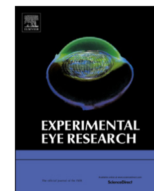
Experimental Eye Research 120 (2014) 167–174

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer



Thiol-dependent antioxidant activity of interphotoreceptor retinoid-binding protein

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ARTICLE INFO

Article history:
Received 21 August 2013
Accepted in revised form 3 January 2014
Available online 12 January 2014

Keywords:
retina
retinoid binding protein
X-ray structure
crystallography
photoreceptors
interphotoreceptor-retinoid binding protein
interphotoreceptor matrix
visual cycle

ABSTRACT

Interphotoreceptor retinoid-binding protein (IRBP), which is critical to photoreceptor survival and function, is comprised of homologous tandem modules each ~300 amino acids, and contains 10 cysteines, possibly 8 as free thiols. Purification of IRBP has historically been difficult due to aggregation, denaturation and precipitation. Our observation that reducing agent 1,4-dithiothreitol dramatically prevents aggregation prompted investigation of possible functions for IRBP's free thiols. Bovine IRBP (bIRBP) was purified from retina saline washes by a combination of concanavalin A, ion exchange and size exclusion chromatography. Antioxidant activity of the purified protein was measured by its ability to inhibit oxidation of 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonate] by metmyoglobin. Homology modeling predicted the relationship of the retinoid binding sites to cysteine residues. As a free radical scavenger, bIRBP was more active than ovalbumin, thioredoxin, and vitamin E analog Trolox. Alkylation of free cysteines by N-ethylmaleimide inhibited bIRBP's antioxidant activity, but not its ability to bind all-*trans* retinol. Structural modeling predicted that Cys 1051 is at the mouth of the module 4 hydrophobic ligand-binding site. Its free radical scavenging activity points to a new function for IRBP in defining the redox environment in the subretinal space.

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1. Introduction

As the major soluble protein component of the interphotoreceptor matrix (IPM), interphotoreceptor retinoid-binding protein (IRBP) surrounds the individual rod and cone outer and inner segments occupying a critical interface between the retina and the RPE. Absence of IRBP results in photoreceptor degeneration in transgenic mice, and Asp1080Asn is associated with a form of recessive retinitis pigmentosa (den Hollander et al., 2009). Little is known about why IRBP is important to photoreceptor survival.

IRBP carries endogenous all-*trans* and 11-*cis* retinols, and 11-*cis* retinal in a light-dependent manner suggesting a role in facilitating the exchange of visual cycle retinoids between the rod, cone, RPE and Müller cell (Gonzalez-Fernandez and Ghosh, 2008; McBee et al., 2001). IRBP is known to promote outer segment release of all-*trans* retinol (Wu et al., 2007), and its delivery to the RPE. IRBP also enhances both the release of 11-*cis* retinal from the RPE (Carlson and Bok, 1992), and its return to the outer segments. Finally, an emerging concept is that IRBP may be important to protecting visual cycle retinoids from photodecomposition (Crouch et al., 1992; Parker et al., 2011; Tsin et al., 2013).

Visual cycle retinoids are vulnerable to photo-oxidation while crossing through the IPM. The primary alcoholic group and the double bonds in the side-chain or ring make 11-*cis* and all-*trans* retinol particularly susceptible to oxidative damage. Photodegradation of vitamin A results in the formation of retinal, and a mixture of epoxy derivatives of vitamin A (Crank and Pardijanto, 1995; Failloux et al., 2003). The retina is particularly susceptible to oxidative stress, due to its high metabolic activity, oxygen

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0014-4835/$ – see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.exer.2014.01.002

tension, and concentration of readily oxidizable polyunsaturated fatty acids, and exposure to light (Chalam et al., 2011). Furthermore, although the RPE and Müller cells are known to have robust antioxidant systems (Bringmann et al., 2006; Cai et al., 2000; Plafker et al., 2012), the photoreceptors themselves and IPM are comparatively lacking in such protection. Although proteomic studies suggest that the IPM may contain neuroprotective proteins including thioredoxin 5 (Hauck et al., 2005), immunohistochemical studies are needed to confirm the localization of these components to the IPM. It is therefore potentially significant that IRBP can protect visual cycle retinoids from photodecomposition. The mechanism of this protection is unknown.

Key to uncovering IRBP's function will be elucidating the structure of the full-length protein. Bovine IRBP (bIRBP) has an overall elongated shape (Saari et al., 1985), and is composed of multiple homologous "modules" that may have diverse roles in the visual cycle. Functionally important structural changes appear to occur upon ligand binding (Adler et al., 1987). However, the preparation of pure bIRBP at concentrations required for crystallization trials proved to an insurmountable task owing to aggregation and precipitation of the protein, possibly through denaturation (Fong et al., 1984). Our early observation that 1,4-dithiothreitol (DTT) prevents aggregation permitted the purification of the pristine bIRBP, and prompted this investigation into the potential role of its free thiols.

2. Experimental methods

This research was approved by the Buffalo Veterans Affairs Medical Center Research & Development Committee, and SUNY Buffalo and Upstate Medical University Biosafety Committee. All chemicals were of highest quality, and obtained from Sigma–Aldrich, unless otherwise specified.

2.1. Purification of native bovine IRBP

Our purification strategy is based methods previously established for the purification of native bIRBP from bovine retina (Adler et al., 1990; Adler and Evans, 1983; Fong et al., 1986, 1984; Redmond et al., 1985; Saari et al., 1985). With the exception of Saari et al. (1985) the use of a reducing agent during the purification of bIRBP is not described. As described below DTT was used throughout the purification.

IRBP is readily extracted from detached bovine retina by simple saline wash, which also affords an initial enrichment. Although some bIRBP remains adherent to the insoluble interphotoreceptor scaffold (Garlipp et al., 2012), at least 50% can be removed from the retina in this way. The extraction and purification were carried out at 4 °C in the presence of 0.5 mM DTT, and protease inhibitors. Retinas were collected under dim red light by WL Lawson Co. (Lincoln, NE), and held at −80 °C. For each purification, 200 retinas were thawed, and soaked for 15min in PBS (2 mM potassium phosphate, 7 mM sodium phosphate, 13.4 mM KCl, 136 mM NaCl, pH 7.4) containing 0.5 mM phenyl sulfonyl fluoride (PMSF), and centrifuged at 2000 × *g* for 5min. The supernatant was removed, and the retinas gently re-suspended in PBS, soaked for 10 min with gentle occasional agitation, and centrifuged for 10 min at 3000 × *g*. The pooled washes were finally cleared at 20,000 × *g* for 30min. A broad spectrum protease inhibitor cocktail was then added along with a 50% concavalin A (ConA) Sepharose 4B slurry (GE Healthcare, Piscataway, NJ) in 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. bIRBP was allowed to bind to the ConA for 4 h with continuous gentle agitation, and finally eluted overnight in 10% (w/v) methyl α-D-mannopyranoside, 50 mM Tris–HCl pH 7.5.

The ConA binding proteins were then loaded on a Q Sepharose high performance (QHP) column (1.6 cm × 12 cm) (GE Healthcare, Piscataway, NJ) equilibrated with 20 mM Tris–HCl pH 7.5, 50 mM NaCl on a Äkta Fast Protein Liquid Chromatography system. bIRBP was eluted at 600 mM NaCl with a linear gradient. The protein was concentrated to 5 mL using an Amicon centrifugal filter, and subjected to size exclusion using Sephacryl S-300HR in a 2.6 cm × 100 cm column on a ÄKTA FPLC chromatography system (GE Healthcare, Piscataway, NJ). The running buffer was 20 mM Tris–HCl pH 7.5, and 100 mM NaCl. The pooled S300 bIRBP fractions were subjected to second QHP column (1.6 cm × 12 cm) and eluted with a NaCl gradient as before. The bIRBP containing fractions were pooled, and the concentration of the purified bIRBP determined by absorbance spectroscopy, and amino acid analysis. Purity was determined by SDS-PAGE analysis. The yield typically was 40–60 mg of 98% pure bIRBP from 200 retinas.

2.2. Crystallization

Crystallization is generally accepted as a validation of integrity of the folded state and monodispersity (Ferre-D'Amare and Burley, 1997; Marmorstein, 1998; Weber, 1997). Bovine IRBP was exchanged into 50 mM Tris–HCl pH 7.5, 100 mM NaCl, and 4 mM DTT using YM-50 centricons and concentrated to 10 mg/mL (0.07 mM). 0.5 mM oleic acid was then added, and the protein solution filtered to remove any particulate matter. Crystal screening was performed using various commercial screens from Hampton Research (San Diego, CA): Crystal Screen 1 and 2; Jena Biosciences (Jena Germany): JBS Membrane Screen 1–3; and Qiagen/Nextal (Montreal, Canada): Classics, SM4, SM5, Mb Class II Suite. The screening was performed at 23 °C and 4 °C in sitting drop plates (Hampton Research). Initial crystals were obtained from 0.1M $MgCl_2$, 0.1M NaCl, 0.1M sodium citrate pH 5.5, 12% polyethylene glycol (PEG) 4000 at 23 °C in a 1:1 protein:cocktail ratio using sitting drop vapor diffusion. This is a significant departure for the conditions used to grow crystal of recombinant IRBP (Loew and Gonzalez-Fernandez, 2002). Optimization of the initial growth conditions resulted in the procedure for routine growth of single crystals from a wide range of PEG concentrations and molecular weights. Single crystals were grown from 12 mg/mL solutions of bIRBP in 0.1M $MgCl_2$, 0.1M NaCl, 0.1M tri-Na citrate pH 5.5 to which the reservoir solution, 10–18% PEG 4K–35K in the same buffer cocktail, was added at 3:1 protein: reservoir volume ratio and vapor diffused in sitting drops against reservoirs at 23 °C. The diffraction data presented here were gathered on a crystal grown against a reservoir of 18% PEG 35K.

Initial characterization of the crystals, and determination of space group and cell dimensions were performed at the Cornell High Energy Synchrotron Source (Ithaca, New York) by collecting low resolution (∼5 Å) diffraction data sets at ambient temperature. Data processing routines MOSFLM (Powell, 1999), and HKL2000 (Otwinowski and Minor, 1995.) were used for this purpose.

2.3. Ligand-Binding assays by fluorescence spectroscopy

The binding of all-*trans* retinol to bIRBP was characterized in titrations using a DM 45 scanning spectrofluorimeter (On-Line Instrument Systems, Inc., Bogart, GA) (Ward, 1985). Titrations monitoring enhancement of retinol fluorescence, quenching of the intrinsic protein fluorescence, and energy transfer were carried out as previously described (Baer et al., 1998). Enhancement of retinol fluorescence was followed by monitoring the increase in retinol fluorescence (excitation, 330 nm; emission, 480 nm). Assays following quenching of protein fluorescence used excitation, and emission wavelengths of 280 nm and 340 nm, respectively. In these

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