

# Selective oxidation of cysteine and methionine in normal and senile cataractous lenses

(cataract/methionine sulfoxide/cysteic acid)

MARGARET H. GARNER AND ABRAHAM SPECTOR

Biochemistry and Molecular Biology Laboratory, Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York, New York 10032

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**ABSTRACT** The oxidation state of methionine and cysteine in normal and cataractous lenses is reported. In young lenses no oxidation was detected in any protein fraction examined. Only the intrinsic membrane fraction and membrane-related components showed evidence of oxidation in old (60-65 years of age) normal lenses. However, in a similar age group, with the development of cataract, progressive, dramatic changes were observed. With severe cataracts, 60% or more of the methionine in membrane-associated components was found in the methionine sulfoxide form, and methionine sulfone was observed in one case. Most of the cysteine was found oxidized to either the disulfide form or putative cysteic acid. Mixed disulfides with glutathione were observed. Oxidative changes in soluble components as illustrated by  $\alpha$ -crystallin occurred more gradually. The data clearly support the viewpoint that extensive oxidation of lens proteins occurs with cataract and that it begins at the lens fiber membrane.

The formation of certain types of cataract appears to be associated with the generation of high molecular weight aggregates which act as scatter points of light (1, 2). Comparison of the quantity and polypeptide compositions of the water-soluble and -insoluble material of normal and cataractous lenses of the same age shows that the amount of water-insoluble material and high molecular weight aggregates increases at the expense of water-soluble protein (3). High molecular weight disulfide-linked aggregates, unique to cataract, can be isolated (4). Partial characterization of these aggregates indicates the presence of a 43,000-dalton polypeptide that is an extrinsic membrane component in the normal lens (5), polypeptides in the 20,000-dalton range including a major component related to  $\gamma$ -crystallin,\* and a 9600-dalton polypeptide fraction (5). Also present in this high molecular weight disulfide-linked aggregate is a diffuse, poorly defined species that contains lipid (unpublished data). This component, present in the normal lens as well, may be membrane associated. The water-insoluble material of both normal and cataractous lenses may also contain non-covalently linked aggregates (6).

Many of the changes associated with cataract may be caused by oxidative posttranslational modification. As first observed by Dische and Zil (7), one of the major posttranslational changes in proteins of cataractous lenses involves oxidation of the thiol group of cysteine. In senile cataract (8, 9), diabetic cataract (†; N. H. Ansari, Y. C. Awasthi, and S. K. Srivastava, personal communication), and certain experimentally induced cataracts (10-12), the number of protein disulfide bonds increases concurrently with the severity of the cataract. A concomitant conversion of methionine to methionine sulfoxide occurs in senile cataract (9). Oxidation products of tryptophan (13) and tyrosine (14) contribute to the abnormal fluorescence and color of the lens.

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The question arises as to whether oxidation of the sulfur found in cataractous lens homogenates is random or, conversely, whether the oxidation progresses in a particular order from specific regions and polypeptides of the tissue.

In the study reported here, methionine and cysteine oxidation in selected fractions of lens tissue homogenates was measured. The data suggest that, in the aging normal lens, oxidation of cysteine and methionine occurs only in the intrinsic membrane fraction and to some extent in the high molecular weight membrane-associated fractions. In the severe cataractous lens, the oxidation occurs not only in the intrinsic membrane fraction but in all fractions examined. Furthermore, higher sulfur oxidation states are observed in membrane-related fractions in cataract lenses.

## MATERIALS AND METHODS

Freshly obtained cataractous and normal lenses were classified according to the procedure of Anderson and Spector (8) and stored at  $-80^{\circ}\text{C}$  prior to use. Decapsulated lenses were used in all studies. The cataractous lenses were of the same age (60-65 years) as those of the old normal population. All chemicals were reagent grade.

Both normal and cataractous lenses were homogenized in 0.1 M KCl/0.05 M Tris/0.05 M iodoacetamide, pH 7.4. The water-soluble protein was separated from the homogenate by centrifugation at  $60,000 \times g$ . The water-soluble proteins were fractionated further by gel filtration on Sephadex G-200 with 0.1 M KCl/0.05 M Tris, pH 7.4 as the eluent. The water-soluble 43,000-dalton polypeptide was isolated from the water-soluble protein by the procedure of Garner *et al.* (15). The water-insoluble fraction, the pellet from the centrifugation, was suspended in 6 M guanidine hydrochloride, treated with citraconic anhydride dialyzed against 0.2% ammonia, and separated into the membrane and the water-insoluble nonmembrane fractions by centrifugation at  $60,000 \times g$ . The water-insoluble nonmembrane fraction was lyophilized and redissolved in 7.6 M urea/0.05 M Tris, pH 7.6 (16). The isolation procedure is shown in Fig. 1.

The urea-soluble high molecular weight, 43,000-dalton polypeptide, 20,000-dalton range, and low molecular weight fractions were isolated from the water-insoluble nonmembrane fraction by gel filtration chromatography on Sephadex G-200 with 7.6 M urea/0.05 M Tris, pH 7.6, as the eluent. The purity of the proteins and polypeptides isolated from the water-soluble and water-insoluble nonmembrane fractions was checked by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis according to the procedure of Weber and Osborn (17); the procedure of

\* Reported in part at the 1979 meeting of the Association for Research in Vision and Ophthalmology.

† Monnier, V. M., Stevens, V. J. & Cerami, A. (1979) in Abstracts of the Association for Research in Vision and Ophthalmology, p. 128.

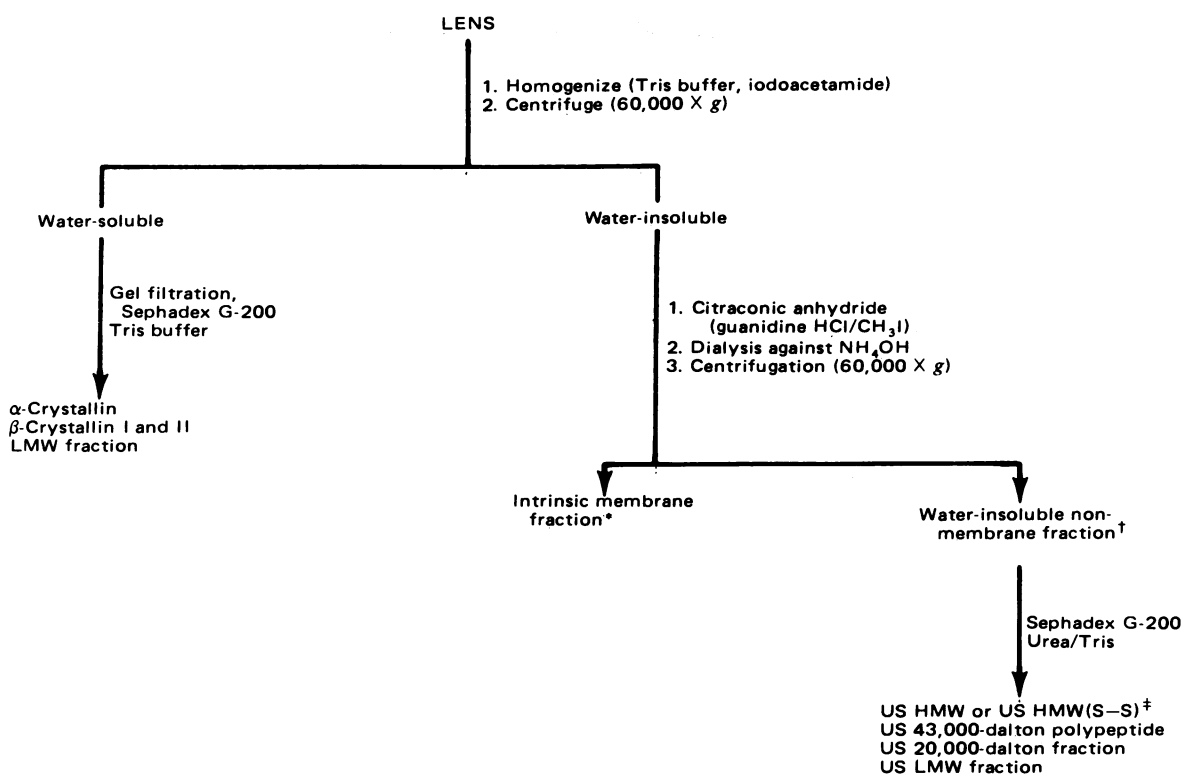


FIG. 1. Preparation of lens protein fractions. US, urea-soluble; HMW, high molecular weight; LMW, low molecular weight. \*In cataract the intrinsic membrane component can be separated into light and heavy fractions. †The water-insoluble nonmembrane fraction was solubilized by the citraconylation procedure. ‡The US HMW component is isolated from both normal and cataractous lenses, but in the latter case it also contains disulfide-linked cytoplasmic and extrinsic membrane components and is designated as US HMW(S-S).

Fairbanks *et al.* (18) was used for the membrane protein fractions.

The rationale and procedures used to determine methionine, methionine sulfoxide, and methionine sulfone in proteins have been described (19). The only alteration in these procedures was the choice of the alkylating agent. The classical alkylating agents for this determination are iodoacetamide and iodoacetic acid. Acid hydrolysis of the sulfonium salts formed by reaction with these alkylating agents yields two major products, *S*-carboxymethylhomocysteine and methionine. When methyl iodide is used (20), the sulfonium salt formed, methyl methionine, reverts back, almost quantitatively, to methionine under normal acid hydrolysis procedures. Furthermore, methyl methionine itself can be detected by amino acid analysis after *p*-toluene-sulfonic acid hydrolysis of the modified protein (21).

The procedure used to determine buried and exposed cysteines, cystines, and more extensively oxidized derivatives involved the use of two alkylating agents, iodoacetamide and methyl iodide, as well as reduction with dithiothreitol. Because all homogenizations and initial dialyses were performed in the presence of iodoacetamide, all exposed cysteine residues were converted to carboxyamidomethylcysteine prior to protein purification. The number of exposed cysteine residues could then be determined directly from the amount of carboxymethylcysteine present in the hydrolysis mixtures of the isolated proteins. The buried cysteine residues were alkylated in denaturing media with methyl iodide. The quantity of methylcysteine was determined directly by amino acid analysis. Finally, reduction and alkylation of the previously alkylated, methylated protein yielded the total amount of disulfide bonds present in the protein as an increase in the carboxymethylcysteine. Those sulfhydryl groups that were oxidized further than the disulfide stage were determined directly as cysteic acid.

Protein-bound glutathione was determined by the loss of cysteine or cysteic acid, glutamic acid, and glycine after reduction and alkylation.

## RESULTS

The oxidation of methionine and cysteine in the protein fractions isolated from normal aged lenses is shown in Table 1. The membrane-associated urea-soluble high molecular weight component contained 20% of its methionine in the sulfoxide form. In the intrinsic membrane fraction of these lenses, 19% of the methionine was detected as sulfoxide and 57% of the cysteine, as disulfide. However, the membrane fraction as well as the water-soluble and water-insoluble fractions isolated from young lenses (12–20 years old) showed no evidence of oxidation.

In most cases, approximately 50% or more of the cysteine groups from the older normal lenses appeared to be buried (inaccessible to alkylation without denaturation). An interesting age-dependent change in the intrinsic membrane protein thiol was observed. In the young lenses, the two thiol groups appeared to be buried whereas in older lenses one was exposed and the other one was in a disulfide linkage.

A number of components isolated from the cataractous lens appeared to be involved in the development of cataract. These fractions included the extrinsic membrane 43,000-dalton polypeptide isolated from both the water-soluble and the water-insoluble material and the disulfide-linked membrane-associated high molecular weight aggregate. There also was a change in the physical state of the intrinsic membrane fraction isolated from cataract. In such lenses, both a light and a heavy component were found. Only one fraction was present in the normal lens. The extent of oxidation of cysteine and methionine in these principal cataract-related components (all associated

Table 1. Oxidation of protein fractions from normal human lenses 60–65 years old\*

Fraction	Met	Met sulfoxide	Cysteine		Protein SH as S—S
			Exposed	Buried	
$\alpha$ -Crystallin	2.1	0	1.6	1.6	0
$\beta$ -Crystallin I	1.9	0	2.0	3.0	0
$\beta$ -Crystallin II	4.1	0	2.0	3.0	0
Low-molecular-weight	2.4	0	3.4	0.6	0
Urea-soluble:					
43,000-dalton	4.3	0	2.0	2.0	0
20,000-dalton	2.3	0	2.6	2.0	0
Low molecular weight	1.8	0	2.6	1.8	0
High molecular weight	2.3	0.6	0	0	0
Intrinsic membrane	4.4	1.0	0.9	0	1.2
Intrinsic membrane†	4.5	0	0	2.0	0

\* Data are expressed arbitrarily as residues per 200 residues.

† Lenses 12–20 years old.

in some way with the membrane) is shown in Table 2. To simplify the discussion,  $\alpha$ -crystallin, a cytoplasmic protein, is included to demonstrate changes that are characteristic of the crystallin polypeptides that remain soluble in cataract lenses.

Protein isolated from cataractous lenses showed increasing oxidation of the soluble and insoluble fractions with the severity of the cataract. Whereas  $\alpha$ -crystallin isolated from 200 cataractous lenses (+2 to +4 in color and opacity) contained no detectable oxidized methionine, approximately 30% of the methionine and cysteine in  $\alpha$ -crystallin from severely cataractous lenses (+4 in color and opacity, nuclear and cortical involvement) was oxidized. The water-soluble 43,000-dalton polypeptide from the pool of 200 lenses had approximately 65% of its methionine in the sulfoxide form. Methionine sulfone was found in the urea-soluble 43,000-dalton polypeptide from the same preparation. In the urea-soluble 43,000-dalton polypeptide isolated from the severe cataracts, virtually all the methionine was in the sulfoxide form. Although these 43,000-dalton fractions are identical immunochemically, there appeared to be inexplicable losses of detectable methionine and cysteine derivatives in the urea-soluble polypeptide, which may indicate transformation of these sulfur-containing residues to other products not yet elucidated.

The disulfide-linked high molecular weight species had a major fraction of its methionines and cysteines in oxidized states. A significant number of the cysteines in this fraction appeared to be disulfide linked to dialyzable constituents. Extensive oxidation also was noted in the membrane fractions: 50–60% of the methionine was in the sulfoxide form. Both the light and

heavy membrane fractions contain amino acids that elute from the amino acid analyzer at the position of cysteic acid. In the heavy membrane fraction, this putative cysteic acid was lost in the dialysis following reduction and alkylation. A concomitant corresponding loss of glutamic acid and glycine was observed, suggesting the presence of a glutathione–protein mixed disulfide (Table 3). Other amino acids showed little change. These data also suggest that the disulfide bond undergoes further oxidation without bond cleavage (22). It has been reported (23) that protein-bound glutathione can be isolated from the human lens.

## DISCUSSION

The results obtained in the study of young and old normal lens constituents indicate that most of the methionine and cysteine is in the unoxidized state. In the old lens only the intrinsic membrane polypeptides and the urea-soluble high molecular weight species, suspected of being associated with the membrane, are oxidized. The oxidation is limited and there is no observable change in protein composition.

In the cataractous lens, sulfur oxidation is found to some extent in all fractions. Oxidation of proteins and polypeptides remaining in the water-soluble fraction of cataractous lenses occurs at a slower rate than that of other components. The membrane-associated fractions, which appear to be involved in the cataract process, are extensively oxidized. In the intrinsic membrane fractions of the +4 cataractous lenses, the cysteine content increases and glutathione–protein mixed disulfides are present. The disulfide bond of this mixed disulfide would ap-

Table 2. Oxidation of protein fractions from cataractous lenses\*

Fraction	Met	Met sulfoxide	Met sulfone	Cysteine		Protein SH as S—S	Mixed SH as S—S	Protein SH as cysteic acid	Mixed SH as cysteic acid
				Exposed	Buried				
$\alpha$ -Crystallin†	2.0	0	0	—	—	—	—	—	—
$\alpha$ -Crystallin†	1.4	0.6	0	1.4	0.8	1.0	0	0	0
WS 43,000-dalton‡	1.4	2.6	0	—	—	—	—	—	—
US 43,000-dalton†	1.0	2.6	0.4	2.0	0	0	0	0	0
US 43,000-dalton‡	0.4	2.2	0	1.6	0	1.0	0	0	0
US HMW S—S†	1.2	1.6	0	1.4	0	2.0	0.4	0	0
US HMW S—S‡	1.0	2.0	0	1.4	0	0.8	2.0	0	0
Light membrane†	2.0	2.5	0	0.4	0	1.6	0	1.0	0
Heavy membrane‡	2.0	2.5	0	0	0	3.0	0	0	3.0

\* Data are expressed arbitrarily as residues per 200 residues. Abbreviations: WS, water-soluble; US, urea-soluble; HMW, high molecular weight.

† From 200 pooled lenses +2 to +4 in color and opacity.

‡ From lenses +4 in color and opacity, with nucleus and cortex involved.

§ This component was isolated from the  $\beta$ -crystallin fraction as described (15).

Table 3. Change in amino acid composition due to reductive cleavage in high-density membrane fraction\*

	Before treatment	After treatment†	Difference
Cysteic acid	3.1	0	3.1
Cysteine or carboxymethyl cysteine	3.2	2.5	0.7
Glutamic acid	24.2	21.4	2.8
Glycine	20.8	17.4	3.4

\* Data are expressed as residues per 200 residues. No significant change was seen in the other amino acids after treatment.

† Preparation was reduced with dithiothreitol, treated with iodoacetamide, and then extensively dialyzed.

pear to be oxidized further because of the loss of a cysteic acid-like component during dialysis after reduction and alkylation. So far, this mixed disulfide has only been detected in the severe cataractous lens. Cysteic acid-like components are also present as nondialyzable entities in one of the intrinsic membrane fractions. In the urea-soluble high molecular weight disulfide-linked aggregate from the +4 cataractous lens, some of the increase in cysteine is also due to mixed disulfides. However, in this fraction the disulfide bonds do not appear to be further oxidized. In the 43,000-dalton polypeptide from the extrinsic membrane, methionine and cysteine oxidation is extensive. In fact, there is evidence that some of the methionine and cysteine in these fractions is converted to a nondetectable product yet to be elucidated.

The degree of oxidation found in old normal and cataractous lenses in this study appears to be approximately 15% lower than that found by using the sodium borohydride-5,5'-dithiobis(2-nitrobenzoic acid) procedure (8). This probably reflects differences in either the methods used for measuring oxidation or the methods used for protein isolation. The general trends in the results are the same for the two procedures.

In summary, oxidation of the sulfur-containing amino acids appears to occur first primarily at the membrane in the aging normal lens. With cataract, oxidation spreads to the extrinsic membrane components and finally to the cytoplasmic proteins. The data are suggestive of but do not prove that oxidation of membrane sulfur is necessary for cataract formation. Not explicit in the preceding data and discussion are the dramatic changes in the amino acid composition of the high molecular weight and intrinsic membrane fractions of the severe cataract. Preliminary data suggest that a cytoplasmic polypeptide, approximately 20,000-daltons containing a significant number of cysteine residues, is covalently attached to the membrane by disulfide bonds. Reduction, alkylation, and centrifugation of the cataractous lens membrane releases this major constituent along with smaller amounts of a 43,000-dalton polypeptide and other components. A similar polypeptide can also be isolated from the high molecular weight disulfide-linked aggregates by reduction and alkylation.

The question arises as to the mechanism leading to the oxidative damage observed in cataractous lens proteins. Several transient oxidative molecules, such as hydrogen peroxide and superoxide, are formed in the electron transport chain, as well as in a number of other enzyme systems, that are known to oxidize protein methionine and cysteine *in vitro* (24). Such oxidants are probably present in the lens. The enzymes catalase, superoxide dismutase, and glutathione peroxidase are believed to convert these transients to innocuous compounds in the normal lens (25, 26). These enzymes may be inactive in the cataractous lens.

Because the lens is normally under photochemical stress,

there is also the possibility that the observed oxidation arises from photochemical reactions. Flavins and other photosensitive compounds such as the *O*- $\beta$ -D-glucoside of 3-hydroxyl-kynurenine are present in the lens (27, 28). Such compounds are known to cause *in vitro* oxidation of protein sulfur in the presence of light (29). The normal lens probably has mechanisms for protecting the structural proteins from photochemical damage. Indeed, vitamin C or superoxide dismutase appears to protect the lens from certain photochemical oxidation damage (30). However, it would appear that in cataract formation such systems may be inactivated, making the lens more susceptible to oxidation.

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