Studies on lens protein polypeptides

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Methods for the separation and identification of the polypeptide chains of each lens crystallin are basic to the study of these proteins on polyribosomes. Rabbit lens proteins have been deaggregated, denatured, and reduced to polypeptide chains, and separated by polyacrylamide gel electrophoresis. Approximate molecular weights of the resultant chains have been calculated by the same system. Vigorous denaturation and reduction with sodium dodecyl sulfate and 2-mercaptoethanol disrupts all structure other than primary structure, degrading each protein to its component polypeptides. The separation method employed is almost exclusively dependent on molecular size. Deaggregated alpha crystallin is a group of chains, each of about 20,000 molecular weight. The beta crystallin region of the DEAE chromatogram yields three polypeptides of estimated molecular weights 21,000, 23,000, and 29,000. Preliminary studies on the four beta crystallin peaks isolated by DEAE-chromatography suggest that one or more may be composed of aggregates of different polypeptide chains. Gamma crystallins appear to be single polypeptide chain proteins of molecular weights from 18,000 to 21,000. Experiments performed on lens epithelium indicate that a significant percentage of their newly synthesized proteins are nuclear and cytoplasmic proteins other than the crystallins.

In recent years, the biosynthesis, structure, and physicochemical properties of a number of proteins have been clarified. Studies on lens proteins have been confined largely to the characteristics of native proteins and have revealed a marked tendency toward aggregation or polymer formation by the alpha and beta crystallin subunits.1-3

It is generally accepted that proteins are synthesized on polyribosomes as polypeptide chains and that the basic coding unit of the genome, the cistron, contains the genetic information for a single polypeptide chain. Assembly of finished polypeptide chains into multichain molecules has been shown to then occur on polyribosomes for several proteins.4-7 Thus knowledge of the polypeptide components of each of the lens crystallins is basic to an understanding of their biosynthesis. An approach to this problem is electrophoresis in polyacrylamide gels in the presence of the anionic detergent sodium dodecyl sulfate (SDS) following extensive denaturation and reduction.8 This method has proven extremely useful in the separation and characterization of both mammalian and viral polypeptides9 and permits rapid calculation of approximate molecular weights of the resultant subunits.10 The extensive disruption of all structure other than primary structure produced by SDS and 2-mercaptoethanol has successfully degraded each protein previously studied by us into its component polypeptide chains.10

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Materials and methods

Both unlabeled soluble lens protein and newly synthesized radioactive proteins were included in this study.

To prepare the latter 1 or 2 whole adult rabbit lenses were washed and then incubated at 37°C in 12 ml of Minimal Essential Medium, Eagle (MEM)1 containing 0.05 to 0.01 the normal concentration of amino acids (to deplete the intracellular pool). They were then labeled with 50 to 100 μC of C14 mixed amino acids (NEC-445) for periods of 8 to 24 hours. Incorporation was stopped by washing in chilled Earle's saline. Soluble proteins (both labeled and unlabeled) were prepared by hand-homogenization of lenses in 0.01M tris pH 7.2 and pelleting of the insoluble residue at 10,000 g for 20 minutes.

Samples of soluble protein weighing 50 to 250 μg were then incubated at 37°C for 2 hours with 1 per cent SDS and 1 per cent 2-mercaptoethanol (2-Me) to disrupt hydrogen, hydrophobic, and disulfide bonds. The samples were then made 0.3M with iodoacetamide to block both interchain and intrachain sulfhydryls exposed by the SDS and 2-Me.

The samples were then dialyzed against 0.01M phosphate pH 7.1 with 0.1 per cent SDS and electrophoresis carried out at 5 v, per centimeter for 16 hours in 200 mm. 10 per cent polyacrylamide gels containing 0.1 per cent SDS and 0.1M phosphate pH 7.1.8 The electrophoresis buffer was 0.1M phosphate pH 7.1 with 0.1 per cent SDS. In preliminary studies, no significant differences in migration were observed in 0.1 per cent, 0.5 per cent, and 1.0 per cent SDS. Similarly, increasing the concentration of mercaptoethanol or the time of reduction made no detectable difference.

Following electrophoresis, unlabeled gels were stained with 0.25 per cent Coomasie Blue for 5 hours, after fixation with 20 per cent sulfosalicylic acid for 16 hours. They were then destained with 7 per cent acetic acid.

Labeled gels were fractionated into 100 equal, sequential fractions by a gel crusher and distributed onto planchettes for counting in an end-window gas-flow counter.

"Pulse-chase" labeling was performed by incubating lenses in amino acid-deficient MEM with 10 μC of C14 each of arginine, lysine, leucine, threonine, and valine (New England Nuclear Corp., 150 to 300 μC per millimole). After 2 hours, a two hundred fold excess of each amino acid (unlabeled) was added and incubation continued 2 to 24 additional hours to "chase" proteins with rapid turnover rates. In some experiments, following suitable incubation periods with isotope, epithelium and capsule were separated from the remainder of the lens for studies of comparative incorporation. Separation was accomplished by incision of the posterior capsule and careful stripping of the capsule with attached epithelium from the underlying fibers. No fiber tags were seen by phase-contrast microscopy. The cortex-nucleus portion was then processed as described above for whole lenses. The epithelium-capsule preparations were washed and then incubated at 4°C for 15 minutes in 0.5 per cent isotonic NP-40 buffer.12 This detergent breaks cell membranes but not nuclear membranes.12 Following dialysis against phosphate-buffered saline, aliquots of soluble epithelial cell protein were prepared for electrophoresis as described previously.

To identify the proteins from which each of the polypeptides demonstrated by this method was derived, DEAE-cellulose chromatography was employed to obtain both labeled and unlabeled rabbit crystallins. The column size was 30 by 2.5 cm and in all cases the samples applied ranged from 100 to 200 μg of protein. Fractions from each eluate buffer concentration were pooled, denatured, and reduced to polypeptide chains and prepared for electrophoresis as previously described. In these studies, the 0.015M and 0.03M eluates were generally not separated but were usually pooled by elution with 0.03M phosphate alone.

Results

A typical experiment is shown in Fig. 1, illustrating the correlation of the subunits of soluble proteins of whole rabbit lens with the subunits obtained by denaturation and electrophoresis of the indicated DEAE-purified protein fractions. Six bands are repeatedly demonstrable when whole lens protein is examined in this manner.

![Fig. 1. Stained 10 per cent polyacrylamide gels comparing subunits of soluble whole lens protein with subunits of lens proteins separated by DEAE chromatography and subsequently denatured. Gamma, beta, and alpha crystallins were representative fractions eluted with 0.001M, 0.03M, and 0.4M phosphate, respectively. The anode is on the right in this and subsequent electrophorograms.](https://lov.s.arvojournals.org)
Band 1 is about 7 per cent of whole lens protein and corresponds to the major fraction eluted from DEAE by 0.001M phosphate. Band 2, a major component, is eluted with 0.4M phosphate, while 3 and 4 correspond to the two major subunits of the 0.03M fraction. Band 5 is a minor component which, along with 3 and 4, is found in the 0.3M DEAE eluate. Band 6 was usually eluted with 0.001M phosphate (Fig. 2), although it cannot be seen in this fraction in Fig. 1.

As previously shown, the approximate molecular weights of polypeptides can be calculated by their rates of migration in this gel electrophoresis system by inclusion of markers of known size. This procedure yielded molecular weights of 19,000, 20,000, 21,000, 23,000, and 29,000 for bands 1 through 5, respectively, when individual bands were isolated and rerun on 125 mm. 5 per cent gels with marker polypeptides.

The electrophoretic pattern of newly synthesized subunits after a 24 hour labeling period is shown in Fig. 2. The subunit composition of pooled eluate fractions of similarly labeled whole lens protein separated by DEAE chromatography is also shown in Fig. 2 for comparison. An additional peak not clearly seen in stained gels appeared at fraction 76 when radioactive whole lens protein was examined in this manner. It was usually eluted from DEAE by 0.001M phosphate and the estimated molecular weight is 18,000. Integration of the area under each peak reflects the relative amount of each subunit present in the sample mixture applied, since under these conditions of denaturation and reduction there is little or no trapping of material at the origin of the gel. These percentages are shown above each peak in Fig. 2A and Fig. 3.

Fig. 3 illustrates the electrophoretic patterns of subunits obtained when denaturation with SDS alone is compared to that produced by SDS and 2-Me. In the absence of 2-Me a new peak appears (fraction 30) and is accompanied by a loss of equivalent area (per cent of counts) under
Fig. 4. Electropherograms of newly synthesized protein after 2 hour label. Homogenized whole epithelium (A), and soluble epithelial cytoplasmic protein (B) produced by NP-40 and "pulse-chase" labeling (2 hour label, 16 hour chase), and cortical fibers (C).

peaks 3 and 4. The estimated molecular weight of the peak at fraction 30 (44,000) is compatible with a disulfide-linked aggregate composed of one molecule each of peaks 3 and 4. The new peak seen at the origin in Fig. 3, B is an artifact representing high molecular weight material excluded by the gel.

Fig. 4 illustrates the comparative electrophoretic patterns of newly synthesized proteins of homogenized whole epithelium, epithelial cell cytoplasm (produced by the "pulse-chase" technique and NP-40 treatment), and cortical fibers.

Discussion

As pointed out by others, alpha crystallin, under suitable conditions of deaggregation, appears to consist of a group of closely related subunits of molecular weight approximately 20,000. The results presented here confirm these findings. It should be pointed out, however, that several experiments with labeled rabbit lens protein have indicated a significant degree of admixture (Fig. 2) of beta crystallin subunits (peak 4) with the 0.4M or alpha crystallin eluate. This observation has been confirmed by double-labeled gels in which the sample was a mixture of C4 beta crystallin and H2 alpha crystallin. Similar contamination was reported by Mason and Hines.

The beta crystallin region of the DEAE chromatogram consists mainly of polypeptide chains of two different sizes (peaks 3 and 4). The early 0.03M eluate fractions contain significant amounts of peak 5 as well. This is more readily appreciated with radioactive polypeptides than with stained specimens because of the typical broadness of stained bands in the SDS-polyacrylamide system and the difficulty in staining protein in the presence of SDS. From the reported molecular weights of native beta crystallins, it may be assumed from the results shown in Fig. 3 that many of the aggregation phenomena associated with them are likely the result of secondary forces (e.g., hydrophobic bonding). The possibility of covalent linkage by disulfide bonding between two chains cannot be completely excluded, however. The appearance of a new peak (fraction 30) in the absence of mercaptoethanol is suggestive evidence that disulfide bonding between the two denatured chains represented by peaks 3 and 4 can occur, but it is not yet known whether beta crystallins can exhibit such linkages in the native state.

Preliminary double-label studies now in progress with the four isolated DEAE beta crystallin peaks indicate that those elutable with 0.03M phosphate are composed almost wholly of peaks 3 and 4. The 0.015M DEAE eluate contains peaks 4 and 5, as well as some of the small, rapidly migrating peak seen at fraction 76 (Fig. 2, A). In some experiments, large amounts of the latter chain (probably a gamma crystallin) are observed in the 0.015M eluate. Thus it seems probable that the beta crystallins consist of at least 3 sizes of polypeptide chains. It is hoped that the
present studies will clarify the polypeptide chain compositions of each beta DEAE peak.

Gamma crystallins are reported to be a relatively homogeneous group of proteins of molecular weights of approximately 20,000. The calculated molecular weight of peak 1 is 19,000 and, as seen in Fig. 2, B, there are significant amounts of material which migrate at approximately the same velocity as peaks 2, 4, 5, and 6. It must be emphasized that complex formation between SDS and protein effectively minimizes native charge differences so that distance or rate of migration in this system is inversely proportional to molecular size. Thus almost all of the material eluted with 0.001M buffer is a group of single polypeptide chain proteins (gamma crystallins) with a spectrum of molecular weights of about 18,000 to 21,000. This technique, however, cannot discriminate between gamma and alpha polypeptides of the same size. The significance of the elution of small amounts of peaks 4, 5, and 6 is obscure since their high molecular weights fall well outside the range of 19,000 to 20,000 described by Björk. It is doubtful that polymer formation between gamma subunits can account for this material because of the disruption of secondary and tertiary forces by SDS and 2-Me and the blockage of resultant exposed sulfhydryls by carboxymethylation. They may, in part, be proteins other than crystallins which are not bound by the column and appear in the void volume.

Preliminary attempts to compare the synthesis of "structural" proteins by epithelium to that by fibers were unsuccessful for two reasons. First, a significant amount of labeled amino acid was incorporated into nuclear proteins, probably reflecting the synthesis of histones and enzymes necessary for the replication of genetic materials (e.g., polymerases). Second, much newly synthesized protein found in the epithelial cell cytoplasm is not identifiable as structural protein (i.e., those with slow turnover rates) and represents, in large part, enzymes necessary for cell metabolism and transport. It is of interest to note that little uptake of isotope by lens cortex could be demonstrated in the absence of capsule and epithelium, suggesting the essential role of the latter in amino acid transport. Combined use of NP-40 buffer to prepare cytoplasmic extracts free of nuclei and the "pulse-chase" technique to progressively "chase" proteins with rapid turnover rates, leaving only structural proteins labeled, permitted a valid comparison of the synthesis of the crystallins by epithelium and by cortical fibers. As shown in Fig. 4, A, a considerable proportion of labeled protein synthesized by lens epithelium is not cytoplasmic structural protein; the shorter the period of labeling, the greater the percentage of nonstructural cytoplasmic protein found to be labeled. Thus in any studies of comparative incorporation into crystallins by epithelium and fibers the possibility of labeled enzymes or nuclear proteins eluting from DEAE along with specific crystallins must be borne in mind. This may in part explain the multiple subunits obtained when DEAE-purified "gamma crystallin" is denatured and electrophoresed.

Although no gross qualitative differences were observed between labeled structural proteins of epithelium and fibers (Fig. 4, B and C), such data are difficult to interpret at present because of the overlap in size between labeled gamma and alpha polypeptides shown in Fig. 2. Experiments to resolve this problem are in progress.

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REFERENCES
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