Non-enzymatic glycosylation in human diabetic lens crystallins

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Summary. Lens crystallins undergo non-enzymatic glycosylation with aging and diabetes mellitus. It is not known, however, whether all crystallins are subject to the same extent of glycosylation. Human diabetic lenses (~80 years of age) were dissected into cortex and nucleus, then fractionated into various crystallins with gel chromatography (Sephacryl S-200, Sephadex G-75 or Bio Gel A-15m). The glycosylated crystallins were then separated from the nonglycosylated crystallins by affinity chromatography on Glyco Gel B boronic acid. The percentage of glycosylated crystallin was about 20–30%, and did not differ much among most crystallins, although γ -crystallin has significantly less ($p < 0.01$) glycosylated protein. The

extent of glycosylation in the glycosylated crystallins, however, was found to be greater in the high molecular weight crystallins. The extent of glycosylation in α -crystallins is approximately two to four times that observed in β - or γ -crystallin. The extent of glycosylation appears to depend not only on the lysine content, which does not vary much among the crystallins, but also on the accessibility of the surface areas where lysine residues are located. This accessibility depends on the protein conformation and appears to correlate with protein unfolding.

Key words: Crystallin, glycosylation, conformation, diabetes.

The human lens contains a very high content of proteins; most of them are represented by crystallins, the structural proteins. They are composed by three antigenically distinct families of polypeptide, called α -, β - and γ -crystallin. They undergo post-translational changes in the normal aging process, and appear to undergo changes to a greater extent in pathology.

The post-translational nonenzymatic glycosylation in haemoglobin and albumin has been suggested as a cause of changes in protein structures and functions leading to the pathogenic complications of diabetic mellitus [1]. The studies of glycosylation of lens proteins, however, have yielded controversial results [2–9]. Stevens et al. [2] suggested that glycosylation partially unfolds lens proteins and facilitates disulfide formation. However, Ansari et al. [5], although reporting an increased glycosylation of lens proteins of cataracts, found no correlation between glycosylation and disulfide bonding. A similar conclusion was reported by Pande et al. [4]. Stevens et al. [2] and Monnier et al. [3] also suggested that glycosylation plays a role in experimental sugar cataract formation, but such a role was disputed by Chiou et al. [7]. Despite these controversies, however, all studies concluded that more glycosylation occurs in lens proteins of senile and diabetic cataracts than in normal lenses.

Recently, Garlick et al. [10] studied the effect of glycosylation of human lens proteins changes on age and diabetes mellitus. They found that glycosylation increases with aging in non-diabetic lenses and is twofold

higher than normal in diabetic lenses. They quantitatively reported results of the extent of glycosylation of both soluble and insoluble proteins of individual lens with aging and diabetes. They also identified lysine as the major site for glycosylation. The extremely slow rate of glycosylation in lens proteins, as compared to haemoglobin and albumin, appears to be regulated by a low concentration of glucose in the lens, a low content of lysine residue, and a unique protein conformation (β -sheet conformation) that restricts molecular rotation.

Although glycosylation was measured in total soluble human lens proteins, it is not known whether the various protein fractions (α -, β - and γ -crystallins) are equally glycosylated. In bovine lens proteins, Kabasawa and Kinoshita [11] reported that γ -crystallin had the highest level of bound sugars of the three crystallins. Chiou et al. [6] studied glycosylation in bovine lens crystallins by [³H] NaBH₄ incorporation and found that the amount of glucitol-lysine increased with age in all crystallins, with HMW α -crystallin showing the greatest increase. In an earlier report, Stevens et al. [2] also showed an increased incorporation of ¹⁴C-labelled glucose into bovine crystallin, especially α -crystallin, in experimental sugar cataract. In diabetic rat lenses, a similar increase in ³H incorporation in α -crystallin was observed [2].

It appears that the extent of glycosylation in a crystallin may depend on its lysine content. However, because of the supramolecular structure of protein, not all surface areas where lysine residues are located are ac-

cessible to sugar molecules. All crystallins, except γ -crystallin, exist in an aggregated state, with α -crystallin showing the greatest extent. This tertiary or quaternary structural difference may affect the extent of glycosylation. Differences in tertiary structure as studied by spectroscopy have been reported among bovine lens crystallins [12–13]. Similar differences also exist among human lens crystallins [14], as the high sequence homology between the mammalian species would predict [15]. In this study, we have determined the extent of glycosylation in various crystallins isolated from diabetic lenses, and have tried to find a possible correlation to their protein conformation.

Materials and methods

Human lenses of diabetic patients (~80 years of age) were obtained from the National Diabetes Research Interchange (NDR), Philadelphia, PA, USA. They were kept frozen prior to their use. Each lens was thawed, decapsulated and dissected into cortical and nuclear portions. Five to eight lenses were pooled together. Each pool was homogenized in 5 ml of 0.01 mol/l phosphate buffer, pH 7.6. The supernatants were applied to a column (2.6 × 100 cm) of Sephacryl S-200 superfine gel (Pharmacia Chemicals, Uppsala, Sweden). All experiments were performed at 4 °C. Detailed procedures for the lens protein isolation have been described elsewhere [13, 14, 16–18]. Six fractions were obtained, and they were rechromatographed on a smaller column (1.0 × 50 cm) of Bio Gel A-15 m (fraction I), Sephacryl S-200 (fractions II–IV), or Sephadex G-75 (fractions V and VI) to minimize cross-contamination.

The separation of glycosylated crystallins was made on Glyco-Gel B boronic acid (Pierce Chemical, Rockland, IL, USA) [17, 19–20]. The separation was based on the interaction of the glyco moiety with boronate. Crystallins (10–15 mg) in 0.25 mol/l ammonium acetate buffer, pH 8.5, were applied to a column (3 × 20 cm) of Glyco Gel B. Nonglycosylated crystallin was eluted from the column with the starting buffer. The effluent was read at 280 nm. When the reading reached less than 0.01, the column was eluted with 0.2 mol/l sorbitol in 0.25 mol/l ammonium acetate, pH 8.5. Each crystallin obtained from gel filtration was applied to the Glyco Gel B column and separated into glycosylated and non-glycosylated crystallins.

Glycosylation was estimated using the thiobarbituric acid (TBA) procedure of Flückiger and Winterhalter [21–22], based on the reaction of TBA with the released 5-hydroxymethylfurfural (5-HMF). Two ml of crystallin solutions were added to 1 ml of 1 mol/l oxalic acid and hydrolyzed at 100 °C in a heating block for 4.5 h. The hydrolysates were cooled on ice and 1 ml of 40% trichloroacetic acid was added. The precipitates were filtered through 0.45 mol/l filter. Aliquots (2 ml) of the supernatants were incubated with thiobarbituric acid (0.5 ml, 0.05 mol/l) at 37 °C for 30 min. The absorbance at 443 nm was read. Reference curve using various concentration of 5-hydroxymethylfurfural (Aldrich Chemical, Milwaukee, WI, USA) was made for the estimation of the released 5-HMF. Samples of nonglycosylated crystallin were used to correct for nonspecific color development. Protein concentration was determined by the Lowry method [23].

Statistical analysis. For the statistical analysis, the *t* value was first calculated; from the *t* value the probability level was determined from the table of the distribution of *t*.

Results

The fractionation of the soluble proteins on Sephacryl S-200 gel has been described in previous papers [18, 24]. Five main peaks and one small shoulder after the last peak were observed, corresponding to fractions I–VI. Fraction I was rechromatographed on Bio Gel A-15 m

column to separate the high and low molecular weight (HMW and LMW) α -crystallin. Fraction I of the nucleus appears to be composed mainly of HMW α -crystallin, the second peak on the Bio Gel A-15 m elution profile being very small. This small amount of LMW α -crystallin in the nucleus may be derived from the inner cortex. Roy and Spector [25] and Horwitz et al. [26] have reported that no LMW α -crystallin was found in the core nucleus. Fractions II–IV were each rechromatographed on Sephacryl S-200 gel to minimize the cross-contamination. Fraction V was rechromatographed on Sephadex G-75 to remove the minor fractions [27–28]. Fraction VI was not further studied because of the low yield.

For the purpose of convenience, we used the conventional classification (α -, β - and γ -crystallin). It has to be pointed out that this classification may not be ideal for diabetic cataracts, since many changes have occurred in proteins resulting from post-translational modification. Fraction I contains the HMW and LMW α -crystallin. Fractions (II–IV) are β_1 -, β_2 - and β_3 -crystallin. Fraction V is γ -crystallin, and based on the elution profile on Sephadex G-75, the major component is the γ -crystallin [27]. The last fraction (VI) is the 11,000 dalton protein [24].

The separation of the glycosylated crystallin from the nonglycosylated crystallin was achieved on Glyco Gel B boronate gel that binds vicinal hydroxyl groups of covalently linked sugar [19–20]. The binding appears to be specific, since the first fraction, which is nonglycosylated protein, gave only an insignificant reading in the TBA assay. This reading was used to correct in the assay for glycosylated crystallin. The percentage of each form was estimated by planimetry, assuming that both forms have the same absorbance at 280 nm.

Percents of total protein glycosylated are listed in Table 1. They do not differ much among the crystallins, with the exception of the significantly less glycosylated γ -crystallin ($t = 2.77$, $0.02 < p < 0.01$).

The extent of glycosylation as estimated by the TBA procedure is given in Table 2. It is expressed either as nmol of glucose per nmol of protein monomer, assuming the monomeric molecular weight of 20,000, or as nmol of glucose per nmol of protein polymer, based on the previous reported molecular weights of 800,000 (α_L); 140,000 (β_1); 82,500 (β_2); 39,600 (β_3) and 17,000 (γ) [24]. The figures are the total hexose released after hydrolysis, representing the actual extent of protein glycosylation. The calculation using polymeric molecular weight is included to show that the crystallins exist as polymers in the physiological state, and that the degree of glycosylation should be reflected under this condition.

Discussion

It has been reported that lens proteins (unfractionated) are more glycosylated in the cortex than in the nucleus [9]. Our results appear to agree with this observation for

Table 1. Relative percentage of glycosylated crystallins in pooled human diabetic lenses^a

	Cortex (%)	Nucleus (%)
α_H	29 ± 1	19 ± 1
α_L	19 ± 3	14 ± 3
β_1	29 ± 10	32 ± 11
β_2	29 ± 5	29 ± 8
β_3	30 ± 3	14 ± 5
γ	11 ± 1	9 ± 1

^a The values are the average of three experiments, and expressed as mean ± SD

Table 2. Extent of glycosylation in the glycosylated crystallins isolated from human diabetic lenses^a

	Cortex	Nucleus
α_H	0.150 ± 0.011 ^b (---) ^c	0.094 ± 0.012 ^b (---) ^c
α_L	0.127 ± 0.012 (5.06 ± 0.49)	0.075 ± 0.017 (2.98 ± 0.68)
β_1	0.053 ± 0.008 (0.37 ± 0.06)	0.057 ± 0.010 (0.40 ± 0.08)
β_2	0.034 ± 0.012 (0.14 ± 0.05)	0.032 ± 0.008 (0.13 ± 0.03)
β_3	0.033 ± 0.010 (0.066 ± 0.019)	0.070 ± 0.009 (0.139 ± 0.017)
γ	0.075 ± 0.012 (0.064 ± 0.010)	0.042 ± 0.009 (0.036 ± 0.007)

^a Two pooled samples were measured; the values are their average expressed as mean ± SD;

^b expressed in nmol per nmol of monomer, assuming the molecular weight of subunit polypeptide to be 20,000;

^c expressed in nmol per nmol of polymer, based on the molecular weight of (α_L) 800,000; (β_1) 140,000; (β_2) 82,500; (β_3) 39,600; (γ) 17,000 (24). The molecular weight of α_H -crystallin is undetermined; the extent of glycosylation cannot be calculated in terms of polymeric form

most crystallins. The γ -crystallin and the β_3 -crystallin of the nucleus, however, show a significant decrease in the amount of glycosylated protein. This may result from their decreased content with aging and cataract formation, as reported for the γ -crystallin [24, 29–31].

The extent of glycosylation of amino acids, mainly lysine residues [10], in glycosylated crystallins show a general decreasing order from the high molecular weight crystallins to the low molecular weight crystallins. Under an identical condition, the extent of glycosylation should be related directly to the lysine content in each crystallin. The amount of lysine residue in the LMW α -crystallin has been reported to range from 4.8 to 5.2 mol% [32–34]. In γ -crystallin the values ranged from 2.4% [28], to 2.9% [33] and 4.4% [34]. The amino acid composition of β -crystallin has been reported for the second fraction of DEAE-cellulose chromatography and the lysine content is 4.1–4.5% [34]. No values have been reported for the individual β -crystallin. The HMW α -crystallin isolated in this study is HM₂ according to Harding [15], since we only used agarose gel filtration in

the separation. It has been reported that this HMW α -crystallin contains other crystallins and is partly cross-linked by disulfide bonds [15]. The lysine content was reported to be 4.0% [35]. The discrepancy between the lysine content and the extent of glycosylation apparently indicates that other factors also affect glycosylation.

One of the main factors is probably the protein conformation, i.e. the tertiary and quaternary structures. The secondary structure does not play a major role, since all crystallins have the same amount of β -sheet conformation [14]. The higher order of structures deal with the interacting properties and thus the aggregating property. The extent of glycosylation in this aspect will then depend on the accessible surface areas where lysine residues are located. This accessibility is enhanced when proteins become partially unfolded. A number of mechanisms have been proposed for the unfolding, including oxidation of tryptophan, oxidation of methionine, mixed disulfide formation with glutathione and glycosylation [15]. Spectroscopic studies on crystallins of both bovine [12] and human lenses [14] indicate that the susceptibility of tryptophan to oxidation is in the order of $\alpha > \beta > \gamma$. Therefore, it is possible that the more damage in α -crystallin, which causes protein unfolding [36], may render it more susceptible to glycosylation. The conformational study indicates that HMW α -crystallin has more exposed tryptophan than LMW α -crystallin and the oxidation of tryptophan proceeded further [18]. Whether other posttranslational mechanisms also contribute to the difference in the extent of glycosylation is difficult to explore. However, it is possible that the initial glycosylation facilitated by tryptophan oxidation may further enhance the unfolding and thus the glycosylation, in view of the finding that in vitro glycosylation partially unfolds the protein [17].

The reactivity of lysine residue, i.e. its pK value, may also play a role in glycosylation. The pK value of lysine in proteins is about 10.0, and can be altered by a change in the local charge environment. The slightly higher extent of glycosylation than expected in γ -crystallin may be due to the presence of free NH₂ terminus. In haemoglobin, the free NH₂ terminus was found to be a major site of glycosylation [20], because of their lower pK values.

Nonenzymatic glycosylation is a normal aging process, and is enhanced in diabetic patients. Its role in the pathogenesis of the complications of diabetes is not fully established [review, 1]. In vitro studies appear to indicate that glycosylation impairs the normal biological function of protein. For example, glycosylated serum albumin showed a partial loss in drug or other ligands (such as fatty acid) binding capability [37–38] and glycosylated collagen also showed a decrease in its cross-linking ability in fibril formation [39–40]. The present studies demonstrate that crystallins that tend to aggregate were more glycosylated. This finding, coupled with the evidence that the crystallins are more glycosylated in the diabetic lenses [10], suggests that glycosylation might induce a secondary change in protein. This in-

cludes a conformational change, as shown by spectroscopic studies on α -crystallin isolated from diabetic cataract [41].

Acknowledgement. Human lenses were obtained from National Diabetes Research Interchange (NDRI), Philadelphia, PA, USA. This work was supported by grants from the National Institute of Health (EY 4969) and the Brigham Surgical Group Foundation.

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Received: 5 August 1985
and in revised form: 5 February 1986

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