Human alpha-crystallin. I. The isolation and characterization of newly synthesized alpha-crystallin

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Studies of the incorporation of $^{14}$C amino acids into human lens proteins demonstrate that an alpha-crystallin fraction takes up more than six times as much radioactivity as any other lens protein. Based on analyses with a calibrated Bio-Gel A-1.5 m column, a molecular weight of $4.9 \times 10^5 \pm 5$ per cent was obtained for this protein while sedimentation equilibrium analyses indicated a weight average molecular weight, $M_w$, of $7.5 \times 10^5 \pm 4$ per cent at 10,000 r.p.m. Gel electrophoresis in sodium dodecyl sulfate revealed two components with molecular weights of 22,000 and 20,000, values similar to those found with calf alpha-crystallin. Alkaline urea gel electrophoresis indicated one major polypeptide with a mobility similar to the B chain of calf alpha-crystallin and two major bands with mobilities between those of the calf alpha-crystallin A, and A', chains. Amino acid analyses of this newly synthesized alpha-crystallin gave a composition which with a few exceptions is very similar to that of calf alpha-crystallin. All three major polypeptides contained $^{14}$C amino acids. However, from the present data, it cannot be determined whether the three polypeptides were independently synthesized or a rapid transformation produced one of the labeled polypeptides in the A region. There appears to be between three and four times as many presumptive A as B polypeptides.

Key words: human, alpha-crystallin, newly synthesized, $^{14}$C amino acid incorporation.

Alpha-crystallin isolated from bovine lens is a physically heterogeneous macromolecule composed of polypeptides with molecular weights of approximately $2 \times 10^4$. A number of different sized populations of the protein have been observed ranging from approximately $7 \times 10^4$ daltons to more than $50 \times 10^4$ daltons. The latter population has been found primarily in the nuclear region and increases dramatically with the aging of the lens. This age-dependent change in the aggregate size of alpha-crystallin is of particular interest since it has been proposed that macromolecules of $50 \times 10^4$ daltons or larger are capable of obstructing the passage of light through the lens and causing opacification. While it is now known that the human lens also produces...
a high molecular weight fraction in the nuclear region, its chemistry has not been elucidated. Indeed, characterization of human alpha-crystallin and its transformation with aging has only recently been undertaken.

A newly synthesized alpha-crystallin has been isolated from calf lenses, which have been incubated in the presence of labeled amino acids. This protein was found to have a specific activity 8 to 10 times greater than that of any other lens protein. The newly synthesized alpha-crystallin appears to be physically homogenous with a $M_w$ of approximately $7 \times 10^5$ and is the only species of bovine alpha-crystallin with these properties. It also has a much simpler polypeptide composition than older alpha-crystallin preparations.

Preliminary experiments indicated that a highly labeled alpha-crystallin fraction also is present in the human lens. Because of its probable simplicity, an attempt has been made to isolate and characterize this protein so that it can be used as a reference for studies on alpha-crystallin obtained from older lenses and perhaps help to elucidate the mechanism of aggregation to higher molecular weight species.

Methods

Normal human eyes were obtained within 30 hours of death. The lenses were immediately removed and treated in the following manner. The lenses were usually incubated in pairs at $37^\circ$ C. for 20 hours in 2 ml. of Difco TC-199 containing 10 $\mu$Ci of $^{14}$C-labeled amino acids (New England Nuclear L-amino acid mixture ($^{14}$C [u.]) 80 units of penicillin, and 2 mg. of streptomycin. Before incubation the medium was adjusted to pH 7.4 with a solution containing 3.97 Gm. NaHCO$_3$, 806 mg. NaCl, and 174 mg. KCl in 500 ml. of H$_2$O. All preparations were sterile. After incubation, the lenses were rinsed with distilled H$_2$O and frozen at $-70^\circ$ C. until use. Over a period of approximately 12 months, 14 pairs of lenses were incubated ranging in age from one day to one year and eight months. The lenses were divided into two sets of seven pairs for further study. Set one contained three pairs of lenses, one pair, two days old; one pair, three days old; and one pair, four days old. Set two contained one pair, one and one half months old; one pair, two and one half months old; two pairs, three months old; one pair, five months old; one pair, six months old; and one pair, one year and eight months old. All lenses were clear and had no apparent pathology.

Each set of lenses was homogenized in ten times its weight of H$_2$O, centrifuged at 15,000 r.p.m. for 15 minutes, and exhaustively dialyzed to remove the free labeled amino acids against 0.002 M phosphate buffer, pH 7.0. Each supernatant was then applied to a 2.3 by 25 cm. column of DEAE cellulose (Brown Company) and fractionated at $4^\circ$ C. by stepwise elution with phosphate buffers prepared from a stock 0.5 M solution, pH 0.8, as previously described. Following fractionation, the peaks were pooled, dialyzed against H$_2$O, and lyophilized.

Gel filtration was performed at $4^\circ$ C. on Bio-Gel A-1.5m columns, 2.5 by 80 cm., in 0.1 M KCl, 0.01 M Tris, pH 7.6. For molecular weight determinations the column was calibrated with proteins of known molecular weight. The standard proteins were added at intervals of 40 to 50 ml. to avoid interaction with each other.

Ultracentrifuge and electrophoresis experiments were conducted according to previously described methods. For the preparative fractionation of the labeled polypeptides by urea gel electrophoresis, 10 per cent polyacrylamide gels at pH 8.7 utilizing the Tris-glycine, 7 M urea buffer system of Ornstein were employed. Electrophoresis was carried out at 6 ma. per tube for three hours with 6 mm. by 10 cm. gel columns. One milligram of the purified highly labeled fraction, specific activity 5,800 c.p.m. per $A_{280}$, was dissolved in 0.5 ml. of the Ornstein buffer and 60 $\mu$l was applied to each of eight gels. After electrophoresis the gels were scanned at 280 nm. in a Gilford spectrophotometer and then stained with amido black. The stained bands were cut out and each band from all eight gels was dissolved in 1.0 ml. of the Ornstein buffer and 60 $\mu$l was applied to each sample and they were then counted with a Packard Tricarb scintillation counter.

Results

Following incubation of the young normal human lenses with $^{14}$C-labeled amino acids, they were divided into two groups of seven pairs. One group contained lenses less than five days of age and the other ranged from one month to one year and eight months. The younger group incorporated approximately three times more labeled amino acids into its soluble protein than the older group, giving specific activities of 3,160 c.p.m. per $A_{280}$ and
Fig. 1. DEAE cellulose fractionation of labeled soluble lens proteins. The bars denote the pooled fractions.

1,050 c.p.m. per A280 nm., respectively. Such observations suggest a marked decrease in protein synthesis during early postnatal life.

The soluble proteins were fractionated on a DEAE cellulose column by stepwise elution with increasing concentrations of phosphate buffer. More than 90 per cent of the A280 nm. and the radioactivity added to the column were recovered with both preparations. The two experiments also gave similar chromatographic profiles. Fig. 1 illustrates the profile obtained with the soluble protein from the younger lenses. The 0.055 M fractions had the highest specific activity while the 0.002 M and the 0.03 M peaks contained little radioactivity. Table I summarizes the results of this experiment. The first peak eluted with 0.55 M phosphate has a specific activity of 10,700 c.p.m. per A280 nm. and the second peak 6,200 c.p.m. per A280 nm. These values are two to five times greater than that observed for any other component. While 19 per cent of the radioactivity was found...
in these fractions, they represented only 5 per cent of the total material. It is of interest to note that the DEAE cellulose profiles were similar to those obtained with calf lens soluble proteins.

The two peaks eluted with 0.055 M phosphate were, respectively, combined with similar material obtained from the second experiment with the older lenses and further purified on Bio-Gel A-1.5m. Figs. 2, 1 and 2, 2 illustrate the profiles obtained from gel filtration of the combined peak I and peak II. Both fractions gave three similar peaks with essentially identical elution volumes suggesting a close relationship between the unfractionated proteins. The only discernible difference between the two 0.055 M eluted fractions appears to be the relatively lower specific activity of the A peak from the pooled peak I. Because of the limited material available, the highest specific activity major B peaks were combined and rerun on the Bio-Gel A-1.5 m column. This procedure was, in part, necessitated by the unusually low recoveries from the Bio-Gel A-1.5 m column which ranged from approximately 40 to 60 per cent.

As shown in Fig. 2, 3, approximately 90 per cent of the eluted protein was isolated in a single peak with a relatively constant specific activity. The results of these ex-

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**Figure 2.** Fractionation of 0.055 M eluted components from DEAE cellulose on Bio-Gel A-1.5 m. 1. 0.055 M peak I pooled from the two DEAE cellulose experiments. 2. 0.055 M peak II similarly pooled from the two DEAE cellulose experiments. 3. Rerun of pooled peak B from 1 and 2.
experiments are summarized in Table II. The Bio-Gel A-1.5m fractionation increased the specific activity of the major peak more than 40 per cent. In comparison to other soluble protein fractions the overall increase in specific activity was approximately six-fold. The recovered protein from peak 2 of the final gel filtration run represented approximately 2 per cent of the unfractionated material. This highly labeled protein was characterized by the following experiments.

The molecular weight of the highly labeled peak 2 fraction was determined by gel filtration utilizing a calibrated Bio-Gel A-1.5m column (Fig. 3). An average molecular weight of approximately $4.9 \times 10^6 \pm 5$ per cent was obtained on the basis of four analyses. Similar experiments with the first peak isolated from the A-1.5 m column gave a molecular weight of approximately $1.2 \times 10^6 \pm 10$ per cent. Equilibrium sedimentation experiments with the highly labeled fraction indicated a relatively homogeneous material with a $M_w$ of $7.5 \times 10^5 \pm 4$ per cent at 10,000 r.p.m. A typical experiment is shown in Fig. 4. The explanation for the difference in the molecular weights determined by gel filtration and the ultracentrifuge is not apparent at the present time.

The amino acid composition of the highly labeled material was compared to other fractions isolated by DEAE cellulose and to calf alpha-crystallin (Table III). With the exception of a few amino acids such as aspartic acid, threonine, proline, glycine, methionine, and CM-cysteine, excellent agreement between calf alpha-crystallin and the purified fraction was obtained. All amino acids of the 0.1 M and 0.4 M fractions from DEAE cellulose have values similar to the highly labeled protein except for glycine which appears unusually high in the purified fraction.
Table III. Amino acid composition of purified highly labeled protein and other lens proteins

<table>
<thead>
<tr>
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<th>Highly labeled protein</th>
<th>DEAE cellulose fractions</th>
<th>Calf α-crystallin</th>
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<tr>
<td></td>
<td></td>
<td>0.1M</td>
<td>0.04M</td>
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<tr>
<td>Aspartic acid</td>
<td>96</td>
<td>97</td>
<td>91</td>
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<tr>
<td>Threonine</td>
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<tr>
<td>Serine</td>
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<td>Glutamic acid</td>
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<tr>
<td>Proline</td>
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</tr>
<tr>
<td>Glycine</td>
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<tr>
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<td>Valine</td>
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<td>11</td>
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<tr>
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<tr>
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<td>Phenylalanine</td>
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<td>Arginine</td>
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<td>76</td>
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<tr>
<td>CM-cysteine</td>
<td>13</td>
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<td>10</td>
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</table>

Values are expressed as residues per 1,000 residues. The analyses were performed after hydrolysis in constant boiling HCl as described previously.

Levels of glycine have been found in alphacrystallin isolated from older human lenses (Roy, D., and Spector, A.: Manuscript in preparation.) The values for most amino acids in the purified highly labeled material differ markedly from the 0.002 M and 0.03 M DEAE cellulose fraction. These results suggest that the highly labeled purified material is a newly synthesized alphacrystallin closely related to the 0.1 M and 0.4 M DEAE cellulose fractions. The amino acid composition of human alphacrystallin has recently been reported from a number of laboratories.10, 11, 15, 19 However, in certain cases there is a considerable variation in some of these values suggesting contamination with other protein fractions. Preliminary experiments with the Bio-Gel A-1.5m peak 1 material gave acid compositions similar to the highly labeled protein suggesting that this fraction is a higher molecular weight alphacrystallin.

The highly labeled material was further characterized by urea and SDS gel electrophoresis. In Fig. 5, A, the urea gel patterns obtained with the purified highly labeled protein (E) are compared to some of the DEAE cellulose fractions (A through D) and calf alpha-crystallin (F). The highly labeled protein has three major bands, one corresponding to the slowest moving band of calf alpha-crystallin, the B2 polypeptide, and two in the more rapidly migrating A polypeptide region with mobilities between those of the calf alpha-crystallin polypeptides A2 and A1. The gel patterns obtained with the 0.1 M and 0.4 M fractions from DEAE cellulose are identical to those of the highly labeled material but differ markedly from the 0.002 M and 0.03 M DEAE cellulose fractions. The latter patterns are somewhat similar to those observed for calf gamma- and beta-crystallins, respectively.

Further confirmation of the relationships suggested by the urea gels was obtained from the SDS gel patterns (Fig. 5, B). No difference in gel pattern was observed between the highly labeled protein, calf alpha-crystallin, and the 0.1 M and 0.4 M DEAE cellulose fractions suggesting that these polypeptide chains all have similar molecular weights in the 20,000 range. The 0.002 M and 0.03 M DEAE cellulose fractions have patterns which vary considerably from the aforementioned profiles and from each other.
In order to ascertain the extent to which the major polypeptides of the highly labeled protein have incorporated $^{14}$C amino acids, preparative urea gels were run. The gels were then scanned in a Gilford spectrophotometer at 280 nm. After staining, the bands were cut out, dissolved, and counted. Fig. 6 illustrates a typical preparative gel pattern and the composite results obtained from eight gels. All the polypeptide bands appear to contain radioactivity with most of the $^{14}$C label being present in the major B and A region bands. Approximately 600 c.p.m. were found in the major B band and 1,700 and 1,000 c.p.m. for the major A peaks. Based on the 280 nm. scans of the gels before staining, the specific activity of the major B band was 4,400 c.p.m. per A$_{280}$ nm. and 6,300 and 4,000 c.p.m. per A$_{280}$ nm. were calculated for the major A bands. The minor bands have specific activities ranging from 1,500 to 2,200. Although there is a slight amount of streaking on the gels, the results clearly suggest that all the polypeptides in the highly labeled protein contain radioactivity.

**Discussion**

The newly synthesized protein is clearly a member of the alpha-crystallin family on the basis of its amino acid composition, aggregate size, and the molecular weight of its polypeptide chains. However, there are a number of definitive differences between calf and human newly synthesized alpha-crystallin. The bovine protein contains essentially two major polypeptides B$_2$ and A$_2$ in contrast to the general population of low molecular weight bovine alpha-crystallin which has four polypeptide chains B$_2$, B$_1$, A$_2$, and A$_1$. $^{19}$ Labeling experiments with calf lenses indicate that in the newly synthesized alpha-crystallin only the A$_2$ and B$_2$ chains incorporate amino acids. On the basis of amino acid composi-
tion and fingerprinting it is quite apparent that B₁ and A₁ are very similar to their B₂ and A₂ counterparts. The modified polypeptides appear to arise over an unknown but relatively short period of time.

Differing from the bovine protein, newly synthesized alpha-crystallin isolated from very young human lenses has three major polypeptide chains. One corresponding to bovine B₂ and two polypeptides in the A region with somewhat different urea gel electrophoretic mobilities than the bovine A chains. Based on studies with human alpha-crystallin isolated from old lenses, the faster moving polypeptides appear to be A type polypeptides (Roy, D., and Spector, A.: Manuscript in preparation.). Utilizing sepharose 6B, an apparent alpha-crystallin has recently been isolated from fetal and neonatal human lenses which appears to have a similar electrophoretic pattern on cellulose-acetate membranes in the presence of 7 M urea to the urea gel electrophoretic profiles reported for the newly synthesized alpha-crystallin in this communication. However, the amino acid compositions of the proteins differed in some respects from the material described in this communication.

It is not clear whether all three chains are independently synthesized or if one of the A-chains is derived from the other. The presence of a significant amount of a second A-chain with a specific activity of approximately 60 per cent of the primary A polypeptide suggests either a very rapid transformation to this structure or its direct synthesis. Based on studies with the bovine lens, the former explanation is favored. Investigation of alpha-crystallin from older human lenses and from cataractous lenses indicate the formation of many more modified A- and B-chains than have been detected in the bovine lens and an unusual instability of some of the modified chains (Roy, D., and Spector, A.: Manuscript in preparation.).

Based on the 280 nm. scans of the preparative gels, there appear to be four times as many major A-chains as B-chains. The incorporation data also indicate that the major A-chains are synthesized four times more rapidly than the major B-chains. However, if it is assumed that all detected polypeptide bands are associated with alpha-crystallin, an incorporation ratio of 3:1 is observed. In the bovine lens the abundance ratio of A-chains to B-chains is 2:1 suggesting a relative increase in the synthesis of A-chains in the human lens.

Another indication of the changes which occur with aging in the human lens is depicted in the DEAE cellulose patterns of the total soluble protein. Only with very young lenses is it possible to obtain patterns which are comparable to those observed with the calf lenses. Even with lenses obtained from twenty-year-old in-
individuals, only a small gamma-crystallin fraction and reduced beta-crystallin components are found. Dilley and Harding\(^\text{19}\) have also found significant age-dependent changes in the relative proportions of the soluble protein fractions eluted from DEAE cellulose.

Newly synthesized bovine alpha-crystallin has an \(M_w\) of approximately \(7 \times 10^5\), while the size of the human protein is uncertain at present ranging from \(4.9 \times 10^5\) daltons observed by gel filtration to \(7.5 \times 10^5\) daltons obtained by sedimentation equilibrium studies. However, in both species, the newly synthesized protein is the smallest and most homogeneous of the alpha-crystallin family. From experiments reported in this communication and other recent observations in our laboratory, it also appears that the age-dependent size transformation, noted with bovine alpha-crystallin, also occurs in the human lens.

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REFERENCES


