Human Lens Coloration and Aging

EVIDENCE FOR CRYSTALLIN MODIFICATION BY THE MAJOR ULTRAVIOLET FILTER, 3-HYDROXY-KYNURENINE O-β-D-GLUCOSIDE*

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Brian D. Hood, Brett Garner, and Roger J. W. Truscott‡

From the Australian Cataract Research Foundation, University of Wollongong, Wollongong, New South Wales 2522, Australia.

The human lens becomes increasingly yellow with age and thereby reduces our perception of blue light. This coloration is associated with lens proteins (crystallins), but its molecular basis was unknown. Here we show that the coloration occurs because of the interaction of crystallins with a UV filter compound, 3-hydroxykynurenine glucoside (3-OHKG). Crystallin modification results from deamination of the 3-OHKG amino acid side chain, yielding an unsaturated ketone that is susceptible to nucleophilic attack by cysteine, histidine, and lysine residues. This novel protein modification contributes to age-related lens coloration and may play a role in human nuclear cataractogenesis.

As part of the normal process of aging, the human lens becomes progressively more yellow and fluorescent (1–3), leading to a concomitant increase in light absorption in the 300–500 nm range (1) and thus diminishes our perception of violet and blue light. The age-related increase in lens coloration and fluorescence is associated with the major proteins of the lens, the crystallins, and is particularly prominent in the lens nucleus (2, 3). Because there is little or no protein turnover in the lens nucleus (4), the proteins are as old as the individual. The post-translational modifications that result in crystallin coloration, therefore, accumulate throughout life and may eventually contribute to age-related nuclear cataract.

The latter condition is characterized by a brown coloration of the lens nucleus and extensive protein oxidation (5, 6). The human lens also contains a family of Trp-derived UV filter compounds, of which 3-OHKG7 is present at the highest concentration (~500 μM) (7, 8). These UV filters are thought to play a protective role by preventing potentially damaging UV light from reaching the retina. Several investigators have considered the possibility that the UV filters could covalently modify lens crystallins with deleterious consequences, including increased protein denaturation and increased sensitivity to UV light (9–11). However, the mechanism leading to crystallin modification by 3-OHKG was not determined nor was there any evidence that 3-OHKG induces protein modification in vivo.

We have recently elucidated a novel pathway that leads to the formation of a glutathione (GSH) adduct of 3-OHKG in the human lens (12). The GSH-3-OHKG adduct was formed via deamination of the 3-OHKG amino acid side chain to form an α, β-unsaturated carbonyl that was highly susceptible to nucleophilic attack by the cysteine of GSH (12). The aim of the present studies is to assess the relevance of analogous reactions in the modification of crystallins and also to probe for evidence of crystallin-3-OHKG adducts in human lenses.

Experimental Procedures

Materials—All organic solvents were HPLC grade (Ajax, Unichrom, Auckland, NZ, Australia). Poly-L-lysine was from Sigma, trifluoroacetic acid (>99% pure) was from Aldrich, and acetic acid (>99.5% pure) from BDH (Poole, UK). 3-hydroxykynurenine O-β-D-glucoside (3-OHKG) and 2-amino-3-hydroxyacetophenone O-β-D-glucoside (AHAG) were synthesized (13, 14) in our laboratory. Mills-Q water (purified to 18 megohms cm−1) was used in the preparation of all solutions. Calf lenses were obtained from Parish meats, Yallah, NSW, Australia.

Human Lens Treatments—Human lenses were obtained from donor eyes used for corneal grafting with ethical approval from the Eastern Sydney Area Health Service-Research Ethics Committee (Ref. 90/057) and the University of Wollongong Human Ethics Committee (Ref. HE96/145). A total of 55 lenses, ranging in age from 14 to 85 years, were obtained from the Sydney Lions Eye Bank, the Queenslands Lions Eye Bank, or kindly provided by Dr B. Ortwerth (University of Missouri, Columbia, MO.). Lenses were homogenized in 0.5 ml of 80% ethanol, placed on ice for 30 min, and then centrifuged (10,000 × g, 10 min, 4 °C) and the supernatant discarded. This procedure was repeated an additional three times to remove low molecular weight compounds including unbound UV filters. The protein pellets were lyophilized, and approximately 30 mg was weighed into screw-capped glass vials. Potassium hydroxide 5% (w/v) in 80% (v/v) ethanol/water (10 ml) was added, and the vial was wrapped in foil to exclude light, bubbled with argon, and sealed prior to incubation (25 °C, 48 h). An aliquot (5 ml) was then adjusted to between pH 5 and 7 with 3 M HCl and lyophilized. One ml of 50 mM acetic acid (pH 4) in water was added to the dried pellet, the mixture centrifuged for 10 min at 10,000 g, and a 50-μl sample analyzed by HPLC.

Incubation of Calf Lens Protein (CLP) or Polylysine with 3-OHKG—One hundred mg of lyophilized CLP or polylysine was dissolved in 25 ml sodium carbonate/bicarbonate buffer, pH 7 or 9 (10 ml). Synthetic 3-OHKG (10 mg) was added together with 40 μl of chloroform. The tube was wrapped in foil, bubbled with argon, sealed, and incubated for up to 24 days at 37 °C. Aliquots (2 ml) were removed every 48 h (or at longer intervals beyond 8 days) and chromatographed through Sephadex G25 (Amersham Pharmacia Biotech) equilibrated in distilled water. The protein fraction was extracted with ethanol four times, and in two experiments the ethanol-extracted lens protein was lyophilized, dissolved in 6 M guanidine hydrochloride, and dialyzed against 1000 volumes of water to ensure that any non-covalently associated UV filters were removed. Because the additional dialysis step did not affect the recovery of AHAG, we concluded that the multiple ethanol extraction was sufficient to remove all low molecular weight compounds. Lyophilized proteins were redisolved in 1 ml of 6 M guanidine hydrochloride prior to measuring UV absorbance and fluorescence. 3-OHKG-modified CLP and polylysine samples were also subjected to base hydrolysis and HPLC analysis of AHAG as described below.

HPLC Analysis of AHAG—HPLC analysis was performed using a Varian (Microsorb-MV C-18, 4.6 × 250 mm, 300 Å) column with the following mobile phase conditions: 0.05% trifluoroacetic acid for 5 min
followed by a linear gradient of 0–80% acetonitrile/0.05% trifluoroacetic acid over 15 min with a flow rate of 1 ml/min. Detection was at 365 nm, and AHAG eluted at 13 min. Confirmation of the identity of AHAG in the base hydrolysate from human lens proteins was obtained using LC-ESIMS (see below) and by comparison with an authentic standard of AHAG (14). Synthetic AHAG was used to construct a standard curve for quantification of lens-derived AHAG by HPLC. The recovery of AHAG after base treatment was assessed by hydrolyzing a synthetic sample of 3-OHKG that had been conjugated previously with GSH (12) and by extending hydrolysis times of modified crystallins until no further AHAG was liberated. The yield of AHAG from modified crystallins after 48 h of hydrolysis was thus estimated to be 85%.

Mass Spectrometry and LC-ESIMS—Peaks that eluted from the Microsorb HPLC column were collected and further analyzed by microbore HPLC (Applied Biosystems, Model 172 Separation System, Foster City, CA) using an Alltima 250 × 2.1 mm, C18 column (catalog no. 88571, Alltech, Deerfield, IL). Samples were routinely eluted using an acetonitrile gradient in aqueous 4 mM ammonium acetate (pH 5) and a flow rate of 200 μl/min. Eluted compounds were detected by monitoring absorbance at 360 nm and by in-line electrospray ionization mass spectrometry (LC-ESIMS). Mass spectra were obtained on a VG Quattro quadrupole mass spectrometer (VG Biotech, Altrincham, UK) equipped with an upgraded ESI source. Mass spectra were acquired in positive ion mode with a scan rate of 100 m/z/s. The source was maintained at 150 °C.

Fluorescence and UV-visible Absorption Spectrophotometric Measurements—UV-visible absorbance spectra were obtained using a Shimadzu UV-265 spectrophotometer (Kyoto, Japan), and fluorescence spectra were recorded on a Hitachi F-4500 fluorescence spectrometer (Tokyo, Japan) in three-dimensional scan mode.

RESULTS AND DISCUSSION

Modification of CLP with 3-OHKG—Our initial studies focused on the ability of 3-OHKG to form covalent adducts with crystallins. 3-OHKG was therefore synthesized (15) and examined for its reactivity toward proteins. Following incubation with 3-OHKG, lens proteins developed coloration (365 nm) and fluorescence (Ex 380 nm/Em 490) linearly with time of incubation (Fig. 1a). Binding of the UV filter was increased approximately 10-fold if the pH was increased from 7 to 9 (Fig. 1b), consistent with our observations on the effect of pH on GSH-3-OHKG formation (12).

It is known that the amino acid side chain of kynurenine is susceptible to hydrolysis by strong base, producing an acetoephone derivative (15). In agreement with this we found that 3-OHKG, and molecules containing this moiety (i.e. GSH-3-OHKG and protein-3-OHKG adducts), are cleaved to release AHAG. This result is illustrated in Reaction 1, where a cysteinyl adduct is given as an example and “X” and “Y” represent amino acid residues in the protein. The extent of binding of 3-OHKG to protein was therefore also assessed by base hydrolysis of the protein followed by quantification of AHAG released. Fig. 1c shows that AHAG was released from the 3-OHKG-modified crystallins, suggesting that the covalent modification was analogous to that of the GSH-3-OHKG adduct (12).

Liberation of AHAG from Human Crystallins—To determine whether human crystallins are also covalently modified by 3-OHKG in vivo, lenses of various ages were examined, and the quantity of AHAG released from the proteins was plotted as a function of age (Fig. 2). AHAG has been isolated previously from cataractous lenses following treatment with base, although its origin was not investigated (16). In the present work, an age-dependent increase in AHAG was observed that was more pronounced in lenses over 40 years old, although considerable scatter was evident (Fig. 2). The inset in Fig. 2 illustrates that, in the case of GSH-3-OHKG, base hydrolysis is complete within 48 h with quantitative recovery. More than half (85%) of the AHAG was released from the modified crystallins within 48 h (Fig. 2, inset). Duplicate samples of 22 of the lenses over 40 years old were also incubated for 144 h. This action resulted in a 15% increase in AHAG detected, on average, but did not reduce the variability (scatter) of the data depicted in Fig. 2. Significant variation in the extent of UV filter-mediated modification of crystallins do, therefore, exist in our study population. We estimate, based on the molar absorptivity of GSH-3-OHKG (12) compared with the known increase in lenticular absorption of light at 360 nm (1), that at least 50% of the increase in age-related lenticular color in humans may be attributed to binding of 3-OHKG.

Confirmation of the structure of liberated AHAG was obtained via microbore HPLC with LC-ESIMS and by comparison with an authentic standard synthesized (14) in our laboratory. Fig. 3 shows that the AHAG released from the isolated human lens proteins displayed a positive ion at m/z 314, with a fragment ion at m/z 152, consistent with the molecular mass of AHAG (313 Da) and its aglucone (151 Da). This mass spectrum was identical to the synthetic AHAG (Fig. 9). The lens-derived AHAG and the synthetic standard also co-eluted when they were mixed together and analyzed by LC-ESIMS. These data provide the first chemical evidence that 3-OHKG forms adducts with human lens proteins according to the mechanism we have proposed, which involves addition at the βC of the side chain (see Reaction 1).

Three-dimensional Fluorescence Plots of 3-OHKG-Modified Crystallins—A well-known feature of human lenses is the development of non-Trp (or “blue”) fluorescence, which increases in intensity with age (17). Examination of CLP incubated with
3-OHKG at pH 7 for 16 days revealed that they became fluorescent (Fig. 1a). A comparison of the three-dimensional fluorescence spectra of 3-OHKG-treated CLP with crystallins isolated from older human lenses showed that they were almost identical (Fig. 4). In both samples, several prominent fluorophores were identified. A major fluorophore exhibited maximum intensity at Ex 380 nm/Em 490 nm, consistent with previous observations (18). The intensity of this fluorophore, which has been documented to increase with age (18), also increased with the time of incubation in our model system, and this may be attributed to binding of 3-OHKG to protein (Fig. 1). The protein fluorescence spectra are considerably more complex than 3-OHKG itself, which displays a single peak (Ex 357 nm/Em 500 nm) (19).

The attachment of 3-OHKG appears to take place via initial deamination of the amino acid side chain of 3-OHKG, yielding an unsaturated ketone that is then susceptible to attack by nucleophilic amino acids such as histidine, cysteine, or lysine residues in the protein. In support of this proposal, when polylysine was incubated in the presence of 3-OHKG, it showed a similar time-dependent increase in fluorescence and color as well as AHAG release following base hydrolysis. Derivatives of 3-OHKG such as $\alpha$-N-acetyl 3-OHKG (13) and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-$\beta$-D-glucoside (AHBG) (19, 20), which cannot undergo deamination, failed to bind to lens proteins. Two factors account for the increased covalent modification of crystallins by 3-OHKG when pH is increased from 7 to 9. First, deamination of the kynurenine side chain is favored at the higher pH (21), and second, the nucleophilicity of amino acids (including cysteine, histidine, and lysine) is closely related to their $pK_a$ values (22).

The intrinsic instability of the kynurenine side chain to deamination, which underlies the binding to lens proteins reported here, also appears responsible for the formation of other

![Fig. 2. AHAG liberated from human lens proteins as a function of age. Fifty-five lenses were extracted with 80% (v/v) ethanol/water four times to remove unbound UV filters. The lyophilized protein was incubated for 48 h in 5% (w/v) potassium hydroxide in 80% (v/v) ethanol to liberate AHAG, which was quantified by HPLC. The inset shows time course experiments for the base hydrolysis of the GSH-3-OHKG adduct (■) and lens proteins (○). In the latter case, the data are means with the error bars showing S.E., where $n = 2, 3, 22, 3, 22$ samples for the times 0.5, 1, 2, 4, and 6 days, respectively. The percentage recovery of AHAG from lens proteins was shown to increase by approximately 15% when the incubation time was increased from 2 to 6 days. For reasons of practicality, the 48-h incubation was adopted as the routine hydrolysis procedure.](image)

![Fig. 3. Mass spectra of synthetic and lens protein-derived AHAG. The AHAG was either derived from base hydrolysis of human lens crystallins (a) or synthesized (b) as described under “Experimental Procedures.” Samples were analyzed using microbore HPLC with in-line electrospray ionization mass spectrometry. The positive ion mass spectra shown are for the single 380 nm-absorbing compounds present and were determined directly after elution from the HPLC column. The $y$ ordinates show relative signal intensity.](image)

![Fig. 4. Fluorescence spectra of 3-OHKG-modified crystallins. Three-dimensional fluorescence spectra of calf lens protein following incubation with 3-OHKG at pH 7 for 16 days (a) (slit widths, Ex 10 nm/Em 5 nm) and 75-year-old human lens protein (b) (slit widths, Ex 5 nm/Em 5 nm) are shown. Both samples were isolated after removal of low molecular weight compounds by Sephadex G25 chromatography followed by multiple ethanol extractions and dialysis against water. The concentration of both samples was 1 mg of protein/ml in 6 M guanidine hydrochloride. Calf lens proteins did not exhibit non-tryptophan fluorescence prior to incubation.](image)
UV filter compounds such as AHBG (20). Thus, conjugation with GSH (12) or reduction to form AHBG may effectively compete with lens crystallins for the reactive product that results from deamination of 3-OHKG. The observed increase in the extent of binding of 3-OHKG to lens protein after the age of 40–50 (Fig. 2) may result in part from a diminished concentration of reduced GSH in the nuclear region of the lens (23, 24). This feature may result from the development of a barrier to the diffusion of GSH, from its site of synthesis (or reduction) in the lens cortex to the interior of the lens (23).

Because other UV filters present in the human lens, such as kynurenine and 3-hydroxykynurenine (7, 8), contain the same amino acid side chain as 3-OHKG, it would be expected that these would also bind to lens proteins. We have shown this to be the case using kynurenine and isolated α, β, and γ crystallins, all of which bound the UV filter. Peptide sequence analysis has revealed that the major sites of modification in lens crystallins in vitro are at histidine, cysteine, and lysine residues. We are currently mapping specific kynurenine- and 3-OHKG-modified sites in human crystallins.

Another interesting aspect of the mode of 3-OHKG-mediated protein modification is that it does not require oxidation and can therefore take place in the normal human lens. Oxidation is, however, a hallmark of age-related nuclear cataract (5, 6), and we speculate that the pronounced coloration that characterizes age-related nuclear cataractous lenses may result from oxidative reactions involving protein-bound UV filters (e.g. 3-hydroxykynurenine) that have accumulated over the lifetime of the individual.

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