Calcium and high molecular weight protein aggregates in bovine and human lens*

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The calcium content of bovine and human lens proteins has been determined. High molecular weight (HMW) bovine α-crystallin contains about three times more calcium than other soluble bovine lens proteins, while the calcium content of HMW human lens protein is approximately 10 times greater than its low molecular weight (LMW) counterpart. The calcium could not be removed by exhaustive dialysis at 4° C. and a pH of 7.6. At more alkaline pH's and higher temperatures most of the calcium could be eliminated from both bovine and human HMW protein. Higher pH's were required to obtain a decrease of the calcium levels of HMW human protein comparable to that observed with bovine HMW α-crystallin. Investigation of a group of potential calcium-binding compounds indicated that threonine and penicillamine were most effective in reducing the calcium level of HMW human lens protein at neutral pH, removing approximately 58 per cent and 52 per cent, respectively. When the calcium level of bovine HMW α-crystallin was decreased to that found in LMW protein, most of the protein was converted to LMW species. The partial removal of calcium from HMW human lens protein produced a small but significant shift to LMW protein. Deaggregation of human HMW protein under conditions which removed most of the calcium followed by reaggregation in the presence or absence of 5 to 8 mM of calcium indicates that this cation is needed for reaggregation of most of the protein to HMW aggregates. The above observations strongly suggest that calcium is required for the formation of HMW lens protein and that removal of this cation will cause a reversion to LMW species.

Key words: high molecular weight, protein aggregates, α-crystallin, calcium, chelation, deaggregation, bovine, human, lens.
Ca and HMW protein aggregates

in the bovine lens the HMW species is α-crystallin.7,8 Newly synthesized bovine α-crystallin is physically homogeneous with a molecular weight of approximately 7 × 10⁵.9 With aging, there is a transformation in the aggregate size of this protein first to a heterogeneous population with a weight average molecular weight of approximately 1 × 10⁶ designated low molecular weight (LMW) α-crystallin and then to populations of higher molecular weights and finally to the HMW species.10 Interest in this transformation process has been stimulated by the development of the concept that HMW protein aggregates are capable of scattering light and if present in sufficient concentration, of producing lens opacity.7,11,12 Recent work upon bovine α-crystallin indicates that there are a number of age-dependent changes in the chemistry of the macromolecule which may be associated with the increase in molecular size.10,12 Such changes include the modification of the A₂ and B₂ chains to A₁ and B₁, respectively,10,13 the partial degradation of some of the polypeptides,10,12 and the masking of the SH groups of the A chains.8 Recently, the role of calcium in the aggregation process has been investigated because of observations that the calcium level in the lens increases during aging and in the development of galactose-induced cataracts.15,16 Studies upon the reaggregation of deaggregated bovine HMW α-crystallin indicate that calcium causes a reassembly of the polypeptide chains to HMW aggregates.17 In the absence of calcium only LMW aggregates were formed. Examination of the isolated polypeptides of the HMW aggregates indicates that only A chains which have masked SH groups appear to interact with calcium to produce high molecular weight species. Jedziniak and co-workers18 have shown that when calcium was added to bovine α-crystallin aggregates, it caused a shift to HMW species. Other protein fractions were not affected. Studies with human lens proteins also indicate that calcium may be involved in causing aggregation to HMW species.4

While it is apparent that calcium can have a pronounced effect on the formation of HMW aggregates, the mechanism is still not understood. In this communication, it is demonstrated that there is much more calcium associated with bovine HMW α-crystallin and HMW human lens protein than with their LMW counterparts. It is also reported that calcium is required for reaggregation of most of the deaggregated HMW human lens protein polypeptides to HMW macromolecules. Removal of the calcium from HMW aggregates under mild conditions causes a shift to LMW species.

Materials and methods

HMW bovine α-crystallin was usually isolated from the nuclear region (the inner 30 per cent on a wet weight basis) of approximately two-year-old steer lenses. The protein was purified by DEAE cellulose chromatography as described previously.8 The 0.4 M phosphate fraction was dialyzed against H₂O and then lyophilized. The material was dissolved in 2 to 4 ml. of 0.1 M KCl, 0.01 M Tris, pH 7.6 (buffer A) and passed through a Bio-Gel A-50m, 100 to 200 mesh, column (3.5 by 45 cm.) utilizing buffer A as the eluant. The material eluting in the void volume is defined as HMW α-crystallin. LMW α-crystallin was usually obtained by a similar procedure but with a Bio-Gel A-5m column.

Human protein fractions were prepared from the lenses of patients 60 to 70 years of age. In all cases, normal lenses were used. All protein fractions were isolated from the inner 100 mg. (wet weight) of the lens. The material was homogenized in a Ten Broeck tissue grinder with 3.5 to 5 ml. of H₂O or buffer A per gram of tissue. The homogenate was centrifuged at 4° C. for 12 minutes at 20,000 × g. The supernatant was then added to the Bio-Gel A-50m column to obtain HMW protein which was eluted in the void volume. The second peak which contained the LMW protein was purified further on Bio-Gel A-15m columns.

The precipitates obtained after centrifugation of the lens homogenates were prepared for calcium determinations in the following manner. In all cases, normal lenses were used. All protein fractions were isolated from the inner 100 mg. (wet weight) of the lens. The material was homogenized in a Ten Broeck tissue grinder with 3.5 to 5 ml. of H₂O or buffer A per gram of tissue. The homogenate was centrifuged at 4° C. for 12 minutes at 20,000 × g. The supernatant was then added to the Bio-Gel A-50m column to obtain HMW protein which was eluted in the void volume. The second peak which contained the LMW protein was purified further on Bio-Gel A-15m columns.

The precipitates obtained after centrifugation of the lens homogenates were prepared for calcium determinations in the following manner. In the case of bovine lenses, the precipitate was suspended in 10 times its volume of H₂O and centrifuged at 27,000 × g for 15 minutes. This procedure was repeated one time. The precipitate was dried to constant weight in a vacuum oven at 80° C. Samples were then taken for protein and calcium determinations.

The precipitate obtained from the human lens
homogenates was resuspended in 10 volumes of water, shaken, and then centrifuged at 3,000 × g for five minutes. The white precipitate remains suspended and was decanted off and spun once more under the above conditions. The remaining suspension was then centrifuged at 20,000 × g to isolate the white precipitate. It was washed two times with H2O and then dried. The yellow precipitate from the two 3,000 × g centrifugations were combined, washed two times with H2O, and dried.

Calcium was determined by a modification of the fluorometric method of Kepner and Hercules utilizing calcein W (fluorescein iminodiacetic acid disodium salt) obtained from Fisher Scientific Co., Springfield, N. J. A Farrand ratio fluorometer with a Farrand primary filter No. 7-39 and a Kodak secondary filter, No. 47 was used. Reagents were made fresh daily with deionized water. Plastic labware was used. Standard curves were constructed daily. Protein samples were dried in plastic vials and then dissolved in 2 N KOH to give concentrations of 1 mg per 0.2 ml. The vials were covered and hydrolyzed at 37° C. for 16 hours. Aliquots of the hydrolyzed solution were used for calcium determinations.

The assays were performed in Farrand cuvettes. The components were added in the following order: 1.9 ml of H2O, 0.5 ml of 0.4 N KOH, up to 50 μl of the sample plus H2O, 100 μl of calcein W 1 mg per 10 ml. (the calcein is dissolved in a minimum of 0.4 N KOH and then brought up to the appropriate volume with H2O). Several determinations with H2O blanks were performed to obtain baseline blank values. Protein samples were added and their fluorometric contribution determined before the addition of the calcein W. After the addition of calcein W, fluorometric readings were taken immediately. Three separate internal standards of 0.05 μg of calcium (prepared from the Fisher Scientific Company calcium reference solution) were added to each assay solution and readings obtained after each addition. From the internal standard curve, the fluorometric units per microgram of calcium were obtained and the calcium content of the unknown determined.

In the case of the determination of the calcium content of lens water, the lens fractions were homogenized in a final concentration of 10 per cent trichloroacetic acid (TCA). The preparations were centrifuged at 27,000 × g for 15 minutes at 4° C. The supernatant was then decanted and centrifuged a second time to remove any residual precipitate. Aliquots of the supernatant were assayed as described above except that TCA blanks were also tested. Dry weights of the different lens fractions were determined by quickly weighing freshly dissected material and then drying to a constant weight at 80° C. in a vacuum oven.

Protein determinations were performed by either ninhydrin determination or by amino acid analysis. In both cases, samples were first hydrolyzed for 18 hours in sealed tubes with constant boiling HCl redistilled three times. The solutions were flushed with nitrogen and taken to a pressure of 50 mm. Hg before sealing the hydrolysis tubes. Following hydrolysis the solutions were taken to dryness under vacuum and redissolved and dried two times with a few milliliters of H2O. Protein content was then determined.

Deaggregation-reaggregation experiments were performed with slight modification of previous procedures. Deaggregation was carried out with 0.01 M Tris, pH 7.4, 0.1 M KCl, 1 mM mercaptoethanol, and 7 M urea. The urea was passed through amberlite MB-3 (Mallinckrodt Corporation, Jersey City, N. J.) immediately before use. The protein was dissolved in the above solution and dialyzed for two hours at 35° C. against a 500-fold excess of the same solution. An additional two-hour dialysis against a fresh solution with or without 5 to 8 mM calcium was then performed at 4° C. Reaggregation was carried out by dialysis of the protein solution at 4° C. with a solution containing 0.01 M Tris, pH 7.4, and 1 mM mercaptoethanol with or without 8 mM CaCl2. The solution was changed after three hours and the dialysis continued for periods of 16 to 65 hours. When calcium was present, an additional one-hour dialysis with the same buffer solution minus calcium was utilized. With the reaggregation experiments in the presence of calcium, a 15 to 20 per cent increase in the HMW species was observed after a 65-hour dialysis in comparison with a 16-hour dialysis. The relative proportions of HMW and LMW protein were determined by gel filtration with Bio-Rad A-50m columns.

**Results**

While calcium has been shown to cause aggregation to HMW species, determination of the amounts of calcium associated with high and low molecular weight fractions has not previously been published. Calcium determinations of such fractions have therefore been made utilizing the calcein fluorometric method. The results obtained with bovine and human lenses are shown in Table I. In the bovine lens, the HMW protein (α-crystallin) contains approximately three times as much calcium
Table I. Calcium content of lens proteins
fractions

<table>
<thead>
<tr>
<th>Sample</th>
<th>µmoles Calcium (± 10%)</th>
<th>20 mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bovine</td>
<td>Human</td>
</tr>
<tr>
<td>HMW protein</td>
<td>1</td>
<td>9-10</td>
</tr>
<tr>
<td>LMW protein</td>
<td>0.35</td>
<td>0.9-1.5</td>
</tr>
<tr>
<td>White precipitate</td>
<td>1.1</td>
<td>5-6</td>
</tr>
<tr>
<td>Yellow precipitate</td>
<td>—</td>
<td>5-6</td>
</tr>
</tbody>
</table>

Bovine HMW and LMW protein are α-crystallin fractions. Total human soluble protein was separated into HMW and LMW fractions for calcium analyses. See Materials and Methods for further information.

as LMW α-crystallin. The other bovine lens proteins contain levels of calcium in the same range as LMW α-crystallin, i.e., about 0.3 µmole of calcium per 20 mg. of protein. Little change in the observed calcium values were found with protein isolated from either the total or nuclear region of bovine lenses of different ages. Since the average molecular weight of the α-crystallin polypeptide chains are approximately 20,000, these values for the bovine protein may be interpreted to indicate the average number of micromoles of calcium bound per micromole of polypeptide. The insoluble protein fraction in the bovine lens (designated as the white precipitate) contains about the same level of calcium as the HMW species.

HMW human lens protein contains approximately 9 to 10 µmole of calcium per 20 mg. of protein, about ten times more calcium than its LMW counterpart. There appears to be much more calcium associated with the human lens proteins than the bovine lens proteins. Unlike the insoluble protein of the bovine lens, that of the human lens can be divided into a white and yellow fraction. Both of these fractions contain approximately 5 to 6 µmole of calcium per 20 mg. of protein. Dialysis at 4°C. and pH 7.6 failed to reduce the calcium levels of any of the bovine or human lens protein preparations.

Although it has been reported that calcium will cause the deaggregated polypeptide chains of HMW α-crystallin to reaggregate to HMW species, the effect of calcium upon the polypeptide chains of human HMW protein has not been previously investigated. The results of such experiments are shown in Fig. 1. If the HMW protein fraction isolated with Bio-Rad A-50m is rerun on the same column, all the material elutes in the void volume (Fig.
Fig. 2. The effect of temperature upon the binding of calcium to bovine HMW α-crystallin. HMW α-crystallin was dialyzed for five hours under the indicated conditions in 0.01 M Tris. The preparations were then dialyzed 16 hours at 4° C. with 0.01 M Tris, pH 7.6, and assayed for calcium. See Materials and Methods for further details.

Table II. Effect of pH and 7 M urea upon calcium binding to lens proteins

<table>
<thead>
<tr>
<th>pH</th>
<th>Bovine</th>
<th>Human</th>
</tr>
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<tbody>
<tr>
<td>7.6</td>
<td>1.0</td>
<td>9.4</td>
</tr>
<tr>
<td>8.2</td>
<td>0.56</td>
<td>9.0</td>
</tr>
<tr>
<td>8.4</td>
<td>0.35</td>
<td>8.6</td>
</tr>
<tr>
<td>8.6</td>
<td>0.30</td>
<td>5.8</td>
</tr>
<tr>
<td>9.5</td>
<td>0.30</td>
<td>5.0</td>
</tr>
<tr>
<td>9.8</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>10.3</td>
<td>0.18</td>
<td>2.6</td>
</tr>
<tr>
<td>7 M urea</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

Approximately 1 to 2 mg. per milliliter of protein was dialyzed for five hours at 44° C. at the indicated pH's in 0.01 M Tris utilizing a 200-fold excess of dialysis solution. The preparations were then prepared for calcium determination as described in the Materials and Methods section. With urea experiments, the protein was dialyzed for two hours at 25° C. against a 200-fold excess of 7 M urea, 0.02 M Tris, pH 7.8, and 0.001 M mercaptoethanol. The preparation was then dialyzed two times for additional two-hour periods at 25° C. against a 500-fold excess of 0.02 M Tris, pH 7.8, and then against a 1,000-fold excess of the same buffer at 4° C. for 16 hours.

...continues to be eluted in the HMW fraction. Thus about 13 per cent of the HMW species cannot be shifted to the LMW fraction by this procedure.

Such experiments suggest that if calcium could be removed from the HMW protein aggregates without prior deaggregation, conversion of most of the protein species might occur. An approach to this problem was suggested by previous observations, that with increasing pH or temperature there appears to be a change in the architecture of α-crystallin so that the sulfhydryl groups become more accessible to reagents such as hydroxymercuribenzoate. It was, therefore, of interest to investigate the effect of temperature and pH upon the binding of calcium to bovine HMW α-crystallin.

The results are shown in Fig. 2. No reduction in calcium binding was observed below 25° C. At higher temperatures, a gradual decrease in calcium content was observed. These results were more dramatic at pH 8.4 than at 7.6. Dialysis at 44° C. and pH 8.4 produced calcium levels approximating those observed with LMW α-crystallin.

The effect of pH upon the calcium level of the HMW α-crystallin preparations dialyzed at 44° C. is shown in Table II.
With increasing pH the calcium values decreased, although between 8.2 and 9.5 little change was found. At pH 10.3, only 0.18 μmole of calcium per 20 mg. of protein was still present.

Has the removal of the calcium caused a shift to LMW protein species? To answer this question the mildest conditions under which the calcium level was reduced to that of LMW α-crystallin was chosen, dialysis at 44° C., and pH 8.4. A control sample of the same protein was subjected to the same conditions but in the presence of 0.1 mM calcium. Both samples were then passed through a Bio-Rad A-50m column. Typical results are shown in Fig. 3. The control contained primarily HMW α-crystallin while the protein dialyzed in the absence of calcium had been altered so that most of the material eluted in the region of LMW α-crystallin. Determination of the proportions of protein in the two-column fractions indicates that when the calcium was removed to a very considerable extent only 20 per cent of the protein remained in the HMW form while in the control approximately 74 per cent of the protein was found in the HMW fraction. Such experiments suggest that calcium does influence the size of the α-crystallin aggregates.

On the basis of such observations it was of interest to determine if a similar situation existed in the human lens, although, in this species the HMW fraction may contain other proteins besides α-crystallin. The binding of the calcium to the human protein appears to be much stronger than to the bovine α-crystallin. From Table II, it can be seen that at pH 8.2 and 44° C. little of the calcium was removed. Under similar conditions approximately two-thirds of the bound calcium was lost from bovine HMW α-crystallin. At a pH of 10.3, 2.6 μmoles of calcium per 20 mg. of protein was still present in human HMW protein. Even after dialysis with 7 M urea, 1.3 μmoles of calcium per 20 mg. of protein remained. Such data indicate that only at rather alkaline pH could an appreciable reduction in the calcium levels be attained.

Another approach to the problem is to examine the effectiveness of compounds which might be expected to chelate calcium. Studies with 5 to 10 mM EGTA indicated that this compound was not effective. It had previously been shown that certain amino acids and sulfhydryl containing compounds are capable of preventing the effect of calcium upon the re-aggregation of bovine α-crystallin polypeptide chains to HMW aggregates. It was therefore of interest to investigate the effect of such compounds. Examination of a number of sulfhydryl compounds such
as glutathione, mercaptoethanol, dithioerythritol, and cysteine indicate that, except for cysteine which removes about 28 per cent of the calcium, these compounds are not particularly effective in reducing the calcium level of HMW protein (Table III). However, D,L-penicillamine (β-mercaptovaline) removed approximately 50 per cent of the calcium. A group of non-sulfhydryl-containing amino acids including glycine, leucine, valine, and threonine were also investigated. Only valine and threonine were effective, removing approximately 35 per cent and 60 per cent of the calcium, respectively. The addition of dithioerythritol, valine, and penicillamine to the same preparation was no more effective than penicillamine alone. As previously noted, EGTA had no effect.

It was of interest to determine if the removal of a substantial amount of the bound calcium affected the aggregate size of the human HMW macromolecules. Therefore, HMW protein which had been treated with D,L-penicillamine was passed through a Bio-Rad A-50m column. As shown in Fig. 4, approximately 20 per cent of the protein was now found in the LMW region. Since it is conceivable that the conditions of the experiment, five hours at 44°C, might have caused this shift in size, control experiments in the absence of penicillamine were also carried out. No effect upon the size of the aggregates was observed under such conditions. Thus it appears that the removal of even 50 per cent of the calcium causes a shift of some of the HMW protein to LMW species.

**Discussion**

The experiments reported in this communication support the concept that calcium is involved in the aggregation of lens proteins to HMW aggregates. Since this reaction occurs almost entirely in the nuclear region of the lens it implies that some aspects of lens chemistry related to the process must differ in this region. It is conceivable that a calcium concentration gradient exists in the lens and that only

### Table III. Effect of certain compounds upon the calcium content of HMW human lens proteins

<table>
<thead>
<tr>
<th>Addition</th>
<th>μmoles Calcium (\times 10^\pm 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.5</td>
</tr>
<tr>
<td>EGTA</td>
<td>9.0</td>
</tr>
<tr>
<td>Glutathione</td>
<td>9.5</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>8.8</td>
</tr>
<tr>
<td>Dithioerythritol</td>
<td>7.6</td>
</tr>
<tr>
<td>Cysteine</td>
<td>6.9</td>
</tr>
<tr>
<td>Penicillamine</td>
<td>4.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.2</td>
</tr>
<tr>
<td>Valine</td>
<td>6.2</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.0</td>
</tr>
</tbody>
</table>

The compounds indicated above were added to 1 to 2 mg per milliliter of HMW human protein at a 10 mM concentration. All isomers were in a L-configuration except for penicillamine which was a racemic mixture. The solutions also contained 0.01 M Tris, pH 7.0. The preparations were dialyzed at 44°C against a 200-fold excess of the chelating Tris solution for five hours and then for 16 hours at 4°C against a 500-fold excess of 5 mM Tris, pH 7.6. EGTA-ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetra-acetic acid. See Materials and Methods for further information.
in the nuclear region is the concentration of calcium high enough to cause the observed aggregation. Examination of the calcium content of the lens water of the cortex and nucleus of human and bovine lenses was therefore undertaken. The average range of calcium in the lens water samples investigated, varied from 1 to 4 mM. The calcium content of the water from the nuclear region varied from the same level to approximately two times that observed in the cortical region. Such observations suggest that while there is a tendency toward higher calcium levels in the water of the nuclear region, it is probably not the determining factor in causing the transformation to HMW protein. Other studies also lend further support to this contention. The reaggregation of LMW bovine α-crystallin polypeptides is not effected by calcium. Bovine HMW aggregates can only be obtained by the addition of calcium to a reaggregation system containing polypeptides derived from HMW α-crystallin. Thus, calcium appears to act in a cooperative manner with modified polypeptides to produce the HMW species. A number of reports have appeared which indicate that with aging chemical changes occur in bovine α-crystallin and that such changes occur predominantly in the nuclear region. However, it is not yet clear how such alterations are related to the binding of calcium nor is it understood how calcium is involved in the aggregation to such huge macromolecules.

Most of the prior work on the effect of calcium has been done with bovine α-crystallin and caution is required in applying such information to the human lens. The HMW human protein has not yet been definitely defined. Human HMW protein binds almost 10 times more calcium than HMW bovine α-crystallin. Furthermore, the binding characteristics are not the same. The calcium can be released from bovine HMW α-crystallin with milder conditions than are required for human HMW protein. Penicillamine which removes a considerable amount of the bound calcium from the human HMW protein chelates almost none of the calcium bound to HMW α-crystallin. Thus the calcium binding sites of the two HMW species must differ considerably.

It is of interest to consider some of the structural aspects of the compounds that have been examined for their ability to remove calcium from human lens HMW protein. The most effective compounds are threonine and penicillamine. Both chelators are α-amino acids with hydrophobic methyl groups on their beta-carbon. The penicillamine contains a sulfhydryl instead of a hydroxyl on the beta-carbon as well as an additional methyl group. Valine, the third most effective compound, but considerably poorer than penicillamine and threonine, is identical to penicillamine except for deletion of the sulfhydryl group. Such data suggest that the hydroxyl or sulfhydryl groups improve the effectiveness of the compound. The requirement for at least one methyl group is demonstrated by the relatively ineffective removal of calcium by cysteine which lacks a methyl group on the beta-carbon. The removal of the beta-carbon yields glycine which is still less effective. An additional methylene group between the hydrophobic and the charged groups as exemplified by leucine produces an ineffective compound comparable to glycine. The requirement for the charged end of the molecule is demonstrated by the almost complete lack of effectiveness of mercaptoethanol. Since cation binding is required to chelate the calcium, it is probable that the positively charged amino group does not enhance the effectiveness of the compound. Screening of a number of compounds such as isopropylmalonic acid and butyric acid derivatives has therefore been undertaken. The unusual requirements for calcium binding described above as well as the ineffectiveness of compounds such as EGTA, normally a highly effective calcium chelator, strongly indicate the need to consider the characteristics of the calcium-binding sites of the protein in the
search for a more effective calcium chela-
tor. The relative effectiveness of some
amino acids in chelating the calcium bound
to HMW protein suggests that their
concentration in the lens may play a role in
controlling the transformation to HMW
species.

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