

# Identification and Characterization of a Pi Isoform of Glutathione S-Transferase (GSTP1) as a Zeaxanthin-binding Protein in the Macula of the Human Eye\*

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Uptake, metabolism, and stabilization of xanthophyll carotenoids in the retina are thought to be mediated by specific xanthophyll-binding proteins (XBPs). A membrane-associated XBP was purified from human macula using ion-exchange chromatography followed by gel-exclusion chromatography. Two-dimensional gel electrophoresis showed a prominent spot of 23 kDa and an isoelectric point of 5.7. Using mass spectral sequencing methods and the public NCBI database, it was identified as a Pi isoform of human glutathione S-transferase (GSTP1). Dietary (3R,3'R)-zeaxanthin displayed the highest affinity with an apparent  $K_d$  of 0.33  $\mu\text{M}$ , followed by (3R,3'S-meso)-zeaxanthin with an apparent  $K_d$  of 0.52  $\mu\text{M}$ . (3R,3'R,6'R)-Lutein did not display any high-affinity binding to GSTP1. Other human recombinant glutathione S-transferase (GST) proteins, GSTA1 and GSTM1, exhibited only low affinity binding of xanthophylls. (3R,3'S-meso)-Zeaxanthin, an optically inactive non-dietary xanthophyll carotenoid present in the human macula, exhibited a strong induced CD spectrum in association with human macular XBP that was nearly identical to the CD spectrum induced by GSTP1. Likewise, dietary (3R,3'R)-zeaxanthin displayed alterations in its CD spectrum in association with GSTP1 and XBP. Other mammalian xanthophyll carrier proteins such as tubulin, high-density lipoprotein, low-density lipoprotein, albumin, and  $\beta$ -lactoglobulin did not bind zeaxanthins with high affinity, and they failed to induce or alter xanthophyll CD spectra to any significant extent. Immunocytochemistry with an antibody to GSTP1 on human macula sections showed highest labeling in the outer and inner plexiform layers. These results indicate that GSTP1 is a specific XBP in human macula that interacts with (3R,3'S-meso)-zeaxanthin and dietary (3R,3'R)-zeaxanthin in contrast to apparently weaker interactions with (3R,3'R,6'R)-lutein.

Carotenoids are ubiquitous highly colored pigments synthesized exclusively by plants and microorganisms. Over 600 carotenoids have been isolated from natural sources, and ~370 of these carotenoids are chiral, bearing one to five asymmetric carbon atoms (1). Many carotenoids are classified as xanthophylls because they contain at least one oxygen atom along their  $\text{C}_{40}\text{H}_{56}$  isoprenoid core structure. Higher animals cannot synthesize carotenoids, but they can ingest them in their diet and subsequently utilize them for important physiological functions. Whereas >15 different dietary carotenoids are detectable in human serum, only lutein ((3R,3'R,6'R)- $\beta,\epsilon$ -carotene-3,3'-diol) and zeaxanthin (a mixture of (3R,3'R)- $\beta,\beta$ -carotene-3,3'-diol and (3R,3'S-meso)- $\beta,\beta$ -carotene-3,3'-diol) (see Fig. 1) and their metabolites are found to any substantial extent in the retina (2–4). High macular levels of these antioxidant and blue light-screening xanthophyll carotenoids are associated with decreased risk of age-related macular degeneration (5, 6), the leading cause of blindness in the developed world.

In the foveal region of the macula of the primate retina, lutein and zeaxanthin concentrations are at their highest, and they are spatially localized to the outer plexiform layer (also known as the receptor axon or Henle fiber layer) and to the inner plexiform region (7). The concentration of the macular carotenoids falls precipitously outside of the foveal region, so that the concentration of lutein and zeaxanthin in the peripheral retina per unit area is 1% of the concentration at the fovea (8), and a considerable portion of the extrafoveal carotenoids is associated with the rod outer segments (9, 10).

Whenever a tissue exhibits highly selective uptake and deposition of biological molecules, it is likely that specific binding proteins are involved. These binding proteins may act as cell surface receptors, transmembrane transport proteins, metabolic enzymes, intracellular mediators of the biological actions of the ligand, or sites for the deposition and stabilization of the ligand. Carotenoid-binding proteins have been described in plants (11), microorganisms (12, 13), and invertebrates (14, 15), but relatively little information has been available about specific carotenoid-binding proteins in any vertebrate system. In 1997, Rao *et al.* (16) reported the existence of a  $\beta$ -carotene-binding protein in ferret liver, although no sequence data have been provided yet. More recently, we have described the properties of a partially purified membrane-associated xanthophyll-binding protein (XBP)<sup>1</sup> found in human retina and macula (17,

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<sup>1</sup> The abbreviations used are: XBP, xanthophyll-binding protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione S-transferase; GSTA, glutathione S-transferase Alpha; GSTM, glutathione S-transferase Mu; GSTP, glutathione S-transferase Pi; HDL, high-density lipoprotein; IEF, isoelectric focusing;

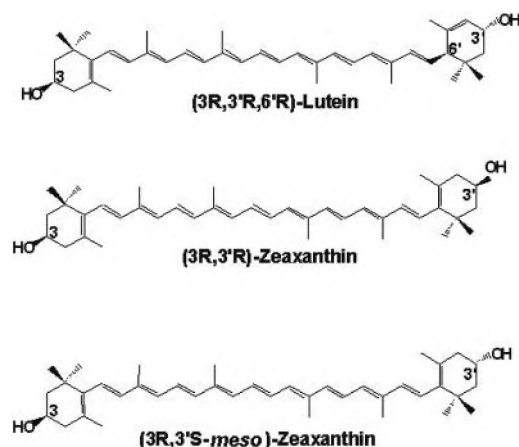


FIG. 1. Structures of xanthophyll carotenoids found in human retina.

18). We have also reported that retinal tubulin exhibits carotenoid binding properties (19, 20), but with less specificity and affinity than XBP (17). Likewise, several other mammalian proteins such as high-density lipoprotein (HDL), low-density lipoprotein (LDL), albumin, and  $\beta$ -lactoglobulin can act as carrier proteins for carotenoids, although there is no evidence that they do so with high specificity or affinity (17, 21–23).

In this study, we further purify human macular XBP and identify the major protein spot on two-dimensional gels as a Pi isoform of glutathione *S*-transferase (GSTP1). Pharmacological and spectroscopic binding studies with human recombinant GSTP1 demonstrate that its interactions with zeaxanthin closely match those of XBP purified from human macula.

#### EXPERIMENTAL PROCEDURES

**Preparation of Solubilized Membrane Extracts from Human Macula**—Preparation of XBP was based on previously published methods (17), with minor modifications. Thawed human maculae (6–8 mm punches) originating from 15 postmortem eyes (average age,  $40 \pm 10$  years) were homogenized in 1.0 ml of a cell lysing solution containing protease inhibitors (Roche Diagnostics) in 20 mM Tris buffer (pH 7.4) supplemented with 1 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , and 15% (w/v) sucrose. Sucrose density gradient (5–50% (w/v)) centrifugation at  $5,000 \times g$  for 30 min at 2 °C resulted in a low-density yellow-colored band containing XBP. The yellow fraction was diluted with Tris buffer (pH 7.4) to bring the sucrose concentration down to 5% (w/v).

A high-speed ultracentrifugation of the diluted yellow fraction was performed at  $200,000 \times g$  for 60 min at 2 °C. The high-speed supernatant was discarded, and the yellow high-speed pellet was solubilized with brief sonication on ice into 500  $\mu\text{l}$  of 25 mM CHAPS in 20 mM MES buffer (pH 5.5) containing 1 mM  $\text{CaCl}_2$  and 2 mM  $\text{MgCl}_2$ , followed by centrifugation at  $100,000 \times g$  for 60 min at 2 °C. The CHAPS-insoluble pellet was resolubilized into CHAPS/MES buffer several more times, followed by high-speed centrifugation as described above. The pale yellow CHAPS-solubilized supernatants were combined and concentrated to 500  $\mu\text{l}$  using an Ultrafree centrifugal filtration system (Millipore, Woburn, MA; molecular mass cutoff, 10 kDa).

**Ion-exchange Chromatography and Silica Gel Chromatography of XBP**—Protein chromatography was performed on a BioLogic liquid chromatography system (Bio-Rad). Detergent-solubilized human macular membrane extracts were loaded immediately after preparation onto an Amersham Biosciences S cation-exchange column (7  $\times$  35 mm,  $\text{SO}_3^-$  exchanging groups). Initial buffer conditions were 20 mM MES buffer containing 8 mM CHAPS at pH 5.5 run at 0.5 ml/min at 4 °C for 15 min, followed by a linear gradient from 0 to 1 M NaCl over 15 min. At later purification stages, anion-exchange chromatography was performed on an Amersham Biosciences Q column (7  $\times$  35 mm,  $\text{N}(\text{CH}_3)_3^+$  exchanging groups). Initial buffer conditions were 20 mM Tris buffer

(pH 7.4) containing 8 mM CHAPS, followed by a linear gradient from 0 to 1 M NaCl over 20 min. Silica gel chromatography was done using a silica gel filtration column (BIOSEP-SEC-S 3000 PEEK;  $300 \times 7.80$  mm; separation range, 5–700 kDa; Phenomenex, Torrance, CA). Concentrated fractions (up to 200  $\mu\text{l}$ ) from the ion-exchange columns were loaded onto the gel filtration column, and 50 mM sodium phosphate buffer (pH 7.0) containing 8 mM CHAPS was used as the eluant at a 0.3 ml/min flow rate. At all stages of chromatographic purification, the eluates were monitored by a UV6000LP photodiode array spectrophotometer equipped with a high sensitivity 50 mm pathlength light-pipe flowcell with an internal volume of 10  $\mu\text{l}$  (Thermo Separations, San Jose, CA), and 300  $\mu\text{l}$  fractions were collected. The fractions of interest containing proteins associated with endogenous macular carotenoids exhibited strong 280 nm protein absorbance and prominent triple peak vibronic structure centered at  $\sim 460$  nm, typical of most xanthophyll carotenoids.

**Carotenoid Extraction and HPLC**—Fifty  $\mu\text{l}$  of purified fractions at each stage of column chromatography were treated with 100  $\mu\text{l}$  of methanol to disrupt ligand-protein associations, and they were then extracted into 200  $\mu\text{l}$  of hexane. Phase separation was promoted by the addition of 20  $\mu\text{l}$  of saturated sodium chloride. The organic extracts were dried by vacuum evaporation in a Speedvac Plus (SC110; Savant, Cambridge, MA) and redissolved in 1 ml of HPLC mobile phase (hexane:dichloromethane:methanol:*N,N'*-di-isopropylethylamine, 80:19.2:0.7:0.1 (v/v)). HPLC separation was carried out at a flow rate of 1.0 ml/min on a cyano column (Microsorb 25-cm length  $\times$  4.6-mm inner diameter; Varian Inc., Palo Alto, CA). The column was maintained at room temperature, and the HPLC detector was operated at 450 nm. Peak identities were confirmed by photodiode array spectra and by coelution with authentic standards as necessary. Peak areas were integrated and quantified with an external standardization curve.

**Two-dimensional Isoelectric Focusing and Electrophoresis**—Isoelectric focusing (IEF) separations were done using precast immobilized pH gradient gel strips (pH 5–8; strip length, 11 cm; Bio-Rad). The immobilized pH gradient strips were rehydrated overnight in trays loaded with the protein samples in the manufacturer's sample buffer. Isoelectric focusing was performed in a Bio-Rad Protean IEF cell unit at 20 °C, with a maximum current of 50  $\mu\text{A}$ /strip. The end voltage for the IEF run was 8,000 V. The strips were equilibrated with buffers containing dithiothreitol and iodoacetamide. After SDS-PAGE on a 1-mm-thick 4–20% gradient Tris-HCl gel, protein spots were stained with fluorescent Sypro Ruby protein stain (Bio-Rad). Gels were then visualized and documented (AlphaDigiDoc 1201; Alpha Innotech, San Leandro, CA) using a UV light source. Detected spots were processed for mass spectral identification.

**Mass Spectral Protein Identification**—Excised protein samples were digested in-gel with sequencing-grade modified trypsin (Promega), according to a previously reported method (24). Digests were desalted and concentrated to 4  $\mu\text{l}$  by solid-phase extraction using  $\mu\text{C18}$  ZipTips (Millipore, Billerica, MA). The matrix-assisted laser desorption/ionization (MALDI) plate was then spotted with 1  $\mu\text{l}$  of sample and 1  $\mu\text{l}$  of  $\alpha$ -cyano-hydroxycinnamic acid as the matrix. Mass spectrometry employed a QSTAR Pulsar instrument equipped with a MALDI source (Applied Biosystems, Framingham, MA). Fragmentation by collision-induced dissociation was performed on parent ions representing each peptide. The collision-induced fragmentation spectrum for each parent ion was then submitted to the Mascot web site (Matrix Science, London, United Kingdom) for database searching.

**Carotenoid Binding Assays**—Synthetic (3*R*,3'*R*)-zeaxanthin and (3*R*,3'*S*-*meso*)-zeaxanthin were gifts from DSM Nutritional Products (Kaiseraugst, Switzerland), and (3*R*,3'*R*,6'*R*)-lutein prepared from marigold flowers was a gift from Kemin Foods (Des Moines, IA). XBP requires CHAPS detergent to maintain solubility and stability during purification, and the CHAPS detergent also aids in solubilizing the xanthophyll carotenoid ligands; therefore, all carotenoid binding experiments for XBP and other proteins were done in the presence of identical concentrations of CHAPS. In a typical binding experiment, 10  $\mu\text{l}$  of concentrated carotenoids (1–250  $\mu\text{M}$ ) dissolved in tetrahydrofuran (THF) were added to 490  $\mu\text{l}$  of 50 mM Tris-CHAPS (8 mM) buffer containing 1  $\mu\text{g}$  of protein ( $\sim 0.1 \mu\text{M}$ , based on a subunit molecular mass of 23 kDa). After brief mixing, the mixture was incubated overnight (16 h) at 4 °C. Kinetic binding studies with (3*R*,3'*R*,6'*R*)-lutein, (3*R*,3'*R*)-zeaxanthin, and (3*R*,3'*S*-*meso*)-zeaxanthin demonstrated that equilibrium was reached within 12–14 h. Unbound carotenoids were removed by four cycles of extraction with 200  $\mu\text{l}$  of hexane, followed by centrifugation for 5 min at  $1,800 \times g$  in a microcentrifuge and removal of the organic solvent layer. Residual hexane was removed from the extracted sample under a stream of argon. THF and hexane were chosen in the

LDL, low-density lipoprotein; MALDI, matrix-assisted laser desorption ionization; MES, 4-morpholineethanesulfonic acid; THF, tetrahydrofuran; HPLC, high-performance liquid chromatography; PBT, phosphate buffer containing 0.1% Triton X-100.

protocol as the preferred organic solvents for carotenoid delivery and extraction because they do not typically lead to denaturation of carotenoid-binding proteins (16, 17). During all experimental procedures, the samples remained clear, with no signs of precipitation. In many cases, UV-visible absorption and CD spectra were measured before extraction and HPLC carotenoid analysis.

Computerized nonlinear regression analyses of binding parameters using one- and two-site models were performed using PRISM version 3.0 (GraphPad Software Inc., San Diego, CA). All binding curves and calculations were corrected for nonspecific binding by subtracting parallel incubations in which protein had been omitted. Binding assays were performed in a similar manner at equivalent protein concentrations with human recombinant glutathione *S*-transferase isoforms GSTP1, GSTA1, and GSTM1, and with other mammalian carotenoid carrier proteins, bovine brain tubulin, human serum HDL, human serum LDL, bovine milk  $\beta$ -lactoglobulin, and human serum albumin (essentially fatty acid-free). All of these commercially prepared proteins were obtained from Sigma.

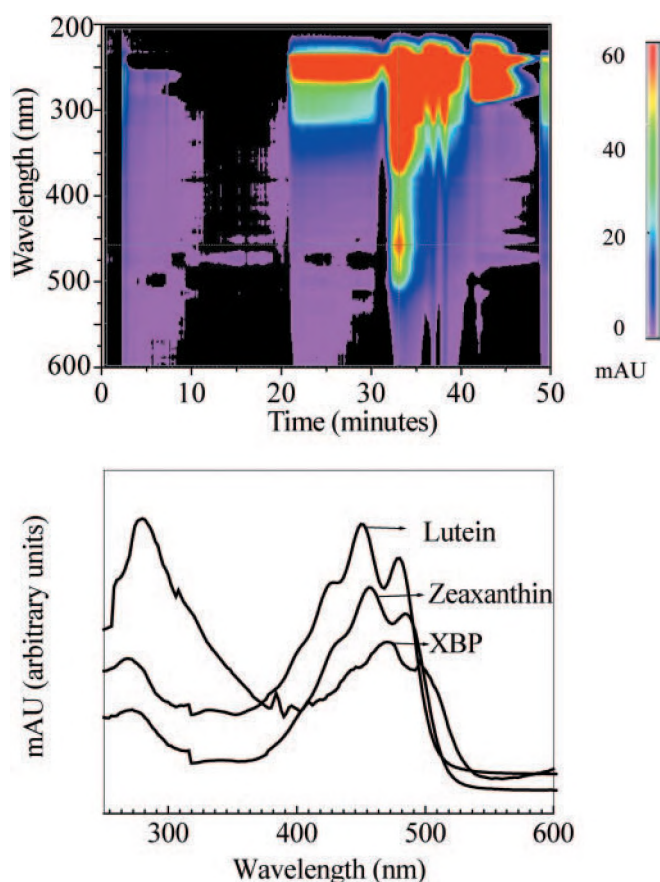
**Spectral Measurements**—CD spectra were recorded at  $4\text{ }^{\circ}\text{C} \pm 0.2\text{ }^{\circ}\text{C}$  with an Aviv Associates CD spectrophotometer (Model 62 DS; Lakewood, NJ). Spectra were recorded at a scan speed of 10 nm/min and a spectral bandwidth of 1.0 nm. Quartz cells with pathlengths of 1 mm were used for all CD measurements, and the spectra were obtained in the wavelength range of 200–700 nm. Each spectrum was the average of 10 replicate scans in steps of 1 nm. All measurements were reported in millidegrees of ellipticity ( $\text{m}^{\circ}$ ). The UV-visible absorption measurements on the samples were done using an absorption spectrophotometer (Lambda 9; PerkinElmer Life Sciences) at room temperature at a scan speed of 30 nm/min with 1 nm resolution in quartz cells with pathlengths of 1 mm.

**Immunocytochemistry and Western Blotting**—Human donor eyes were obtained from the Utah Lions Eye Bank (Salt Lake City, UT). After removal of the anterior segment and vitreous, the eye cup from a 23-year-old multiple organ donor was fixed 3 h post-mortem using freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 90 min, rinsed, and cryoprotected overnight in 30% sucrose in buffer. Subsequently, a rectangular piece of retina centered approximately on the fovea was isolated, embedded in TBS tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC), and frozen. Cryosections (14  $\mu\text{m}$  thick) were cut, rinsed in 0.1 M PBT, and blocked for 1 h using 10% normal donkey serum in PBT. Antibodies to GSTP1 (Alpha Diagnostic International, San Antonio, TX; GSTP1-S; 1:200 dilution) and cone arrestin (7G6 from Peter R. MacLeish; Morehouse School of Medicine, Atlanta, GA; 1:500 dilution) were applied overnight at  $4\text{ }^{\circ}\text{C}$ . The polyclonal antibody against GSTP1 was raised in rabbit using HPLC-purified rat protein as the immunogen. After rinsing in three 10-min PBT washes, rhodamine- and fluorescein isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA; catalog no. 711-295-152 and 715-096-150; each used at a 1:200 dilution) were applied for 2 h at room temperature to visualize sites of label. The immunolocalization was imaged using a Zeiss LSM 510 confocal microscope set to an optical slice of  $<0.9\text{ }\mu\text{m}$ . Control sections, in which incubation in primary antibodies was omitted, were processed in parallel and found to be negative for immunoreactivity.

Protein samples were separated on SDS-PAGE and transferred to nitrocellulose filters using a Bio-Rad *trans*-blot apparatus at 100 V for 2 h. Nonspecific binding was blocked by immersing the membrane in 5% nonfat dried milk, 0.1% Tween 20, in 1% donkey serum in phosphate-buffered saline for 1 h at room temperature on an orbital shaker. The membrane was briefly rinsed with two changes of wash buffer and incubated with diluted primary antibody (1:400; rabbit anti-rat GSTP1) for 1 h at room temperature on an orbital shaker. After two changes of wash buffer, peroxidase-conjugated affinity-purified secondary antibody was diluted in phosphate-buffered saline (1:800; donkey anti-rabbit IgG; catalog no. 711-035-1520; Jackson ImmunoResearch Laboratories). The membrane was incubated in the diluted secondary antibody for 1 h at room temperature on an orbital shaker and then developed using ECL Plus Western blotting detection reagents (Amersham Biosciences).

## RESULTS

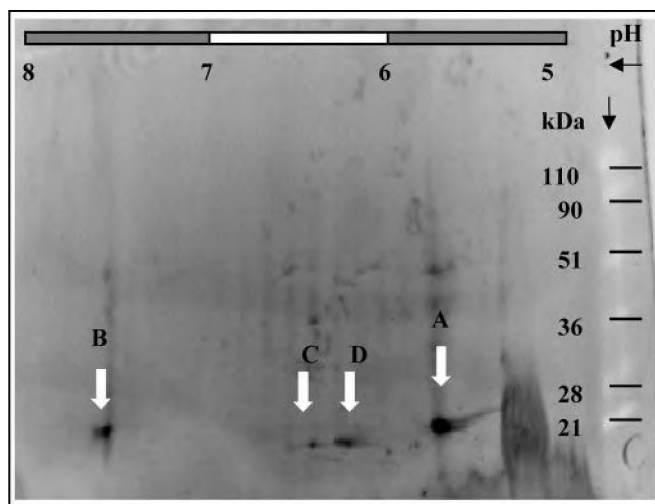
**Purification of Human Macular XBP**—We have reported previously that partially purified membrane preparations from human macula have saturable and specific binding sites for xanthophyll carotenoids (17). As described under “Experimental Procedures,” we have improved the XBP purification proto-



**FIG. 2. Elution pattern of human macular XBP on a silica gel chromatographic column.** Contour view of the on-line photodiode array scan of the chromatographic purification of XBP (*top panel*). Absorbance spectrum of purified XBP eluting from the silica gel column at 32 min compared with that of (3*R*,3'*R*)-zeaxanthin and (3*R*,3'*R*,6'*R*)-lutein standards dissolved in THF (*bottom panel*). There is a bathochromic shift of the absorbance peaks of XBP (438, 468, and 488 nm) relative to zeaxanthin (425, 456, and 478 nm) and lutein (425, 450, and 476 nm).

col by the addition of a sucrose gradient centrifugation step before detergent solubilization and by enhanced chromatographic separations. XBP is a membrane-associated protein; therefore, it is necessary to use detergents to solubilize the protein during chromatographic separations. Our previously reported protocol for purification of XBP utilized CHAPS, an excellent detergent for studying membrane-associated proteins (17, 25). CHAPS, like most detergents useful in protein purifications, is a chiral molecule (26). Because these chiral detergents can complicate interpretation of circular dichroism studies performed for characterization of proteins, we also explored the use of nonchiral detergents such as Triton X-100 for purification. Unfortunately, XBP was unstable in all nonchiral detergents that we examined. Thus, all protein studies reported here included CHAPS in the buffer, and suitable controls were always incorporated in the experimental protocols to correct for the presence of a chiral detergent.

The light yellow XBP fraction that elutes from the final silica gel chromatography step exhibits a characteristic carotenoid absorbance profile, displaying a primary peak at 468 nm, with secondary peaks at 438 and 488 nm (Fig. 2). HPLC analysis of the most purified fractions confirms that both zeaxanthin and lutein are present in a ratio of 2.5:1. XBP purified from human macula carrying only endogenous carotenoids displayed a consistent  $A_{460}/A_{280}$  of  $\sim 0.5$ . Addition of saturating amounts of exogenous lutein or zeaxanthin to XBP, followed by hexane extraction to remove unbound excess carotenoids, raised the



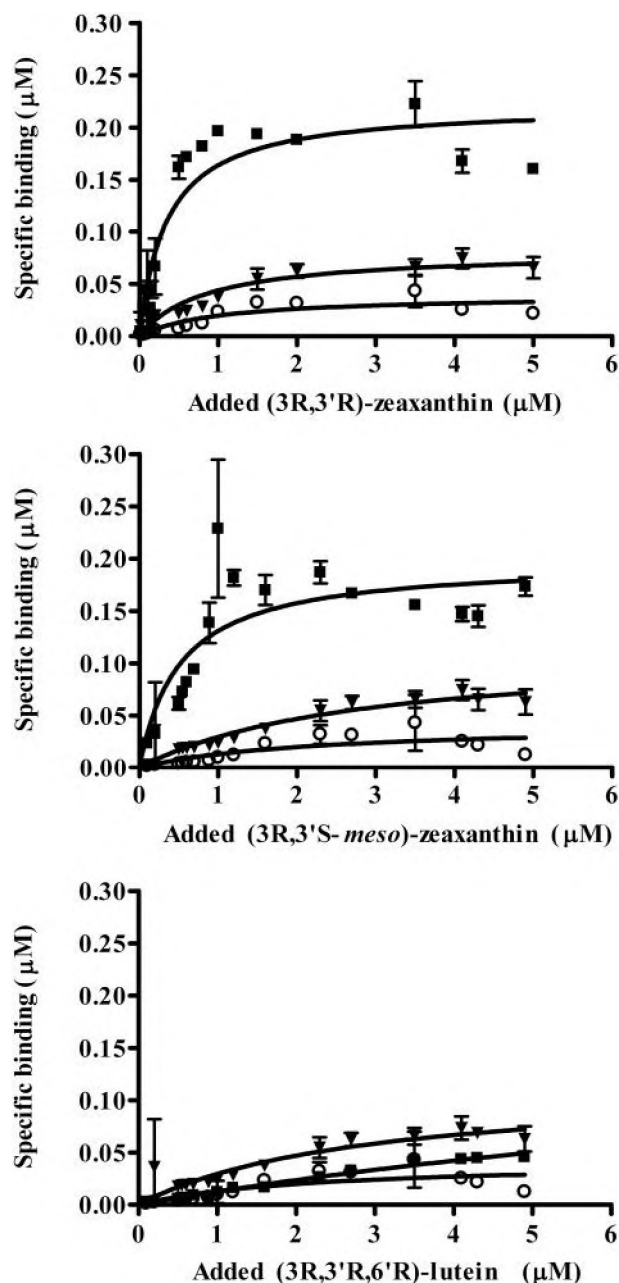
**FIG. 3. Two-dimensional IEF and SDS-PAGE of human macular XBP.** An aliquot of highly purified XBP was subjected to isoelectric focusing and SDS-PAGE analysis according to the procedure detailed under "Experimental Procedures." Protein spots were stained with fluorescent Sypro Ruby protein stain and then visualized using a UV light source. Spot A is GSTP1. The other spots (B–D) have not yet been identified.

$A_{460}/A_{280}$  ratio to 1.5, indicating that chromatographically purified macular XBP is ~30% saturated with endogenous carotenoids.

**Mass Spectral Analysis of Highly Purified Human Macular XBP**—One-dimensional SDS-PAGE gels of chromatographically purified human macular XBP have only a single major band at ~23 kDa, but two-dimensional IEF/SDS-PAGE reveals that there are actually four 21–23-kDa spots with isoelectric points ranging from 5.7 to 7.5 when stained with Sypro Ruby. These are designated as spots A–D in Fig. 3. Because it is the strongest staining and presumably the most abundant protein in our XBP fraction, we first performed mass spectral sequencing on spot A (isoelectric point, 5.7). We reproducibly detected three strong tryptic peptides at  $m/z$  1337.7, 1883.9, and 2126.2 on several two-dimensional gels from independent preparations. These peptides were further analyzed using collision-induced fragmentation techniques to obtain additional sequence information on them. Using a Mascot search engine and the public NCBI database, we were able to positively identify spot A as GSTP1. The three peptides correspond to the sequences PPYTVVYFPVR, FQDGLTLYQSNTILR, and ALPGQLKPFETLLSQNQGGK, covering 22.5% of the 209-amino acid sequence of GSTP1 at positions 1–11, 55–70, and 121–140. Our MS/MS spectra match fragmentation patterns predicted from these sequences.

Spot B was observed to have an isoelectric point of 7.5. Tryptic digestion and mass spectrometry yielded strong molecular ions of  $m/z$  1654.8 and 1745.8. On further examination of their MS/MS spectra, it is clear that these two species are related; the apparent peptide at  $m/z$  1745.8 is actually the 1654.8 peptide with an as yet undefined posttranslational modification. Neither of the collision-induced fragmentation spectra from these parent ions matches any fragmentation patterns predicted for peptides in the public or private databases, and the MS/MS spectra have not been suitable for definitive *de novo* sequencing. Spots C and D with molecular masses of 21–23 kDa and isoelectric points of 6.2 and 6.5, respectively, have not yet been identified.

**Xanthophyll Binding and Spectral Studies with GSTP1 and XBP**—Binding of exogenous dietary (3R,3'R)-zeaxanthin and nondietary (3R,3'S-*meso*)-zeaxanthin to recombinant human



**FIG. 4. Binding studies of GSTP1, GSTM1, and GSTA1 with macular xanthophylls.** Ten  $\mu$ l of concentrated (3R,3'R)-zeaxanthin (top panel), (3R,3'S-*meso*)-zeaxanthin (middle panel), and (3R,3'R,6'R)-lutein (bottom panel) dissolved in THF were added to 490  $\mu$ l of 50 mM Tris-CHAPS (8 mM) buffer containing 1  $\mu$ g of the three GST isoforms (GSTP1,  $\blacksquare$ ; GSTM1,  $\blacktriangledown$ ; GSTA1,  $\circ$ ). After brief mixing, the mixtures were incubated overnight (16 h) at 4°C. Unbound carotenoids were removed by four cycles of extraction with 200  $\mu$ l of hexane. Only GSTP1 exhibited definite saturable binding with a  $K_d$  of 0.33  $\mu$ M with (3R,3'R)-zeaxanthin and a  $K_d$  of 0.52  $\mu$ M with (3R,3'S-*meso*)-zeaxanthin. Two zeaxanthins were bound per 23-kDa GSTP1 subunit. (3R,3'R,6'R)-Lutein did not bind to any of the GST isoforms saturably or with high affinity. The means  $\pm$  S.D. for three independent incubations are shown.

GSTP1 was saturable with apparent dissociation constants of 0.33 and 0.52  $\mu$ M (Fig. 4), respectively, somewhat lower than the dissociation constants of 1–2  $\mu$ M that we reported previously for the interaction of partially purified human macular XBP with lutein and zeaxanthin (17). The ligand/protein ratio for both forms of zeaxanthin to the 23-kDa monomer of GSTP1 was observed to be 2.0 at saturation, and two-site binding modeling provided no evidence for receptor heterogeneity. Our

binding studies generally did not have glutathione present in the buffer, and the addition of up to 1 mM glutathione did not alter zeaxanthin binding to GSTP1. We were unable to assess the impact of xanthophyll binding on the GST activity of GSTP1 because the CHAPS detergent required for xanthophyll binding inhibited its GST activity. When we examined other GST isoforms such as human recombinant GSTM1 and GSTA1, we observed low-affinity binding without definite evidence of saturation (Fig. 4). Although we have demonstrated previously that human macular XBP can bind exogenous lutein (17), we were unable to demonstrate high-affinity specific binding of lutein to any of the three isoforms of recombinant human GST (Fig. 4). We also compared GSTP1 with a series of mammalian proteins thought to interact with xanthophyll carotenoids (tubulin, albumin, HDL, LDL, and  $\beta$ -lactoglobulin). No high-affinity saturable zeaxanthin binding comparable with GSTP1 was detectable (Fig. 5), although albumin did exhibit saturable high-affinity binding for lutein ( $K_d$ , 0.54  $\mu$ M; 3 binding sites/molecule).

The binding behavior of dietary (3*R*,3'*R*)-zeaxanthin and nondietary (3*R*,3'*S*-*meso*)-zeaxanthin to GSTP1 was further characterized using absorption and CD spectroscopy. The absorption spectrum of purified XBP carrying its endogenous carotenoid ligands displayed a bathochromic shift of 9–12 nm as compared with the spectrum of lutein and zeaxanthin in organic solvents (Fig. 2). The visible spectrum of XBP is identical to that of the macular carotenoid pigment *in vivo* (8–10). GSTP1 apoprotein had no visible absorbance.

The far-UV CD spectrum of native XBP displayed a negative Cotton peak at 220 nm, which is typical of a protein with dominant  $\alpha$ -helix structure (27), whereas the human recombinant GSTP1 apoprotein had a far-UV CD peak at 213 nm. The visible-range CD spectrum between 300 and 700 nm of XBP bearing its endogenous ligands was weak and complex with minor positive and negative peaks at 400 and 494 nm, respectively, whereas the GSTP1 apoprotein had no detectable visible-range CD. Addition of exogenous saturating amounts of either form of zeaxanthin to XBP or GSTP1 resulted in substantial alterations of their visible and CD spectra (Figs. 6 and 7). The three visible absorption peaks of XBP had a bathochromic shift of 8–10 nm relative to the spectrum of zeaxanthin solubilized in THF or CHAPS, and the bathochromic shifts of GSTP1 were even larger (19–21 nm). The far-UV negative CD peak of GSTP1 shifted to 219 nm after binding either form of zeaxanthin, closely matching that of human macular XBP carrying its native ligands.

As expected, nonchiral (3*R*,3'*S*-*meso*)-zeaxanthin has no detectable CD when dissolved in THF, a nonchiral solvent. In CHAPS, a chiral detergent, there was likewise no detectable CD, indicating that the (3*R*,3'*S*-*meso*)-zeaxanthin dissolved in the detergent micelles was unconstrained and unaggregated (Fig. 6). (3*R*,3'*S*-*meso*)-Zeaxanthin displayed a definite induced circular dichroism with a positive Cotton effect at 419 nm and a negative Cotton effect at 490 and 529 nm with a zero crossover point at 460 nm when bound to recombinant GSTP1, corresponding well with the somewhat weaker visible-range CD of XBP saturated with exogenous (3*R*,3'*S*-*meso*)-zeaxanthin (Fig. 6). The intensities of the induced Cotton effects increased with the ligand/protein ratio, and saturation was observed at a ligand/protein ratio of 2 in agreement with the binding studies.

Unlike (3*R*,3'*S*-*meso*)-zeaxanthin, dietary (3*R*,3'*R*)-zeaxanthin has a strong CD spectrum in any solvent. In THF, its CD spectrum is strongest in the UV range (Fig. 6), and the weak signals in the visible range are usually considered to be artifactual (28, 29). When dissolved in CHAPS, the UV CD of (3*R*,3'*R*)-zeaxanthin is no longer detectable, whereas a nega-

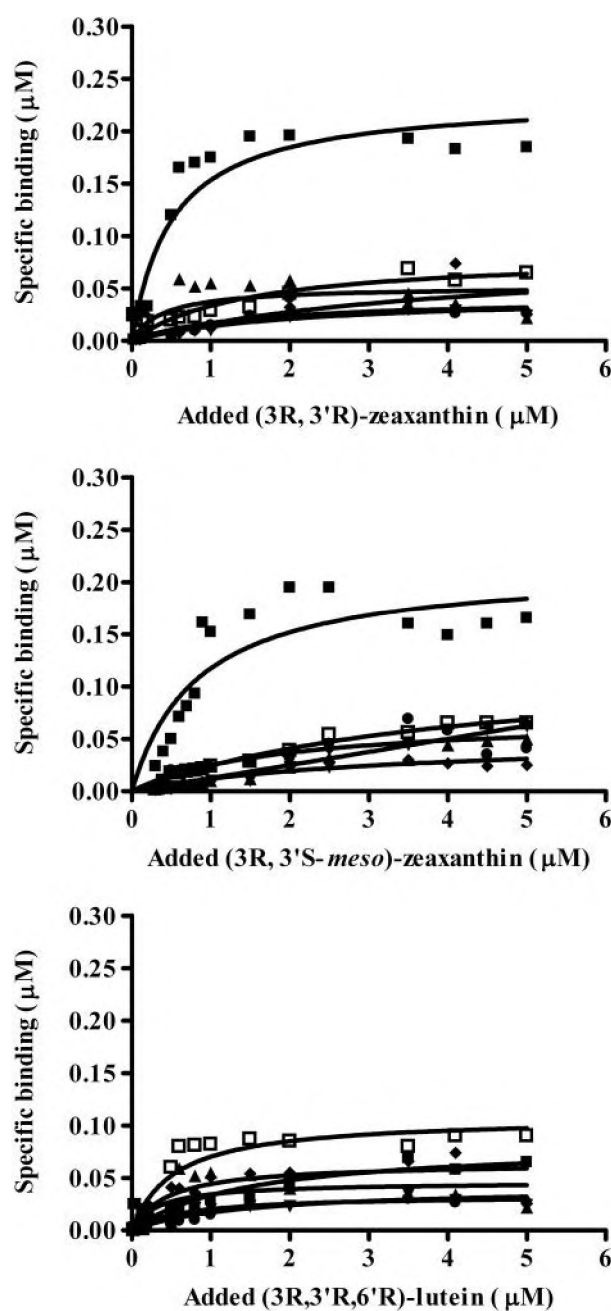


FIG. 5. Binding studies of GSTP1, tubulin, albumin, HDL, LDL, and  $\beta$ -lactoglobulin with macular xanthophylls. Ten  $\mu$ l of concentrated (3*R*,3'*R*)-zeaxanthin (top panel), (3*R*,3'*S*-*meso*)-zeaxanthin (middle panel), and (3*R*,3'*R*,6'*R*)-lutein (bottom panel) dissolved in THF were added to 490  $\mu$ l of 50 mM Tris-CHAPS (8 mM) buffer containing 1  $\mu$ g of protein (GSTP1,  $\blacksquare$ ; bovine brain tubulin,  $\blacktriangle$ ; human serum albumin,  $\square$ ; human HDL,  $\blacktriangle$ ; human LDL,  $\blacklozenge$ ; bovine  $\beta$ -lactoglobulin,  $\bullet$ ). After brief mixing, the mixtures were incubated overnight (16 h) at 4  $^{\circ}$ C. Unbound carotenoids were removed by four cycles of extraction with 200  $\mu$ l of hexane. All data points are single measurements. The only definite saturable high-affinity binding interactions observed for the zeaxanthins were between GSTP1 and (3*R*,3'*R*)-zeaxanthin and between GSTP1 and (3*R*,3'*S*-*meso*)-zeaxanthin (see Fig. 4 for GSTP1 binding constants). Only albumin exhibited high-affinity saturable binding for lutein ( $K_d$ , 0.54  $\mu$ M; 3 binding sites/albumin molecule).

tive Cotton peak is present at 410 nm, and a biphasic positive Cotton peak is present at 480 and 500 nm. The crossover point is at 430 nm, the visible absorption maximum of one of the vibronic peaks. When bound to XBP or GSTP1, these CD peaks shift bathochromically by 10–20 nm. The red-shifted visible absorption spectra (Fig. 7) and the induced/altered chirality of

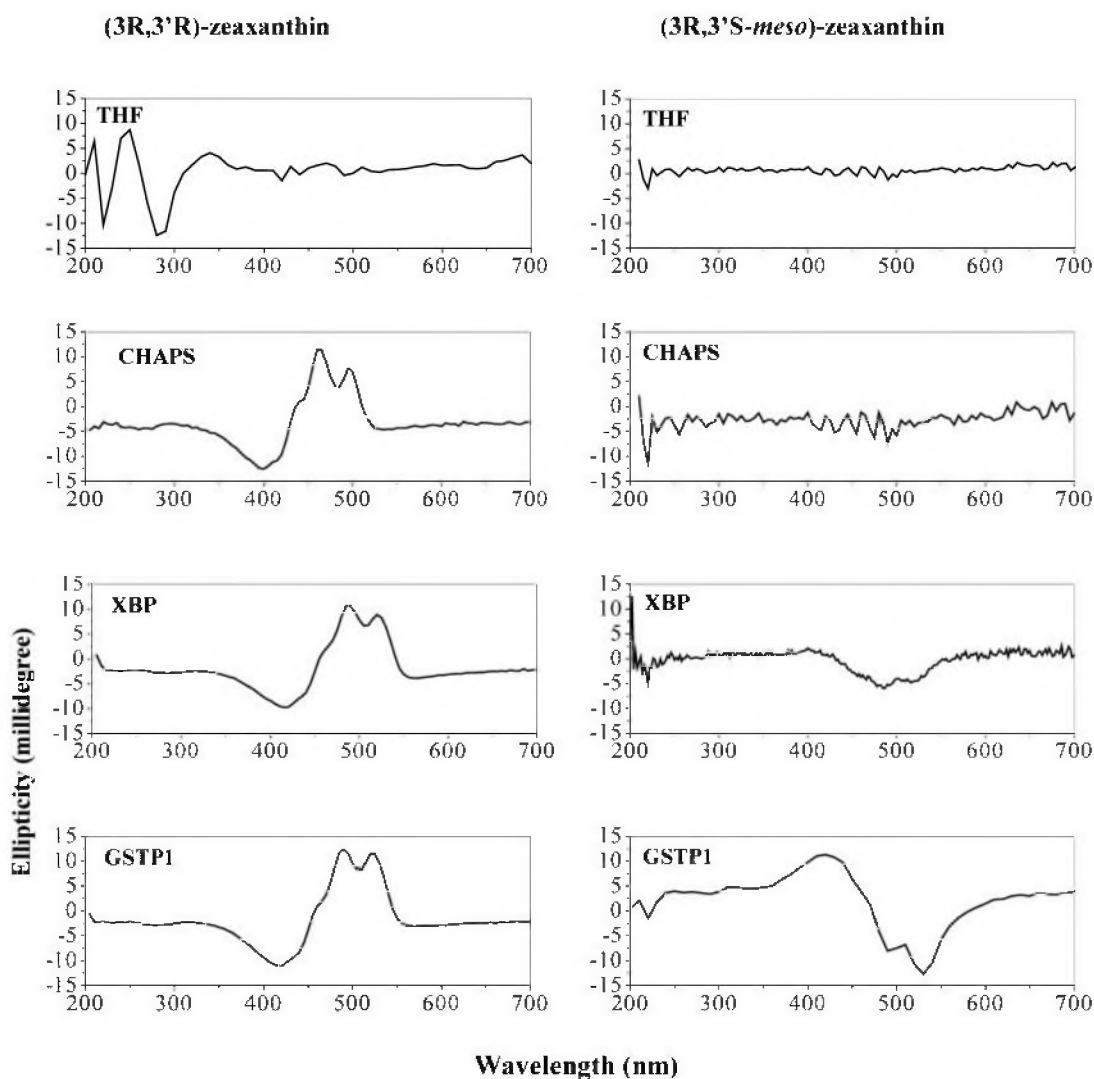


FIG. 6. Induced and altered CD spectra of zeaxanthins bound to GSTP1 and human macular XBP. Saturating amounts of (3*R*,3'*R*)-zeaxanthin (left column) and (3*R*,3'*S*-*meso*)-zeaxanthin (right column) were incubated with 1  $\mu$ g of human recombinant GSTP1 and with human macular XBP overnight at 4 °C, and unbound carotenoids were then removed by hexane extraction. Zeaxanthins dissolved at similar concentrations in THF and in 8 mM Tris-CHAPS buffer (pH 7.8) were used as controls. CD spectra were acquired as described under "Experimental Procedures."

both zeaxanthin diastereomers when bound to GSTP1 or XBP are indicative of a J-type association (loose type, head to tail) with supramolecular coupling between the two bound ligands (30, 31).

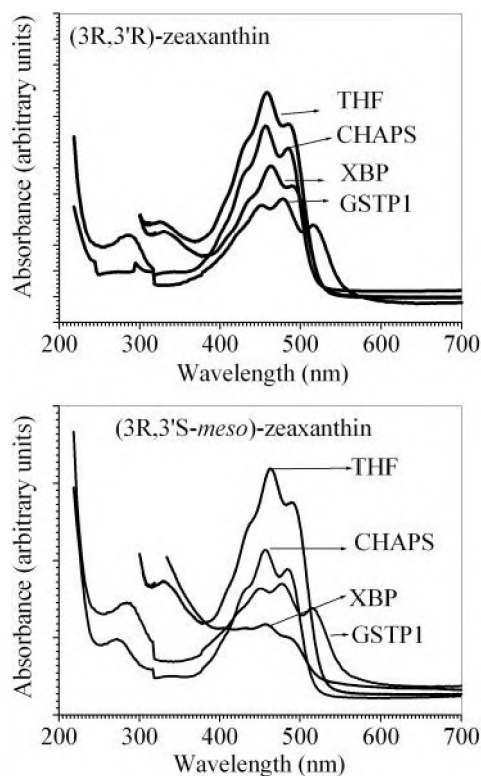
We also examined visible absorption and CD spectra of (3*R*,3'*S*-*meso*)-zeaxanthin and (3*R*,3'*R*)-zeaxanthin with GSTA1 and GSTM1 and a series of other mammalian proteins thought to be relatively nonspecific low-affinity carriers of xanthophyll carotenoids (tubulin, albumin, LDL, HDL, and  $\beta$ -lactoglobulin). In nearly all cases, the proteins induced no significant alterations in the visible absorption or CD spectra relative to the CHAPS control. The only exception was tubulin, which induced modest bathochromic shifts that were much lower in magnitude than those seen with XBP or GSTP1.

**Immunolocalization of GSTP1 in Human Macula**—Immunolocalization with a monospecific polyclonal antibody to GSTP1 on a human parafoveal section revealed that GSTP1 is excluded from nuclei but is otherwise present throughout the human macula, and it is especially concentrated in the outer plexiform layer (also known as the Henle fiber or receptor axon layer) and the inner plexiform layer (Fig. 8, red). Similar sections of more peripheral regions of the retina did not exhibit such intense GSTP1 labeling of the inner and outer plexiform

layers. The labeling with GSTP1 antibodies in the parafovea was contrasted with that of cone arrestin (32) to demonstrate the distribution and density of cone pedicles (Fig. 8, green). The observation of high levels of GSTP1 in the parafoveal plexiform layers of the human macula correlates well with the pigment distribution of the macular carotenoids reported by Snodderly *et al.* (7).

#### DISCUSSION

Of more than 15 carotenoids typically detected in human serum, only 2, lutein and zeaxanthin, are present in the macula of the human eye, and their concentrations at the fovea are extraordinarily high, on the order of 1 mM. Highly selective uptake of these two xanthophyll carotenoids implies the existence of specific XBPs, but until now, no saturable and specific vertebrate XBP has been positively identified. To identify a human macular XBP, we have taken advantage of the very high endogenous levels of intensely colored lutein and zeaxanthin in the human macula as a readily monitored marker for proteins that copurify with xanthophyll carotenoids through multiple biochemical purification steps. In our most purified XBP preparations, we have only one major band at 23 kDa that can be resolved into four spots on two-dimensional gels. The

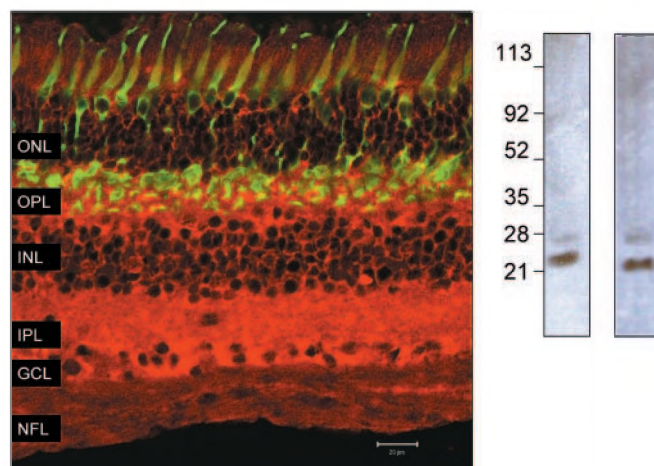


**FIG. 7. Absorption spectra of zeaxanthins bound to GSTP1 and human macular XBP.** Saturating amounts of (3R,3'R)-zeaxanthin (top panel) and (3R,3'S-meso)-zeaxanthin (bottom panel) were incubated with 1  $\mu$ g of human recombinant GSTP1 and with human macular XBP overnight at 4  $^{\circ}$ C, and unbound carotenoids were then removed by hexane extraction. Zeaxanthins dissolved at similar concentrations in THF and in 8 mM Tris-CHAPS buffer (pH 7.8) were used as controls. Absorption spectra were acquired as described under "Experimental Procedures."

most prominent spot has a pI of 5.7, and we were able to positively identify it as GSTP1 by in-gel tryptic digestion and mass spectral sequencing. Biochemical and spectroscopic binding studies with human recombinant protein confirm that GSTP1 binds both physiological forms of zeaxanthin with high affinity and specificity.

GSTs are members of a superfamily of phase II detoxification enzymes that consist of two identical 23-kDa subunits. They are best known to be involved in the conjugation of glutathione to various electrophilic metabolites generated by oxidative processes in the body. Based on their structural and functional properties, GSTs can be divided into at least 12 different classes (Alpha, Beta, Delta, Kappa, Mu, Omega, Phi, Pi, Sigma, Tau, Theta, and Zeta) (33), and within these classes, multiple subisoforms can exist. Only one Pi isoform of GST, GSTP1, is known to be expressed in human tissue, and its gene is localized to chromosomal locus 11q13-qter. GSTP1 is widely expressed in human epithelial tissue, and it is known to be present in the retina, although the cellular location of GSTP1 within the retina has not been described.

GST proteins have many other physiological functions besides their enzymatic conjugation activities. GSTs may act as enzymes in other reactions, gene expression regulators, and binding proteins. For example, GSTA3-3 is thought to be the main enzyme involved in a glutathione-dependent double-bond shift reaction in steroid biosynthesis (34), and GSTP1 can act as a retinoic acid *cis-trans* isomerase in a glutathione-independent manner (35). Other GSTs are involved in gene regulation and therefore used as therapeutic targets in diseases such as bronchial asthma and cancer (36). Reactive oxygen species



**FIG. 8. Confocal immunolocalization of GSTP1 and cone arrestin in human macula near the fovea.** Immunoreactivity with a polyclonal antibody directed against GSTP1 (red) was observed predominantly over the outer and inner plexiform layers and was completely excluded from nuclei. Cone photoreceptors were revealed by coinubination with monoclonal antibody 7G6 (32), which recognizes cone arrestin (green). ONL, outer nuclear layer; OPL, outer plexiform layer (also known as the receptor axon or Henle fiber layer); INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer. Magnification bar = 20  $\mu$ m. Aliquots of recombinant human GSTP1 and partially purified human macular XBP (before the final gel filtration step) were subjected to SDS-PAGE and blotted, and the membrane was incubated with the same polyclonal antibody used for immunocytochemistry. Membranes were developed with an enhanced chemiluminescence Western blot kit to visualize immunoreactive bands. Lane 1, human recombinant GSTP1; lane 2, partially purified human macular XBP. The faint band at 28 kDa in both lanes is presumed to be a splice variant or a posttranslational modification.

activate gene transcription through the regulatory element of GSTs known as an antioxidant-responsive element. Activation of genes containing an antioxidant-responsive element could lead to the induction of enzymes that protect the cells from endogenous and/or exogenous compounds that undergo redox cycling and form reactive oxygen species (36). Much interest is currently being focused on the Pi class of GST because the gene is up-regulated during the early stages of oncogenesis, and it is the most significantly overexpressed GST gene in many human tumors, including gliomas (37).

It has long been known that some GSTs function as binding proteins for small hydrophobic molecules. In fact, as far back as the 1970s, some forms of GST were named "ligandins" due to their abilities to bind bilirubin and other toxic metabolites with high affinity (38). More recently, a GST isoform in maize was reported to bind and protect protoporphyrin from oxidation (39). In plants, some GSTs are reported to be auxin-binding proteins (40).

Before this report, GSTs were not known to have any direct physiological interactions with carotenoids. Using recombinant human GSTP1, we were able to demonstrate that both zeaxanthin diastereomers normally present in the human macula bind saturably and with submicromolar affinity. Absorption and CD spectral studies with dietary (3R,3'R)-zeaxanthin and nondietary (3R,3'S-meso)-zeaxanthin provide further support for a strong and specific interaction with GSTP1. Comparable studies with other commercially available isoforms of human GST (GSTA1 and GSTM1) and other mammalian xanthophyll carrier proteins (tubulin, albumin, HDL, LDL, and  $\beta$ -lactoglobulin) showed little or no evidence of specific interactions with zeaxanthin.

Several common polymorphisms of GSTP1 are known to alter the small molecule binding properties and glutathione S-transferase activity of GSTP1 when the protein is expressed

in *Escherichia coli* (41). Some of these activity-altering polymorphisms have been associated with increased risk of cortical cataracts in the human lens, a tissue known to contain GSTP1 and both forms of zeaxanthin (2, 42). Whereas the association of risk of age-related macular degeneration with polymorphisms of glutathione *S*-transferase Mu and Theta isoforms has been examined (43), no comparable studies of GSTP1 polymorphisms have been reported. If rare or common polymorphisms or mutations in GSTP1 decrease the ability of GSTP1 to bind zeaxanthin and other carotenoids, then elevated risk of age-related macular degeneration might be expected due to abnormally low levels of macular carotenoid pigment.

It is clear from the studies reported here that GSTP1 is a zeaxanthin-binding protein, but the human recombinant protein differs from human macular XBP in several ways. First, XBP purified from human macula copurifies with both lutein and zeaxanthin, whereas GSTP1 binds only dietary zeaxanthin and *meso*-zeaxanthin. Second, human macular XBP is membrane-associated and requires CHAPS detergent solubilization for stability, whereas human recombinant GSTP1 is a soluble protein. In only a few cases have GSTs been reported to be membrane-associated (36). At present, these discrepancies remain unexplained, but it is possible that human macular XBP is a form of GSTP1 that is posttranslationally modified in such a way as to make it more lipophilic. Posttranslational modifications could also alter its ligand binding affinity and specificity. Alternatively, one of the as yet unidentified proteins that copurifies with GSTP1 in our macular XBP preparations may be a lutein-binding protein.

The function of GSTP1 in the macula remains to be explored. If it is acting solely as a zeaxanthin uptake and stabilization protein, then its retinal distribution would be expected to mirror the distribution of zeaxanthin in the retina, with high concentrations in the inner and outer plexiform layers of the macula near the fovea, and much lower levels elsewhere in the retina. Our initial immunocytochemistry studies of GSTP1 localization in human macula support this concept (Fig. 8). It is also possible that GSTP1 may possess an enzymatic activity toward xanthophyll carotenoids. For example, the glutathione-catalyzed double-bond shift reaction mediated by GSTA3-3 is very analogous to the double-bond shift reaction required for the postulated enzymatic conversion of (3*R*,3'*R*,6'*R*)-lutein to (3*R*,3'*S*-*meso*)-zeaxanthin (44, 45). Finally, it is possible that interactions with the GSTP1 protein may enhance the antioxidant function of zeaxanthin within the macula and retina. Our discovery that GSTP1 is a zeaxanthin-binding protein has implications beyond the eye, as well, because GST proteins and carotenoids appear to play important roles in protection against cancer and systemic disorders associated with oxidative stress. Additional studies of the physiological interactions of GST proteins and xanthophyll carotenoids in the eye and elsewhere in the body should prove to be fruitful areas of investigation.

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