Novel Protein Modification by Kynurenine in Human Lenses

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Received for publication, August 7, 2001, and in revised form, November 16, 2001

The Journal of Biological Chemistry
Vol. 277, No. 7, Issue of February 15, pp. 4867–4873, 2002

It is known that human lenses increase in color and fluorescence with age, but the molecular basis for this is not well understood. We demonstrate here that proteins isolated from human lenses contain significant levels of the UV filter kynurenine covalently bound to histidine and lysine residues. Identification was confirmed by synthesis of the kynurenine amino acid adducts and comparison of the chromatographic retention times and mass spectra of these authentic standards with those of corresponding adducts isolated from human lenses following acid hydrolysis. Using calf lens proteins as a model, covalent binding of kynurenine to lens proteins has been shown to proceed via side chain deamination in a manner analogous to that observed for the related UV filter, 3-hydroxykynurenine O-β-D-glucoside. Levels of histidylkynurenine and lysylkynurenine were low in human lenses in subjects younger than 30, but thereafter increased in concentration with the age of the individual. Post-translational modification of lens proteins by tryptophan metabolites therefore appears to be responsible, at least in part, for the age-dependent increase in coloration and fluorescence of the human lens, and this process may also be important in other tissues in which up-regulation of tryptophan catabolism occurs.

The lens of the eye plays a crucial role in vision. Its chemical composition is unusual in that proteins represent ~38% of the wet mass. The high concentration of protein is needed to achieve the refractive index necessary for focusing (1). Crystallins constitute more than 90% of the lens protein and comprise three main classes, α, β, and γ, based on their aggregation behavior and sequence homology (2). The tightly packed and ordered distribution of the crystallins is essential for maintaining lens transparency and therefore vision (3).

The lenses of humans and other primates contain low molecular weight compounds that act as intraocular filters by absorbing UV light in the 300–400 nm region (4, 5), thus preventing UV-induced photodamage to the retina (6). These filters are produced through the catabolism of tryptophan. The first step in this process involves the oxidative cleavage of the pyrrole ring of tryptophan to N-formyl-L-kynurenine, catalyzed by indoleamine 2,3-dioxygenase (7, 8). The major UV filters in primate lenses in decreasing order of abundance are 3-hydroxykynurenine O-β-D-glucoside (3-OHKG), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-β-D-glucoside (AHBG), kynurenic acid (Kyn), and 3-hydroxykynurenine (3-OH-Kyn) (4, 9–12). Recent work in our laboratory has led to the discovery of glutathionyl-3-hydroxykynurenine O-β-D-glucoside, a novel fluorescent UV filter that was found to increase in concentration with the age of the individual (13).

The young human lens is pale yellow in color; however, with age, an increase in lens coloration and fluorescence is observed (14–16). This increase is particularly prominent in the lens nucleus and is associated with changes to the crystallins (15, 17). Because lens proteins, once produced, show little or no turnover (18), any post-translational modifications accumulate with age and may eventually contribute to age-related cataract (19).

Several investigators have considered the possibility that UV filters may covalently modify lens crystallins (4, 20–22), and most have proposed a role for UV light in this modification (23–26). Our approach, however, has focused on the binding of these compounds to lens proteins without the involvement of UV light. Support for this proposal came from the mechanism of formation of novel human UV filters. For example, the glutathione adduct of 3-hydroxykynurenine glucoside, GSH-3-OHKG (10), is formed via deamination of the 3-OHKG amino acid side chain, yielding an α,β-unsaturated ketone that is highly susceptible to nucleophilic attack by the Cys residue of glutathione (GSH) (13). Reduction of the unsaturated side chain in the lens yields another UV filter, AHBG (27). Because Kyn has the same amino acid side chain as 3-OHKG, it should also undergo deamination. Indeed, in a model system, reaction of Kyn with calf lens protein (CLP) under nonoxidative conditions has been found to generate colored, fluorescent protein. Peptide mapping of Kyn-modified crystallins has revealed that all of the colored peptides contained His, Cys, or Lys residues (28–30).

In this paper we demonstrate that proteins from human lenses contain covalently bound Kyn. Furthermore, we show that the Kyn is attached primarily to His, Lys, and to a lesser extent, Cys residues and that this pattern of covalent modifi-

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¶ This work was supported by a grant from the National Health and Medical Research Council of Australia (NHMRC). Grants from the Australian Research Council (ARC), the Ramaciotti Foundation, and the University of Wollongong enabled the purchase of the mass and NMR spectrometers used in this work. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: 3-OHKG, 3-hydroxykynurenine O-β-D-glucoside; Kyn, kynurenine; 3-OHKyn, 3-hydroxykynurenine; MS, mass spectrometry; His-Kyn, histidylkynurenine; Lys-Kyn, lysylkynurenine; Cys-Kyn, cysteinylkynurenine; RP-HPLC, reversed phase high pressure liquid chromatography; ESI-MS/MS, electrospray ionization tandem mass spectrometry; CLP, calf lens protein; LC/MS, liquid chromatography/mass spectrometry; GSH, glutathione; t-Boc, tert-butoxycarbonyl; AHBG, 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-β-D-glucoside; R, retention time; Ex, excitation; Em, emission; HIV, human immunodeficiency virus.
cation can be reproduced by incubation of the lens proteins with Kyn under conditions that promote deamination of the amino acid side chain.

**EXPERIMENTAL PROCEDURES**

**Materials**—All organic solvents and acids were HPLC grade (Ajax, Auburn, New South Wales, Australia). Milli-Q® water (purified to 18.2 megohms/cm²) was used in the preparation of all solutions. Human lenses were obtained from the Sydney Eye Bank (New South Wales, Australia) or from the National Disease Research Interchange (U. S. A.) with ethical approval from the University of Wollongong Human Ethics Committee (HE99/001). Fresh calf lenses (<2 years old) were obtained from Parrish Meats (Yallah, New South Wales, Australia). Amino acids (N-a-t-Boc-t-histidine, N-a-t-Boc-t-lysine, and cytochrome) and Kyn sulphate salt were all obtained from Sigma. Sequencing grade HCl (6 M) was purchased from Pierce.

HPLC—Reverse phase high pressure liquid chromatography (RP-HPLC) was performed on a Beckman System Gold® HPLC system equipped with a 1275 solvent module and a model 166 UV-visible detector. For analytical scale separations, a Varian (MicroborestM C18, 100 A, 5 µm, 4.6 × 250 mm) column was used with the following mobile phase conditions: solvent A (aqueous 4 mM ammonium acetate, pH 6.5) for 5 min followed by a linear gradient of 0–50%; solvent B (80% acetonitrile with 4 mM ammonium acetate) over 20 min followed by a linear gradient of 50–100% solvent B over 15 min and re-equilibration in the aqueous phase for 15 min. The flow rate was 1 ml/min. Semi-preparative separations were performed using the same conditions as those for the analytical separations except that a (Hypersil® BDS C18, 5 µm, 10 × 250 mm) column was used with a flow rate of 3 ml/min.

**Mass Spectrometry**—Electrospray ionization mass spectra were acquired with a VG Quattro triple quadrupole mass spectrometer (VG Biotech Ltd., now Micromass, Altrincham, UK). Samples were dissolved in 50 mM Na2CO3, NaHCO3 buffer, pH 9.5 (10 ml). The sulfate was then homogenized in absolute methanol. After cooling for 1 h at −20 °C, the homogenate was centrifuged for 20 min at 14,000 rpm. The supernatant liquid was removed, and the pellet was re-extracted in 80% ethanol and centrifuged. The supernatant was discarded, and the pellet was vacuum-dried.

**Synthesis and Purification of the Kyn-Amino Acid Adducts**—The sulfite salt of Kyn (50 mg) was dissolved in 50 mM Na2CO3, NaHCO3 buffer, pH 9.5 (30 ml). The amino acids (N-a-t-Boc-t-histidine and N-a-t-Boc-t-lysine or cysteine) were added in 10-fold molar excess. After adjusting the pH to between 4 and 5 with glacial acetic acid, the resulting mixture was separated by semi-preparative or analytical HPLC using the methods detailed above. The respective yields, high resolution exact mass measurements, NMR, and MS data for each adduct are given below.

**Preparation of Protein-bound Kynurenine**—40 mg, 56% yield based on kynurenine. Found: MH+ 438.2239. Calculated for C19H17N2O4S: MH+ 438.2240; 6.7% 1H, d, J 8.2, 1.2, 2.1, 20.6; 7.31 (1H, ddd, J 8.4, 8.2, 1.2, 2.1, 2.4, 2.7, 6.71 (1H, d, J 8.4, 9.3), 6.70 (1H, d, J 8.2, 8.2, 2.1, 2.4, 6.95). 3.97 (1H, "broad t", J −5, 9.9), 3.82 (1H, m, H-15), 3.62 (2H, m, CH2-8), 3.04 (2H, t, J 7.4, CH-11), 1.65 (4H, m, CH2-12, CH2-14), 1.55 (2H, m, CH3-12), 1.52 (4H, s, 3 × CH3); 13C 199.9 (CO-7), 171.3 (CO), 173.3 (CO), 150.7 (C-2), 136.1 (C-4), 131.7 (C-6), 118.4 (C-11), 117.2 (C-3), 116.2 (C-5), 81.4 (C-18), 55.7 (C-9), 55.7 (C-15), 47.4 (C-11), 38.6 (C-8), 31.4 (C-12), 28.0 (3 × CH2), 25.5 (C-14), 22.6 (C-13); ESI-MS/MS of m/z 438 (MH+), 382 (26%), 338 (48%), 203 (100%), 192 (7%), 147 (11%), 136 (54%), 128 (37%).

**N-a-t-Butoxycarbonyl-t-histidyl-t-lysyl-t-kynurenine**—23 mg, 30% yield based on kynurenine. Found: MH+ 447.1880; 6.8% 1H, d, J 8.2, 1.2, 2.1, 2.4, 6.71 (1H, d, J 8.4, 9.3), 6.67 (1H, dd, J 8.2, 8.2, 2.1, 2.4, 5.32 (1H, m, H-9), 4.10 (1H, m, H-15), 3.52 (2H, m, CH2-8), 3.12 (1H, br dd, J 14.5, 4.2, CH-14), 2.85 (1H, dd, J 15.4, 9.8, CH2-14), 1.19 (−4.5H, s, CH3s), 1.14 (−4.5H, s, CH2s); 13C 199.9 (CO-7), 179.3 (CO), 177.3 (CO), 150.7 (C-2), 136.1 (C-4), 131.7 (C-6), 118.4 (C-11), 117.9 (C-1), 117.2 (C-5), 81.4 (C-18), 60.6 (C-9), 55.5 (C-15), 54.9 (C-14), 42.1 (C-8), 28.2 (C-14), 28.1 (C-14), 27.9 (C-19), 27.8 (C-19); ESI-MS/MS of m/z 447 (MH+), 391 (35%), 347 (100%), 192 (7%), 156 (1%); t-Cysteinyl-t-t-kynurenine: 25 mg, 49% yield based on kynurenine. Found: MH+: 313.0865. Calculated for C13H12N2O3: MH+: 313.0868; 6.0% 1H, d, J 8.1, 1.2, 2.1, 2.4, 6.71 (1H, d, J 8.2, 9.3), 7.27 (1H, m, H-14) (CO-16), 156.0 (C-2), 135.3 (C-11), 135.3 (C-11), 131.6 (C-6), 130.6 (C-12), 119.8 (C-13), 118.4 (C-3), 117.9 (C-1), 117.2 (C-5), 81.4 (C-18), 60.6 (C-9), 55.5 (C-15), 54.9 (C-14), 42.1 (C-8), 28.2 (C-14), 28.1 (C-14), 27.9 (C-19), 27.8 (C-19); ESI-MS/MS of m/z 447 (MH+), 391 (35%), 347 (100%), 192 (7%), 156 (1%).

**Lens Preparation**—Three-dimensional scans were recorded at 30 s per scan. The spectrum was then summed to obtain the final spectrum.

**Acid Hydrolysis of Lens Protein and Kyn-Amino Acid Adducts**—Lens proteins (10 mg) or Kyn-modified amino acids (N-a-t-Boc-t-histidine or N-a-t-Boc-t-lysyl-t-kynurenine) were hydrolyzed with 6 M HCl (1 ml) for 24 h at 110 °C in an evacuated hydrolysis tube. After hydrolysis, the samples were lyophilized overnight and then dissolved in 400 µl of 0.1 M NaH2PO4 and 200 µl of 1 M NaHPO4 (pH ~ 5). The solution was then examined by RP-HPLC.

**Incubation of Calf Lens Protein with Kyn**—With 50 mg of CLP was dissolved in 50 mM Na2CO3, NaHCO3 buffer, pH 9.5 (10 ml). The sulfate Japan in three-dimensional scan mode. Slit widths were 5 nm for excitation and 5 nm emission, and the scan speed was 12 000 nm/min. UV-visible absorbance spectra were obtained using a Shimadzu UV-265 spectrophotometer (Kyoto, Japan). Milli-Q® water was used as the solvent in all experiments.
salt of Kyn (10 mg) was added, and the pH of the resulting solution was readjusted to 9.5 with 0.1 M NaOH if required. The tube was bubbled with argon, sealed, wrapped in foil, and incubated at 37 °C for 4 days.

The resulting mixture was separated on a Sephadex G25 PD-10 column (Amersham Biosciences Inc.) in Milli Q® water. The resulting protein fraction was extracted twice with ethanol to ensure that all unreacted Kyn was removed.

RESULTS

Initial model studies involving Kyn and calf lens proteins had shown that Kyn was capable of binding to the proteins and suggested that Lys, His, and Cys may be the sites of covalent attachment (28). Hence, the first stage in our investigation, to determine whether such Kyn adducts were present in human lenses, involved the synthesis of authentic standards of Kyn adducts of these amino acids. As we had demonstrated that basic conditions promote deamination of the Kyn side chain (27), the Kyn amino acid adducts of N-\textit{t}-Boc-L-lysine (\textit{t}-Boc-Lys), N-\textit{t}-Boc-L-histidine (\textit{t}-Boc-His), and Cys were prepared at pH 9.5. \textit{t}-Boc-protected amino acids were used in the case of Lys and His to prevent reaction of the \textit{N}-amino group. Cys was left unprotected, as the sulfhydryl group was the preferred site of reaction with Kyn (30). The reaction mixtures were analyzed by RP-HPLC, and the adducts were identified by ESI-MS of isolated peaks or by direct LC/MS. The major components were found to be the unreacted amino acid, Kyn, and the Kyn-amino acid adduct (\textit{1:1 diastereomeric mixture}) along with deaminated Kyn and kynurenine yellow, the product resulting from intramolecular cyclization of deaminated Kyn (31, 32).

The Kyn adducts of \textit{t}-Boc-Lys, \textit{t}-Boc-His, and Cys were purified by semi-preparative RP-HPLC. The diastereomers were poorly resolved and therefore were not separated. Characterization of the adducts was achieved using a combination of UV-visible spectroscopy, three-dimensional fluorescence spectroscopy, tandem mass spectrometry, and NMR spectroscopy. One-dimensional and two-dimensional NMR spectra were acquired for each of the three Kyn adducts to confirm the site of covalent attachment. The one-dimensional $^1$H-NMR chemical shifts for each of the adducts are shown in Table I. The one-dimensional $^1$H-NMR spectra revealed four aromatic protons (H-3, H-4, H-5, and H-6) with chemical shifts and coupling patterns consistent with unmodified Kyn aromatic ring. The side chain CH$_2$-CH spin system of Kyn was observed in the COSY spectra of each adduct, with the diastereotopic methylene protons clearly discernible for the Cys-Kyn adduct. The downfield chemical shifts for CH-9 ($\delta$ 3.88–5.32 ppm) and CH$_2$-8 ($\delta$ 3.54–3.82 ppm), were indicative of covalent attachment of amino acids at C-9 of the Kyn side chain. The site of these modifications was confirmed by ROESY experiments. The diagnostic cross-peaks observed in these spectra are shown in Fig. 1. In each case, cross-peaks were observed between H-9 of Kyn and the pertinent protons of the amino acids Lys, His, and Cys. These modifications were confirmed by heteronuclear multiple bond correlation experiments (data not shown), where the corresponding carbon-proton cross-peaks to those in Fig. 1 were observed. In the case of the \textit{t}-Boc-His-Kyn adduct, cross-peaks between both the primary imidazole carbons and C-9 were also present. The structures determined via NMR spectroscopy were consistent with those predicted from a mechanism involving nucleophilic attack by the sulphydryl or amino groups of the amino acids on

| Adducts | H-3  | H-4  | H-5  | H-6  | H-8  | H-9  | H-11 | H-12 | H-13 | H-14 | H-15 | H-19 |
|---------|------|------|------|------|------|------|------|------|------|------|------|------|
| A       | 6.77 | 7.31 | 6.70 | 7.22 | 3.62 | 2.97 | 3.04 | 1.65 | 1.55 | 1.65 | 3.82 | 1.32 |
| B       | 6.72 | 7.27 | 6.67 | 7.11 | 3.82 | 5.32 | 8.69 | 7.27 | 3.12 | 2.85 | 4.10 | 1.19 | 1.14 |
| C       | 7.19,7.17  | 7.56 | 7.27 | 8.01 | 3.70,3.59,3.54 | 3.88 | 3.37,3.31,3.23,3.13 | 4.17 |

*See Fig. 1 for numbering of the protons ($^1$H).
$^a$ Diastereotopic protons.
$^b$ Resolved peaks of diastereomers.
Lys-Kyn 96
/H11006
alyzed in high yield. For Cys-Kyn, recovery was 96
RP-HPLC. Although a small amount of decomposition occurred
Aliquots were removed every 6 h and the adducts analyzed by

**Fig. 2.** UV-visible and three-dimensional fluorescence spectra of His-Kyn (**inset**). Fluorescence spectra for all of the adducts were similar.

the unsaturated side chain of deaminated Kyn.

ESI tandem mass spectrometry (ESI-MS/MS) of the adducts
t-Boc-Lys-Kyn, t-Boc-His-Kyn, cysteinylkynurenine (Cys-Kyn),
and the t-Boc-deprotected adducts lysylkynurenine (Lys-Kyn)
and histidylkynurenine (His-Kyn) was also investigated. The
ESI-MS/MS of both t-Boc-Lys-Kyn and t-Boc-His-Kyn exhibited
a significant protonated molecular ion for Lys-Kyn (m/z 338)
and His-Kyn (m/z 347), respectively, because of loss of the
t-Boc group. Most of the major ions for the t-Boc and depro-
ected Kyn adducts of Lys and His were the same, thus facilitat-
ing the later identification of the Kyn adducts in the acid
hydrolysates. For each of the Kyn adducts, the presence of
the respective amino acid was confirmed by the observation of a
product ion for the protonated amino acid (m/z 147, 156, and
d22 for Lys, His, and Cys, respectively). The ESI-MS/MS spe-
tra of all of the Kyn adducts showed product ions characteristic
of the Kyn moiety. This included a diagnostic peak at m/z 192
for the deaminated form of Kyn.

The UV and three-dimensional fluorescence spectra of His-
Kyn following removal of the t-Boc-protecting group are shown in
Fig. 2. Removal of the t-Boc group did not alter appreciably the
spectral characteristics. The UV spectrum of His-Kyn dis-
played an absorbance maximum at 361 nm. The His-Kyn ad-
duct was also fluorescent, demonstrating a maximum fluores-
cence intensity at 410 nm excitation (Ex) and emission (Em) at
490 and 525 nm (Fig. 2, **inset**). Lys-Kyn and Cys-Kyn displayed
similar UV and fluorescence spectra (Lys-Kyn UV maximum
361 nm, fluorescence Ex maximum 410, Em maximum 490,
527; Cys-Kyn UV maximum 357 nm, fluorescence Ex maximum
392, Em maximum 490, 527). By contrast, Kyn absorbs at
360 nm but is barely fluorescent at neutral pH.

Because we wished to quantify the amounts of these Kyn
derivatives in proteins, we first investigated the stability of the
synthetic adducts under the conditions used for total hydrolysis
of proteins. Therefore, each Kyn-modified amino acid adduct
was subjected separately to acid hydrolysis (6 M HCl, 110 °C).
Aliquots were removed every 6 h and the adducts analyzed by
RP-HPLC. Although a small amount of decomposition occurred
over the 24-h hydrolysis period, each of the adducts was recov-
ered in high yield. For Cys-Kyn, recovery was 96 ± 2%, for
Lys-Kyn 96 ± 2%, and for His-Kyn 99 ± 1%.

To establish whether Kyn-modified amino acids could be
recovered from proteins that had been exposed to Kyn, CLP
was first incubated with Kyn at pH 9.5 to promote the reaction
of Kyn with CLP. After 24 h, the Kyn-modified CLP was clearly
yellow in color. It was purified and hydrolyzed as described
under “Experimental Procedures.” The hydrolysate was then
examined by LC/MS. Fig. 3 shows the LC/MS data for the
hydrolysate. The major colored peaks were identified by corre-
lation between the UV absorbance at 360 nm (Fig. 3A) and the
ion currents for the protonated molecular ions of His-Kyn at
m/z 347, R<sub>t</sub> = 16.4 min (Fig. 3B), Lys-Kyn at m/z 338, R<sub>t</sub> =
15.8 min (Fig. 3C), and Cys-Kyn at m/z 313, R<sub>t</sub> = 18.4 and 18.7
min (isomers) (Fig. 3D). Furthermore, the ESI-MS/MS spectra
of each of these protonated molecular ions were identical to the
spectra of the authentic standards (data not shown). As a
control experiment, Kyn was added to unmodified CLP imme-
diately prior to hydrolysis. No ions corresponding to Kyn ad-
ducts were observed in these experiments, thereby eliminating
the possibility of artifactual formation of these adducts.

Having established the applicability of the acid hydrolysis
method, human lens proteins were then examined. Proteins
from the nuclei of 20 human lenses, with subjects ranging in
age from 16 to 80 years, were subjected to acid hydrolysis. Fig.
4 shows a typical HPLC trace of hydrolyzed human lens protein
in which the presence of two major amino acid adducts corre-
sponding to His-Kyn and Lys-Kyn are evident. It should be
noted that this HPLC system employed acetate buffer, which
provided the best separation of the adducts and is different
from the volatile buffer used for LC/MS shown in Fig. 3. The
identity of these peaks was established by spiking the sample
with the synthetic standards to confirm the retention times. In
addition, the individual peaks were analyzed by ESI-MS and
tandem mass spectrometry following a second HPLC purifica-
tion step to remove salt and to minimize interference by coel-
uting hydrolysis products. In contrast to the model CLP sys-
tem, in which higher levels of the adducts were present, the
Kyn adducts were not detected by direct LC/MS analysis of the
human lens hydrolysate.

A small peak was observed at the retention time expected for
Cys-Kyn (Fig. 4, R<sub>t</sub> = 12.4 min), however, the identity of the
peak could not be confirmed by ESI-MS because of the high
amino acid background. We considered the possibility that the
Cys adduct may be selectively oxidized and thus lost during
handling. To eliminate possible oxidative loss of the Cys-Kyn
adduct, acid hydrolysis of the human lens protein was also
performed in the presence of an antioxidant system (phenol/
mercaptoacetic acid) (33). This added precaution did not, how-
ever, result in an increased yield of the adduct, and so it was
assumed that the observed level of Cys-Kyn reflects the levels present in the lens. One probable explanation for the low concentration of Cys-Kyn in lens protein is that it is inherently unstable at the neutral pH of the human lens. This instability was confirmed by experiments on Cys-Kyn at pH 7.2, where ~50% decomposition was observed at 37 °C over 24 h (data not shown).

All human lenses analyzed contained measurable levels of His-Kyn and Lys-Kyn. The concentrations of both of the Kyn adducts were found to increase as a function of age, as shown in Fig. 5. The concentration of the His-Kyn adduct was more than 10 times greater than that of Lys-Kyn over the age range examined. Both graphs showed a considerable degree of scatter in the Kyn adduct levels from the older individuals. Quantification of Cys-Kyn was complicated by the low levels present, particularly in younger lenses, and by the presence of another compound that eluted nearby. For this reason, the levels of Cys-Kyn are not presented in Fig. 5. The levels of Cys-Kyn in lenses appeared to follow those observed for Lys-Kyn, e.g. in an 80-year-old lens (ca. 0.12 nmol/mg) and in a 20-year-old lens (ca. 0.012 nmol/mg). A notable difference between the human lens protein and the synthetically produced Kyn-modified CLP was that the levels of Kyn-modified amino acids were significantly lower in the human lens protein. This is a consequence of the elevated pH and high Kyn concentration used in the model experiment generating high yields of the reactive deamminated Kyn intermediate. In addition to the effect of pH, it is likely that other processes, such as reduction and conjugation with GSH, compete for the unsaturated ketone derivative of Kyn when it is formed within the lens (34).

Also evident in the chromatograms of human lens protein samples were several late eluting peaks (not shown in Fig. 4). These peaks, which eluted between 10 and 15 min, were also observed at the same level in the acid hydrolysates of unmodified CLP and probably arose from acid degradation products of tryptophan (35).

**DISCUSSION**

In the work presented here we have shown that Kyn is covalently conjugated to human lens proteins and that the amount bound increases with age. Acid hydrolysis of human lens protein revealed that His-Kyn and Lys-Kyn were the major adducts, with lower levels of Cys-Kyn present. If pK_a values only are considered, the order of reactivity of these amino acids toward Kyn adduction, at physiological pH, would be expected to be His (pK_a 6.0) followed by Cys (pK_a 8.33) and then Lys (pK_a 10.53). In agreement with this trend, the levels of the His-Kyn adduct were always found to be higher than the levels of the Lys-Kyn adduct in the human lenses. For example, the concentrations of the His-Kyn and Lys-Kyn adducts in a 71-year-old lens were 0.70 and 0.12 nmol/mg protein, respectively. These levels, and the degree of scatter noted as a function of age, are comparable with those of 3-OHKG bound to human lens protein (36). The concentrations of Kyn-modified amino acids were observed to increase in concentration with the age of the individual. The concentration levels of His-Kyn and Lys-Kyn in the nuclear region of the human lens were found to increase from 0.013 ± 0.005 and 0.009 ± 0.003 nmol/mg protein in lenses younger than 30 to a maximum value of 2.5 and 0.12 nmol/mg protein, respectively, in an 80-year-old lenses.

The lower than expected level of Cys-Kyn implies that a variety of factors may play a role in determining the final concentration of the adduct. In addition to their pK_a values, the extent to which residues are modified is likely to be influenced by the tertiary structure of the protein and accessibility of residues for reaction, as well as the adduct stability at physiological pH. The low level of Cys-Kyn observed may also reflect the lower abundance of Cys residues in crystallins compared with His and Lys (37). At present, the best explanation for the observed HPLC pattern would appear to be the relative stability of the Kyn adducts at physiological pH.

In support of the involvement of Cys in the covalent binding of Kyn, preferential reaction with the Cys residue of the α_A subunit is observed when α-crystallin is incubated with Kyn at pH 7. His and Lys residues comprise 3–4% of total amino acids.
in α, β, and γ crystallin, whereas cysteine represents 0.3% in α, 1.7% in β, and 3.3% in γ crystallin (30). This may indicate that Cys is an initial site of binding in the lens, but because of the inherent instability of the Cys adducts at neutral pH, these adducts decompose, with regeneration of Cys and of dimerized Kyn, which is then available to form other adduct species. The stability of the relevant adducts may therefore be primarily responsible for the pattern of adducts we observed. In experiments on synthetic Cys-Kyn, we found that at pH 7.2 only 50% of the adduct remained after incubation at 37 °C for 20 h. Under these pH conditions, and in the presence of t-Boc-protected His and Lys, the released α,β-unsaturated ketone was then available to react with the protected amino acids to form t-Boc-His-Kyn and t-Boc-Lys-Kyn, which are relatively stable at neutral pH (data not shown). Over a period of many years, a similar process in the lens could cause a shift in the distribution of the Kyn adducts in favor of the formation of His-Kyn and Lys-Kyn and may thus explain why the Cys-Kyn adduct is found only at low levels in the lens.

The majority of covalent modifications of human lens proteins reported to date have been found at very low levels, except for carboxymethyllysine (~300 pmol/mg protein) (38). The levels of the Kyn adducts that we have described here are comparable with those of carboxymethyllysine. These findings are consistent with those from previous model experiments (28) in which the reaction between Kyn and nucleophilic amino acids was investigated. The reaction proceeds via initial deamination of the Kyn amino acid side chain followed by a Michael addition of the nucleophilic amino acid residue to the resultant α,β-unsaturated ketone. The reaction mechanism is summarized in Fig. 6. As noted previously, the rate of Kyn adduct formation is dependent on two factors. First, deamination of the Kyn amino acid side chain; this has been found to be most efficient at pH 9–10, with a reduced, but still significant, rate at physiological pH (27). Second, the nucleophilicity of the amino acids, which is related to their pKa values and attributed to the nucleophilicity of the deprotonated forms (39, 40). NMR spectroscopy revealed that in t-Boc-His-Kyn, one of the imidazole nitrogen atoms reacted to a greater extent with the deaminated Kyn. This finding is in agreement with the reaction of 4-hydroxy-2-nonenal with His (41). Steric hindrance appears to direct reaction to the τ nitrogen (Fig. 1), the nitrogen furthest from the His side chain, for relatively bulky molecules such as Kyn.

It is well known that human lenses become more fluorescent with age. Generally the fluorescence associated with aging has been described as having an excitation of 420–435 nm and an emission of 500–520 nm (15). Covalent binding of Kyn to lens proteins appears to be one mechanism for this increase, because protein-bound Kyn is fluorescent (Ex 410 nm, Em 490 and 525 nm (Fig. 2)). By contrast, free Kyn is barely fluorescent. In recent work (13, 36), we have identified a novel UV filter compound, the 3-OHKG adduct of GSH, which is also formed by a Michael-type addition, and have shown that 3-OHKG is also bound to lens crystallins. Thus it can be inferred that 3-OHKyn, which is also present at significant levels in human lenses, will react in a similar manner to 3-OHKG and Kyn. The consequences of Kyn binding to lens protein are not yet understood. Kyn has the potential to render the protein photoreactive, which we have confirmed in preliminary experiments. The consequences of 3-OHKyn binding to human lens protein will be even more deleterious. When 3-OHKyn binds to protein through the amino acid side chain, as described here, the reactive α-aminophenol functionality is still able to undergo oxidation reactions that, in turn, may lead to localized formation of superoxide and hydrogen peroxide (42). Oxidation of bound 3-OHKyn could also produce protein cross-links (43) and may also result in protein coloration, precipitation, and cataract formation.

Nucleophiles such as GSH that are present in the lens in relatively high concentrations (44–46) may compete with the amino acid residues for the unsaturated ketone derivative of Kyn and therefore protect crystallins from modification. After middle age, the concentration of GSH in the nuclear region of the lens decreases to <1 mM (45, 47), and this diminished protection may therefore contribute to the nonoxidative attachment of UV filters in this part of the lens. It has been proposed that the development of an internal lens barrier after age 40–50 may be the primary event responsible for these changes, which leads to lens coloration and ultimately to age-related nuclear cataract (34). In agreement with this theory, the levels of Kyn adducts in lens nuclear proteins were ~3-fold higher than those found in the cortical proteins from the same lens (data not shown).

Although protein-bound Kyn has been shown in this study to be present in the lens, tryptophan metabolites can also be increased in other tissues. For example Kyn and 3-OHKyn, together with quinolinic acid and kynurenic acid, are found in the central nervous system, and their concentrations increase significantly in response to central nervous system inflammation, systemic immune response, Alzheimer’s disease, and HIV infection (48). The role of Kyn and 3-OHKyn in these conditions is not yet known, but their binding to proteins may play a part in the genesis of these diseases. These modifications may also occur in other disease states where Kyn and Kyn metabolites are increased via the up-regulation of indoleamine 2,3-dioxygenase.

In conclusion, we have identified a novel post-translational
modification of proteins by the tryptophan metabolite, Kyn, and shown that in the lens, Kyn is attached primarily to histidine and, to a lesser extent, lysine and cysteine residues. In the human lens, this process contributes to the normal age-related increase in coloration and fluorescence and may play a role in cataract formation. Its relevance in diseases in which the levels of Kyn and 3-OHKyn can increase substantially remains to be determined.

Acknowledgments—We thank Larry Hick for running the high resolution mass spectra.

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