

# Glycation of crystallins in lenses from aging and diabetic individuals

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Received 14 September 1992

Water-soluble crystallins were obtained from clear human lenses of different age (4-81-year-olds) and lenses of individuals showing senile or diabetic cataracts. Levels of early glycation products were high in the high molecular weight material (HM) and the  $\alpha$ -crystallin fractions, compared with  $\beta$ - and  $\gamma$ -crystallins. This difference becomes more prominent upon aging. The content of total early glycation products in HM and  $\alpha$ -crystallin increases clearly with age, whereas levels remain relatively constant in the  $\beta$ - and  $\gamma$ -crystallins. There is an elevation of early products in cataractous lenses from diabetic individuals compared with those suffering from senile cataract. Specific non-tryptophan fluorescence (excitation/emission wavelengths 370/440 nm), used as an indicator for late glycation products, increased dramatically with age and was 2-fold higher in the diabetic subjects. Levels of fluorescence decreased in the order HM >  $\alpha$ - >  $\beta$ - >  $\gamma$ -crystallins. The results suggest an increase in glycation rate in  $\alpha$ -crystallin as a result of aging and diabetes, while the rate of glycation of  $\beta$ - and  $\gamma$ -crystallins remains almost constant.

Glycation; Diabetes; Aging; Crystallin; Cataract; Lens

## 1. INTRODUCTION

Glycation, which involves the reaction of a reducible sugar with a free amino protein group, is important in diabetic as well as in aging individuals. Possibly, this feature is the cause of cataract which is a common complication in diabetics [1]. Reducing sugars have been shown to be able to initiate crosslinking of crystallins *in vitro*, partly as a result of disulfide bonds [2,3]. Glycation products are divided into early modification products (this includes the NaBH<sub>4</sub>-reducible Schiff base and the ketoamine or Amadori product) and late glycation products. The latter are characterized by fluorescent properties, sometimes combined with protein crosslinking. We investigated glycation on crystallins from lenses ranging from 4- up to 81-year olds. Early products were determined using radiolabeled NaBH<sub>4</sub>, a common method in glycation research. Advanced glycation products were studied using specific fluorescence as a parameter. Additionally, lenses from senile and diabetic cataractous individuals were used in an attempt to clarify the role of glycation in diabetic cataract.

## 2. MATERIALS AND METHODS

### 2.1. Lenses

Clear human lenses (4-81-year olds) were provided by the Ophthal-

mic Research Institute (Amsterdam). Lenses from subjects with senile or diabetic cataract were obtained from the Eye Clinic of the Free University of Berlin. Lenses were decapsulated and homogenized in phosphate buffer (140 mM NaCl, 3 mM KCl, 8 mM KH<sub>2</sub>PO<sub>4</sub>, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8.1). Clear lenses were used individually, cataractous lenses were pooled into two groups: senile cataract (2 of 77 and 3 of 78 years of age) and diabetic cataract (3 of 76 and 2 of 78 years of age). The insoluble material was removed by a 5-min centrifugation in a Beckman Microfuge B.

### 2.2. [<sup>3</sup>H]NaBH<sub>4</sub> reduction

Samples were reduced by a [<sup>3</sup>H]NaBH<sub>4</sub> solution (Amersham) and were allowed to react for 10 min at room temperature, followed by an incubation of 90 min at 4°C. Unreacted NaBH<sub>4</sub> was removed by extensive dialysis against phosphate buffer (140 mM NaCl, 3 mM KCl, 8 mM KH<sub>2</sub>PO<sub>4</sub>, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.3) until radioactivity in the dialysis buffer was negligible. The labeled proteins were separated using high-pressure gel permeation chromatography.

### 2.3. High-pressure gel permeation chromatography

HPGPC was performed using combined Zorbax 450/250 columns (both 28 cm, DuPont) preceded by a guard column (Bio-Rad). Elution was performed using a high-salt phosphate buffer (20 mM sodium phosphate, 100 mM Na<sub>2</sub>SO<sub>4</sub>, 1 mM EDTA, pH 6.9) with a flow of 0.8 ml/min. Detection was done by measuring the absorbance at 280 nm (Hitachi 100-30 spectrophotometer) and fluorescence (excitation wavelength 370 nm, emission wavelength 440 nm; Perkin-Elmer 204-A spectrofluorometer). The eluting material was collected, and incorporated label was determined by counting 0.6 ml of each fraction using a liquid scintillation counter (2200 Tricarb, Packard). Protein concentrations were determined using the BCA assay (Pierce).

## 3. RESULTS

The content of early glycation products in high molecular weight material (HM),  $\alpha$ -,  $\beta$ - and  $\gamma$ -crystallins are presented in Figs. 1 and 2. In all lenses radioactivity was highest in the HM, immediately followed by  $\alpha$ -crystallin. Incorporation of label in  $\beta$ -crystallins was,

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*Abbreviations:* HM, high molecular weight material; HPGPC, high pressure gel permeation chromatography; BCA, bicinchoninic acid; WS, water soluble; EDTA, ethylene diamino tetra-acetate.

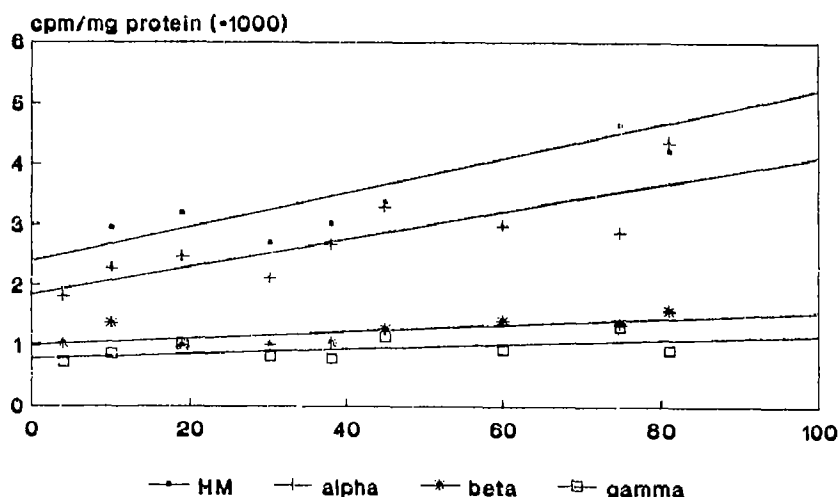


Fig. 1. Incorporation of radioactivity in [ $^3$ H]NaBH $_4$ -treated WS proteins from clear lenses.

roughly, half of that found in  $\alpha$ -crystallin. Radioactivity was lowest in  $\gamma$ -crystallins, slightly below the values found for  $\beta$ -crystallins. For example, the values for the 38-year-old lens were (in cpm/mg): HM 3011,  $\alpha$ -crystallin 2677,  $\beta$ -crystallins 1043,  $\gamma$ -crystallins 778. As a function of age there was a strong increase of early glycation products in both HM and  $\alpha$ -crystallin (slightly less prominent in  $\alpha$ -crystallin). During aging, there was only a slight increase in the content of early glycation products of  $\beta$ -crystallins (Fig. 1). The incorporation of label in  $\gamma$ -crystallins was also almost constant as a function of time, showing slightly lower values compared with  $\beta$ -crystallins. The values for the different crystallin fractions obtained from senile and diabetic cataractous lenses followed the patterns described above. Again, incorporation of label was most prominent in the HM and  $\alpha$ -crystallin (Fig. 2). Levels of radioactivity were clearly higher in lenses obtained from diabetic individu-

als compared with both senile cataractous and clear lenses of similar age (Figs. 1 and 2).

Advanced glycation products were investigated by monitoring fluorescence (excitation/emission wavelengths 370/440 nm): in vitro glycation of crystallins generates fluorophores with these specific wavelengths [3]. Values were expressed as fluorescence per mg protein (as determined by the BCA assay, [4]). The results are presented in Figs. 3 and 4.

With age, we observed a strong increase in specific fluorescence in all crystallin fractions. Fluorescence was highest in the HM material and decreased in the order HM >  $\alpha$ -crystallin >  $\beta$ -crystallin >  $\gamma$ -crystallin. Advanced glycation products were also studied in lenses from patients with senile or diabetic cataract. Senile cataractous lenses showed only slightly higher levels of fluorescence in comparison with clear lenses. However, fluorescence was significantly higher in lenses from individuals with diabetic cataract (Fig. 4). The increase of fluorescence, compared with the lenses with senile cataract, were: HM 108%,  $\alpha$ -crystallin 109%,  $\beta$ -crystallin 83% and  $\gamma$ -crystallin 54% increase.

#### 4. DISCUSSION

Glycation of proteins is considered to be an age-dependent process. However, studies concerning glycation of crystallins do not show consistent results: some investigators report a clear increase of early glycation levels with age [5,6] while others find only minor differences between young and older material [7,8]. We investigated not only water soluble (WS) protein fractions from lenses of different age, but also discriminated between different crystallin fractions. HM and  $\alpha$ -crystallin showed a clear increase in early glycation levels with

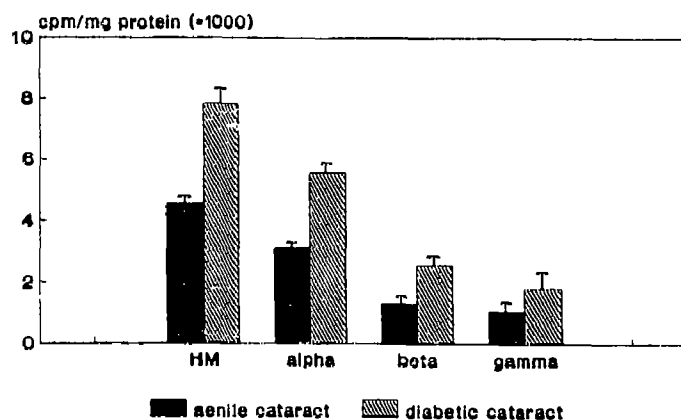


Fig. 2. Incorporation of radioactivity in [ $^3$ H]NaBH $_4$ -treated WS lens proteins of individuals with senile or diabetic cataract ( $\pm$  S.D.).

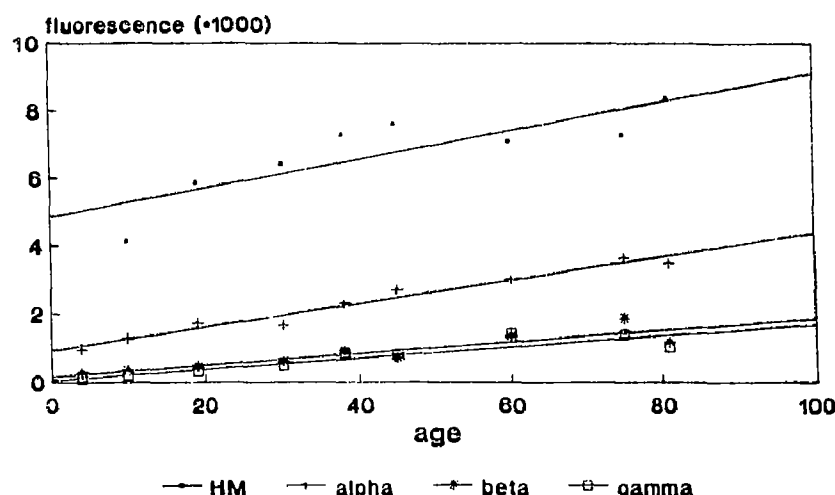


Fig. 3. Specific fluorescence (excitation/emission wavelengths 370/440 nm) of WS proteins from clear lenses.

age, while levels in  $\beta$ - and  $\gamma$ -crystallins remained relatively constant (Fig. 1). HM consists of a considerable amount of  $\alpha$ -crystallin. We therefore suggest a prominent role for  $\alpha$ -crystallin when considering glycation of eye lens proteins. During experimental procedures, the proteins with a relatively high molecular weight show a tendency to aggregate and precipitate. When not handled carefully, protein samples easily lose HM and  $\alpha$ -crystallin, and become enriched in  $\beta$ - and  $\gamma$ -crystallins. Especially old and cataractous lenses are characterized by rapid loss of high molecular weight WS proteins. This could explain the slight increase in early glycation products in old material observed by some investigators [7]. Levels of early and advanced glycation products were significantly elevated in lenses from diabetic patients, compared with clear and cataractous lenses obtained from individuals of comparable age (Figs. 1–4). Remarkably, early glycation products hardly increased in  $\beta$ - and  $\gamma$ -crystallins during aging, while fluorescence increased more prominently (Figs. 1 and 3). Because early glycation products are only the first step in a long series of reactions, the reducible Amadori product and Schiff base can be considered as indicators of the rate of glycation, while the advanced glycation end products represent the final levels of glycation. This interpretation indicates that the glycation rate in  $\beta$ - and  $\gamma$ -crystallins remains constant as a function of age, whereas the glycation rate of  $\alpha$ -crystallin (and eventually HM) speeds up during aging. The  $\alpha$ -crystallin molecule probably first undergoes, in the course of time, conformational changes which make lysine residues more accessible for glycation. These changes could be initiated by early glycation or other age-dependent protein modifications, resulting in changes in the tertiary structure of the molecule. Conformational changes are more relevant in molecules with a high molecular weight, such as

$\alpha$ -crystallin. These findings are confirmed by in vitro glycation of crystallins with glucose, showing a rapid initial glycation of  $\gamma$ -crystallins. On the other hand, in vitro glycation of  $\alpha$ -crystallin started slowly and was followed by a steep increase [9]. While investigating glycation of bovine  $\alpha$ -crystallin subunits we found a preferential glycation of  $\alpha A$  compared with  $\alpha B$  chains [10]. Remarkably, post-translational modifications (as shown for oxidation and racemization in  $\alpha$ -crystallin) do not occur *at random* at all potential sites in the protein chain, but take place at preferential residues [11,12]. These preferential sites are probably, because of their location and micro-environment, more susceptible to modification. The presence of highly glycation-sensitive residues could explain the preferential modification of  $\alpha A$  subunits. Our investigations suggest a prominent role for  $\alpha$ -crystallin as far as glycation is concerned. Reaction of crystallins with reducing sugars, intensified by other modifications, could easily lead to conforma-

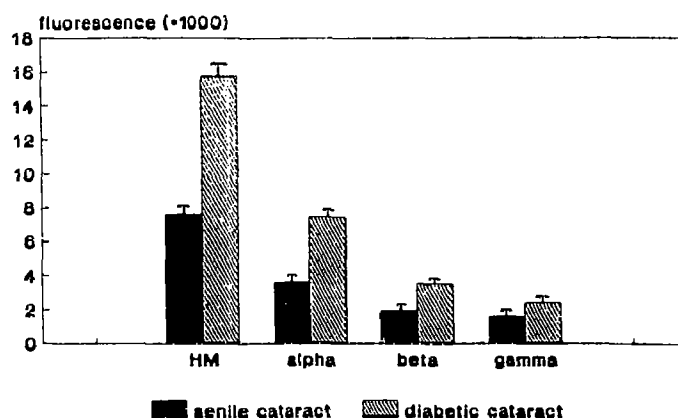


Fig. 4. Specific fluorescence (excitation/emission wavelengths 370/440 nm) of WS lens proteins from individuals with senile or diabetic cataract ( $\pm$  S.D.).

tional changes, crosslinking and aggregation, which have shown to be the initiating processes leading to cataract [13].

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