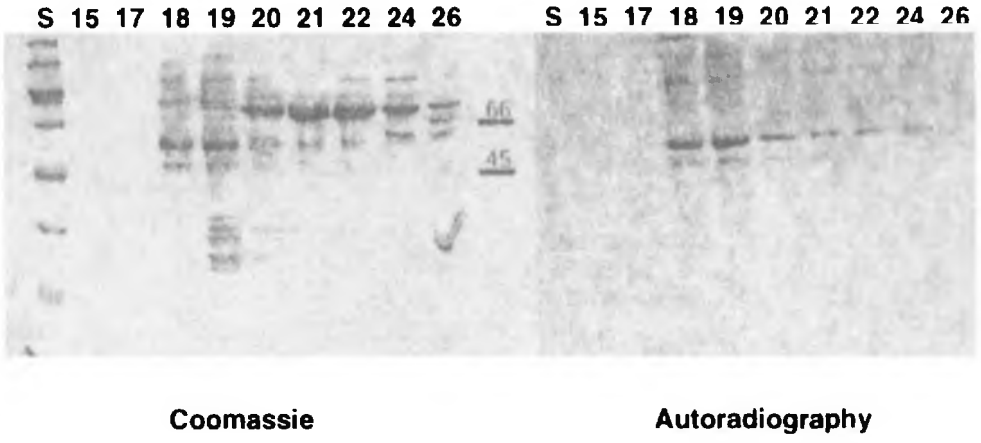

FIGURE 4. Photoaffinity labeling of purified bovine retinal carotenoid-binding protein. Photoaffinity-labeled samples of the indicated fractions from the experiment in Figure 3 were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis and autoradiography. The protein band at 55 kDa was strongly labeled.

indeed be photolabeled with radioactive canthaxanthin (Fig. 5).

Absorbance spectra of the carotenoid-binding protein with canthaxanthin, lutein, or zeaxanthin bound to it were obtained by incubating the nonradioactive carotenoids with soluble bovine retinal extract and purifying the protein through the ion exchange step. Spectral shifts and peak splitting, similar to those seen in carotenoid-binding proteins from lower organisms, were observed (Fig. 6). It should be noted that these protein-bound carotenoid spectra are similar to human macular pigment spectra obtained by microspectrophotometry and psychophysical methods.[1,24] Spectroscopy of these carotenoids bound to purified bovine brain tubulin demonstrated identical shifts.

Purification of Carotenoid-Binding Proteins From Human Macula

Based on the encouraging results of the experiments using bovine retinal tissue and exogenous radioactive carotenoids to detect potential carotenoid-binding proteins, attention was turned to the human macular system, which selectively concentrates very high levels of lutein and zeaxanthin. Typically, a single human macula contains 5 to 40 ng of each carotenoid, an amount that easily permits detection and quantification by HPLC analysis, in which the limit of detection is below 1 ng.[4] The endogenous carotenoid content was thought to be sufficient to allow for detection of potential carotenoid-binding proteins without the addition of exogenous carotenoids.

Soluble extracts of human maculae were loaded onto an anion exchange column in a manner similar to the experiments on bovine retinal tissue described in Figure 1, except that no exogenous carotenoids were added. Absorbance was monitored at 280 nm for protein and 436 nm for carotenoids. The experiment described in Figure 7 showed that it is possible to detect peaks of protein with absorbance at 436 nm

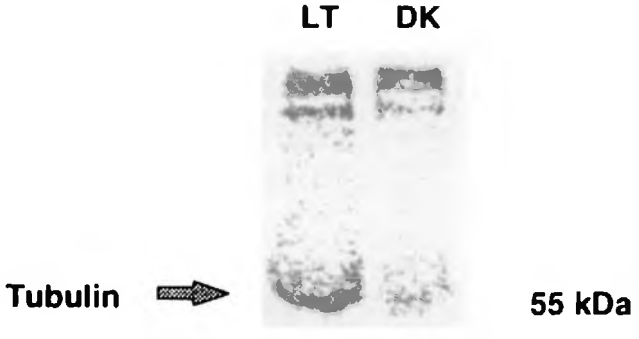

FIGURE 5. Photoaffinity labeling of bovine brain tubulin by ^{14}C-canthaxanthin (CTX). Two 100-μg portions of bovine brain tubulin were incubated with 20 μM ^{14}C-CTX (1 μCi) for 1 hour in the dark. One sample was exposed to intense visible light for 1 hour, whereas the other was kept in the dark. Both samples were extracted with $CHCl_3$–MeOH and analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis and autoradiography. The sample exposed to light (LT) showed strong labeling, whereas the sample kept in the dark (DK) did not.

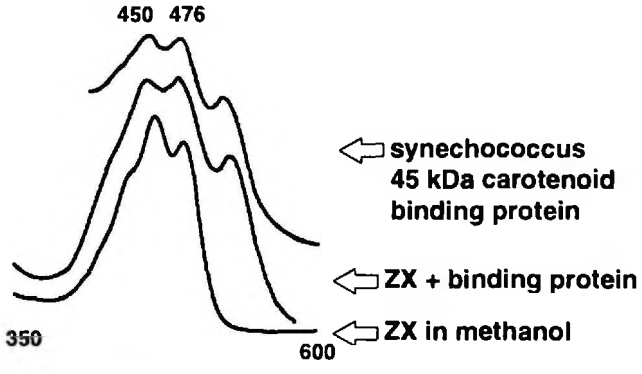

FIGURE 6. Zeaxanthin-binding protein absorption spectra. The visible absorption spectrum of ^{14}C-zeaxanthin (ZX) in methanol is compared to the spectrum of bovine retinal cytosol that had been incubated with exogenous zeaxanthin and purified through the ion exchange step (see Fig. 1 for a comparable purification using CTX). Note the broadening of the peaks and the shifts to absorbance at longer wavelengths. A spectrum of the 45-kDa carotenoid-binding protein from the cyanobacterium Synechococcus is strikingly similar.[14]

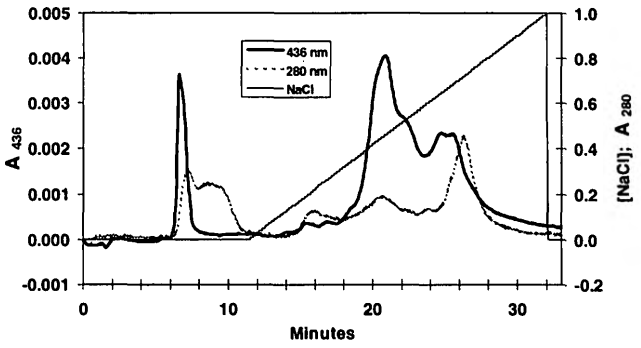

FIGURE 7. Purification of carotenoid-binding proteins from human macula. Soluble proteins from three human maculae were loaded onto a HiTrap Q ion exchange column and eluted with a gradient from 0 to 1 M NaCl with monitoring at 280 nm for protein (*broken line*) and 436 nm for carotenoids (*solid line*). Extraction and high-performance liquid chromatography analysis of the 436-nm peak at 21 minutes showed that lutein and zeaxanthin were present in substantial amounts (see companion experiment in Table 1). The peak at 7 minutes had no detectable carotenoids associated with it, and the double peak at 25 minutes contained only trace amounts of carotenoid. Carotenoids were detected in no more than trace amounts in all other fractions examined.

from soluble extracts of human maculae. The peak eluting at 21 minutes with 0.5 M NaCl has retention characteristics nearly identical to the peak of radioactivity seen when radioactive canthaxanthin is incubated with soluble bovine retinal extracts (Fig. 1). Extraction and HPLC analysis confirm that endogenous lutein and zeaxanthin are bound to this protein fraction from human macula. Carotenoid analyses of the other fractions, with significant absorbance at 436 nm, demonstrated that the peak of unbound protein eluting at 7 minutes had no carotenoid, whereas trace amounts of lutein and zeaxanthin were associated with the broad double peak at 25 minutes. Western blots using a monoclonal antibody to tubulin demonstrated that the major peak of carotenoid-associated protein coincides with the elution of tubulin immunoreactivity (Table 1).

DISCUSSION

Age-related macular degeneration is the leading cause of legal blindness among the elderly in the United States. Initially, patients may have the the atrophic or "dry" form of AMD, with the gradual progressive loss of visual acuity associated with retinal pigmentary changes and the formation of subretinal deposits of oxidized lipid material known as drusen. At virtually any time, the disease can convert to the exudative or "wet" form characterized by subretinal neovascularization and lipid exudation, which can lead to devastating and permanent loss of central vision in a matter of days. The lack of truly effective interventions to prevent or treat AMD has made it one of the most frustrating diseases commonly encountered in ophthalmologic practice.

Carotenoids may play an important role in the pathogenesis and prevention of AMD.[6] Within the retina, light and the high levels of oxygen create an environment in which reactive free-radicals of oxygen and related species may cause damage to the highly polyunsaturated lipids of the photoreceptor outer segment membranes, as well as proteins, DNA, and carbohydrates. Recently, it has been shown that persons with high dietary intakes of carotenoids and with high serum carotenoid levels are at lower risk for macular degeneration.[5,25] The correlation between high carotenoid levels and decreased relative risk for AMD is particularly provocative in light because of the integral role of carotenoids in normal retinal function. β-carotene is an effective antioxidant, and it is a major dietary precursor for the vitamin A used in the visual

TABLE 1. Copurification of Macular Carotenoids With Tubulin*

Fraction Number	*Lutein (μV/second)*	*Zeaxanthin (μV/second)*	*Tubulin Immunoreactivity*
18	Undetectable	Undetectable	Undetectable
19	Undetectable	Undetectable	Undetectable
20	Not determined	Not determined	Undetectable
21	1850	1160	Trace
22	1890	1190	Positive
23	2247	2970	Positive
24	1410	720	Positive
25	1200	680	Trace
26	Undetectable	Undetectable	Trace

* Soluble proteins from six human maculae were purified by ion exchange chromatography as described in Figure 7. Fractions corresponding to the major peak of absorbance at 436 nm eluting at 0.5 M NaCl were analyzed for carotenoid content by HPLC and for tubulin immunoreactivity by Western blotting with a monoclonal antibody to α-tubulin.

cycle. Two other dietary carotenoids, lutein and zeaxanthin, though unable to act as precursors for vitamin A, appear to be especially important for normal macular function. These two compounds, of the approximately 10 carotenoids found in normal human serum, are concentrated in high amounts in the cells of the human macula.[1-4] These pigments account for the characteristic yellow coloration of the primate macula, and it is thought that they may function as filters to attenuate photochemical damage, image degradation associated with bright short-wavelength light, or both, and that they may function as a large store of antioxidant power within the retina.

The biochemical mechanisms that mediate the selective uptake, concentration, and stabilization of the macular carotenoids are unknown. In lower animals, such as lobsters and cyanobacteria, specialized carotenoid-binding proteins perform these tasks. It is hypothesized that comparable carotenoid-binding proteins may have a similar role in the human macula.

To study the interactions of carotenoids and proteins in the vertebrate eye, initial experiments focused on the bovine retina, which contains lutein and zeaxanthin at levels comparable to those found in human peripheral retina. Exogenous radioactive carotenoids were added to soluble bovine retinal extracts to enhance the sensitivity of detection. As shown in Figure 1, purification by hydrophobic interaction chromatography, followed by anion exchange chromatography, demonstrated that the radioactive carotenoid ligand eluted as a single discrete protein-associated peak.

The partially purified carotenoid-binding protein preparation obtained in Figure 1 was a complex mixture of proteins. It was thought that photoaffinity labeling with the bound radioactive carotenoid ligand would be an exceedingly useful means to help identify the proteins within the mixture that were responsible for carotenoid binding. This method has been used to identify retinoic acid-binding proteins in bovine retina.[22] An important aspect of this purification technique relies on the fact that canthaxanthin fits a key criterion of a photoactivatable compound with its α,β-unsaturated carbonyl structure. This allows for the covalent photolabeling of proteins that interact with the carotenoid. Covalently labeled proteins thus can be followed during the purification process without worry about the loss of ligand from the protein, and the molecular weight of the labeled protein is assessed easily by SDS–PAGE and autoradiography. Using radioactive zeaxanthin rather than canthaxanthin in the purification process yielded similar results; however, it proved to be more susceptible to degradation, and it did not work efficiently as a specific photoaffinity-labeling agent.

Photoaffinity-labeling experiments (Fig. 2) demonstrated that four major bands of photoaffinity-labeled protein were present in bovine retina cytosol. The most heavily labeled of these proteins had an apparent molecular weight of 55 kDa and was responsible for the majority of carotenoid-binding activity in the peak fractions after ion exchange chromatography. After further purification by gel filtration chromatography (Figs. 3, 4), a sufficient amount of this protein was harvested and submitted for proteolysis and peptide sequencing, which demonstrated that the labeled material consisted of a mixture of α- and β-tubulin. Purified bovine brain tubulin (Sigma) was successfully photoaffinity labeled by ^{14}C-CTX (Fig. 5).

Attention was then turned to human macular tissue, which concentrates many times more lutein and zeaxanthin on a per milligram basis than human peripheral retina or bovine retina.[4] It was feasible to study macular carotenoid-binding proteins that had no added exogenous carotenoids. The experiment depicted in Figure 7 showed several major peaks with absorbances at 436 nm eluted from the anion exchange column. Of course, it cannot be assumed that macular carotenoids are the only chemical compounds in the fractions responsible for absorbance at this wavelength. High-performance liquid chromatography analysis of portions of the peak fractions showed that the early peak of unbound material contained no detectable carotenoid. The peak eluting at 21 minutes contained substantial amounts of lutein and zeaxanthin, whereas the double peak at 25 minutes contained only trace amounts of carotenoid. Western blot analysis of a subsequent human macular preparation showed that tubulin immunoreactivity coincided with the peak of carotenoid-associated protein (Table 1).

The finding that carotenoids can bind to retinal tubulin has not been described before. It is expected that Henle's fiber layer of the macula is particularly rich in tubulin because the fibers consist primarily of cone axons. Immunocytochemical studies have shown that microtubules are oriented axially along the cone myoid and axon and are virtually nonexistent in the outer segment.[26] Interestingly, the association of carotenoids and microtubules has been documented in the squirrelfish erythrophore. In these nonocular pigment organelles, droplets of carotenoid migrate along microtubular structures.[27]

It has been speculated that macular carotenoids are located primarily in the axonal membranes[24,28]; however, there is no direct evidence for this, and it could not explain the selective accumulation of carotenoids into the Henle's fiber layer of the macula. On the other hand, an abundant structural protein, such as tubulin, could bind and stabilize the enormous concentration of lutein and zeaxanthin in the fovea in much the same way that astaxanthin is deposited on the actin of salmon muscle fibers.[18]

The association of carotenoids with a protein such

as tubulin, which can form highly ordered macromolecular arrays, provides a possible explanation for the phenomenon of Haidinger's brushes. Psychophysical and microspectrophotometric investigations have demonstrated that carotenoids in Henle's fiber layer are the likely chromophore for the dichroic absorbance properties of the human macula.[28-30] If the protein-bound macular carotenoids are perpendicular to the long axis of the microtubules, they are properly oriented to generate Haidinger's brushes. In addition, a protein-bound mechanism for Haidinger's brushes provides a better explanation than does a pure membrane mechanism because there is evidence that the number of dichroic chromophore sites in the macula is subject to saturation.[29] Support for the protein-bound carotenoid mechanism for Haidinger's brushes would likely require crystallography of lutein or zeaxanthin bound to tubulin to determine whether the carotenoid is bound in the correct orientation.

The interaction between carotenoids and tubulin may have implications for cancer chemotherapy: Carotenoids have antitumor activities that appear to go beyond their antioxidant effects, and tubulin is the target of a number of important antitumor agents, such as taxol, vincristine, vinblastine, and colchicine. Further studies to identify and characterize the carotenoid binding site or sites on tubulin and to investigate the effect of carotenoids on cycles of microtubule polymerization and depolymerization should prove interesting.

Preliminary binding-affinity and specificity studies using bovine brain tubulin have indicated that many carotenoids, including β-carotene, can bind to tubulin. Unless cone tubulin has a particularly high affinity and specificity for zeaxanthin and lutein, it is unlikely that tubulin is the mediator of the specific uptake of the macular carotenoids. It would not be surprising to find that the proteins responsible for the specific uptake of carotenoids are much less abundance. Because the human macula is only several millimeters in diameter, the relatively small amount of starting biologic material is a major problem in attempting to identify additional macular carotenoid-binding proteins. It is likely, too, that some carotenoid-binding proteins are membrane-bound insoluble proteins whose purification is more difficult. Only fellow primates have an equivalent macular anatomy, and macular cells cannot be grown in culture. Hence, certain mammalian macular proteins that might be in low abundance, such as receptors or transport proteins, are more challenging to identify. Further studies to identify additional carotenoid-binding proteins are in progress.

The experiments discussed here provide new insights into the biochemical basis of the specific uptake and concentration of lutein and zeaxanthin into the macula. Derangements of the mechanisms of uptake and stabilization of the macular carotenoids could have profound impact on the progression of age-related macular degeneration and inherited retinal dystrophy. Anticipated interventional clinical studies may use the specific uptake systems to increase the level of macular carotenoid pigment and perhaps to retard or prevent the progressive blindness produced by these diseases.

Key Words

binding protein, carotenoid, macula, photoaffinity labeling, tubulin

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