Fluorophors and Chromophors from Rat Lens Crystallins in UV with Hydroxykynurenine

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Isolated α-, β-, and γ-crystallins from young rat lenses were incubated in solution for 16 hr with 3-hydroxykynurenine under ultraviolet (366 nm) light. Controls included: incubation without light, without kynurenine, and with 2-mercaptoethanol. These procedures generated several chromophors (with absorption maxima or shoulders at 340, 370, and 470 nm) and fluorophors (with excitation/emission at 407/515, 458/550, 515/555, 647/664, and 647/740 nm). The formation of these pigments was inhibited by 2-mercaptoethanol. The findings are discussed in relation to the chromophors and fluorophors found in aged and brunescent human lenses. Invest Ophthalmol Vis Sci 25:581–585, 1984

The origin of the chromophors and fluorophors in human lenses long has been a subject of great interest and some controversy. Because of the nature of these substances, it is apparent that they are probably produced by oxidation or photoreaction, although there are other possibilities such as adduct formation between glucose and amines in the lens or the accumulation within the lens of a metabolite diffusing in from the aqueous humor. The identity of the precursors has been established in only a few instances, primarily because their extremely low concentrations make them difficult to isolate and purify. Among the compounds whose occurrence in the lens is supported by good evidence are: kynurenine derivatives,1 anthranilic acid,2–4 β-carbolines,5 bityroine,6 and oxindolyl alanine.7

From a consideration of the structures of the known compounds found in the lens, the ultimate precursor of the majority is tryptophan. This amino acid is extremely sensitive to oxidation and also to photoreaction. Furthermore, it absorbs in that range of the ultraviolet (UV) to which the cornea is transparent. Thus, much in vitro experimentation has been focused on tryptophan derivatives, not only as to their production in the lens but also their reactions with lens proteins. This paper concerns the reaction of 3-OH-L-kynurenine with isolated rat crystallins, both in the dark and under near-UV irradiation, to produce colored and fluorescent products. The young rat lens is a good system to use in such a study because it lacks significant absorption of visible light and has no prominent fluorophor. This investigation is an extension of earlier work6,9 showing that the calcium-induced aggregation of rat lens soluble proteins is accelerated by photo-oxidation with 3-OH-L-kynurenine (and its glucoside) and 8-methoxypsoralen.

Materials and Methods

Fresh lenses of 5-week-old rats were homogenized in 30 or 40 times their weight of ice-cold 2 mM K-phosphate, pH 7.2, 10 mM 2-mercaptoethanol (2-ME), and the soluble fraction was separated from the insoluble fraction by centrifuging at 15,000 g for 1 hr at 4°C. Preparation of α-, β-, and γ-crystallin of the soluble fraction was performed by the ion exchange chromatography (DEAE-cellulose) method of Satoh10 and Spector,11 using serial elution with 2, 50, and 400 mM K-phosphate, pH 7.2 containing 10 mM 2-ME as elution buffer. Low molecular weight substances were removed completely by dialysis at 4°C against 10 mM K-phosphate, pH 7.2.

Reagents and chemicals used were of the purest grade available from commercial sources; 3-OH-L-kynurenine (3-HK) was purchased from Calbiochem-Behring Corporation (La Jolla, CA). Purity of the 3-HK solution was found to be more than 95% by thin-layer chromatography as described by Bando et al.12 The 3-HK solution rapidly oxidized in air at neutral pH,13 but at acid pH (in 0.1 N HCl) it could be stored without much oxidation at −20°C for several weeks or more.

The solutions for irradiation were made by adding to 5 ml of each crystallin solution (about 0.7 mg/ml in 10 mM K-phosphate, pH 7.2) 0.1 ml of 11.4 mM

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Supported in part by NIH Grants EY-01746 and EY-00260 and associated with the Cooperative Cataract Research Group. Research to Prevent Blindness has given a departmental grant to Department of Ophthalmology, Emory University.

Submitted for publication: May 24, 1983.

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3-HK in 0.1 N HCl. The final pH was 7.0. A control solution was identical except that it lacked 3-HK. The solutions were irradiated by near-UV light for 16 hr at 30–32°C or incubated in the dark. The light source was a Black-ray UVL-21 fluorescent lamp (ULTRAVIOLET Products; Gabriel, CA) with a peak output at 366 nm (3-HK absorbs maximally at 370 nm). The sample with cylindrical dimensions of 4 cm² (area) × 1.25 cm (depth) was in a beaker; lamp suspended 5 cm above the sample surface, giving a light intensity of 300 μW/cm². After incubation, low molecular weight substances in each sample were removed by dialysis against 1 L of 10 mM K-phosphate, pH 7.2 for at least 24 hr at 4°C with one volume change. Insoluble protein precipitated occasionally during the incubations, especially in γ-crystallin. Such precipitates were dissolved by adding 0.5 ml of 1% sodium dodecyl sulfate (SDS) to 4.5 ml of each dialyzed sample with warming.

Fluorescence and absorption spectra of the clear crystallin solutions were measured by a laser Raman spectrometer, as described by Yu and East,14 and a Cary 14 spectrometer (Cary Instruments; Monrovia, CA), respectively. Fluorescence spectra were recorded at a scan speed of 5 cm⁻¹/sec using a slit width of 800 nm, time constant of 2.5 sec and sensitivity of 1 × 10⁴ to 1 × 10⁵ counts/sec full scale. Excitation wavelengths at 406.7 nm (10 mW), 568.2 (150 mW), and 647.1 (250 mW) were provided by a krypton ion laser (Spectra-Physics Model 171–01; Mountain View, CA) excitation wavelengths at 457.9 (10 mW) and 514.5 (30 mW) were provided by an argon ion laser (Coherent Radiation Model CR-8; Palo Alto, CA). Measured fluorescence intensities were normalized by dividing by the intensity of the Raman water line at 3350 cm⁻¹. Such normalized intensities for the different crystallin solutions were, however, directly comparable only for the same fluorophor excited at the same wavelength because of the variation with wavelength of phototube sensitivity and frequency of the water signal.

Protein concentrations in the crystallin solutions were determined by the Bradford dye-binding assay15 using bovine serum albumin as the standard. The dry weights of protein contained in two or three samples of each crystallin solution were measured in order to correct for differences in color yield in the dye-binding assay between the standard and each crystallin. Such dried protein samples were prepared by precipitation with cold 5% trichloroacetic acid (TCA) and the precipitate, collected on filter paper, was washed free of TCA with ethanol. The precipitate was then dried at 110°C and weighed.

Results

Figure 1 shows absorption spectra at 300–600 nm of chromophors formed in α-, β- and γ-crystallins of...
rat lens by near-UV photoreaction with 3-OH-L-kynurenine compared with various controls. Both UV and 3-HK are needed for appreciable formation of the chromophors. The shapes of individual curves seem to depend on different rates of formation of at least three chromophors (absorption maxima at 340, 370, and 470 nm). The effect of 3-HK and UV light (Fig. 1, curve 1) are abolished almost completely by the addition of 2-ME (10 mM); the resultant absorption spectra (not shown) are very similar to the corresponding curve 3 (light only) or curve 4 (nontreated) in each crystallin.

Irradiation of α-, β- and γ-crystallin with 3-HK also gives fluorescence with emission maxima at 515, 550, and 664 nm when excited at 406.7, 457.9, and 647.1 nm, respectively. The production of these fluorophors is greatly reduced in the dark control and the control lacking 3-HK as shown in Figures 2–4 and Table 1. In all cases of figures and table concerning fluorescence, the fluorescence has been normalized by dividing the measured value by the Raman water signal. Thus, each curve in Figures 2–5 has the same value for the water signal (as measured from the base line of that curve) and has a fluorescence intensity measured along the ordinate.

There is much variation among the 3 crystallins with respect to the intensity of photoinduced fluorescence. The green fluorescence (515 nm) is most intense in γ-crystallin, the yellow-green (550 nm) is greatest in β-crystallin, and the red (664 nm) is greatest in the α-crystallin. Formation of these fluorophors also is inhibited by 2-ME, suggesting a mechanism involving oxidation. The far-red fluorophor (647/740) seems to be formed chiefly in a dark reaction between 3-HK and the crystallins but is not formed in the presence of 2-ME.

Figure 5 and Table 1 show that another yellow-green fluorophor (515/555) appears either in the dark or under irradiation when the crystallins are incubated with 3-HK in the absence of 2-ME. Formation of this fluorophor in the dark is most striking with α-crystallin and least with γ-crystallin. The yellow-green fluorescence intensity from dark reaction (Figure 5, curve 2) is somewhat higher than that from UV effect (curve 1); it may suggest that the dark-generated fluorophor is being destroyed slowly by UV light (curve 1).

Fluorescence at 600–650 nm (excitation at 568 nm) also was produced by photoreaction with 3-HK, but the results were highly variable and will not be presented here.
Table 1. Comparison of relative fluorescence intensities (measured by taking the ratio between fluorescence peak intensity and water Raman intensity)* in rat lens crystallins treated with 3-HK

<table>
<thead>
<tr>
<th>Excitation/emission (nm)</th>
<th>Crystallin</th>
<th>+3-HK and light</th>
<th>+3-HK, light, and 2-ME</th>
<th>+3-HK and dark</th>
<th>Nontreated</th>
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</thead>
<tbody>
<tr>
<td>407/515</td>
<td>α</td>
<td>0.8</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>1.7</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>γ</td>
<td>2.3</td>
<td>0.4</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>458/550</td>
<td>α</td>
<td>0.8</td>
<td>0.3</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>1.8</td>
<td>0.5</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>γ</td>
<td>0.9</td>
<td>0.2</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>647/664</td>
<td>α</td>
<td>70</td>
<td>7</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>β</td>
<td>30</td>
<td>5</td>
<td>11</td>
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<tr>
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<td>γ</td>
<td>12</td>
<td>6</td>
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<td>3</td>
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<tr>
<td>515/555</td>
<td>α</td>
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<td>1.5</td>
<td>8.7</td>
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<tr>
<td></td>
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<td>4.0</td>
<td>6.9</td>
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</tr>
<tr>
<td></td>
<td>γ</td>
<td>2.0</td>
<td>0.3</td>
<td>1.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* All measurements were based on a protein concentration of 0.5 mg/ml.

Discussion

Kurzel et al\textsuperscript{16} found several fluorophors in intact human lenses and cataracts: 300/350 (tryptophan), 350/440, 400/475, 450/520, and 500/550. They believe these to be related to tryptophan and kynurenine. Zigler and Goosey\textsuperscript{17} showed that human lens soluble proteins in solution with 3-hydroxykynurenine exhibited enhanced cross-linking under irradiation by near UV. The mechanisms seem to involve the production of singlet oxygen by photosensitizer (3-HK) and UV. Kuck et al\textsuperscript{18}, Yu et al\textsuperscript{19}, and Yu et al\textsuperscript{20} have found six fluorophors in the human lens. These were detected at 407/496, 458/527, 515/556, 568/591, 568/633, and 647/672 where the exciting wavelengths are those of convenient laser lines and not necessarily those giving maximum excitation. The weaker fluorophors were observed only by taking advantage of the greatly increased sensitivity of a laser Raman spectrometer as compared with a conventional fluorometer. The last (red) fluorophor at 647/672 accumulates remarkably in brunescence.\textsuperscript{19} Cataractous and aged human lenses also absorb throughout the visible region and the absorption curve, which goes up gently from longer to shorter wavelength, has a shoulder at 340 nm.\textsuperscript{21} There is some experimental evidence that the well-known blue fluorophor (340–60/420–50) of human and animal species is a photooxidation product.\textsuperscript{22–28} However, Yu et al\textsuperscript{20} recently have shown that the concentration of a blue-green fluorophor (407/496), the only detectable fluorophor in the normal mouse lens in the visible region, is the same for animals reared in the dark as for animals reared in the light.

This paper indicates that several of the fluorophors of human lenses are similar spectrographically to fluorophors of 407/515, 458/550, 515/555, 647/664, and 647/740 found in rat lens crystallins by photoreaction (photooxidation) or dark incubation (dark-oxidation) with 3-HK, and that absorption spectra similar to those of human lenses can be represented as some combination of spectra of chromophors at 340, 370 and 470 nm formed by photoreaction of crystallins. Van Heyningen\textsuperscript{21} has demonstrated the appearance of a blue fluorophor (360/445) in human lens protein exposed to sunlight in the presence of kynurenine derivatives extracted from the same lens. We also observed that rat lens crystallins treated by photoreaction with 3-HK emitted blue fluorescence when excited by light peaking at 366 nm.
It is known that the blue fluorescence appears in mammalian lens proteins photooxidized by near-UV alone or in the presence of other photosensitizing pigments as well as kynurenine derivatives. Accordingly, photooxidation of tryptophan or other amino acid residues in lens proteins may be responsible for the formation of the various chromophors and fluorophors including the blue fluorophor. However, the present investigation suggests another possibility. Chemical or physical binding of oxidized products of 3-HK to rat lens crystallins may occur, because the presence of absorption bands at 370 and 470 nm of the chromophors formed in the treated rat lens crystallins is consistent with absorption maxima of free 3-HK and an absorption shoulder of its oxidized product, respectively. Furthermore fluorophors of 515/555 and 647/740 nm are formed in the treated rat crystallins by dark oxidation with 3-HK. Zigman et al. have reported that photooxidized tryptophan, which emits blue fluorescence at 440 nm when excited at 360 nm, binds to bovine lens γ-crystallin. Detailed mechanisms for such pigment formation in rat lens crystallins by photoreaction or dark incubation are at present under investigation.

In addition to the oxidative reactions discussed above to explain lens pigment formation, two other possibilities not considered here should be mentioned: lipid peroxidation and the Maillard reaction.

**Key words:** lens, rat, crystallins, chromophors, fluorophors, 3-OH-L-kynurenine, photooxidation, dark-oxidation

**References**


