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GAMMA-CRYSTALLIN, A MAJOR CYTOPLASMIC POLYPEPTIDE DISULFIDE LINKED TO MEMBRANE PROTEINS IN HUMAN CATARACT*

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Summary: Examination of human cataract has revealed the presence of a number of unique complexes containing cytosol and membrane components: the high molecular weight disulfide linked aggregates and membrane preparations containing disulfide linked cytosol polypeptides. It is now shown that a major cytosol species associated with these complexes is gamma-crystallin. This conclusion is based upon investigation of polypeptides released by reduction and comparisons based on amino acid, immunochemical and sequence analyses. It is suggested that two types of complexes may be closely related.

Introduction: Recent work has demonstrated that there are a number of unique molecular species associated with the development of cataract (1-3). Among these species are HMW(SS) aggregates (4) and membrane polypeptide complexes (5) both of which contain cytosol polypeptides. The HMW(SS) aggregates are found in the water insoluble fraction (3) and together with the linkage of cytosol polypeptide to the membrane (4) contribute to the accelerated production of water insoluble protein (6). While the HMW(SS) aggregates have been found in the opaque regions of all types of cataract, the disulfide linkages of polypeptides to the isolated membrane fraction has been observed only in the nuclear region of nuclear cataract (7).

It is of considerable importance to elucidate the proteins involved

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Abbreviations: HMW(SS), high molecular weight disulfide; IAA, iodoacetamide; ANOVA, analysis of variance; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin; DBM, diazobenzyloxymethyl; R, reduced; NR, non-reduced.

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in the processes described above. Attention has been drawn to gamma-crystallin because of its loss from the water soluble protein during cataract formation (8). It has been suggested that the observed loss may be due to leakage from the lens, degradation and insolubilization.

Leakage of gamma-crystallin from the lenses of Nakano mice during formation of opacity has been established by Kinoshita and co-investigators (9). Sandberg and Closs have shown that gamma-crystallin antigens are normally present in the aqueous of normal human lenses and that the level increases with cortical cataract formation (10). These investigators also found that with nuclear cataract formation, gamma-crystallin was decreased in human aqueous fluid (10). Our laboratory has recently established that the 10,000 dalton polypeptide population, which increases with aging and cataract formation (11), contains antigens that cross-react with gamma-crystallin antiserum (12) suggesting that degradation of gamma-crystallin occurs and contributes to the disappearance of native gamma-crystallin. This degraded 10,000 dalton species is found primarily in the water insoluble fraction (11). These overall results would suggest that degradation and insolubilization of gamma-crystallin are primarily processes involved with the disappearance of gamma-crystallin particularly during nuclear cataract formation.

While the young human lens contains gamma-crystallin of 19,500 daltons, the predominant species synthesized in older lens is 22-24,000 daltons (13). These polypeptides have been denoted $gamma_L$ and $gamma_H$, respectively (14). Observations from this laboratory based on SDS gels suggest a major polypeptide species associated with both the HMW(SS) aggregates and the disulfide linked membrane-cytosol polypeptide complex have molecular weights in the 22-24,000 range (4). In this communication evidence is presented indicating that a gamma-crystallin is the predominant component in this size range.

Materials and Methods: Freshly obtained cataractous and normal lenses were classified according to the procedure of Chylack (15) and stored at -80°. Lenses were decapsulated prior to experiments. The nucleus and cortex were separated with a 6mm corneal trephine which yields a 60:40 distribution (7). All chemicals were reagent grade.

Lenses were homogenized in standard buffer (3) containing 10mM IAA. The water soluble supernatant (60,000xg; 15 min.) was fractionated in Sephadex G-75 (13). The $gamma_L$ and $gamma_H$ crystallins were isolated from the last two major fractions from this column, respectively (14). The HMW(SS) was isolated according to the procedure of Spector and Roy (3). The heterogeneous 22-24,000 dalton polypeptide was isolated from HMW(SS) as described by Spector *et al* (4). The intrinsic membrane fraction was isolated from the water insoluble pellet using the procedure of Roy *et al* (16). The normal intrinsic and

cataract cytosol proteins comprising the lens fiber membrane can be further separated on Sepharose 6B at 40° using 1% SDS, 10mM Tris, 0.05% 2-mercaptoethanol (pH 8.0) as the eluent (5).

Antiserum to calf gamma-crystallin fraction II (17) was prepared using the procedure previously described (12). When the antiserum was tested against calf alpha, beta and gamma-crystallin, only gamma was shown to react (12). Specific gamma antigens were detected in SDS polyacrylamide gels by the procedure of Renart, Reiser and Stark using the DBM paper (18). Protein A was ^{125}I labeled using the lactoperoxidase-glucose oxidase method (19).

Automated Edman degradation was performed on a Beckman 890 Sequencer and the PTH amino acid derivatives were identified by back hydrolysis and subsequent amino acid analysis (20).

Results: When the nuclear region of cortical or nuclear cataractous lenses were examined, certain polypeptides could be released from the nuclear cataract membrane fraction by reduction and alkylation (7). Fairbanks' gels (21) of non-reduced nuclear cataract membrane show all the polypeptides at the top of the gel; however, after reduction, most polypeptides enter the gel (7). If the membrane fraction is re-isolated after reduction and alkylation, the supernatant contains released material that on polyacrylamide-agarose gel electrophoresis is shown to contain major protein bands which correspond to molecular weights of 22-24,000 and 10,000 daltons (7). The membrane fractions from normal and nuclear cataract treated as described above were hydrolyzed and the corresponding amino acid compositions, determined as mole percent, are compared in Table I. Comparison of the normal membrane fraction by two-way ANOVA (12) between either non-reduced (NR) cortical or reduced (R) nuclear membrane indicates no significant difference. However, a similar comparison of normal membrane with NR nuclear membrane indicates a significant difference. Therefore, the question arises as to the nature of the abnormal material that is bound to the nuclear membrane components and can be removed by subsequent reduction. The amino acid composition of the material released (see Materials and Methods) from the nuclear cataract membrane by reduction and alkylation is shown in Table II. When the human $gamma_L$ and $gamma_H$ are isolated and compared to similar sized components released by reduction from the nuclear membrane, $gamma_H$ is found to be more closely related to these components. These polypeptides are also compared with a heterogeneous 22-24,000 dalton polypeptide isolated from the cataract HMW(SS) complex previously isolated and reported from this laboratory (4). Again, the $gamma_H$ is more closely related, but some specific differences are noted in a few amino acids sensitive to oxidative damage including cysteine and methionine. Such oxidation has

TABLE I: LENS MEMBRANE FRACTIONS
COMPARISON OF AMINO ACID COMPOSITIONS[a]

	NORMAL	NUCLEAR CATARACT		
	NR	Cortical NR	Nuclear NR	Nuclear R
Asp	5.5	6.0	9.0	6.5
Thr	5.0	5.0	4.0	4.0
Ser	7.0	7.0	7.0	6.0
Glu	7.0	8.0	12.0	10.0
Pro	5.0	5.5	7.0	5.5
Gly	10.5	11.0	8.0	10.0
Ala	11.0	11.0	6.5	9.0
Cys	1.0	1.5	2.0	1.0
Val	7.5	5.5	5.0	5.5
Met	2.5	2.5	2.5	2.5
Ile	3.5	4.0	5.0	4.0
Leu	12.5	13.0	8.0	12.5
Tyr	4.0	3.0	6.5	4.5
Phe	7.0	7.0	5.5	6.5
Lys	2.5	2.5	3.5	2.5
His	2.5	2.5	2.5	2.5
Arg	5.5	5.0	8.0	5.5
ANOVA (F_s)	--	ns (1)	** (20)	ns (5)

**: variance $\geq F_{.01}$
ns: not significant (beyond $F_{.01}$)

a) Residues expressed as mole percent

been observed during cataractogenesis (22).

By composition data alone, these disulfide-linked polypeptides obtained after reduction appear to be gamma-crystallins. Further proof of this relationship to gamma was obtained using immunochemical techniques. Before utilizing the antiserum for defining the identity of polypeptides, it was necessary to determine if the antiserum would react with both $gamma_L$ and $gamma_H$ from both bovine and human sources. Using the blot technique modified by Renart, Reiser and Stark (18), the gamma and $gamma_L$ proteins from calf, cattle, and human lenses isolated from Sephadex G-75 were separated on SDS-slab gels and transferred to DBM paper. These proteins covalently linked to the paper were then tested

TABLE II: HUMAN CATARACT (SS) POLYPEPTIDES ISOLATED AFTER REDUCTION & ALKYLATION;COMPARISON OF AMINO ACID COMPOSITIONS[a,b]

	Nuclear Membrane(SS) Polypeptide	HMW(SS) Polypeptide	$Gamma_L$ Polypeptide	$Gamma_H$ Polypeptide
Asp	9.0	8.5	8.0	9.0
Thr	3.0	3.5	2.5	3.5
Ser	6.5	6.0	4.0	6.0
Glu	12.5	13.5	9.0	13.5
Pro	6.0	6.0	6.0	7.5
Gly	8.5	9.0	7.0	8.0
Ala	4.5	6.1	1.0	4.0
Cys	2.0	1.5	3.2	.5
Val	6.0	5.5	3.5	3.0
Met	2.5	1.5	3.5	3.0
Ile	5.0	5.0	3.5	5.0
Leu	8.0	8.0	6.5	6.5
Tyr	7.0	5.5	4.0	5.0
Phe	5.5	5.5	4.0	5.0
Lys	3.5	4.0	2.5	4.5
His	2.5	2.5	3.5	2.5
Arg	7.5	8.0	9.0	8.0
ANOVA (F_s)	--	ns (1)	**(23)	ns(1)

**: variance $\geq F_{.01}$

ns: not significant (beyond $F_{.01}$)

a) Residues expressed as mole percent

b) 22-24,000 dalton polypeptides

for gamma-crystallin antigens. In Figure 1, the radioautographs obtained from the ^{125}I protein-A binding to anti-calf $gamma_L$ is shown and compared with the conventionally visualized Coomassie blue-stained gel. It can be seen that both $gamma_H$ and $gamma_L$ isolated from the calf, steer and human show similar reactivity. These results demonstrate that this antiserum can cross-react with both gamma species and can also react with human gamma antigens. This gamma antiserum was then tested using the Ouchterlony immunodiffusion technique against the isolated disulfide-linked polypeptides. The polypeptides were obtained from the HMW(SS) complex after reduction and alkylation followed by fractionation on Sephadex

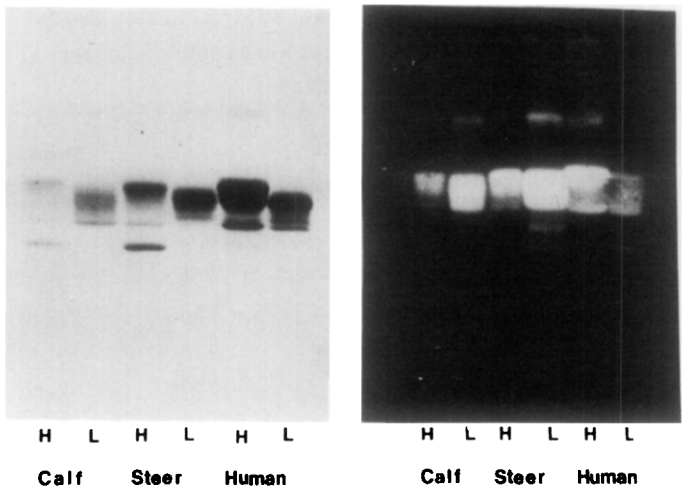

Fig. 1. Comparison of Coomassie-blue stained SDS polyacrylamide gels (left) with radioautograph (right) of blot-transfer (18) DBM paper after detection with antiserum against purified gamma$_L$ (25) and ^{125}I labeled protein-A. The gamma$_H$ (H) and gamma$_L$ (L) fractions were isolated from the water soluble fraction of calf, steer (cattle), and human (cataractous) lenses as described in the Materials and Methods section. Certain differences are noted, in addition to the cross-reaction of gamma$_L$ and gamma$_H$ described in the text. The 10,000 dalton polypeptide (lowest band) observed by staining in calf and steer gamma$_H$ fractions are not detected with gamma$_L$ antiserum. The converse of this situation is found in the steer and human gamma$_L$ fractions: no stained 10,000 dalton polypeptide but 10,000 gamma antigens detected by radioautogoraphy. In addition, minor level of polymerization of gamma is noted by radioautography as evidenced by molecular weights $\geq$ 40,000 daltons.

G-200 (4). As indicated previously, six fractions were isolated. Fraction 1 representing material that does not penetrate SDS gels and fraction 5 representing material in the 22-24,000 dalton range were examined and found to cross-react with the gamma-crystallin antiserum (wells 1 and 2, Figure 2). In a similar type of fractionation (see Materials and Methods) comparable peaks obtained from the nuclear cataract membrane fraction after reduction were also found to react with the gamma antiserum (wells 3 and 4, Figure 2). The normal membrane fractions prepared by identical procedure contained no gamma reacting material (wells 5 and 6, Figure 2).

Since both composition and immunochemical data indicate that the disulfide-linked polypeptides are related to gamma-crystallin, sequence analysis by automated Edman degradation using a Beckman 890 Sequencer was carried out to further substantiate this conclusion. In Table III, the comparative sequence results obtained from calf gamma$_L$ and gamma$_H$

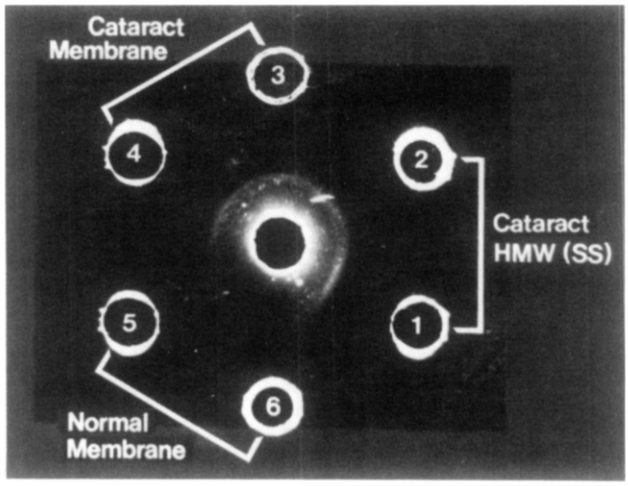

Fig. 2. Ouchterlony double immunodiffusion with antibody to calf gamma$_L$ in center well against lens antigens: well 1, 22-24,000 dalton fraction from Sephadex G-200 of reduced HMW(SS) complex; well 2, non-reducible covalent HMW complex peak 1 from Sephadex G-200 (cataract lenses); well 3, peak 1 membrane protein (cataract lenses, 60-70 years); well 4, peak 3 membrane protein (cataract lenses, 60-70 years); well 6, peak 3 membrane protein (normal lenses, 60-70 years); well 6, peak 3 membrane protein (normal lenses, 60-70 years).

and the 22-24,000 dalton polypeptide reduced from nuclear cataract membrane are summarized. These results show significant sequence homology between these various isolated fractions and the calf gamma$_L$ sequence previously reported by Croft (23). Human gamma$_H$ sequence was found to contain a glycine to valine or threonine substitution at position 10.

Discussion: On the basis of amino acid compositions, immunochemical reactivity and amino acid sequence analyses, it is evident that both the nuclear cataract nuclear fiber membrane disulfide-linked polypeptides and the HMW(SS) complex contain cytoplasmic gamma-crystallin. Indeed, work from this laboratory suggeststhat the disulfide-linked polypeptides associated with the nuclear fiber membrane may be similar to those observed in the HMW(SS) aggregate (5). Observations from this laboratory based on SDS gels suggest a major polypeptide species associated with both the HMW(SS) aggregates and the disulfide linked membrane-cytosol polypeptide complex have molecular weights in the 22-24,000 range (4,5). In this communication, evidence is presented indicating that gamma-crystallin is the predominant component in this size range. This pathological state observed in cataract probably arises following some insult in mem-

TABLE III: SEQUENCE ANALYSIS

	1	2	3	4	5	6	7	8	9	10	11	12	13
Calf Gamma$_L$[a]	GLY	LYS	ILE	THR	PHE	TRY	GLU	ASP	ARG	GLY	PHE	GLU	GLY
Calf Gamma$_L$	GLY	LYS	ILE	THR[b]	PHE	(TRY)	(GLU)	(ASP)	(ARG)	GLY	(PHE)	GLU	GLY
Human Gamma$_H$	GLY	LYS	ILE	THR[b]	PHE	TYR	GLU	ASP	ARG	VAL[c]	PHE	GLU	GLY
Membrane(SS) Polypeptide	GLY	LYS	ILE	THR[b]	PHE	TRY	GLU	(ASP)	(ARG)	GLY	(PHE)	(GLU)	GLY

[a] Fraction II, see reference (25).

[b] THR identified as Gamma-aminobutyric acid (GABA)

[c] VAL and GABA elute as a single peak.

brane permeability that allows oxidative changes that cause disulfide cross-linking between membrane proteins and cytoplasmic crystallins, including gamma-crystallins (4,5). Data concerning exposure of sulfhydryl groups indicate that gamma-crystallin contains proportionately more exposed SH groups than any other crystallins (24,25); therefore, involvement of gamma-crystallin in intermolecular disulfide cross-links following membrane disintegration is not surprising.

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