Disulfide-linked high molecular weight protein associated with human cataract

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ABSTRACT A major high molecular weight disulfide-linked protein has been isolated from cataractous lenses. It is only present in the water-insoluble protein fraction. This species has not been found in normal lenses of comparable age. Upon reduction of this fraction, polypeptides having molecular weights of approximately 60,000, 43,000, and 20,000 as well as a non-characterized heterogeneous species are released. Similar sized polypeptides have been found in various noncovalently linked aggregates in both normal and cataractous lenses. Examination of the disulfide content of the high molecular weight disulfide-linked protein fraction indicates that approximately 70% of the sulfhydryl groups are in the oxidized state. Although little change in the sizes of the other major polypeptides in the water-insoluble fraction is observed upon reduction, these components were also found to contain an appreciable disulfide content. Such results indicate that the only major lens fraction containing disulfide-linked polypeptide is the high molecular weight species and that the disulfides present in the remaining fractions are either intrachain disulfides or link polypeptides to small peptides.

One of the least understood protein fractions of the human lens is the water-insoluble protein component. Only relatively small amounts of this material are present in young lenses (1, 2) but, with aging, this component becomes increasingly prominent (3-5). In cataract, particularly of the senile type involving the inner regions of the lens, there is a marked increase in this fraction and it may represent as much as 90% of the total protein (6-7).

In the bovine lens the water-insoluble protein is relatively simple, being composed primarily of @ crystallin (8). However, in the human lens this is not the case and it would appear that this fraction may contain components arising from possibly all of the major structural lens proteins (9). There are a number of chemical characteristics besides solubility that distinguish the water-insoluble fraction from the remainder of the lens. It probably contains the oldest protein in the tissue. It is normally present in higher concentrations in the older inner region of the lens (7) where little protein synthesis is found (10), it contains a relatively high level of protein-associated nontryptophan fluorescence (2), and it has a markedly higher abundance of D-aspartic acid than does the soluble protein fraction (11, 12).

It has been suggested that the so-called high molecular weight protein is a precursor to the water-insoluble component because of similarities in its chemistry and its behavior upon differential centrifugation (9). Based upon light scattering theory (13) and the observed relationship between loss of lens transparency and accumulation of these protein species, particularly in the nuclear region, it has been proposed that certain types of cataract may be directly related to the production of these components (14).

The present communication reports studies of aspects of the protein of cataractous and normal lenses not previously observed. An important difference was found in the chemistry of the water-insoluble fraction. In addition to an increase in this fraction in cataract, it is demonstrated that the water-insoluble protein isolated from such lenses contains a major high molecular weight species composed of disulfide-linked smaller polypeptides. This component is not found in normal lenses, it may account for a significant proportion of the increased abundance of the water-insoluble fraction in cataractous lenses, and it may possibly contribute to the opacity of such tissue.

METHODS

Normal and cataractous human lenses were obtained from patients 55-65 years old within 24 hr of operation or death and were stored at -70°C. Cataractous lenses were graded according to Anderson and Spector (7). Lenses used in the experiments described in this communication were +3 to +5 in color and +3 to +4 in opacity. The transparency of presumptive normal lenses was always confirmed by inspection (7). Pooled lenses (groups of five) were homogenized in 0.1 M KCl/0.01 M Tris, pH 7.6, and the lens proteins were fractionated into water-insoluble, high molecular weight, and soluble fractions as described (9). Alkylation of insoluble protein was performed in 9 M guanidine-HCl/0.2 M Tris, pH 8.6/2 mM EDTA at 10°C under argon with iodoacetamide. The alkylated insoluble or freshly prepared water-insoluble protein was chromatographed on Sephadex G-200 equilibrated with 7.2 M urea/10% acetic acid or on Sephadex G-100 equilibrated with 7 M guanidine-HCl/0.2 M Tris, pH 8.6. Columns were run at room temperature.

For sodium dodecyl sulfate (NaDodSO4) gel electrophoresis of individual normal and cataractous lenses, the tissue was homogenized in the guanidine buffer described above, containing 0.1 M iodoacetamide. After alkylation, the protein was dialyzed, lyophilized, and then subjected to electrophoresis as described below with and without reduction with 2-mercaptoethanol in 1% NaDodSO4 at 10°C.

NaDodSO4/polyacrylamide gel electrophoresis was carried out according to the procedure of Weber and Osborn (15). It also was conducted in a medium containing agarose and polyacrylamide in the following manner. A 1% solution of agarose (Sigma, electrophoretic grade) was made by dissolving the agarose in boiling water and then refluxing for 20 min. Twenty milliliters of this agarose solution was mixed with 2.5 ml of 6% 3-dimethylaminopropionitrile, 10 ml of NaDodSO4 buffer (1 g of NaDodSO4, 3.9 g of NaH2PO4·H2O, and 19.3 g of

Abbreviations: NaDodSO4, sodium dodecyl sulfate; CM-Cys, carbamoylmethylcysteine.
Na₂HPO₄·7H₂O made up to 250 ml in water), 6 ml of acrylamide solution (19 g of acrylamide and 1 g of methylene bisacrylamide or 38 g of acrylamide and 1 g of methylene bisacrylamide made up to 100 ml in water), and 1.25 ml of 1.4% ammonium persulfate. The solution was held at 45-50°C. Electrophoresis tubes were 0.5 X 15 cm. Tubes were layered with water and polymerization was carried out at room temperature. Running buffer for gel electrophoresis was the same as for regular acrylamide gel electrophoresis (15). Gels were run at room temperature at 8 mA per tube for 4 hr or 2 mA per tube for 16 hr. Under the above conditions the marker dye (bromphenol blue) moves with the 20,000 molecular weight protein, when the crosslinkage is 19:1. Sample loads and staining of these gels are the same as for regular acrylamide gels (15).

Double gels of agarose and polyacrylamide were made by filling half of the electrophoresis tube with regular polyacrylamide solution and inducing polymerization. Then, the inside of the tube was rinsed with NaDodSO₄/phosphate running buffer and the upper half of the tube was filled with the agarose/polyacrylamide solution. The gels were run for 1 or 6 hr at 6 mA per tube. Reduction, alkylation, and amino acid analysis of proteins were performed as described (16).

RESULTS

Almost all of the water-insoluble fraction of normal and most cataractous lenses can be dissolved in 1% NaDodSO₄/0.1 M phosphate, pH 7.2, at a concentration of 2 mg/ml. When this material is analyzed by 0.1% NaDodSO₄/polyacrylamide gel electrophoresis, a number of major polypeptide bands were observed with molecular weights of 43,000, ~20,000 range, and 9600 as well as material that did not enter the gels (Fig. 1, lane A). A few minor bands also could be observed. The patterns obtained for material from normal and cataractous lenses were generally similar. Reduction with dithioerythritol or 2-mercaptoethanol before electrophoresis produced a number of changes in the profiles but, again, protein from normal and cataractous lenses gave similar results. After reduction, the bands were sharper and there appeared to be an increase in the relative abundance of the 43,000-dalton component, a decrease in minor components, and an increase in the 22,000-dalton band which in some cases was resolved into two components.

In attempts to isolate these components by chromatography for further characterization, it was found that NaDodSO₄ was difficult to work with because of problems in removal of the detergent and in column flow rate (17). Concentrated solutions of urea or guanidine, although not as effective solvents as NaDodSO₄, do not cause the problems noted with the detergent and therefore were used in fractionation of the insoluble lens proteins. Most of the results reported in this communication were obtained with material solubilized in 7.2 M urea/10% acetic acid. Although a significant amount of protein was not solubilized with urea, the overall NaDodSO₄ electrophoresis patterns appeared to be the same for urea-solubilized and urea-insoluble fractions (Fig. 1, lanes B and C). Again, normal and cataractous lenses generally gave the same results and the effect of reduction was the same as for the unfraccionated material.

Decreasing the crosslinking and increasing the pore size of the polyacrylamide gels did not cause the material normally found at the top of the gel to move into the gel column. However, in some cases, migration into the gel could be achieved with 0.5% agarose gels containing 2.9% acrylamide (acrylamide:methylene bisacrylamide, 19:1). From Fig. 2 it can be seen that, in the case of the total water-insoluble material from normal lenses, no material remained at the top of such gels. A diffuse background stain could be observed as well as bands representing the expected polypeptides. With similar material from cataractous lenses, a band was detected that did not penetrate the gel. However, this band disappeared after reduction, suggesting that this component is at least in part composed of interchain disulfide-linked polypeptides. Reduction did not appear to produce any new polypeptides on the basis of the size distribution observed after agarose/polyacrylamide gel electrophoresis. Normal or cataractous lenses that were carboxymethylated during homogenization to prevent disulfide formation gave results similar to those reported above.

To isolate these polypeptides, the carboxymethylated urea-solubilized fraction of the water-insoluble material was chromatographed on a Sephadex C-200 column. (The dextran columns appear to give a better flow rate than the polyacrylamide columns.) A typical profile is shown in Fig. 3. Four peaks were obtained. Of particular interest is the markedly greater abundance of peak 1 material insolated from cataractous lenses: approximately 19% of the material isolated from normal lenses compared with approximately 38% from cataractous lenses. A number of experiments utilizing this procedure gave variations of not more than ±10% in these values.

Upon electrophoresis in agarose/polyacrylamide, a striking difference in behavior again was observed between normal and cataractous lenses (Fig. 4). The carboxymethylated peak 1
material obtained from the urea-solubilized water-insoluble protein from cataractous lenses gave a prominent band that remains at the surface of the gel and a diffuse streak. After reduction, the surface band disappeared and was replaced by a major component in the 20,000-dalton range and two minor components of 43,000 and 60,000 daltons. (The latter component is obscured by the background stain.) Thus, it would appear that peak 1 from cataractous material contains two species, one held together by interchain disulfides and one that gives a diffuse background streak. The peak 1 carboxymethylated material isolated from the urea-solubilized component of normal water-insoluble protein had no band at the top of the gel but contained the same diffuse streak observed in the material isolated from the cataract. The other peaks obtained from the water-insoluble fraction from normal and cataractous lenses were similar. Peak 2 was composed primarily of material in the 40,000-dalton range, a diffuse staining material, and occasionally a 60,000-dalton component. After reduction, particularly with cataractous material, the minor components and most of the background stain disappeared and a new 20,000-dalton species was observed. Peak 3 contained polypeptides in the 20,000-dalton range. A minor component in the range of 40,000 disappeared after reduction. The peak 4 fraction contained components in the 10,000-dalton range.

In an attempt to estimate the size of the disulfide-linked peak 1 material, the gel crosslinking was decreased by increasing the acrylamide/methylene bisacrylamide ratio to 38:1. Under such conditions, the material continued to remain at the top of the gel (Fig. 5, upper left, lane B). With similar conditions myosin (220,000 daltons) moved more than half way through the gel (Fig. 5, upper left, lane A). These data suggest that this peak 1 component probably has a molecular weight greater than $1 \times 10^6$. This viewpoint is supported by preliminary light scattering experiments.

In order to explore the anomalous behavior of the reduced peak 1 material on gel electrophoresis, experiments were performed with double-gel columns composed of the agarose/polyacrylamide support (acrylamide/bisacrylamide, 19:1) in their upper half and conventional polyacrylamide in their lower
essentially did not penetrate the polyacrylamide gels. As can be seen by the behavior with \( \alpha \) crystallin which contains two different polypeptide chains, the double-gel system does not affect the migration of most polypeptides.

These experiments suggest that at least some of the peak 1 material from cataractous lenses is not disulfide-linked to the major peak 1 component. However, from analyses of the gels in Figs. 3 and 5 it would appear that some of this heterogeneous material is also present in disulfide linkage because there appears to be a more intensely stained background in gels obtained from reduced peak 1. To examine this possibility more critically the following experiment was carried out. Peak 1 material from cataractous lenses was electrophoresed on agarose/polyacrylamide gels for 18 hr (under such conditions, the diffuse staining component moves through the gel). Then, 50 \( \mu l \) of 1% NaDodSO\(_4\)/2% 2-mercaptoethanol/0.01 M phosphate, pH 7, was added to the top of the gels. After 10 min the gels were electrophoresed for an additional 4 hr. Reduction was found to release material that gave a broad band with the same mobility as previously observed. Thus, it can be concluded that this apparently heterogeneous fraction is also involved in the interchain disulfide-linked macromolecular population.

An estimate of the relative abundance of the components present in the reduced peak 1 material from the urea-solubilized water-insoluble fraction of cataractous lenses was made by scanning Coomassie blue-stained gels similar to the one shown in Fig. 1 at 560 nm. Such scans indicated three peaks of approximately 60,000 and 43,000 daltons and a broad component in the 20,000-dalton range with abundances of approximately 25, 23, and 53%, respectively. (The broad background staining material was not included in this analysis.) The 60,000-dalton component is difficult to visualize in the figure because of the background staining. Isolation of the 20,000-dalton fraction by gel filtration on Sephadex G-100 followed by conventional gel electrophoresis and scanning of the gels indicated that approximately 56% of the 20,000-dalton fraction was composed of a presumptive 27,000-dalton fraction with 25% a 22,000-dalton component and 19% a 20,000-dalton component.

To define the disulfide content in some of the components of normal and cataractous human water-insoluble protein, the insoluble protein was solubilized in 9 M guanidine-HCl/0.2 M Tris, pH 8.6/0.2 mM EDTA at 100° in the presence of iodoacetamide (present at a 10-fold excess based on one of SH per 20,000 daltons) and then incubated for 2 hr at 37°. The alkylated protein was then fractionated on Sephadex G-100 in 7 M guanidine-HCl/0.2 M Tris, pH 8.6. With this system, as previously reported (17), the 43,000-dalton fraction was eluted with the high molecular weight component. The fractions were hydrolyzed and carboxymethylcysteine (CM-Cys), representing free SH, was determined by amino acid analysis. A portion of each carboxymethylated polypeptide component was reduced with dithioerythritol in 9 M guanidine-HCl/0.2 M Tris, pH 8.6, at 100° for 5 min and then at 37° for 4 hr under nitrogen. Alkylation was then carried out as before except that additional iodoacetamide was added to compensate for the presence of the dithioerythritol. After hydrolysis, CM-Cys was again determined to obtain the total SH content.

It is apparent from the results (Table 1) that the insoluble protein from normal lenses contained essentially no disulfide whereas the material from the cataractous lenses contained polypeptides with disulfide contents ranging from 30 to 70%, with the highest level of disulfide being observed in peak 1. Reduction caused only minor changes in NaDodSO\(_4\) electrophoretic profiles of all components except peak 1. It can be assumed, therefore, that the disulfides associated with the 9600- and 20,000-dalton range polypeptides represent predominantly...
intrachain disulfides or disulfide between the polypeptides and low molecular weight components. However, in the case of the peak 1 material from cataractous lenses, much of this disulfide must involve interchain linkages between 20,000-, 43,000-, and 60,000-dalton components as well as the nondescript background staining fraction. It is of interest to note that the recovery of protein in peak 1 from guanidine-solubilized water-insoluble protein is greater than would be expected from the urea-solubilized material. Such results suggest that greater proportions of the 45,000- and 20,000-dalton fractions are present as disulfide-linked peak 1 components in the urea-insoluble fraction.

**DISCUSSION**

It was first suggested by Dische and Zil (18) in 1951 that, with cataract formation, there is a marked increase in protein disulfide. They found that bisulfite reduction of the water-insoluble protein from cataractous lenses caused the appearance of a greater amount of thiol than with such protein isolated from normal lenses. Since that time, similar suggestions have been made by others (7,19,20). This communication confirms the validity of this concept. An interchain disulfide-linked high molecular weight species is found but only in cataractous lenses and only in the water-insoluble fraction. It should also be noted that Giblin and Reddy (21) have recently observed high molecular weight disulfide-linked aggregates associated with x-ray-induced rabbit lenses.

Changes in the chemistry of the presumptive precursor of the water-insoluble fraction, the high molecular weight protein, reflects the results reported in this communication. Thus, in the normal lens no disulfide-linked component is present in this fraction. In contrast, the high molecular weight protein isolated from cataractous lenses contains a major disulfide-linked component with the same characteristics as its counterpart in the water-insoluble fraction.

It is clear that high molecular weight and water-insoluble proteins represent distinct populations of lens macromolecules. However, there is some question as to the in vivo size of these macromolecular species. It can be argued that in the lens in vivo no high molecular weight proteins are present—they are formed during isolation. Such an argument cannot be applied to the high molecular weight component of the water-insoluble protein from cataractous lenses. In such lenses, the large macromolecular structure is stabilized by disulfide links and cannot be attributed to a rearrangement of noncovalently bonded components.

Preliminary light scattering studies of the disulfide-linked material as well as its behavior in other systems suggest that this fraction may be much larger than 1 × 10^6 daltons. It is possible that this material may indeed be large enough to scatter visible light and not require noncovalent linkage with other components to attain a critical light scattering mass. Most of the polypeptides released from the disulfide-linked macromolecule upon reduction are similar in size to polypeptides previously observed in the soluble and insoluble proteins. However, it cannot be assumed on this basis that they are the same group of polypeptides. Indeed, polypeptides similar in size to some of the components observed in this investigation have been reported in membranes isolated from lens and other tissues (22–24). It is therefore possible that this disulfide-rich material represents a covalently linked aggregate between structural lens polypeptides and fiber membrane components.

It has been suggested that the high disulfide content found in cataractous lenses is an artifact produced by autoxidation during isolation (25). It is unlikely that this is the case with the work described in this report. Results similar to those reported in this communication are obtained when homogenization is conducted in the presence of iodoacetamide in a nitrogen or argon atmosphere. Furthermore, little disulfide is detected in similarly treated normal lenses.

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