Non-Tryptophan Fluorescence of Crystallins From 
Normal and Cataractous Human Lenses

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The soluble proteins of a newborn human lens exhibit almost no non-tryptophan fluorescence. On aging, an increase in the fluorescence of all native crystallins is found except for \( \gamma_1 \)-crystallin. With formation of nuclear cataract, a further increase is seen for \( \gamma_1 \)-crystallin. The fluorophore, excitation 355 nm/emission 420 nm, is mainly associated with one species of the \( \gamma_1 \)-crystallin population. It is also present as such in the soluble fraction and increases significantly with nuclear cataract formation. At least one of the \( \gamma_1 \)-crystallins seems to play an important role in the cataractogenic process. Invest Ophthalmol Vis Sci 28:1157–1163, 1987

Many modifications of the proteins observed with age and cataract formation could be due to oxidative processes. Garner and Spector have shown that no sulfur oxidation in lens proteins is detectable in young human lenses, whereas in older ones membrane proteins are oxidized; in cataractous lenses extensive sulfur oxidation of membrane proteins as well as cytoplasmic proteins was demonstrated. These changes are the consequence of the oxidation of protein thiol groups to the disulfide form and further to cysteic acid. In addition, methionine residues are oxidized to the sulfoxide and the sulfone, as was also shown by others. Besides sulfur oxidation, other oxidative processes can be observed in the human lens, such as coloration and formation of protein-associated fluorescence (extrinsic or non-tryptophan fluorescence, NT-fluorescence) by compounds other than the aromatic amino acid residues. These fluorescent compounds become especially abundant in the nucleus of the human lens. Some of these fluorophores, which may be involved in protein crosslinking, have been identified as kynurenine, \( \beta \)-carboline, anthranilic acid and bityrosoine, whereas others are present but unidentified.

The mechanisms involved in the oxidative protein changes are obscure at present. Several suggestions have been put forward to explain the occurrence of the pigments and fluorophores, including direct photolysis and photosensitized oxidation. Nonenzymatic glycosylation of long-lived proteins may also lead to coloration, NT-fluorescence and crosslinking; this is substantiated by in vitro experiments with bovine lens crystallins.

The major NT-fluorescence of human lenses is found in the insoluble protein fraction. However, when exposing whole human lens homogenates to near-UV irradiation in the presence of tryptophan photoproducts, the induced fluorescence accumulates mainly in the soluble proteins.

This study presents some observations on changes in NT-fluorescence of the native soluble proteins in individual human lenses as a function of age and formation of nuclear cataract. The particular role of a \( \gamma_1 \)-crystallin, to which a low-molecular weight fluorophore is loosely bound, will be discussed.

Materials and Methods

Lenses

Human cataractous lenses were stored at \(-70^\circ C\) immediately after surgery in the Berlin Eye Clinic. Only pure nuclear cataractous lenses, classified in vivo into four groups on the basis of color, were used. Normal human lenses were obtained from the Rotterdam Eye Clinic; extraction of the lens was done within 10 hr after death of individuals. The lenses were thawed, decapsulated and dissected into a nuclear and a cortical portion; the inner 30% (by weight) was chosen as the nucleus.

High-Pressure Gel Permeation Chromatography (HPGPC)

The lens parts were weighed and homogenized gently (Potter-Elvehjem) in two volumes of elution buffer (0.1 M Na sulfate, 0.02 M Na phosphate and 1 mM EDTA, pH 6.9) and centrifuged at 10,000 g for 10 min at 4°C (Beckman Instruments Inc., Micro-
The supernatant fractions were separately pooled, desalted and lyophilized. Part of the supernatant was applied onto a TSK G3000 SW column (60 × 0.75 cm; Toyo Soda Co., Ltd., Tokyo, Japan) and fluorescence with excitation at 360 nm and emission at 420 nm (Perkin-Elmer 204-A, Norwalk, CT). The same instrumental settings were used throughout this study. Data reduction was performed with a Hewlett-Packard 3353 data system (Palo Alto, CA); peak areas were established by drawing perpendiculars in the minima between the detector signals for a- and γ2-crystallin, especially in a- crystallin, especially in the nucleus, is observed: the peak position of the fluorescence is shifted towards a shorter retention time with respect to that of the A280 signal. The shift in the peak position of the two detector signals for a- and γ1-crystallin is obvious for all crystallins with the exception of γ2-crystallin. Second, an HM-crystallin fraction is present in the void volume of the column system (23 min). Third, a shift in the peak position of the two detector signals for a- and γ1-crystallin, especially in the nucleus, is observed: the peak position of the fluorescence is shifted towards a shorter retention time with respect to that of the A280 signal. The shift is absent if the intrinsic tryptophan fluorescence (285/330 nm) is recorded. This means that the NT-fluorescence of native crystallins exhibit almost no fluorescence. Compared with the newborn lens, the profiles of the soluble part from cortex and nucleus of a 29-yr-old lens (Fig. 2) demonstrate several interesting changes. First, a relatively large fluorescent signal is obvious for all crystallins with the exception of γ2-crystallin. Second, an HM-crystallin fraction is present in the void volume of the column system (23 min). Third, a shift in the peak position of the two detector signals for a- and γ1-crystallin, especially in the nucleus, is observed: the peak position of the fluorescence is shifted towards a shorter retention time with respect to that of the A280 signal. The shift is absent if the intrinsic tryptophan fluorescence (285/330 nm) is recorded. This means that the NT-fluorescence is shifted towards the higher molecular weight part of the heterogeneous fractions. High-pressure ion-exchange chromatography on a TSKgel DEAE-5PW column of γ1-crystallin revealed at least eight subfractions. The fraction with the largest molecular weight (24 kDa by SDS polyacrylamide gel electrophoresis; not shown) exhibited the major part of the fluorescence and no shift in peak position of the two detector signals. Fourth, the relative proportions of γ1- and γ2-crystallin are reversed compared with those of the newborn lens; this may be attributed to a switch in the synthesis of the monomeric crystallins, as demonstrated in aging bovine lenses. This phenomenon is also observed using a mass-sensitive detector (differential refractive index), implying...
that the large increase in the $\gamma_1$-crystallin peak is not due to the presence of new chromophores that absorb at 280 nm.

The profiles of the soluble fraction from a 70-yr-old lens are shown in Figure 3. The nuclear profile is less distinct than the cortical one, especially in the $\beta$-crystallin range. In the nucleus, $\alpha$-crystallin has disappeared almost completely, as also demonstrated previously by several investigators.\textsuperscript{25,28,29} Again, the shift in both signals of the peaks from $\alpha$- and $\gamma_1$-crystallin is clearly seen.

Figure 4 shows the elution patterns of the crystallins from a 70-yr-old nigra cataractous lens. Because of the low amount of soluble proteins in the nucleus, the A280 response is low (the same instrumental settings were used for this parameter and the fluorescence measurements throughout this study). The fluorescence intensity is relatively large for nuclear $\gamma_1$-crystallin. Moreover, a low molecular weight species with a strong fluorescence, but no A280 signal, elutes at 59 min.

Several lenses, normal as well as nuclear-cataractous, have been analyzed in this way. Changes in the fluorescence intensities, relative to the A280 response (F420/A280) of the crystallins as a function of age and formation of nuclear cataract, are depicted in Figure 5. The fluorescence of all crystallins is larger in the nucleus than in the cortex; the highest ratios are found for HM-crystallin. In addition, all crystallins exhibit a gradual increase in fluorescence upon aging with the exception of $\gamma_2$-crystallin, which has no NT-fluorescence at all. With formation of nuclear cata
Fig. 4. HPGPC of the cortical and nuclear water-soluble fraction of a 70-yr-old nigra cataractous lens. For further explanation see Fig. 1. A280 (- - -). Fluorescence 360/420 (□ □ □).

Fig. 5. Variation in the relative fluorescence intensity (F420/A280) in arbitrary units of the water-soluble proteins from single human lenses upon aging and formation of nuclear cataract. Normal (no): yellow (ye); brown (br); dark brown (db); nigra (ni). Cortex (○ — — — O), nucleus (● — — ●).

extract. The relative fluorescence of the cortical crystallins does not change significantly, but in the nucleus an increase is apparent, especially for γ1-crystallin (lens age was 71 ± 4 years). It should be noted that because of the highly heterogeneous mixture, no quantitative conclusions can be drawn from the fluorescence measurements. Nevertheless, trends in the F420/A280 ratio are clearly visible; they may be valuable with respect to alterations in structure of the crystallins and/or the possible involvement of new fluorophores.

Rechromatography of crystallin fractions, isolated by HPGPC of extracts from pooled cataractous lenses on a TSK G3000 SW column, revealed an interesting phenomenon. Two fluorescent compounds (F1 and F2) are separated from γ1-crystallin (Fig. 6). The other crystallins did not exhibit a release of fluorophores. Gel chromatography of F1 and F2 on a calibrated Bio-Gel P2 column in 0.1 M NH4HCO3 (not shown) revealed a molecular weight of approximately 500 and 400 Da, respectively. These molecular weights, however, may be underestimated because of retardation of aromatic structures, which are presumably part of the fluorophores. On RP-HPLC, using a methanol/water gradient, F1 and F2 elute at 15 and 24.5 min, respectively (Fig. 7).

From Figure 4 it is clear that a fluorescent compound with a molecular weight lower than that of γ-crystallin is present in a nigra cataractous lens. Applying the aforementioned method of RP-HPLC, we have analyzed a number of normal and cataractous lenses in order to find out whether fluorophores are present in the soluble part. A typical example of an elution pattern obtained from a 70-yr-old clear lens nucleus is shown in Figure 8. Although sometimes more fluorescent peaks are seen, the peak at 24.5 min (F2) is always present in lenses older than about 50 yr, but absent in younger ones. With formation of nuclear cataract the fluorescence intensity increases significantly, especially in the nucleus (P > 0.01), as depicted in Table 1. The excitation and emission maxima of F2 are 355 and 420 nm, respectively. Based on retention time, F2 is not identical with the known lens fluorophores anthranilic acid (13.2 min), kynurenine (16.0 min), N-formyl-kynurenine (18.1 min) and 3-hydroxy-kynurenine (11.8 min). The presence of the fluorescent 3-hydroxy-kynurenine-glucoside, identified in the human lens, was not determined; based on hydrophobicity, this com-

![Diagram of HPGPC](http://example.com/hpgpc_diagram.png)
Fluorescent chromophores may be involved in the insolubilization process of lens proteins. This could be inferred from the simultaneous increase of fluorescence and the amount of insoluble proteins in human lenses upon aging and, more pronounced, with formation of nuclear cataract. It is not clear whether the fluorophores are directly involved in the insolubilization process by means of acting as a crosslink between crystallin subunits, or indirectly by altering the conformation of the proteins leading to aggregation.

All crystallin species show an age-related increase in fluorescence; this is observed within the lens (cortex vs nucleus), as well as on comparison of lenses of different ages. HM-crystallin has the most intense fluorescence with respect to the A280; it should be contrasted will have a shorter retention time than tryptophan and, thus, cannot be identical to F2.

**Discussion**

Fluorescent chromophores may be involved in the insolubilization process of lens proteins. This could be inferred from the simultaneous increase of fluorescence and the amount of insoluble proteins in human lenses upon aging and, more pronounced, with formation of nuclear cataract. It is not clear whether the fluorophores are directly involved in the insolubilization process by means of acting as a crosslink between crystallin subunits, or indirectly by altering the conformation of the proteins leading to aggregation.

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Table 1. Fluorescence in arbitrary units of the 24.5 min compound in single normal and nuclear-cataractous human lenses

<table>
<thead>
<tr>
<th></th>
<th>Cortex</th>
<th>Nucleus</th>
<th>Age (yrs)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear</td>
<td>1.1 ± 1.4</td>
<td>1.7 ± 1.9</td>
<td>29-90</td>
<td>16</td>
</tr>
<tr>
<td>Yellow</td>
<td>4.1 ± 1.5</td>
<td>7.8 ± 4.9</td>
<td>62-82</td>
<td>4</td>
</tr>
<tr>
<td>Brown</td>
<td>3.7 ± 1.9</td>
<td>8.6 ± 1.4</td>
<td>65-83</td>
<td>4</td>
</tr>
<tr>
<td>Dark brown</td>
<td>11.3 ± 15.1</td>
<td>21.3 ± 8.7</td>
<td>61-79</td>
<td>4</td>
</tr>
<tr>
<td>Nigra</td>
<td>26.7 ± 10.7</td>
<td>103.4 ± 52.7</td>
<td>65-78</td>
<td>5</td>
</tr>
</tbody>
</table>

N = number of lenses.

sidered that this phenomenon is still larger if a correction is made for the contribution of scattering to the A280 response. An interesting phenomenon is the shift in elution time of the fluorescence signals, observed for α- and γ1-crystallin. In the case of α-crystallin, the explanation is that stronger fluorescence is associated with the higher molecular weight part of the α-crystallin population. Thus, the size of α-crystallin is increased due to fluorescent substances which become bound to the protein. This would nicely fit the model proposed for the quaternary structure of bovine α-crystallin, where a partially-filled outer layer of subunits in young α-crystallin becomes more occupied with aging.30 The major part of the fluorescence in the γ1-crystallins is due to a 24 kDa component. No fluorescence is associated with γ2-crystallin. This fraction is the main γ-crystallin species in the newborn lens, but decreases in proportion in favor of γ1-crystallin with age.26

With formation of nuclear cataract, striking changes are observed for γ1-crystallin: a decrease in proportion (Figs. 3 and 4) and an increase in fluorescence intensity (Fig. 5). Moreover, a fluorophore (F2), loosely bound to γ1-crystallin, increases significantly with cataract formation, especially in the colored nucleus (Table 1). The generation of the fluorophores may be attributed to photochemically induced changes in which protein-bound tryptophan appears to be the initial absorbing component.16,31 Migration of electrons from nearby tyrosine residues, generating tyrosine radicals, would then bring about the return of the excited tryptophan to its ground state.32 Such a process might occur in γ-crystallin since several tryro- sine residues are located close to tryptophan.33,34 Thus, there is accumulating evidence that γ-crystallin plays a significant role in the formation of nuclear opacities.

The nature of the F2 fluorophore (355/420 nm) needs to be established. RP-HPLC analysis indicates a greater hydrophobicity than that of tryptophan; this argues against an oxidation product of free tryptophan, because oxidation generally leads to a more hydrophilic nature. Exposing human lens homoge-

References

11. Dillon J, Spector A, and Nakanishi K: Identification of β-car-