Nonenzymatic Glycosylation of Bovine Lens Crystallins

EFFECT OF AGING*

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We have investigated nonenzymatic glycosylation of crystallins from calf and mature bovine lenses (2-6 years old). The lens homogenates were treated with 200-fold molar excess of [3H]NaBH4 and the incorporation of radioactivity was determined. The extent of glycosylation was more precisely determined from the 6 N HCl hydrolysate of [3H]borohydride-treated proteins by analyzing the glucitol-lysine adduct on a high pressure cation exchange column. We found that the [3H]NaBH4 incorporation and the amount of glucitol-lysine detected increased with age, particularly in HMa crystallin, a high molecular weight aggregate which accumulates with aging. This age-related increase in nonenzymatic glycosylation was also demonstrated by a comparison of crystallins isolated from the cortex and nucleus of a single lens. Nonenzymatic glycosylation of lens crystallins exemplifies a new form of posttranslational modification of long-lived proteins in vivo.

Human hemoglobin undergoes post-translational modification as a result of nonenzymatic condensation of glucose with amino groups on the protein subunits (1). The most abundant minor hemoglobin component, Hb A2, contains glucose covalently attached to the NH2 terminus of the β chains by a ketoamine linkage. In addition, glucose is linked in a similar way to certain ε-amino groups of lysine residues on both the α and β chains (2). Other proteins, such as lens crystallin (3), red cell membrane proteins (4, 5), albumin (6), collagen (7), and the basic myelin protein of nerve (8), also contain this glucitol-lysine adduct. Interestingly, ketoamine-linked glycosylation of these proteins is enhanced in patients with diabetes.

A previous report by Stevens et al. (3) suggested a role of nonenzymatic glycosylation in the formation of diabetic cataracts. We have initiated a series of in vivo and in vitro studies (9) in order to examine the role of nonenzymatic glycosylation in the pathogenesis of sugar-induced cataract formation. In this study, we have used crystallins of normal clear bovine lenses as a model to study in vivo nonenzymatic glycosylation of these long-lived proteins. We have demonstrated that there is an age-dependent increase of [3H]NaBH4 incorporation which is corroborated by the detection of increased amount of glucitol-lysine in the α-crystallin of older lens. The uptake of radioactivity incorporation among the different lens crystallin fractions reflects the age-dependent modification by nonenzymatic glycosylation.

MATERIALS AND METHODS

At a local slaughter house, bovine lenses and aqueous humor samples were obtained from eyes of animals of different ages. They were frozen and stored at −40°C immediately after removing from the eye. The decapsulated lenses were homogenized in 0.05 M phosphate buffer, pH 6.8, containing 0.5% β-mercaptoethanol. The suspension was centrifuged at 27,000 × g for 1 h. The supernatant was adjusted to a concentration of 20–30 mg of protein/ml before treating with [3H]borohydride. In one of the experiments, a calf lens was first separated into cortex and nucleus and each was homogenized separately. The nuclear portion contains about ½ of the total wet weight of the lens.

[3H]Borohydride Reduction—The protein solutions of lens crystallins in phosphate buffer were reacted with 200-fold molar excess (based on Mr = 20,000) of [3H]NaBH4 for 30 min at 4°C. Specific [3H]borohydride activity was adjusted by adding different amounts of [3H]NaBH4 to unlabeled NaBH4. Excess borohydride was removed by acidification of the reaction mixture with acetic acid, followed by extensive dialysis against several changes of distilled water. The reaction mixtures were then lyophilized.

Ionexchange and Separation of Lens Crystallins by Gel Chromatography—The lyophilized protein sample (about 100–150 mg each) was dissolved in 0.05 M Tris/0.05 M NaH2PO4/20 mM EDTA buffer, pH 7.7, and then applied to Sepharose CL-6B column (2.5 × 170 cm) equilibrated with the same buffer, and run at a constant flow rate (8.1 ml/tube/12 min) maintained by a peristaltic pump. The absorbance and H radioactivity of each fraction were determined by Cary 118 spectrophotometer and liquid scintillation counter (Isocap 300, Searle Analytical), respectively.

Analysis of Glucitol-Lysine by High Pressure Cation Exchange Column—Lyophilized protein samples were hydrolyzed in 6 N constant boiling HCl at 110°C for 24 h. The dried hydrolysate was applied to a DC-6A cation exchange column (0.9 × 17 cm) (Durrum Chemical Co., Sunnyvale, CA) eluted with a 200-ml linear gradient of 0.2 M pyridinium acetate, pH 3.2, to 1.5 M pyridinium acetate, pH 5.0. Column pressure was maintained at 200–400 p.s.i. The elution position of glucitol-lysine was located with a standard prepared from a hydrolysate of synthetic ε-[(14C)Glucose-polyl-lysine) reduced with unlabeled NaBH4 as described before (2).

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate—The procedure of Laemmli (10) was used to check the effect of borohydride reduction on the isolated lens crystallins and the distribution of H radioactivity among the subunits of lens crystallins.

Protein concentration was determined by Lowry’s method (11) using bovine serum albumin as standard.

Intracellular glucose concentration was determined according to Bergmeyer et al. (12).

RESULTS AND DISCUSSION

Lens crystallins comprise more than 90% of the dry weight of lens fiber cells. They are excellent candidates for the study
of aging and post-translational modifications of structural proteins because of their extreme longevity compared to other proteins (13, 14). Nonenzymatic glycosylation, a well established post-translational modification of hemoglobin (1), is gaining a lot of attention due to its possible role in the pathogenesis of the complications of diabetes. We have investigated the nonenzymatic glycosylation of lens crystallins from normal bovine lenses of different age groups in order to establish a model system for the future study of human diabetic and cataractous lenses.

Lens proteins were reacted with [3H]NaBH₄, a relatively specific reagent to locate the Schiff base and ketoamine linkage between reducing sugars and amino groups of proteins (2), and then separated into five crystallin fractions on a Sepharose CL-6B column (15). The elution pattern is shown in Fig. 1. It is clearly demonstrated that the elution peaks of radioactivity correspond very well with the absorbance peaks in spite of the fact that radioactivity distribution manifests a slightly broader profile, indicative of the heterogeneous nature of nonenzymatic glycosylation of lens crystallins. The resolution between each of the protein peaks and the subsequent elution of unreacted [3H] label is sufficient to allow the unambiguous determination of radioactivity uptake/mg of protein. Absorbance of each peak fraction was converted into milligrams of protein content by use of the molar extinction coefficients of the five crystallin fractions (16). Since γ-crystallin eluted with the highly radioactive peak of low molecular weight molecules, it was necessary to isolate and dialyze the fraction first before an accurate activity/protein ratio could be estimated. Determination of specific activity by absorbance and by Lowry's method gave about the same specific radioactivity within experimental error. The results of these specific radioactivities from the lenses of four different ages are shown in Table I. There was a slow increment in radioactivity uptake with increasing age. This age-related increase is most prominently demonstrated when one compares the lens crystallins isolated from the cortex and nucleus of a single calf lens as shown in Table II. The crystallin fractions from nucleus consistently show higher specific radioactivity than from those in the cortex. It is well known that the fiber cells of nucleus accumulate in the center of the lens as newer cortical fibers are laid down in the lens periphery. Since no fiber cells are lost from the lens, the nuclear core contains the oldest lens cells. The analysis of the [3H] radioactivity in this study corroborates the morphological development of lens fiber cells. The fact that nonenzymatic glycosylation can differentiate proteins of different ages within a 3-month-old calf lens suggests it may be a general process for all proteins exposed to glucose and may provide a probe for determining age of proteins.

The evidence for the presence of glycosylamine linkage in lens crystallin was further strengthened by analyzing the hydrolysates of HMa- and α-crystallins on DC-6A cation exchange column as shown in Fig. 2. The identified glucitol-lysine peak (fractions 38-41) and the unbound radioactive peak (fractions 2-4) comprise more than 70% of total radioactivity recovered from the column. In contrast to the previous reports on collagen (17, 18) and connectin (19) in which histidinohyderoxymerodesmosine and hydroxylysinosinonul-eucine were the major adducts, hexosyl-lysine adduct seems to be the major [3H]borohydride-reducible compound in lens crystallin. There is a 2.4-fold increase of glucitol-lysine specific radioactivity (from 6,720 to 16,100 cpm/mg) with increasing age when α-crystallin of a 2-year-old lens is compared to that of a 6-year-old lens (Fig. 2A).

Reduction of the aldmine and ketoamine linkages with [3H]NaBH₄, is the major tool available to detect the hexosyl-lysine adduct in native proteins. The reported side reactions derived from borohydride reduction are: 1) cleavage of peptide bonds (20), 2) reduction of indole group of tryptophan (21), and 3) reduction of carboxyl groups (22). In this study, the reaction was done at low temperature (4°C) and the reaction time (30 min) was kept short in order to avoid the above-mentioned side reactions. As can be seen in Fig. 3, the fact that the polypeptide pattern on gel electrophoresis of the crystallins after reduction is the same as that of the unreduced crystallins rules out the first side reaction. The nonspecific tritium incorporation into the first (void volume) peak and into several smaller and variable peaks may be due to the other side reactions. It is likely that exchange of the tritium in the hexosyl-lysine with solvent and amino acids in the hydrolysate can occur when the sample is hydrolyzed in 6 N HCl at 110°C (23). This may account for part of the loss of labeled [3H]glucitol-lysine after the acid hydrolysis (see Fig. 2).

It is noteworthy that the radioactivity distribution and incorporation (Fig. 1; Tables I and II) are not uniformly labeled among the five crystallin fractions. HMa incorporated 2- to 3-fold more [3H]-radioactivity (Table I) than that of α-crystallin in spite of the fact that they have the same amino acid composition and subunit structures (Fig. 3). This may reflect differences in the local pKa values of lysine residues.

![Graph](http://www.jbc.org/)

**Fig. 1.** Chromatography of 3-year-old bovine lens soluble proteins on a Sepharose CL-6B Column (2.5 x 170 cm). The tritium-labeled lyophilized protein sample (about 100 mg) was dissolved in 5 ml of equilibration buffer (0.05 M Tris/0.05 M NaHSO₄/20 mm EDTA, pH 7.7) and the elution of proteins was run in the same buffer at a flow rate of 8.1 ml/tube/12 min. Column eluents were monitored for optical activity (A₂₈₀, O—O) and [3H] radioactivity (●—●). The elution profiles for the lens proteins of other age groups were similar except that the relative proportion of HMa increased with advancing age. The arrows indicate the elution positions for five crystallin fractions.
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TABLE I
Effect of aging on incorporation of NaB3H4 into bovine lens crystallins

Bovine lens homogenate (20-30 mg/ml) was reacted with 200-fold molar excess of [3H]borohydride (8.8 mCi/mmol) and the reaction mixture was fractionated into five crystallin fractions on Sepharose CL-6B (2.5 x 170 cm). The radioactivity incorporation is expressed as counts per min/mg of protein content (Lowry's protein determination). Values shown are the mean of duplicate analyses. These radioactivity data include nonspecific uptake of H at sites on lens crystallins other than ketoamine linkages (Fig. 2). Extrapolation of specific radioactivity to age zero indicates significant background radioactivity.

<table>
<thead>
<tr>
<th>Age</th>
<th>HMα</th>
<th>α</th>
<th>βH</th>
<th>βL</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>yr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4900 ± 230</td>
<td>2480 ± 70</td>
<td>2020 ± 60</td>
<td>2720 ± 90</td>
<td>4920 ± 230</td>
</tr>
<tr>
<td>3</td>
<td>5720 ± 280</td>
<td>2990 ± 90</td>
<td>2700 ± 90</td>
<td>2990 ± 100</td>
<td>5630 ± 270</td>
</tr>
<tr>
<td>4</td>
<td>6220 ± 400</td>
<td>3090 ± 100</td>
<td>2900 ± 100</td>
<td>3300 ± 120</td>
<td>6120 ± 310</td>
</tr>
<tr>
<td>6</td>
<td>9660 ± 550</td>
<td>3280 ± 100</td>
<td>3220 ± 100</td>
<td>3370 ± 130</td>
<td>6340 ± 340</td>
</tr>
</tbody>
</table>

TABLE II
Incorporation of NaB3H4 into crystallins from a single calf lens

Results are expressed as counts per min/mg of protein content (Lowry's protein determination). Values shown are the mean of duplicate analyses.

<table>
<thead>
<tr>
<th>Source</th>
<th>HMα</th>
<th>α</th>
<th>βH</th>
<th>βL</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>62,500 ± 3,300</td>
<td>9,660 ± 390</td>
<td>4,920 ± 140</td>
<td>8,230 ± 370</td>
<td>7,420 ± 380</td>
</tr>
<tr>
<td>Nucleus</td>
<td>71,200 ± 3,400</td>
<td>12,100 ± 430</td>
<td>6,580 ± 230</td>
<td>12,100 ± 580</td>
<td>11,200 ± 660</td>
</tr>
</tbody>
</table>

Fig. 2. Separation of 3H-labeled α-crystallin (A) and HMα-crystalline (B) hydrolysates by high pressure cation exchange column. A: Upper panel, α-crystallin hydrolysate from a 2-year-old bovine lens (0.41 mg of protein content); lower panel, α-crystallin hydrolysate from a 6-year-old bovine lens (0.91 mg of protein content). The hydrolysate was applied to the column in the starting equilibration buffer (0.2 M pyridinium acetate, pH 3.2) and the elution was run for the first nine fractions followed by a 200-ml linear gradient of 0.2 M pyridinium acetate, pH 3.2, to 1.5 M pyridinium acetate, pH 5.0. Fractions were collected at 2.8 ml/tube/2.5 min. The slight shift of glucitol-lysine peak in the lower panel is due to a delay in the elution gradient. Total radioactivity recovery from the column is about 75% in each case. B: upper panel, HMα-crystallin hydrolysate from a 2-year-old bovine lens (0.51 mg of protein content); lower panel, HMα-crystallin hydrolysate from a 6-year-old bovine lens (0.47 mg of protein content). The elution conditions are the same as in A.
and/or conformational rigidity of lysine residues in these two crystallins. It is known that the amount of HMa-crystallin increases with advancing age of the lens (13). Circular dichroism has revealed that HMa is denatured α-crystallin aggregate (24). The higher incorporation of tritium label in HMa may be due to the combined effects of aging and conformational accessibility of lysine to glucose. The higher radioactivity of HMa is due to nonspecific [3H]borohydride binding. The radioactivity in this peak was fully recovered in the subunits of HMa separated on sodium dodecyl sulfate gel electrophoresis (Fig. 3B). Moreover, following acid hydrolysis, most of the radioactivity could be recovered as glucitol-lysine (Fig. 2B).

Is the nonenzymatic glycosylation of crystallins described here the causative factor for the aggregation of lens proteins in vivo? Two previous reports (3, 25) suggested that the glycosylation of crystallins imparts an increased susceptibility of the crystallins to sulfhydryl oxidation, resulting in the formation of high molecular weight aggregates. However, in bovine lenses, HMa molecules are aggregates of α-crystallin which are engendered by noncovalent interactions (Fig. 3). The greater extent of glycosylation in HMa is probably unrelated to disulfide-linked aggregates. It is most likely that this slow nonenzymatic glycosylation of crystallins is a secondary event rather than the primary cause of the aggregation process with aging. We have also observed that γ-crystallin, the only crystallin with a free NH2-terminal amino acid, has about equal specific activity as those of α and βL-crystallins (Table II), despite the fact that it contains only one-quarter of the lysine residues of other crystallins (13). On the other hand, the specific radioactivity of βL is higher than βH, which is consistent with the finding (26) that βH is a post-translational protein with a shorter average lifetime than βL.

Previous studies (1) indicate that glycosylated hemoglobin formed in vivo is a function of erythrocyte age and plasma glucose concentration. However, as shown in Tables I and III of this study, the extent of radioactivity uptake, a rough index of nonenzymatic glycosylation, seems to correlate with animal age only. The intracellular glucose concentrations of lenses is approximately ¼–½ that of aqueous humor which, in turn, is about equivalent to plasma glucose concentration (Table III). The lens proteins are constantly exposed to glucose during the animal’s lifetime, as opposed to the 120-day exposure of hemoglobin inside the erythrocyte. The decreased susceptibility of crystallins to nonenzymatic glycosylation compared to that of hemoglobin probably reflects differences of conformational accessibility of lysine residues between these two proteins. It appears that it takes a much longer time for α-crystallin to accumulate a 2-fold increase in glucitol-lysine adduct than for hemoglobin to do so (Fig. 2). Similar conclusions were reached by Pandey et al. (27).

The data shown in Fig. 2 provide firm evidence for the presence of glucitol-lysine adducts in native lens crystallins, comparable to our recent demonstration of galactitol-lysine in crystallins of rats fed a high galactose diet (9). In both of these studies, analyses of hydrosyrlates of [3H]borohydride-reduced crystallins...
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Table III

Glucose concentrations in the aqueous humour and lens tissue of normal bovine eyes.

Each value represents the average of triplicate determinations of a single lens.

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Aqueous humor (µmol/ml)</th>
<th>Lens (µmol/g lens water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.98</td>
<td>0.33</td>
</tr>
<tr>
<td>3</td>
<td>3.94</td>
<td>0.80</td>
</tr>
<tr>
<td>4</td>
<td>2.88</td>
<td>0.70</td>
</tr>
<tr>
<td>6</td>
<td>1.60</td>
<td>0.34</td>
</tr>
</tbody>
</table>

* Assuming 65% (w/w) water content in each intact lens.

lens crystallins give unambiguous demonstration of a prominent well-defined radioactive peak having an elution volume identical with that of the glucitol-lysine standard. In contrast, a comparable analysis of diabetic rat lens crystallins by Stevens et al. (3) revealed a heterogeneous pattern that included a peak of low radioactivity, considerably broader than that of the glucitol-lysine standard. We (9) obtained a similar non-specific pattern in nondiabetic rats fed a normal diet. Pande et al. (27) observed a comparable degree of heterogeneity in their analyses of human lens crystallins. Thus, our current work on bovine crystallins provides a more definitive demonstration of the in vivo formation of glucosyl-lysine than the earlier studies in rat (3) and man (27).

We conclude that there is an age-related increase of non-enzymatic glycosylation in normal bovine lens crystallins. It appears to be a general characteristic of the aging process. The possible physiological significance of the effect of glycosylation on the structural integrity of lens crystallins in vivo is currently under investigation. An improved understanding of the chemical and kinetic bases which underlie nonenzymatic glycosylation of proteins may shed light on some of the long term complications of diabetes.

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