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SUGAR-INDUCED CHANGE IN NEAR ULTRAVIOLET CIRCULAR DICHROISM OF α-CRYSTALLIN

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Summary In order to study the effect of sugar molecules on the structure of α-crystallin, the protein from bovine lenses was isolated and treated with D-glucose and D-glucose-6-phosphate. Structural studies of the products were carried out using circular dichroism (CD) measurements. In the near-ultraviolet (UV) region, three positive and two negative CD bands are observed for α-crystallin. Upon incubation with the saccharides, the positive ellipticities of these near-UV CD bands increase greatly. The CD spectrum of α-crystallin in the far-UV region, which is typical of the β-conformation of a protein, does not change. The increase does not appear to be caused by glycosylation, or by sugar metabolites such as sorbitol or glycolytic products. When the concentration of sugar is higher than that has been used in this study, the protein tends to aggregate. The results strongly suggest that sugar, upon interaction with α-crystallin, induces a change in the tertiary structure of the protein while its secondary structure remains intact. The interaction of sugar molecules causing such change is weak, noncovalent, and temperature-dependent. The near-UV CD of α-crystallin appears to be a useful probe for the study of such structural changes, which may be significant in the pathogenesis of cataract.

Introduction α-Crystallin, one of the major structural proteins of the lens, is composed of approximately 50 subunits and has a weight average molecular weight of about 1 x 10^6 (1). In the native form, α-crystallin and other components are believed to interact and to maintain specific conformation. α-Crystallin, separated from other components by the chromatograph method, has been found to exist in an ordered form, mainly β-conformation (2-4).

Many mechanisms are involved in cataract formation, including formation of high-molecular-weight protein (5), disulfide bonding (6), nondisulfide protein cross-linking (7), and an increasing amount of blue fluorescent α-crystallin (8). It is not known, however,

The abbreviations used are: CD, circular dichroism; UV, ultraviolet; G-6-P, D-glucose-6-phosphate; TMG, 3,3-tetramethylene glutaric acid.

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whether these processes are followed by conformational change of the lens protein under conditions of high sugar level, radiation, or metabolic disorders. In vitro experiments show that incubation of lens protein with glucose causes opacification (9).

Two possible approaches to the etiology of the sugar-induced opacification include glycosylation of lens proteins and the effect of enzymatically reduced sugar, polyol, on the hydration behavior of the lens. Glycosylation of lens proteins has been suggested as one of the causes of cataract (9,10). From the results of in vitro and in vivo experiments, Stevens et al (9) reported that nonenzymatic glycosylation leads to an opacity in lens crystallins. They suggested that glycosylation may cause protein unfolding, resulting in unmasking of the sulfhydryl groups that would lead to an increased susceptibility of the proteins to sulfhydryl oxidation and would then result in aggregation of proteins. This assumption is supported by Harding's observation (11) that the protein thiol groups have higher reactivity in a cataractous lens than in a normal lens; he suggested that this might be the result of protein unfolding during cataractogenesis. Controversies arose when two independent studies found no correlation between the extent of glycosylation and cataract formation (12) or the amount of disulfide formation (13). Chiou et al (14) also reported that glycosylation might not be responsible for the sugar-induced cataract.

When the lens is exposed to a high level of sugar, polyol is formed through the reduction of sugar by aldose reductase (15). Polyol accumulation, which is accompanied by increases in lens hydration and in total electrolytes, leads to osmotic swelling. The development of sugar cataract by means of the osmotic mechanism has been well accepted.

Our primary objective in this study is to determine whether sugar changes the structure of lens protein. Protein aggregation or polymerization has been suggested to occur in the high-molecular-weight α-crystallin, which contains 3% glucose (16,17). Sugar-binding to proteins is common (18-22). Saccharides, on binding to protein, have been reported to cause a change in its conformation that is detectable by CD (19-22). In most cases, interaction of proteins with such ligands affected the tertiary structure of the proteins. In the present study, we investigated CD properties of α-crystallin

upon incubation with sugar; the results strongly suggest a sugar-induced change in the tertiary structure of the protein as manifested by large changes in the near-UV CD.

Materials and Methods α-Crystallin was isolated from bovine lens by gel filtration as described by Li and Spector (23). Fresh bovine eyes from a local slaughterhouse were decapsulated and then homogenated in an equal weight of Tris buffer by stirring until two-thirds of the outer lenses were dissolved. (All steps were performed at 1-4°C.) The resulting suspensions were centrifuged at 2700 g for 15 min. The supernatant was applied to a Bio-gel A-5m column (1.5 x 50 cm) equilibrated with a buffer containing 0.01 M Tris, 0.1 M KCl, 1.0 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6, and eluted with that buffer. The crystallins collected from the column were dialyzed exhaustively against distilled water, lyophilized, and stored in a freezing room. The protein solutions were made by dissolving samples in Tris buffer; the concentrations were determined by the Lowry method (24).

α-D(+) Glucose and α-D-glucose-6-phosphate of high purity were purchased from Sigma.

Protein solutions in 0.05 M Tris buffer, with or without sugar, in screw-cap tubes were added to 0.02% Na-azide to prevent growth of microorganisms. The tubes were tightly sealed and incubated at 37°C in the dark.

D-Glucose-6-phosphate was determined by oxidation of glucose-6-phosphate (G-6-P) to 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase as described by Lang and Michal (25).

Lactate or pyruvate was measured by enzymatic reaction according to the method of Henry (26).

Sorbitol pathway was tested by using the aldose reductase inhibitor, 3,3-tetramethylene glutaric acid (TMG), as described by Kinoshita et al (27).

To measure the degree of nonenzymatic glycosylation, some of the incubated proteins were reduced by borohydride reaction as described by Bookchin and Gallop (28). Incubated samples were reacted with excess $NaBH_4$ for 30 min at room temperature. The samples, both unreduced and reduced by $NaBH_4$, were then dialyzed extensively against distilled water. The glycosylation was estimated by the trichloroacetic procedure of Fluckiger and Winterhalter (29), which measures 5-hydroxymethylfurfural released upon hydrolysis of ketoamine adducts of protein. The ketoamine adducts in the samples reduced by $NaBH_4$ exist in a nonreactive form and can be used as a blank. One milliliter of reduced or unreduced samples was hydrolyzed by mixing with 0.5 ml 0.3 N oxalic acid and heated for 1 hr in a boiling water bath. After cooling to room temperature, 0.5 ml of 40% trichloroacetic acid was added and the resulting precipitate removed by filtration. After reaction with thiobarbituric acid, absorption at 443 nm was read.

CD spectra were measured in a Cary 60 spectropolarimeter with CD attachment. Cell pathlength of 1 cm was used in the near-UV region and 0.1 or 0.05 cm in the far-UV region. Molar ellipticity $[\theta]$ values per residue were computed using 130 as the mean residue weight, and were expressed as deg-cm^2 dmol^{-1}.

The scattering measurements were performed in Perkin-Elmer MPF 44F fluorometer at 37°C. The time-dependent 90° angle scattering was recorded at 400 nm, the wavelength of both excitation and emission.

Results. Various crystallins were separated on an agarose column. A typical elution profile is shown in Fig. 1. Four major peaks (fractions 2, 3, 4, 5) were identified as α-, β_H-, β_L-, and γ-crystallin, respectively (16,30,31). Peak 1, which appears in the void volume of the column, corresponds to high-molecular-weight α-crystallin (30,31). Throughout this study, peak 2 fractions were used.

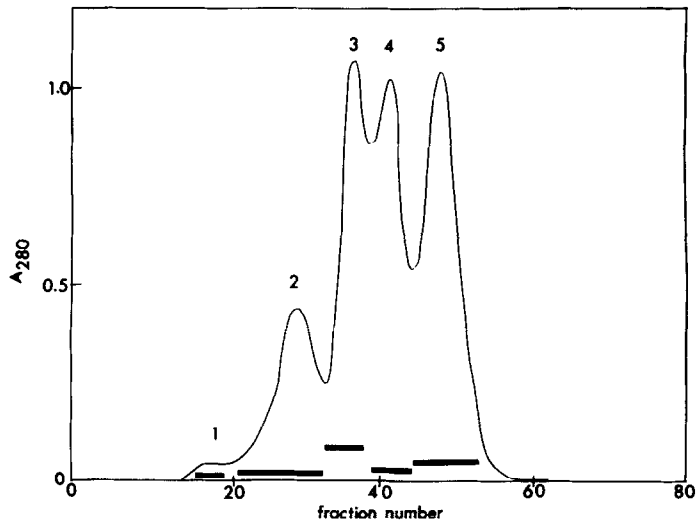

Fig. 1. Gel filtration of lens protein on Bio-gel A-5m column (1.5 x 5.0 cm). The elution buffer is 0.01 M Tris, 0.1 M KCl, 1.0 mM EDTA, pH 7.6. 1.7 ml was collected for each Fraction. Void volume appears in Fraction 18. Crystallins were pooled as indicated by horizontal bars.

CD spectra of α-crystallin in the far-UV and near-UV regions (Fig. 2) are similar to those that have been reported (32). In the far-UV region, there is a negative band centered at 217 nm and a positive band at 197 nm (Fig. 2a). The $[\theta]$ values of these two bands are -5,690 and +13,000, respectively. Using poly-L-lysine as a standard (33), the β-form was estimated to contribute about 50% of the total conformation.

In the near-UV region, the CD shows three positive bands, at 258, 265, and 274 nm, and two negative bands, at 286 and 293 nm (Fig. 2b). The near-UV CD bands are contributed by aromatic side chains, phenylalanine, tryptophan, and tyrosine, and by the disulfide. In α-crystallin, since no disulfide was present (34), all CD bands in the near-UV region arise from the vibronic vicinal effect of the aromatic chromophores (35).

The near-UV CD bands do not change when the pH is between 6 and 10. Above pH 10, however, the positive bands diminish and the negative bands disappear. In addition, a new positive band appears around 253 nm. In an alkaline solution, the tyrosine residues are ionized and the CD undergoes a change similar to that observed in poly-L-tyrosine (36); the far-UV CD indicates that disordered conformation is dominant.

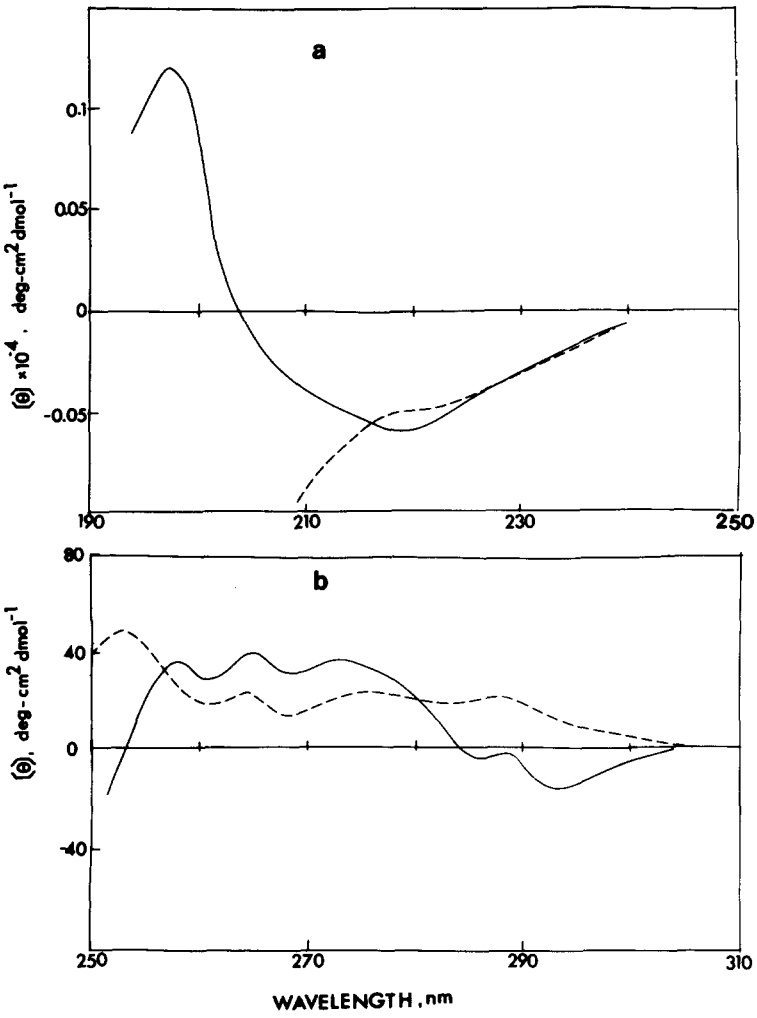

Fig. 2. CD spectra of α-crystallin. (a) Far UV-CD of α-crystallin in 5 mM Tris buffer, pH 8.40 (——), 11.8 (----); protein concentration, 0.16 mg/ml; cell pathlength, 0.5 mm. (b) Near UV-CD of α-crystallin in 5 mM Tris buffer, pH 8.40 (——), and in 0.01 M Na_2HPO_4, pH 11.8 (- - -). Protein concentration, 1.6 mg/ml; cell pathlength, 10 mm.

Addition of glucose or G-6-P to α-crystallin and incubation at 37°C cause the near-UV CD to increase greatly. The change is more effective with G-6-P than with glucose. Both glucose and G-6-P lack any near-UV absorption, and CD changes induced by sugar are attributed to protein CD bands. Fig. 3a shows the increase of CD of α-crystallin due to G-6-P. The addition of G-6-P without incubation caused no increase in CD: the change is detectable after 3 hr of incubation and after 24 hr CD intensity

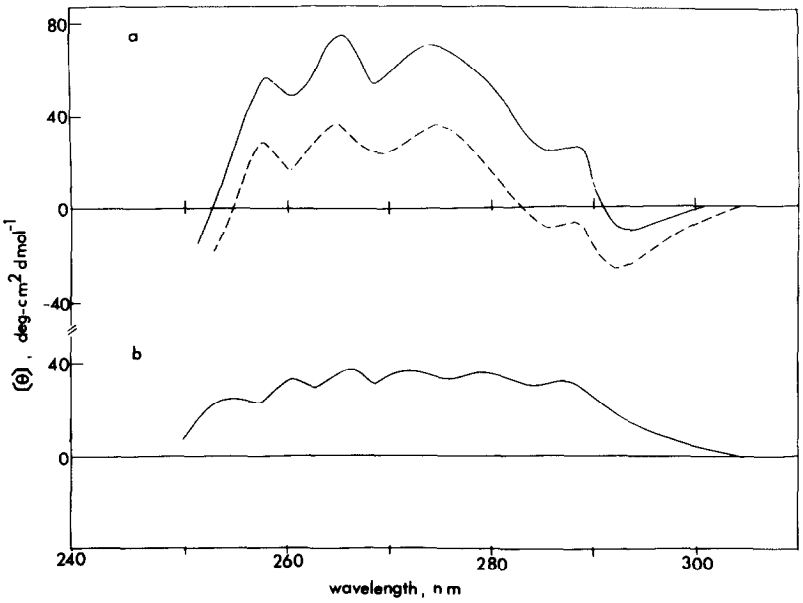

Fig. 3. (a) Near-UV CD increase of α-crystallin caused by incubation with (——) and without (-----) G-6-P. Protein concentration, 2.14 mg/ml; G-6-P, 12 mM; in 0.05 M Tris buffer, pH 8.40. Samples were incubated at 37°C for 2 days. (b) Difference CD spectrum of α-crystallin incubated with and without G-6-P.

almost doubled with no further change upon longer incubation (Fig. 4). The absence of any scattering increase, shown also in Fig 4, indicates that in this duration of incubation no major aggregation occurs. The sugar-induced CD increase is less prominent when incubation is at a lower temperature; there is no change at 4°C, but as the temperature rises from 4° to 37°C there is a gradual CD increase. The CD increase is also dependent on the amount of G-6-P added. However, at higher sugar concentration (>20 mM), increased light scattering can be observed in parallel to CD change. At much higher sugar concentration (>50 mM) CD loses its fine structure.

In contrast, the far-UV CD remains the same after incubation with G-6-P (not shown), indicating that the secondary structure is not affected.

Glycosylation is usually tested by using sugars containing radioactive isotopes such as $\left[{}^{14}C\right]$ and $\left[{}^{3}H\right]$ and measuring the amount of radiolabelled substrate in protein. However, a recent study (37) indicates that radioactive impurities also simulate glycosylation. We have used the thiobarbituric acid procedure (29) to detect and

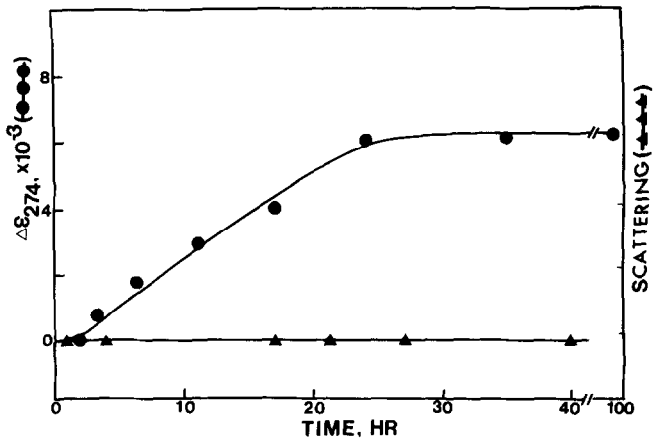

Fig. 4. CD and scattering changes with incubation time. The band at 274 nm was chosen arbitrarily for the plot of CD (●-●). Aliquots of samples were taken out periodically for CD measurements. Conditions were the same as described in Fig. 3 legend. The scattering was recorded at 400 nm of both excitation and emission wavelength. The change of scattering (▲-▲) were plotted as difference between sample and control.

estimate glycosylation. About 15 times more glycosylated proteins were found in the α-crystallin incubated with G-6-P than in the control.

The difference CD spectrum (Fig. 3b) clearly shows seven bands between 250 and 310 nm. Their positions are at 254, 262, 267, 272, 279, 287, and 295 nm. The 287 and 295 nm bands are readily assigned to transitions of tryptophan (35,38). The three lower peaks (254, 262, and 267 nm) can be assigned to the phenylalanine chromophore. The remaining two bands (272 and 279 nm) arise from tyrosine and overlap those from tryptophan (35, 38, 39). The difference spectrum indicates that upon interaction of sugar molecules, disulfide bond formation cannot be detected. CD bands of disulfides usually begin at long wavelengths (320 to 350 nm) and gradually intensify to give one or two broad bands at 240 nm (35).

Glucose-6-P determination shows that there was no continuous decrease of sugar during incubation or appearance of lactate and pyruvate. The sugar-induced CD change remains unaffected in the presence of TMG.

Discussion Several possibilities for the observed changes in the tertiary structure in the presence of sugar have been considered:

Nonenzymatic glycosylation — Sugar-induced change in the CD disappears upon dialysis of the sample whether or not it was reduced by $NaBH_4$. The CD enhancement cannot be observed by adding more sugar unless the sample is incubated again. This indicates that the CD increase is not directly or indirectly due to glycosylation of the protein. However, it does not eliminate the possibility that glycosylation causes some change in the microenvironment of the protein. Since the glycosylation reaction involves only the ε-amino group of lysine and the aldehydric group of sugar, its extent may be too low for detection by CD.

Sorbitol pathway — Even if we assume that some aldose reductase may be contaminating our α-crystallin preparation, the fact that the sugar-induced CD change remains unaltered with prior treatment of aldose reductase inhibitor TMG rules out sorbitol formation.

Adventitious enzymes — The amount of G-6-P decreases when incubated with α-crystallin, and the decrease is proportional to the amount of α-crystallin. The decrease, however, is not continuous with time and no lactate or pyruvate can be detected even after prolonged incubation. Thus the possibility of glycolysis can be safely excluded. The reduction of G-6-P can be accounted for by glycosylation of the protein.

Microorganism growth — This has been prevented by adding the common bacteriostat, sodium azide, before incubation.

It appears, therefore, that the CD increase in the near-UV region is caused by a weak binding of the added sugar to α-crystallin. The saccharide-induced effects are observed only in the region where aromatic chromophores display their Cotton effects. It is interesting to note that the change is very similar to that observed for the binding of N-acetylglucosamine to lysozyme (22). The aromatic transitions may gain or lose CD intensity through vibronic vicinal interactions (μ-μ coupling) that do not affect the backbone peptide chromophores (35). Such interactions are possible when sugar-binding changes the environment and/or orientation of the chromophore groups, which in turn changes the tertiary structure of the proteins.

Since we have not observed the time-dependent scattering increase and the protein concentration dependence of molar ellipticity values, and since the decrease in

CD upon dilution from the maximum CD change is perfectly linear with protein concentration, the change in tertiary structure rather than protein aggregation may be considered as the primary process when sugar interacts with the protein. However, the scattering measurements may not be sensitive enough to detect any dimer formation which is also related to some alterations in the tertiary structure often manifested in the near UV-CD (40). Moreover, protein aggregation in most cases follows a CD decrease (41), particularly in the far-UV region, which we have not observed. However, with a high concentration of sugar, aggregation of protein results, but it is likely to be a secondary step following this initial conformational change. Preliminary results (42) with other crystallins show similar increase in the near UV-CD bands. Physico-chemical studies such as sedimentation equilibrium and column chromatography are in progress to elucidate the nature of aggregates formed upon interaction with sugar.

To our knowledge, no such noncovalent interaction of sugar molecules with the lens protein has been reported. It appears that the lens crystallin tertiary structure change due to sugar-interaction is unique, in that other proteins, such as ovalbumin (chick egg albumin) or hemoglobin, did not show such CD increase when it was incubated with G-6-P. It is too early to speculate that the sugar-induced change in the tertiary structure may eventually lead to the formation of cataract, but the sugar binding appears to be quite extensive, and the resultant conformational change is significant enough to warrant further investigation.

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