Aspartic acid racemization in heavy molecular weight crystallins and water-insoluble protein from normal human lenses and cataracts

(aging/protein conformational changes)

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ABSTRACT High D/L aspartic acid ratios are observed in heavy molecular weight aggregates and in water-insoluble protein extracted from whole lenses and nuclear and cortical regions. Purified α, β, and γ-crystallins have low D/L ratios. Fractionation of urea-solubilized material from the water-insoluble protein yields four molecular weight classes of proteins. Fractions representing crosslinked material or apparently degraded products have high D/L ratios. Racemization within lens proteins may contribute to formation of the water-insoluble fraction seen in aging lenses and cataracts.

The racemization of aspartyl residues has recently been added to the list of age-related changes taking place in the proteins of the human lens (1). D-Aspartyl residues have been shown to accumulate at the rate of $1.25 \times 10^{-3}$ yr$^{-1}$ or 0.14% yr$^{-1}$ in the central nucleus. The extent of racemization in yellow cataracts is equivalent to that observed in age-matched normal lenses, but brunescent cataracts exhibit up to a 2-fold increase in D/L aspartic acid ratios over comparable aged normal lenses or yellow cataracts.

The rationale for investigating lens proteins came from our studies of calcified proteins in human teeth (2, 3). Detectable amounts of D-aspartic acid accumulate during the human lifespan as a result of the spontaneous chemical racemization reaction. These findings suggested that the aspartyl residues in any metabolically stable protein maintained at mammalian body temperature for several decades or longer would be subject to racemization. Because the proteins in the nucleus of the human lens are among the most stable in the body, we decided to test our hypothesis with the lens system.

Numerous studies have shown that structural changes are taking place in lens proteins during the normal aging process and in cataracts. The proportion of water-insoluble protein increases relative to water-soluble protein (4, 5). The increase of insoluble protein is more marked in cataracts (6). When the water-insoluble residue is extracted with 7 M urea, the urea-insoluble portion is greater in cataracts (6) and is derived primarily from the lens nucleus (7). There is some evidence that more exposed thiol groups occur in cataractous proteins, and these proteins may have a greater susceptibility to tryptic digestion (8). All of these observations indicate that changes in native conformation of the lens proteins are occurring in cataract development and in aging.

We have previously suggested that alterations in protein conformation will result from racemization of aspartyl residues (9). If racemization is to any degree responsible for the changes in the properties of lens proteins, then D-aspartic acid should be distributed in a predictable manner. It should occur predominantly in the central nucleus of the lens, and D/L Asp ratios ought to be higher in the insoluble than in the soluble fraction.

The purpose of this study was to survey various components of the lens in order to test these predictions. In this paper, we present the results of racemization analysis of (i) nucleus and cortex samples from normal lenses; (ii) water-soluble and water-insoluble fractions from whole lenses; (iii) the same fractions in nucleus and cortex samples; (iv) purified crystallins from yellow cataracts; (v) heavy molecular weight aggregates from yellow cataracts; and (vi) urea-solubilized proteins from water-insoluble material.

METHODS

Normal human lenses were obtained from eye bank collections at the Duke University Medical Center, Durham, NC, and Lions Eye Bank and Research Foundation, Inc., Washington, DC. Cataracts were obtained after surgical extraction at the Duke University Medical Center and classified as yellow or brunescent. Specimens of the central nucleus were sectioned by the method of Spector et al. (10) so that the central 15% by weight of each lens was sampled. The peripheral layers of the lens, consisting of up to 30% of original wet weight, were taken for the cortex samples.

The proteins of whole lenses, nuclei, and cortex samples were fractionated into water-soluble (WS) and water-insoluble (WI) fractions by homogenizing each specimen in 7 times its wet weight of cold deionized water in a glass tissue grinder. After centrifugation at 25,000 X g for 20 min at 4°C, the soluble fraction was dialyzed exhaustively against deionized water, lyophilized, and stored at −20°C. The insoluble material was resuspended in water and recentrifuged two times to remove residual soluble protein. The insoluble protein was then dialyzed, lyophilized, and stored at −20°C. The percentage yield of WS and WI for each sample was determined from the respective dry weights.

Fractionation of heavy molecular weight (HMW) protein and soluble crystallins was accomplished using two different gel filtration media. For isolation of HMW and α-crystallin, nine yellow cataracts with an average age of 69 years were decapsulated and homogenized together in 7 times their wet weight of cold 0.05 M Tris-HCl buffer, pH 7.4, containing 0.1 M KCl, 5 mM EDTA, and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 X g for 20 min in order to retain the HMW fraction in the supernatant. The supernatant was applied to a column of Bio-Gel A-5m (2.5 X 90 cm) equil-

Abbreviations: WS, water-soluble; WI, water-insoluble; Ms, molecular weight, HMW, heavy molecular weight proteins; LMW, low molecular weight proteins.
Results

D/L Asp ratios were determined for the central nucleus and outer cortex of six normal lenses. These results are shown in Fig. 1A. The central nuclei analyses are taken from the data previously published (1) and are included here for comparative purposes. The straight line represents the least squares fit to the original 17 lenses (1). The extent of racemization in the cortex samples is consistently less than in the respective nuclei. There is considerable scatter in the cortex values, and it is not possible to rule out an age-related but very slight increase in D/L ratios in the outer cortex. Two of the three cortex samples over 60 years in age have D/L ratios greater than the time t = 0 value of 0.047 (1). It is possible that the sectioning procedure may not have produced exactly comparable samples from the lenses. The inner cortex from one yellow cataract has been analyzed (1), and the D/L Asp ratio is essentially the same as the central nucleus. Thus, even slight differences in the amount of the cortex sampled may introduce variability in the cortical D/L ratios.

When WS and WI fractions from whole lenses are compared, the D/L Asp ratios follow basically the same pattern as the nucleus–cortex pairs. These results are plotted in Fig. 1B. The samples were derived from normal lenses, yellow cataracts, and brunescent cataracts. The D/L Asp ratios for soluble proteins in lenses ranging in age from 11 to 84 yr cluster about a mean of 0.060. Soluble protein from one 89-yr-old brunescent cataract yielded a D/L ratio of 0.155, considerably higher than the other 14 samples.

The D/L Asp ratios in insoluble fractions from whole lenses clearly increase with age. The least squares fit to the data yields a line with a slope of 3.24 × 10^{-6} yr^{-1}– Because the slope is equivalent to 2 times the rate constant in the reversible first-order kinetic equation (see ref. 12), the aspartic acid racemization rate in the insoluble fraction is 1.62 × 10^{-3} yr^{-1}.

WS and WI proteins were isolated from both central nucleus and outer cortex of the same normal and cataractous lenses. The extent of racemization in these fractions is shown in Fig. 1C. There is no difference between the WS samples from nucleus and cortex up to 60 years. These D/L ratios are also comparable to the WS results in whole lenses (see Fig. 1B) and approximate the t = 0 value of ~0.05. Two lens nuclei aged 67 and 74 years have D/L ratios of ~0.100, but again it is possible that the sectioning included older proteins.

The amounts of racemization observed in the WI protein from the four nucleus samples are the same as the cortex WI. The slope of the linear least squares fit to all the WI results in Fig. 1C is 3.54 (±0.60) × 10^{-5} yr^{-1}, and the k_{asp} is 1.67 × 10^{-3} yr^{-1}. Table 1 summarizes the information on rate constants. The kinetics of racemization in WI protein is very similar to that in the unfractionated normal proteins: the k_{asp} values both fall within 2 SD of the original rate constant (1). Thus, the D/L Asp ratio in the human lens nucleus is a function of the amount of WI protein present and the D/L ratio in the WI fraction. This association between high D/L Asp ratios and WI protein holds for both the "old" nuclear and "young" cortical regions.

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**Table 1.** Reversible first-order rate constants for aspartic acid racemization in different lens fractions

<table>
<thead>
<tr>
<th>Lenses</th>
<th>No.</th>
<th>k_{asp}, yr^{-1}</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal and yellow nuclei*</td>
<td>17</td>
<td>1.25 × 10^{-3}</td>
<td>0.15</td>
</tr>
<tr>
<td>WI fraction, whole lenses</td>
<td>14</td>
<td>1.62 × 10^{-3}</td>
<td>0.18</td>
</tr>
<tr>
<td>WI fraction, nucleus and cortex</td>
<td>12</td>
<td>1.67 × 10^{-3}</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*From Masters et al. (1).
The D/L ratios for the original, unfractionated nuclear and cortical sections can be reconstructed from the data in Fig. 1C and the percentage yields of the WS and WI fractions. The reconstituted nuclear ratios should be the same as those predicted from the results of our initial study (1), and the cortex ratios ought to approximate the $t = 0$ value. As can be seen in Table 2, the D/L ratios estimated by this procedure are very close to the expected ratios for the three nonbrunescence nuclear samples. The 67-yr-old brunescent cataract ratio is higher, also as expected. The reconstituted ratios for the cortex samples are twice as high as the $t = 0$ value (D/L Asp $\approx 0.05$) and there is no age-related trend. The high cortex value may reflect the type of scatter seen in the older lenses in Fig. 1.

The amount of WI recovered here is higher than that reported in previous studies (4, 13), although the procedural methods are similar. However, the trend of increasing insolubility with age in the nucleus and a marked increase in WI in the brunescent nucleus are both consistent with the earlier studies. Because the reconstituted nuclear D/L ratios agree well with the expected values, the percentage of WI recoveries we have determined are probably accurate.

One other class of lens proteins was of particular interest to us. These proteins are known as heavy molecular weight (HMW) aggregates and are thought to be an intermediate stage in the formation of the insoluble material (14–17). HMW protein was obtained by chromatographic separation using Bio-Gel A-5m. Partially purified $\alpha$, $\beta$, and $\gamma$-crystallins were also isolated from this column or a Sephadex G-200 column in order to compare D-aspartic acid contents with the HMW fraction. The A-5m elution profile of nine yellow cataracts ranging in age from 51 to 83 years is shown in Fig. 2A. The HMW, $\alpha$-crystallin, and low molecular weight (LMW) fractions were analyzed for D-aspartic acid. In Fig. 2B, the C-200 profile of seven yellow cataracts (74 to 83 years) illustrates the three molecular weight classes of $\beta$-crystallin plus a LMW component. The D/L Asp determinations on all of these fractions are listed in Table 3. The HMW fraction from the A-5m column has a D/L ratio much higher than the other soluble proteins, and this ratio of 0.177 is equivalent to that observed in the WI protein (0.184) prepared from the same group of lenses. The LMW components from both columns, representing primarily $\gamma$-crystallin, show the same extent of racemization. The $\alpha$-cat}

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**Table 2. Estimates of D/L Asp ratios in unfractionated nucleus and outer cortex samples based on extent of racemization and relative proportions of soluble and insoluble protein**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% WI</th>
<th>WI D/L Asp</th>
<th>% WS</th>
<th>WS D/L Asp</th>
<th>Reconstituted</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>52.5-yr normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>53.5</td>
<td>0.175</td>
<td>46.5</td>
<td>0.057</td>
<td>0.120</td>
<td>0.121</td>
</tr>
<tr>
<td>Cortex</td>
<td>34.0</td>
<td>0.187</td>
<td>66.0</td>
<td>0.062</td>
<td>0.098</td>
<td></td>
</tr>
<tr>
<td>58-yr yellow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>61.5</td>
<td>0.179</td>
<td>38.5</td>
<td>0.067</td>
<td>0.137</td>
<td>0.128</td>
</tr>
<tr>
<td>Cortex</td>
<td>35.5</td>
<td>0.183</td>
<td>64.5</td>
<td>0.056</td>
<td>0.101</td>
<td></td>
</tr>
<tr>
<td>67-yr brunescent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>88.5</td>
<td>0.197</td>
<td>11.5</td>
<td>0.100</td>
<td>0.186</td>
<td>0.139</td>
</tr>
<tr>
<td>Cortex</td>
<td>40.8</td>
<td>0.143</td>
<td>59.5</td>
<td>0.058</td>
<td>0.093</td>
<td></td>
</tr>
<tr>
<td>75-yr yellow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>60.5</td>
<td>0.184</td>
<td>39.5</td>
<td>0.098</td>
<td>0.150</td>
<td>0.147</td>
</tr>
<tr>
<td>Cortex</td>
<td>34.8</td>
<td>0.192</td>
<td>65.2</td>
<td>0.052</td>
<td>0.101</td>
<td></td>
</tr>
</tbody>
</table>

* D/L Asp ratios for the nucleus and cortex were calculated by summing the products of the percentage yields and the D/L ratios for the water-soluble and -insoluble fractions.

† Expected D/L ratios for the nucleus samples were obtained by substituting the age of the lens(es) in the equation $\ln[(1 + D/L)/(1 - D/L)] = 2.50 \times 10^{-2} \text{yr}^{-1} + 0.112$ (1). The expected D/L ratio for each cortex sample is the $t = 0$ value of $\sim 0.05$.

‡ Mean age of two lenses pooled for this experiment.

§ Mean age of three lenses.

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**Table 3. Aspartic acid racemization in partially purified human lens proteins**

<table>
<thead>
<tr>
<th>Column</th>
<th>Fraction</th>
<th>Protein</th>
<th>D/L Asp</th>
<th>$\ln \left(1 + \frac{D/L}{1 - D/L}\right)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-Gel</td>
<td>31–34</td>
<td>HMW</td>
<td>0.177</td>
<td>0.358</td>
</tr>
<tr>
<td>A-5m</td>
<td>50–55</td>
<td>$\alpha$-Crystallin</td>
<td>0.065</td>
<td>0.130</td>
</tr>
<tr>
<td></td>
<td>77–80</td>
<td>LMW</td>
<td>0.077</td>
<td>0.154</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* Insoluble</td>
<td>0.184</td>
<td>0.372</td>
</tr>
<tr>
<td>Sephadex</td>
<td>77</td>
<td>$\beta_1$</td>
<td>0.038</td>
<td>0.076</td>
</tr>
<tr>
<td>G-200</td>
<td>92</td>
<td>$\beta_2$</td>
<td>0.040</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>$\beta_3$</td>
<td>0.048</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>126</td>
<td>LMW</td>
<td>0.080</td>
<td>0.160</td>
</tr>
</tbody>
</table>

* Insoluble protein, from the same preparation, which was not applied to the column.
crystallin from the A-5m column exhibits an amount of racemization only slightly above the \( f = 0 \) value, while the three molecular weight classes of \( \beta \)-crystallin fall just below this value of \(-0.05\).

Urea extraction of the WI protein from yellow cataracts resulted in soluble proteins as well as membrane-enriched insoluble material. The urea-soluble component was fractionated on a Sephadex G-100 column, and the elution pattern is shown in Fig. 3. Fraction 1 consists of components with \( M_r \) of 80,000 or more, which are apparently formed by covalent crosslinks (11). This fraction is found only in WI protein. Fraction 2 contains proteins of \( \sim 43,000 \) \( M_r \), as determined by sodium dodecyl sulfate gel electrophoresis, and is similar in size to material found in the \( \beta \)-crystallin from human WS protein. It may be a single gene product, or it may result from a postsynthetic process linking together two smaller polypeptides. Fraction 3 contains polypeptides that correspond in \( M_r \) to those of normal lens crystallins (20,000-30,000). When soluble lens proteins are fractionated in this urea system, the predominant peak co-elutes with this material, as is illustrated by the broken line in Fig. 3. Fraction 4 is almost entirely material of 11,000 \( M_r \) and probably represents a degradative product of a normal crystallin polypeptide.

The purpose of urea fractionation of the WI material was to isolate and characterize components enriched in D-aspartic acid. Results on the four urea-solubilized fractions are presented in Table 4. Fraction 2 falls within 2 SD of the D/L ratio expected in WI protein from a 58-yr-old lens. Fractions 1 and 4 are above this range, while fraction 3 as well as the urea-insoluble component lie below this range.

**DISCUSSION**

The distribution of D-aspartic acid in the human lens generally conforms to the predicted pattern. Comparison of D/L ratios in the central nucleus and outer cortex shows that the more highly stable or "older" proteins of the core of the lens are more extensively racemized. In the unfracated crystallin cortex samples, essentially background amounts of racemization are generated by the acid hydrolysis procedure. WI proteins from the whole lens and the nucleus exhibit higher D/L ratios than WS proteins from the same lens, and the extent of racemization in the WI fraction increases with age.

An unanticipated observation is that WI protein isolated from the cortex shows D/L Asp ratios comparable to the WI fraction from the same nucleus. If all of the proteins in the outer layers of the lens are relatively recently synthesized, then the "young" cortex protein should not be as highly racemized as "old" nuclear proteins. However, reproducible sectioning of the outermost cortex is difficult, and small amounts of inner cortex may include more highly racemized proteins (1). Wannemacher and Spector (18) have shown that even in the calf there is low protein synthetic activity in the lens cortex, except for the extreme periphery. Thus, the human cortex WI protein may approximate the nuclear proteins in age.

When the WI protein from yellow cataracts averaging 58 yr old is further dissected by urea extraction, fractions 1 and 4 have D/L ratios higher than the value expected from WI protein from a 58-yr-old lens, while 2 falls within this range. The D/L ratios for fraction 3 and the urea-insoluble component lie just below this range. On the basis of previous characterization of these fractions (11), we can provisionally conclude that the higher D/L ratios are associated with classes of proteins that have apparently been structurally altered. Crosslinked (fraction 1) and presumptively degraded (4) proteins exhibit the highest D/L ratios. The lowest D/L ratios in the WI protein are found in fraction 3, which contains polypeptides corresponding in size to normal lens crystallin subunits, and in the urea-insoluble material, which is probably composed largely of cell membranes. Thus, the greatest extent of racemization is observed in what are thought to be degraded or crosslinked proteins from yellow cataracts.

In our initial study (1), the brunescent lenses showed a significantly greater extent of racemization than yellow cataracts or normal lenses. Yet the WI proteins from both the whole lens (Fig. 1B) and the nucleus (Fig. 1C) of brunescent cataracts have D/L ratios indistinguishable from the other types of lenses. Consequently, there must be yet another fraction(s) with a D/L ratio high enough to produce the elevated ratios seen in brunescent cataracts. We have some preliminary evidence on fractionated WI material from brunescent cataracts. Four brunescent lenses averaging 77.5 yr were extracted with 6 M guanidine-hydrochloride, 50 mM dithiothreitol. The protein solubilized by this procedure has a D/L ratio of 0.199, while the insoluble residue shows 0.222. These values fall within the range of D/L ratios observed for WI protein from whole brunescent lenses: 0.173–0.247 (see Fig. 1B). Because none of the protein fractions account for the elevated ratios previously seen in brunescent nuclei, it is likely that very high D/L ratios are to be found in the LMW dialyzable components (free amino acids and small peptides).

D-aspartic acid is seen to be associated primarily with WI protein and HMW aggregates in normal lenses and yellow cataracts. Within the WI fraction, high D/L ratios occur in material that is apparently both degraded and crosslinked. It is not yet known whether racemization initiates any of these changes. However, several of these observations are consistent.
with the prediction that racemization of aspartyl residues would induce conformational changes in a protein, and these in turn could alter its physicochemical properties (9). Because HMW aggregates are thought to be precursors to the WI protein, the high D/L Asp ratio in the HMW fraction (Table 3) suggests that racemization precedes the formation of WI protein. A possible mechanism might be that the conformational changes resulting from racemization of a few aspartyl residues would expose the hydrophobic amino acids, which compose a very high proportion of the total amino acids in α-crystallin (the major component in HMW), leading to aggregation through hydrophobic interactions. Once aggregation has taken place, other reactions may serve to covalently crosslink certain components or degrade others, resulting in formation of the types of fractions isolated from WI material by urea (Fig. 3).

Instead of racemization triggering the development of WI protein, the reverse mechanism should also be considered. It is possible that aspartyl residues become more susceptible to racemization once the protein has become aggregated, partially degraded, or crosslinked. However, there are several lines of evidence that do not support this second mechanism. If the HMW protein is a precursor to the WI fraction, then extensive racemization has taken place prior to WI formation. It would also seem to be highly coincidental that the kinetics of aspartic acid racemization in lens proteins so closely approximate those previously observed in calcified dental tissue (2, 3) if separate mechanisms are involved. Finally, we have preliminary results on trout lenses which indicate that racemization of aspartyl residues is not associated with the appearance of WI protein in these poikilotherms. Racemization should not be occurring in trout due to their low temperature, but WI protein can be extracted from fish lenses. There may be more than one means of producing this WI fraction, but in fish lenses, development of WI protein does not generate D-aspartyl residues. Thus, the results reported here are consistent with the first suggested mechanism, that racemization of aspartyl residues in human lenses may lead to the HMW aggregates, which are then converted to WI protein by various reactions, including degradation and formation of covalent bonds.

Conclusions. Proteins from human lenses provide a system for testing our predictions of the effects of in vivo racemization of aspartyl residues. Conformational changes producing alterations in the properties of affected proteins were expected to result from racemization. Aggregated, WI, crosslinked, and presumptively degraded material are all found to have high D/L Asp ratios. Racemization may trigger aggregation of lens proteins, which are then converted by other reactions to WI material seen in aging lenses and in cataracts.

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