Thiol oxidation in the crystalline lens

I. The rate-limiting role of hexokinase in aging rat and human lenses

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Reduction of nonprotein disulfides required both glucose or glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide phosphate (NADP). However, hexokinase (HK) was found to be the rate-limiting step: the glucose-supported reduction rate was only 50% of that of G6P-supported activity. This disulfide-reducing activity seemed to decline in the aging lens. Further, the glucose-supported activity dropped substantially if HK was deactivated with diamide; the deactivation was partially reversible. HK activity in aging clear and cataractous human lenses had greatly diminished. This might explain the disproportionate decrease in glucose-supported reduction in the aging lens. The components of the disulfide-reducing mechanism in human lens were reviewed and discussed.

Key words: lens, rat, human, aging, cataract, thiol oxidation, disulfide reduction, hexokinase regulation

Recent evidence indicates that protein disulfide formation may play an important role in human senile cataractogenesis. Harding[1] and Spector and Roy[2] have found that human nuclear cataracts contain water-insoluble high-molecular-weight protein aggregates that are rich in disulfides. Anderson and Spector[3] documented the accumulation of protein disulfides in the nucleus of human cataracts. And in addition to the nuclear change, Truscott and Augusteyn[4, 5] also discovered increasing disulfides in the cataractous lens cortex. With Raman spectroscopy, East et al.[6] were able to demonstrate an age-dependent increase of disulfides in the nucleus of intact rat lens.

The origin of these disulfides is not entirely known, although it is quite clear that thiol oxidation occurs in aging lens and that the oxidation is not being counteracted effectively.

Other reports show that in human senile cataracts there is an age-dependent loss of glutathione (GSH) and increased protein-GSH mixed disulfides.[7, 8] These data also support the concept that disulfide-reducing activity in aging lens is diminished.

Harding[7] suggested that maintenance of protein sulfhydryl was mediated through GSH, which was a product of the G6P dehydrogenase (G6PD)/NADP$\leftrightarrow$NADPH/GSH reductase (GSSG-R) system. Previous studies have shown that, in early cataracts, the activities and the kinetic properties of

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Table I. Some metabolites and enzymes in lenses (mean wet weight, 22 mg) from 1-month-old rats

Metabolite	*nmol/lens**	*Mean conc. (μM) in lens dispersion†*	*Enzyme*	K_m(μM)
Glucose	16 ± 5 (5)	75	HK	Glucose 100
ATP	72 ± 2.5 (6)	337.7		ATP 400
G6P	1.6 ± 1 (8)	7.5	G6PD	G6P 17
NADP	0.69 ± 0.07 (6)	3.2		NADP 4
NADPH	0.53 ± 0.1 (6)	2.5	GSSG-R‡	GSSG 21
				NADPH 5

*Values shown are mean ± S.D. (number of lenses). Each lens was dispersed in 0.2 ml buffer.
†To compute the final concentration of each metabolite in the dispersion (in μM), the per lens values are multiplied by a factor of 4.69 (corrected for 60% lens water).
‡Partially purified enzyme (Cheng, H-M, unpublished work).

Table II. NPSH regeneration in tBHP-treated rat lens.

*Addition**	*(1) Control lens*	*(2) Regeneration lens*	*(2) − (1) Net change*
Glucose	0.050 ± 0.010	0.077 ± 0.015	+0.027 ± 0.009
NADP	0.059 ± 0.007	0.093 ± 0.011	+0.034 ± 0.007
Glucose + NADP	0.060 ± 0.005	0.108 ± 0.008	+0.048 ± 0.003
G6P + NADP	0.058 ± 0.008	0.101 ± 0.010	+0.043 ± 0.004
NADPH	0.056 ± 0.006	0.099 ± 0.008	+0.043 ± 0.006
Untreated†	0.117 ± 0.013	0.112 ± 0.008	−0.005 ± 0.012

*Glucose and G6P, 5.5 mM; NADP and NADPH, 1 mM; final concentration.
†Lenses without tBHP treatment were processed similarly to tBHP-treated lenses. The values represent maximal NPSH contents in the lens.
Values shown are micromols of NPSH per lens (mean ± S.D.); 6 lens pairs were used in each determination.

G6PD and GSSG-R do not seem to vary significantly from those of the clear aging lens.[9–11] In this article we report that the disruption of the reducing system may have occurred at HK, the enzyme which controls the supply of G6P in aging human lens.

Materials and methods

Sprague-Dawley albino rats were purchased from Charles River Breeding Laboratories, Wilmington, Mass. Human cataracts were collected in the operating rooms of the Massachusetts Eye and Ear Infirmary, and noncataractous human lenses were obtained through the New England Eye Bank. Human lenses were classified according to Chylack.[12]

To test the substrate/cofactor requirements for nonprotein sulfhydryl (NPSH) (or GSH, which comprised more than 90% of the NPSH) regeneration (Table II), lens pairs from 1-month-old rats were incubated in 4 mM t-butyl hydroperoxide (tBHP), prepared in Dulbecco's phosphate-buffered saline (Microbiological Associates, Bethesda, Md.), at room temperature for 15 min to oxidize approximately 50% of NPSH. One lens from each lens pair was immediately acidified with 0.3 ml of 1.67% metaphosphoric acid–30% NaCl, after the lens had been dispersed in 0.2 ml of 0.1M Tris-Cl, pH 7.4 with a glass rod. The fellow lens was dispersed in 0.2 ml of 0.1M Tris-Cl, pH 7.4, containing various substrates/cofactors, and incubated at 37.5° C for 20 min before acidification. A preliminary time study showed that NPSH regeneration reached a plateau after 10 to 15 min of incubation. NPSH was determined according to the method of Beutler et al.,[13] i.e., measurement of A_{412nm} as a result of reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by NPSH or GSH. Treatment with tBHP did not alter the activities of HK, G6PD, or GSSG-R.

The regulatory role of HK (Fig. 1 and Table III) was tested with an incubating medium containing 1 mM each of ATP and NADP and 0.5 mM of GSSG; glucose or G6P was added to a final concentration of 5.5 mM. The medium was prepared in 0.1M Tris-Cl with a final pH of 7.15 at 37.5° C. GSH produced was determined by the method of Beutler et al.[13] To test the effect of HK loss on GSSG reduction, diamide was used to deactivate epithelial HK in rat lens (Tables IV and V). The lenses were incubated in 5 mM diamide (in Dul-

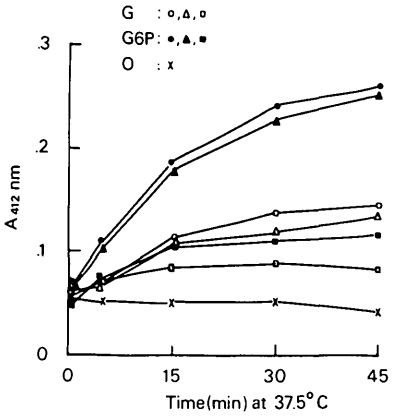

Fig. 1. Reduction of GSSG. Pooled rat lens epithelia were homogenized and incubated in medium containing GSSG at 0.1 (□, ▪), 0.5 (△, ▲), or 1 (○, •) mM and glucose *(G)* or G6P, each at 5.5 mM. All media contained 1 mM each NADP and ATP. Control *(O)* medium contained 1 mM each GSSG, ATP, and NADP, but no glucose or G6P. Final pH at 37.5° C, 7.15. The ordinate indicates GSH produced, as determined with DTNB (see Materials and methods).

becco's phosphate-buffered saline solution) at room temperature for 15 min. Loss of epithelial HK was verified by detecting the residual HK activity on Cellogel strips following electrophoresis. Rat lenses were usually dissected into epithelial and cortical-nuclear fractions. Dissection of human lenses was performed with a cork borer (5 mm, id) into cortical and nuclear fractions. Only the epithelial fractions were collected in the case of diamide-treated rat lenses. These fractions were homogenized in ice-cold 0.1M Tris-Cl, pH 7.4, before testing for GSSG reducing activity.

HK and G6PD activities were assayed according to Chylack et al.[14] and Cheng and Chylack,[15] respectively. Assay medium for GSSG-R was prepared with 0.11 mM NADPH and 0.5 mM GSSG in 0.05M Tris Cl, pH 7.4. Protein was determined according to Lowry et al.[16]

ATP was measured with the procedures of Strehler and Totter,[17] and NADPH and NADP were measured according to Allig et al.[18] Measurements of glucose and G6P were performed according to Bergmeyer.[19]

Cellogel (Chemetron, Milan, Italy) electrophoresis for separation of HK isozymes was performed with 0.25M barbital buffer pH 8.4, containing 0.1M glucose and 0.63 mM EDTA, at 200 V for 2 hr at 4° C. HK activity was detected with the medium of Chylack et al.[14]

Diamide and GSSG were products of Sigma Chemical Co. (St. Louis, Mo.), tBHP was purchased from Matheson, Coleman, and Bell (Norwood, Ohio), DTNB was from Calbiochem (San Diego, Calif.), and most of the auxiliary enzymes, substrates, and cofactors were from Boehringer-Mannheim (New York, N. Y.).

Results

NPSH regeneration in rat lens. It has previously been shown that glucose stimulates NPSH regeneration in lenses treated with tBHP,[20] although the involvement of G6PD/GSSG-R scheme has not been demonstrated directly.

Rat lenses pretreated with tBHP were dispersed and allowed to regenerate NPSH in the presence of various substrates and/or cofactors. The final concentration of each endogenous substrate or cofactor in lens dispersions was below that of the K_m of related enzymes (Table I), except that ATP, at the concentration indicated, was sufficient for sustaining lactate production from glucose for at least 1 hr at 37.5° C.[21] Exogenous ATP was therefore omitted from the incubating medium.

Table II shows that, with both glucose (or G6P) and NADP in the medium, the maximal NPSH regenerated was equivalent to that with NADPH. Regeneration with glucose or NADP alone was significantly lower than that with both glucose (or G6P) and NADP, or with NADPH only (in each case, $p < 0.05$ with unpaired Student's t test).

The assumption of an HK/G6PD/GSSG-R sequence in NPSH regeneration was apparently a valid one.

Role of HK. Homogenates of rat lens epithelium and cortex-nucleus were used to determine the rate of GSSG reduction with glucose or G6P as the substrate.

A typical GSSG reduction graph is shown in Fig. 1. It could be seen that the reduction rate with glucose was approximately 50% of that with G6P at GSSG concentrations ranging from 0.1 to 1 mM.

To show that the reduction was not due to nonenzymatic activity, rat lenses were incubated in medium containing no or 12 mM glucose for 20 hr at 37.5° C.[22] The epithelia

Table III. GSSG reduction with the epithelial and cortical-nuclear preparations from the rat lens (see Materials and methods)

Age (month)	Wet lens weight (mg)	Epithelium*			Cortex-nucleus†		
		(1) With glucose	(2) With G6P	(1)/(2)	(3) With glucose	(4) With G6P	(3)/(4)
1	21	0.034	0.071	0.48	0.742	1.291	0.57
2.5	37	—	—	—	0.348	0.738	0.47
8	52	0.013	0.032	0.41	0.139	0.374	0.37

*Values shown are net GSSG reduced (in micromols) per milligram of protein per 10 min at 37.5° C. Determined from plots similar to those shown in Fig. 1.
†Values shown are net GSSG reduced (in micromols) per gram wet weight per 10 min at 37.5° C.

Table IV. Epithelial HK and G6PD in diamide-treated rat lens (see Materials and methods)

Treatment	Time of incubation (min) in glucose medium	%HK activity*	%Residual*† G6PD activity	Whole-lens‡ NPSH (μmol/lens)
None (fresh lens)	—	100	88.1	0.104 ± 0.012 (6)
Diamide	0	15.7	43.2	0.067 ± 0.006 (6)
	10	14.7	67.7	0.076 ± 0.015 (6)
	30	30.7	88.6	0.078 ± 0.015 (6)
	60	37.6	94.1	0.094 ± 0.011 (6)

*The initial activity was 0.041 and 0.055 (as ΔA_{340nm}/5 min/0.5 ml enzyme preparation) for HK and G6PD, respectively.
†Percent activity after a 1 hr incubation at 37.5° C, pH 7.15.
‡Values shown are mean ± S.D. (number of lenses tested) determined from incubated intact lenses.

were pooled and tested. The epithelia from glucose-deprived lenses were devoid of HK and G6PD activities, and no GSSG reduction was observed. In contrast, the control lenses (incubated in 12 mM glucose) retained the reducing activity, although with a high background due to accumulation of glucose and G6P in the cells (Cheng, H-M., unpublished data).

Effect of aging. There appeared to be an age-related decline of GSSG-reducing activity in the rat lens, either with glucose or G6P as the substrate (Table III). In the cortical-nuclear fraction, the glucose-supported GSSG reduction showed a greater decrease than with G6P, i.e., a drop of 81.3% (from 0.742 to 0.139 μmol GSSG reduced/gm/10 min) vs. 63% (from 1.291 to 0.374 μmol/gm/10 min) from 1 to 8 months of age. Further, the ratio of glucose-/G6P-supported activity decreased from 0.57 to 0.37 (Table III). Comparative study of epithelial fractions from 1- and 8-month-old lenses, on the other hand, showed that both the glucose- and G6P-supported GSSG-reducing activities in the old lenses decreased to about 40% of the level in young lens (Table III).

These data seemed to suggest that HK in aging lens had undergone certain functional changes that were not apparent from measurement of the specific activity of HK per se since the HK activity remained relatively constant in lenses of these ages.[14]

Studies with aging normal and cataractous human lenses showed a disproportionate loss of HK-mediated reduction of GSSG. Some typical results are shown in Fig. 2. All 16 human lenses that we tested exhibited a similar pattern. These lenses contained no detectable G6P, the HK inhibitor. An age-dependent study was not possible due to the nonavailability of fresh young human lenses.

Pooled human lens epithelia also showed GSSG-reducing patterns similar to those shown in Fig. 2. Nuclear fraction, however, has no reducing activity at all. Further, if the lenses were stored at −80° C for a few days, the GSSG-reducing activity was totally lost.

Effect of HK loss. Since HK has been found to be decreased in the cortex (and nu-

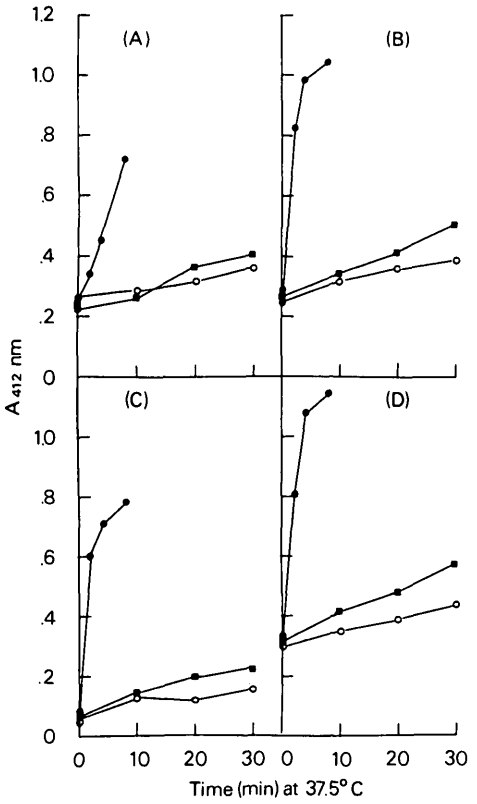

Fig. 2. Reduction of GSSG. Human lens cortex was homogenized and incubated in medium containing 0 (o), 5.5 mM glucose (■), or G6P (●). All media contained 0.5 mM GSSG and 1 mM each NADP and ATP. Final pH at 37.5° C, 7.15. The four lenses shown here are representatives of 16 lenses tested. *A*, 70 years old, clear lens; *B*, 78 years old; $I,CXE_4N_4NS_y$; *C*, 76-year-old diabetic; $I,CXE_2SCP_{69}N_1NS_y$; *D*, 82 years old; $I,CXA_1CXE_1N_2NS_y$. The ordinate indicates GSH produced.

cleus) of human cataracts,[23, 24] the effect of HK loss on GSSG reduction was examined with a simulated rat lens study by selectively deactivating HK with diamide.

Cellogel electrophoresis of diamide-treated rat lenses showed that type II and a fraction of type I HK isozymes in the epithelium were lost; this pattern was remarkably similar to the HK of freshly extracted human aging lens.[23] Both type I and type II HKs were found in the cortical-nuclear fraction, although with slightly diminished activities as compared to lenses without diamide treatment. The residual cortical-nuclear HKs, however, appeared to be adequate for restoring the portion of NPSH oxidized by diamide (Table IV).

Table V. Initial rate of GSSG reduction in the epithelial fraction of rat lenses treated with 5 mM diamide, followed by a 1 hr incubation in 5.5 mM glucose (See Table IV)

Treatment	*(1) With glucose**	*(2) With G6P**	*(1)/(2)*
None	0.039	0.066	0.59
Diamide	0.003	0.051	0.059

*Values shown are net GSSG reduced (in micromols) per milligram of protein per 10 min at 37.5° C from two determinations.

Diamide treatment did not affect GSSG-R; however, G6PD was oxidized. Oxidized G6PD retained activity and kinetic properties that were similar to those of the native enzyme, except that the activity was lost at a faster rate when incubated at 37.5° C, pH 7.15.[15] This thermal sensitivity was used as the index of the extent of G6PD oxidation (Table V). Oxidized G6PD could be reverted back to the native form with the treatment of dithiothreitol.[15]

Other changes following diamide treatment included a 15% loss of NADP (but not NADPH) and oxidation of NPSH.

Diamide-treated 1-month-old rat lenses were transferred to 5.5 mM glucose (in Dulbecco's saline solution) and further incubated for up to 1 hr at 37.5° C. The results are shown in Table IV. After 1 hr, the whole lens NPSH returned to 90% of normal level, and epithelial G6PD was completely reduced, whereas the activity of HK remained depressed. The epithelia were pooled and tested for GSSG reduction. The results are shown in Table V. The loss of HK, mainly type II, activity indeed retarded GSSG reduction with glucose as the substrate; in contrast, the control lenses (without diamide treatment) stayed normal, with the glucose-supported activity reaching 59% of that with G6P.

Loss of type II HK in human cataracts possibly resulted in a depressed GSSG reduction, as shown in Fig. 2.

Discussion

Our studies indicate that in rat lens there is an age-related decline of GSSG-reducing activity and that the decline seems to affect the glucose-supported reduction more than that with G6P as the substrate in the cortical-nuclear fraction (Table III). In all cases, it is evident that HK regulates disulfide reduction in the lens.

We have also shown that the loss of type II HK activity tends to further restrict glucose-supported activity (Table V). This, in fact, may have occurred in aging human lens (Fig. 2).

A review of the components of the disulfide reducing mechanism in human lens revealed the following:

1. We have tested 36 cataracts and found that the lowest endogenous ATP concentration in immature cataracts was sufficient for HK saturation (i.e., more than 0.4 mM).
2. There was no significant loss of GSSG-R in early cataracts.[10, 11]
3. There was only a slight loss of G6PD in cataracts as compared to lenses of similar ages.[9]

Fig. 2, moreover, suggests that both GSSG-R and G6PD are in excess.

4. NADP(H) content in human aging lenses was extremely low (0 to 8.5 nmol NADPH per lens and 0 to 5 nmol NADP per lens, as determined on eight lenses frozen in liquid N_2 immediately following cataract extraction). However, a whole-lens incubating study indicated that the sorbitol pathway was activated in high (35.5 mM) glucose medium,[25] suggesting that the NADPH-generating system was functional in human aging lens, although it is not known if the capacity of the system meets the requirement of disulfide reduction.
5. HK activity decreased in the cortical-nuclear fraction of human cataracts.[22, 23] Chylack[23, 24] has shown that, only in fetal lens, the specific activity of HK in the cortex is equivalent to that in the epithelium. An age-dependent HK loss in human lens is possible.

The mechanism of HK loss is not clear. Preliminary data show that HK, especially type II isozyme, is extremely sensitive to oxidation and that, depending on the duration/extent of oxidation, the reversibility (reactivation) varies. This is quite unlike G6PD, which is apparently readily reduced together with NPSH regeneration (Table IV). It is interesting to note that Na,K-ATPase is also sensitive to thiol oxidation. The resultant activity loss is irreversible.[26, 27] Alteration of Na,K-ATPase activity in human senile cataracts has been previously reported.[28] Correlation of GSSG-reducing capacity and protein disulfide reduction is currently under investigation.

It is tempting to speculate that thiol oxidants such as H_2O_2 may create a demand for G6P (1) to reduce disulfides resulting from thiol oxidation and (2) to counteract the ionic imbalance resulting from partial destruction of Na,K-ATPase. Since thiol oxidation also destroys HK, the G6P demand may not be fully satisfied. Additional stress, such as that from the accumulation of sorbitol in diabetic lens, besides competing for NADPH, may overwhelm the already depressed HK activity in aging human lens. Furthermore, the GSH function as a free radical scavenger would be severely limited due to decreased GSSG reducing activity in HK-deficient lenses.

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