Identification of Glutathionyl-3-hydroxykynurenine Glucoside as a Novel Fluorophore Associated with Aging of the Human Lens*

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A novel fluorophore was isolated from human lenses using high performance liquid chromatography (HPLC). The new fluorophore was well separated from 3-hydroxykynurenine glucoside (3-OHKG) and its deaminated isofom, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-glucoside, which are known UV filter compounds. The new compound exhibited UV absorbance maxima at 260 and 365 nm, and increased in concentration with age. Further analysis of the purified compound by microcobe HPLC with in-line electrospray ionization mass spectrometry revealed a molecular mass of 676 Da. This mass corresponds to that of an adduct of GSH with a deaminated form of 3-OHKG. This adduct was synthesized using 3-OHKG and GSH as starting materials. The synthetic glutathionyl-3-hydroxykynurenine glucoside (GSH-3-OHKG) adduct had the same HPLC elution time, thin-layer chromatography R<sub>p</sub> value, UV absorbance maxima, fluorescence characteristics, and mass spectra as the lens-derived fluorophore. Furthermore, the <sup>1</sup>H and <sup>13</sup>C NMR spectra of the synthetic adduct were entirely consistent with the proposed structure of GSH-3-OHKG. These data indicate that GSH-3-OHKG is present as a novel fluorophore in aged human lenses. The GSH-3-OHKG adduct was found to be less reactive with β-glucosidase compared with 3-OHKG, and this could be due to a folded conformation of the adduct that was suggested by molecular modeling.

The human eye lens is composed largely of elongated fiber cells that are derived from epithelial cells located in a thin layer at the anterior surface. The innermost layers of the lens are formed during embryonic development, and throughout life, the lens continues to grow as newly differentiated fiber cells are formed (1). The fiber cells are rich in crystallins, which form a highly ordered transparent structure that permits light transmission and thus vision. It is well known that aging of the lens is associated with certain biochemical changes. In particular, the lens becomes yellow and fluorescent (2). Previous work has aimed to identify exactly which biochemical changes lead to the increased yellow color of the lens, with a focus mostly on modification of the crystallins (e.g. Refs. 3 and 4). However, the exact mechanisms responsible for the development of lens color and fluorescence remain to be defined. Furthermore, less is known about age-related changes in lens fluorescence that occur in the non-protein (aqueous) environment of the lens.

The lens is equipped with two major Trp-derived UV filter compounds, 3-hydroxykynurenine glucoside (3-OHKG) (5) and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-glucoside (AHBG) (6), which are thought to be present to protect the lens and retina from UV-induced photodamage and/or to reduce chromatic aberration. It has also been suggested that under certain conditions, UV filters can form covalent links with lens crystallins and in this way play a damaging role. Several theories have been proposed to account for the modification of lens crystallins by UV filters. In most cases, some form of photexitation, oxidation, or enzymatic modification of the UV filters is required to render them reactive (7–10).

In this study, we report the isolation and characterization of a novel fluorophore that was determined to be a GSH adduct of deaminated 3-OHKG. Unlike the other UV filter compounds, which remain relatively stable or decrease slightly in concentration during adult life (11–13), the concentration of the GSH-3-OHKG adduct, relative to the other UV filters, was found to increase with age.

**EXPERIMENTAL PROCEDURES**

**Materials—**All organic solvents were HPLC grade (Ajax, Unichrom, Auburn, New South Wales, Australia). Reduced glutathione was from Sigma; trifluoroacetic acid (>99% pure) was from Aldrich; and acetic acid (>99.8% pure) was from BDH. Me<sub>2</sub>SO-d<sub>6</sub> was from Cambridge Isotope Laboratories (Andover, MA). Milli-Q water (purified to 18 megohms cm<sup>−</sup>) was used in the preparation of all solutions.

**Isolation of Lens UV Filters—**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). Lenses were homogenized in 80% ethanol (0.5 ml/lens) and left on ice for 1 h before centrifugation (12,000 <i>x</i> g, 15 min, 4 °C) to remove precipitated protein. The pellet was re-extracted, and the combined supernatants were lyophilized. The residue was redissolved in water, and the UV filters were analyzed using the isocratic reversed-phase HPLC system described previously (6). The mobile phase used in this method is initially 20 mM sodium acetate (pH 4.5), followed by 20% (v/v) methanol in water.

Peaks that eluted from the isocratic HPLC system in 20% methanol were collected and further analyzed by microbore HPLC (Model 172 separation system, Applied Biosystems, Foster City, CA) using an Alltima 250 × 2.1-mm C<sub>18</sub> column (catalog no. 88371, Alltech Associates

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* The abbreviations used are: 3-OHKG, 3-hydroxykynurenine glucoside; GSH-3-OHKG, glutathionyl-3-hydroxykynurenine glucoside; AHBG, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-glucoside; NMR, nuclear magnetic resonance; 1H NMR and 13C NMR, proton and carbon nuclear magnetic resonance; TMS, trimethylsilyl; MS, mass spectrometry; HPLC, high performance liquid chromatography; UV, ultraviolet; GSH-3-OHKG, glutathionyl-3-hydroxykynurenine glucoside; ESI-MS, electrospray ionization mass spectrometry; DQF-COSY, double quantum-filtered correlated spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum correlation; Kyn, kynurenine; DOPA, 3,4-dihydroxyphenylalanine.
Inc., 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. The gradient was run linearly from 0 to 80% acetonitrile over 40 min. In some experiments (where indicated), the same system was employed using a more shallow gradient (0–40% acetonitrile over 40 min), with trifluoroacetic acid (0.05%, v/v) instead of ammonium acetate. Samples were detected by monitoring absorbance at 360 nm and by in-line liquid chromatography-electrospray ionization mass spectrometry (LC-ESIMS). Mass spectra were obtained on a VG Biotech Quattro quadrupole mass spectrometer equipped with an upgraded electrospray ionization source. Mass spectra were acquired in positive ion mode with a scan rate of 1060 m/z per s. The source was maintained at 150 °C.

**Thin-layer Chromatography—** Samples (0.5–1 μl) were separated on Silica Gel 60 TLC plates (Merck, Darmstadt, Germany) using a mobile phase of butanol/acetic acid/water (12:3:5, v/v/v) and visualized under UV light (365 nm).

**Fluorescence and UV-visible Absorbance Spectrophotometric Measurements—** UV-visible absorbance spectra were obtained using a Shimadzu UV265 spectrophotometer, and fluorescence spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer in three-dimensional scan mode.

**Synthesis of the GSH-3-OHKG Adduct—** OHKG thus isolated was dried under vacuum, dissolved in Me2SO-d6, and analyzed by NMR spectroscopy (see below). Reaction mixtures contained 3-OHKG (15 mg) and GSH (150 mg) in 3 ml of 25 mM carbonate buffer (pH 9.1) and were incubated as described above for 24–48 h. The reaction mixture was then analyzed by gradient-reversed-phase HPLC (250 × 10-mm C18 column, Hypersil, Cheshire, United Kingdom) using an acetonitrile–H2O gradient in 0.05% (v/v) trifluoroacetic acid. The percentage acetonitrile in the gradient was 0% for 25 min, 0–40% over 15 min, 40–100% over 5 min, and 100–0% over 15 min. The flow rate was 5 ml/min for 20 min, 4 ml/min for 25 min, and 3 ml/min for 15 min. Salt, GSH, and GSSG eluted within the first 15 min; 3-OHKG and GSH-3-OHKG adduct eluted at 32 and 36 min, respectively (the identity of these species was confirmed by LC-ESIMS). The GSH-3-OHKG thus isolated was dried under vacuum, dissolved in Me2SO-d6, and analyzed by NMR spectroscopy.

**Nuclear Magnetic Resonance Spectroscopy—** One- and two-dimensional 1H NMR spectra were acquired at 400 MHz and 25 °C using a Varian Unity-400 spectrometer. GSH-3-OHKG was dissolved in Me2SO-d6 to give a concentration of 4.4 mM. DQF-COSY, TQF-COSY, TOCSY, NOESY, and ROESY spectra (mixing times of 200 and 500 ms) and NOESY (mixing time of 250 ms) 1H NMR experiments were acquired in the phase-sensitive mode using time-proportional phase incrementation (16). Typically, 512 t1 increments, with up to 96 scans/increment, were acquired over 2048 data points, which were zero-filled to 2048 data points in both dimensions and multiplied by a gaussian window function prior to Fourier transformation. The gradient heteronuclear single quantum correlation (HSSQC) experiment (17) was acquired in the phase-sensitive mode with 256 t1 increments and 256 scans/increment. The spectral delay was set to 1/2(JCH), with JCH = 140 Hz. All chemical shift values (δ) are given in ppm. Spectra were referenced to residual Me2SO methyl resonances at δ 2.49 and 39.5 ppm for 1H and 13C, respectively.

**Treatment of UV Filter Compounds with β-Glucosidase—** Lens extracts were digested for 18 h at 37 °C in 10 mM citrate buffer (pH 5) containing 1 mg/ml (4 units) almond β-glucosidase (EC 3.2.1.21; catalog no. G0395, Sigma). 1 unit of activity will liberate 1.0 μmol of glucose from salicin/min at pH 5 and 37 °C. Digested extracts and controls (incubated in the absence of enzyme) were then analyzed using isocratic HPLC. The rate of glucose hydrolysis from isolated UV filters was also assessed by continuous fluorescence monitoring at Ex = 300 nm/Em = 400 nm at 37 °C. Since the deglucosylated UV filters had no longer fluoresced (6, 11), the rate of fluorescence loss can be used to monitor the course of the reaction. For these experiments, substrate concentrations were adjusted to give the same absorbance at 365 nm. Enzyme concentration was routinely 0.2 mg/ml in the kinetic studies.

**Molecular Modeling—** The three-dimensional structures of GSH-3-OHKG and 3-OHKG were investigated using molecular mechanics and molecular dynamics methodology as incorporated in Insight II/Discover Version 97.0 software (Molecular Simulations Inc., San Diego, CA) and using a Silicon Graphics O2 workstation. The default force field was used (consistent-valence forcefield), and the modeling was performed on isolated molecules (in vacuo, constant dielectric of 1). The amino acids were modeled both in the neutral and charged forms. Conformational searching was achieved by using a repeated routine of molecular dynamics, then sampling, and then minimization. Details were as follows: time step for molecular dynamics = 1 fs, temperature = 600 K, and initialization period = 100 fs, followed by 50 × 1000 iterations of dynamics. A sample was taken after each 1000 iterations and minimized by 100 iterations of steepest descents, followed by 1000 iterations of conjugate gradients (or until the maximum derivative was <0.0001 kcal/Å). All other parameters were left at the default values specified in the software. Hydrogen bonding was defined to be present in the various conformers only where the distance between the bonding atoms was <2.5 Å and the bond angle was between 120° and 180°. The initial structure of GSH-3-OHKG was not folded, and the energetics of every conformer was compared.

**RESULTS**

**Identification of a Novel Chromophore in Older Human Lenses—** Ethanol extracts of human lenses contain two major UV filter compounds, 3-OHKG and AHBG, and at least three additional chromophores, 3-hydroxykynurenine, Kyn, and another compound of unknown identity that elutes after AHBG on isocratic reversed-phase HPLC (5, 6). In the present study, we identified an additional chromophore (“Unknown-18 min”) that eluted before AHBG and appeared to be present predominantly in lenses taken from older subjects (Fig. 1, A versus B). When the UV filter compounds were extracted from lenses ranging in age from 18 to 83 years and analyzed by HPLC, a significant linear increase (r2 = 0.62, p = 0.0003, 18–83 years, n = 17) in the relative abundance of Unknown-18 min was observed as age increased (Fig. 2). The data are expressed relative to AHBG levels, which do not change significantly in concentration during adult life.

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2 A. M. Wood, J. F. Jamie, J. Zhu, and R. J. W. Truscott, unpublished observation.
FIG. 2. Correlation of the relative abundance of Unknown-18 min with age. The relative abundance of Unknown-18 min was determined from HPLC chromatograms and plotted as a function of age. Data points show values derived from individual lenses and are expressed as ratios of Unknown-18 min peak area to AHBG peak area. ♂, males; ♀, females. The R value for simple regression analysis is given. 

Characterization of the New Chromophore, Unknown-18 min—To examine the possibility that Unknown-18 min was related to the other kynurenine-derived UV filters, its fluorescence and absorbance characteristics were determined. Maximum fluorescence intensity was observed at Ex_357 nm/Em_500 nm (Fig. 3A), and two absorbance maxima were observed at 260 and 365 nm (Fig. 3B). These spectral characteristics were very similar to those of the major UV filters (i.e. 3-OHKG: λ_max at 365 and 264 nm and maximum fluorescence at Ex_360 nm/Em_500 nm; AHBG: λ_max at 358 and 262 nm and maximum fluorescence at Ex_357 nm/Em_500 nm).

LC-ESIMS was then employed to elucidate the molecular mass of Unknown-18 min. Thus, the compound was collected from the column, and its UV-visible and fluorescence spectra were analyzed. The data shown were derived from the lens of an 83-year-old male. A, fluorescence contour plot revealing maximum intensity at Ex_360 nm/Em_500 nm; B, UV-visible wavelength scan revealing absorbance maxima at 260 and 365 nm.

FIG. 3. Fluorescence and UV-visible absorbance spectra of Unknown-18 min. Individual human lenses were analyzed for UV filter content using isocratic HPLC as described under “Experimental Procedures.” The compound eluting at ~18 min in 20% (v/v) methanol/H_2O was collected from the column, and its UV-visible and fluorescence spectra were analyzed. The data shown were derived from the lens of an 83-year-old male. A, fluorescence contour plot revealing maximum intensity at Ex_360 nm/Em_500 nm; B, UV-visible wavelength scan revealing absorbance maxima at 260 and 365 nm.

The synthetic GSH-3-OHKG adduct was collected from the HPLC column and subjected to the same analytical techniques used to characterize Unknown-18 min. The characteristics of Unknown-18 min and the synthetic GSH-3-OHKG adduct are given in Table I. Both compounds exhibited the same absorbance and fluorescence characteristics and migration pattern on TLC plates (note that the R_f values for 3-OHKG and AHBG were 0.36 and 0.63, respectively), eluted at the same time.
within experimental error) on the isocratic HPLC system, and had the same mass spectral properties (Fig. 6; cf. Fig. 4C). In particular, for both the synthetic GSH-3-OHKG adduct and Unknown-18 min, the appearance of the major ion at m/z 677 was accompanied by minor fragments with m/z values of 515, 548, and 530, which are explicable by the loss of glucose (162 Da), Glu (129 Da), and Glu plus H2O (147 Da in total) from the structure proposed in Fig. 5. In summary, Unknown-18 min and the synthetic GSH-3-OHKG adduct appeared to be identical for all parameters tested.

Isolated Unknown-18 min was also reanalyzed by the isocratic HPLC method, and this was followed directly by analysis of the synthetic GSH-3-OHKG adduct. In this case, both eluted with a retention time of 18.3 min. Furthermore, when synthetic GSH-3-OHKG was added to isolated Unknown-18 min and analyzed by LC-ESIMS, there was a dose-dependent increase in a single 360 nm-absorbing chromophore with a corresponding increase in signal for the ion at m/z 677. We interpret these results as indicating that Unknown-18 min was a GSH-3-OHKG adduct. The molar extinction coefficient of GSH-3-OHKG was determined to be ε365 = 6759 M⁻¹ cm⁻¹ (note: 3-OHKG, ε365 = 4340 M⁻¹ cm⁻¹; and AHBG, ε360 = 3400 M⁻¹ cm⁻¹). Using data derived from Fig. 2, we calculate that GSH-3-OHKG is present in older lenses at concentrations of up to 0.62 μmol/g of lens protein.

Reaction of GSH-3-OHKG with β-Glucosidase—In a previous study from our group, treatment of the ethanolic extracts of human lenses with β-glucosidase (1 mg/ml for 3 h at 37 °C) resulted in the loss of 3-OHKG and AHBG from the HPLC traces and the appearance of the corresponding aglucon moieties (6). In these earlier studies, another peak that eluted before AHBG was also detected, and this was only partially removed (~60%) after such β-glucosidase treatment (see Fig. 1 of Ref. 6). We believe that this peak probably corresponds to the GSH-3-OHKG adduct as identified in the present report. In agreement with this, we found, in the present study, that it was necessary to extend β-glucosidase incubation times from 3 to 18 h to completely hydrolyze the O-glucosidic linkage in GSH-3-OHKG (data not shown). In addition, kinetic studies of the hydrolysis of glucose from GSH-3-OHKG suggested that its affinity for almond β-glucosidase was lower than for 3-OHKG. This is illustrated in Fig. 7, which shows that the rate of fluorescence loss from lens-derived GSH-3-OHKG was lower than from 3-OHKG. An initial faster rate of hydrolysis was followed by a slower (linear) phase from 8 to 20 min (Fig. 7, inset). The loss of fluorescence was lower in the GSH-3-OHKG incubations during both phases.

Molecular Modeling of GSH-3-OHKG and 3-OHKG—To investigate how the addition of GSH to the Kyn side chain might influence the interaction of GSH-3-OHKG with glucosidase, we used molecular modeling techniques to predict the structure of GSH-3-OHKG. All of the low energy conformers (either
TABLE I
Characteristics of Unknown-18 min and synthetic GSH-3-OHKG

|                     | Unknown-18 min | GSH-3-OHKG |
|---------------------|----------------|------------|
| HPLC                |                |            |
| RT<sup>a</sup> in 20% MeOH | 18 ± 1 min (17)<sup>b</sup> | 16 ± 5 (5) |
| UV-vis              | 260, 365 nm    | 260, 365 nm |
| Fluorescence        |                |            |
| Ex<sub>360 nm</sub>Em<sub>500 nm</sub> | Yes | Yes |
| TLC                 |                |            |
| RF                  | 0.11 ± 0.01 (2) | 0.13 ± 0.03 (2) |
| Fluorescence (Ex<sub>360 nm</sub>) | Green | Green |
| Mass spectrometry   |                |            |
| ESI +ve m/z         | 677 ± 0.3 (5)  | 677 ± 0.1 (6) |
| 515 m/z fragment    | Yes            | Yes        |

<sup>a</sup> RT, retention time; ESI, electrospray ionization.
<sup>b</sup> Quantitative data show means ± S.E. with the number of experiments given in parentheses. There were no significant differences between the values for Unknown 18 min and GSH-3-OHKG for any of the parameters measured and assessed using the two-tailed Student's t test for unpaired data.

Fig. 6. Mass spectrum of the synthetic GSH-3-OHKG adduct. The GSH-3-OHKG adduct was synthesized using GSH and 3-OHKG as starting materials. Details of incubation conditions and purification are given under “Experimental Procedures.” The purified adduct was then analyzed using microbore HPLC with in-line electrospray ionization (+ve) mass spectrometry. The mass spectrum shown is for the single 360 nm-absorbing synthetic adduct and was determined directly after elution from the HPLC column. The y ordinate shows relative signal intensity.

Fig. 7. Comparison of GSH-3-OHKG and 3-OHKG as substrates for β-glucosidase. 3-OHKG and GSH-3-OHKG were isolated from lenses and subsequently incubated at pH 5 and 37 °C in the presence of 0.2 mg/ml almond β-glucosidase for the times shown. Loss of fluorescence indicates hydrolysis of glucose from the 3-position of the kynurenine ring. The inset shows the rate of fluorescence loss after the reaction reached a linear phase (8 min).

The novel fluorophore (Unknown-18 min) and synthetic GSH-3-OHKG were purified using isocratic HPLC. The isolated samples were then characterized using UV-visible spectroscopy (UV-vis), fluorescence scanning (Fluorescence), TLC, and microbore HPLC with in-line mass spectrometry (mass spectrometry) as described under “Experimental Procedures.”

**Structural Characterization of GSH-3-OHKG by NMR Spectroscopy**—To confirm that the GSH-3-OHKG structure indicated by the mass spectrometric experiments was correct, a series of one- and two-dimensional NMR experiments were conducted on the synthetic compound. All 1H and protonated 13C resonances of GSH-3-OHKG were assigned by standard two-dimensional through-bond (DQF-COSY, TOCSY, and HSQC) and through-space (NOESY) experiments. Fig. 9 shows the HSQC spectrum of GSH-3-OHKG. The predicted structure of the adduct (Fig. 5) contains 17 protonated carbon atoms, and these were all accounted for in this spectrum (Fig. 9). The aromatic region of the one-dimensional 1H NMR spectrum revealed three resonances at δ 6.50 (1H, dd), 7.30 (1H, d), and 7.51 (1H, d), which were coupled to each other (from the DQF-COSY and TOCSY data) and arose from the protons at C-5, C-4 and C-6, respectively (Fig. 5). Their presence indicates that no addition occurred at the aromatic ring. The aliphatic side chain of the Kyn moiety contains an isolated CH<sub>2</sub>—CH moiety with coupled resonances at δ 2.22, 2.55 (2H, dd) and δ 3.72 (1H, t), which were therefore assigned to the protons at C-8 and C-9, respectively. This isolated three-spin system was also clearly resolved in the TOCSY (28) spectrum. The doublet of doublets at C-8 is due to the presence of two diastereotropic protons and therefore confirms that GSH addition is not at C-8. The spin systems of the three amino acids in the GSH moiety were clearly resolved in the TOCSY experiments (e.g. via correlations from the three NH resonances). In addition, the chemical shifts of the cysteine β-CH<sub>2</sub> protons and carbon (δ H-β 3.10 and 2.78 and δ C-β 33.7) indicated that the amino acid was not reduced, consistent with its presence as a thioether (Fig. 9). The DQF-COSY, TOCSY, and NOESY spectra showed that the GSH moiety was intact, charged or neutral) exhibited a folded structure with various degrees of hydrogen bonding between glucose and the Glu and Gly residues of GSH. Fig. 8 shows the lowest energy conformer of the adduct (Fig. 5) contains 17 protonated carbon atoms, and these were all accounted for in this spectrum (Fig. 9). The aromatic region of the one-dimensional 1H NMR spectrum revealed three resonances at δ 6.50 (1H, dd), 7.30 (1H, d), and 7.51 (1H, d), which were coupled to each other (from the DQF-COSY and TOCSY data) and arose from the protons at C-5, C-4 and C-6, respectively (Fig. 5). Their presence indicates that no addition occurred at the aromatic ring. The aliphatic side chain of the Kyn moiety contains an isolated CH<sub>2</sub>—CH moiety with coupled resonances at δ 2.22, 2.55 (2H, dd) and δ 3.72 (1H, t), which were therefore assigned to the protons at C-8 and C-9, respectively. This isolated three-spin system was also clearly resolved in the TOCSY (28) spectrum. The doublet of doublets at C-8 is due to the presence of two diastereotropic protons and therefore confirms that GSH addition is not at C-8. The spin systems of the three amino acids in the GSH moiety were clearly resolved in the TOCSY experiments (e.g. via correlations from the three NH resonances). In addition, the chemical shifts of the cysteine β-CH<sub>2</sub> protons and carbon (δ H-β 3.10 and 2.78 and δ C-β 33.7) indicated that the amino acid was not reduced, consistent with its presence as a thioether (Fig. 9). The DQF-COSY, TOCSY, and NOESY spectra showed that the GSH moiety was intact,
e.g. the predicted sequential inter-residue nuclear Overhauser effects from the glycine and cysteine NH protons were observed (data not shown). The presence of glucose was apparent in the HSQC spectrum (Fig. 9). In summary, the structure that was predicted by theory and by the mass spectrometric studies was confirmed using NMR spectroscopy.

The NOESY spectrum also provided additional information about the structural arrangement of the various moieties within GSH-3-OHKG. Thus, a nuclear Overhauser effect was observed across the glycosidic linkage between the protons at the glucose H-1’ (δ 4.60) and C-4 (δ 7.30) of the Kyn aromatic ring. A nuclear Overhauser effect was also observed between the cysteiny1 α-hydrogen (δ 4.54) and the proton at C-9 (δ 3.72) of the Kyn side chain, consistent with the addition of GSH at C-9 (Fig. 5). The molecular modeling data suggested that the glucose moiety could be interacting with the amino acid side chains via hydrogen bonding (distances of 1.6–2.2 Å were calculated); however, no correlations consistent with this arrangement were observed in the NOESY experiment.

DISCUSSION

These studies describe the isolation and characterization of a novel GSH-containing fluorophore that is present in aged human lenses. Whereas the lenticular concentrations of the major UV filters, 3-OHKG and AHBG, either decline slightly with age or remain constant (11-13), the novel fluorophore increases in relative abundance with age. The newly discovered fluorophore shared similarities with the major UV filters of the lens, and by comparison with a synthetic adduct of GSH and 3-OHKG, we concluded that the novel fluorophore was GSH-3-OHKG. Although the mechanism underlying the generation of GSH-3-OHKG in the lens has not been addressed here, it is likely that it results via the formation of an αβ-unsaturated carbonyl derivative of 3-OHKG (as was induced in the in vitro synthesis reaction). This deamination of 3-OHKG could occur slowly at physiological pH. In support of this, we observed a small degree of adduct formation at pH 7 when 3-OHKG was added to GSH. The position of nucleophilic attack in the Kyn side chain was confirmed to be at C-9 by NMR studies. We also used molecular modeling software to conduct a lowest unoccupied molecular orbital plot (29) of the αβ-unsaturated carbonyl derivative of 3-OHKG, and this showed clearly that C-9 was susceptible to attack, whereas C-8 was not (data not shown). The deamination of the Kyn side chain to yield an αβ-unsaturated carbonyl is implicated in the pathways leading to the production of yellow pigments in insects, indicating that it is a physiologically relevant reaction (25). We speculate that a deaminated form of 3-OHKG, which contains a reactive αβ-unsaturated carbonyl, forms transiently in the lens and that this reacts with GSH to form the novel fluorophore we have detected here. Related to this, we have observed that the lens UV filter AHBG can be formed via a similar pathway involving deamination followed by reduction of the unsaturated carbonyl.

Alternatively, 3-OHKG deamination could initially be due to enzymatic action. It has been reported previously that 3-hydroxykynurenine transaminase (kynurenine aminotransferase, EC 2.6.1.7) activity increases in the human lens...
throughout adult life, thus producing xanthurenic acid derivatives that may contribute to lens fluorescence (10). Since a xanthurenic acid:UDP-glucosyltransferase is known (at least in insects) (30), one could envisage a possible reopening of the xanthurenic acid ring of this glucoside, perhaps by a glutathionyl radical (as occurs in other systems) (31), to generate the GSH-3-OHKG adduct. However, we have no experimental evidence to support this pathway at present.

GSH has been shown to add to aromatic ring structures under some physiological conditions. An example is the formation of 5-S-glutathionyl-DOPA from the amino acid DOPA (32). In this case, DOPA must first be oxidized (e.g. by tyrosinase) to form DOPA quinone before GSH will undergo addition (33). However, the 1H NMR studies showed that no addition to the aromatic ring occurred in GSH-3-OHKG. Also, attempts to synthesize a GSH adduct were unsuccessful when 3-OHKG was replaced by AHAG, which is truncated in the amino acid side chain to give an acetophenone, but otherwise identical to 3-OHKG. We therefore conclude that GSH addition did not occur at the 3-OHKG aromatic ring.

The well characterized nucleophilic addition of thiols to activated double bonds, such as αβ-unsaturated carbonyls, is known to involve reaction of the thiolate anion (mercaptide) in preference to the undissociated thiol (26). The pK of cysteine in proteins and peptides is generally ~8.5 (26). Therefore, the addition of thiols to αβ-unsaturated carbonyls is facilitated by base. Indeed, an established method for the estimation of αβ-unsaturated carbonyls is performed by the addition of thiol in the presence of base (26). Given the facile nature of this reaction, we considered the possibility that a proportion of the GSH-3-OHKG adduct isolated from human lenses could be formed as an artifact during UV filter extraction (in the ice-cold 80% (v/v) ethanol/water (pH 6)). However, when the extraction solution was buffered to give final pH values ranging from 3 to 8 (i.e. from low to high thiolate anion concentration with respect to cysteine residues), there was no difference in the re-
covery of the GSH-3-OHKG adduct. In addition, a high level of GSH-3-OHKG was detected only in the older lens when young and old lenses (which both contain GSH and 3-OHKG) were extracted under the same conditions in parallel (Fig. 1). The most plausible explanation for the existence of GSH-3-OHKG is that it is formed in the lens in vivo.

The reason why GSH-3-OHKG increases with age is probably due to the increased lifetime of 3-OHKG in older lenses. This has been shown by several researchers. In most cases, oxidative stress has been proposed to be necessary for UV filter-induced modification to occur. However, the precise mechanisms responsible for these types of modification in vivo have still not been determined. Given that GSH forms an adduct with a Kyn-derived UV filter in the lens, we hypothesize that a similar (non-oxidative) mechanism involving cysteine residues of lens proteins may contribute to the gradual increase in crystallin coloration that occurs in the normal human lens with age. It is known that protein oxidation (based on levels of Phe and Tyr oxidation products) in the normal aging human lens is not extensive (43). In contrast, there is direct evidence for drastic radical-mediated lens protein oxidation in cataractous lenses (44). It is possible that the prior non-oxidative formation of 3-OHKG-crystallin adducts in the normal lens could also contribute to the lens coloration observed at a later stage in senile nuclear cataract. These hypotheses are currently under investigation by our group.

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