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ON THE STABILITY OF BOVINE GAMMA II CRYSTALLIN

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Bovine gamma II crystallin, undergoes structural changes when lyophilized. The lyophylized powder does not readily dissolve in buffer, although it can be taken up in distilled water. The lyophylized sample, as opposed to a sample concentrated by vacuum distillation at 30°C, does exhibit different migration on isoelectric focusing gels. The hydration and denaturation properties of the two preparations are different. The lyophylized sample possesses a higher non-freezable water content as a function of concentration than its counterpart. The lyophylized sample also shows less denaturation in differential scanning calorimetry scans, up to 110°C, than its counterpart. This indicates that lyophylization may induce a slight denaturation, due to structural-conformational change. On the other hand, the CD spectra of lyophylized and non-lyophylized gamma II crystallins do not differ significantly. This implies that the conformational changes upon lyophylization do not involve the secondary structure of gamma II crystallin.

Gamma crystallins are the monomeric, about 20kD, components of the structural proteins of the lens of the eye, commonly called crystallins (1). They are unevenly distributed in mammalian lenses, largely concentrating in the nucleus (2,3). It has been alleged that gamma crystallins are cryoproteins (4,5) and mainly responsible for the cold cataract formation observed in young animal lenses, reported most often in calf lenses (6,7). Gamma crystallins are isolated from lens homogenates by gel permeation chromatography and ion-exchange chromatography (8,9). After isolation the samples are often lyophylized and stored in the powder form. We made the chance observation that gamma II crystallin does not dissolve completely in buffer after lyophylization. This would indicate that lyophylization itself may cause some conformational change and thus influence the studies on

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the properties of gamma II crystallins obtained by lyophylization. The following study was designed to shed some light on the changes induced by lyophylization.

MATERIALS AND METHODS

Frozen fetal bovine eyes were obtained from Pel-freez Biologicals, Rogers, AS. After the eyes were thawed, the lenses were removed, decapsulated and homogenized in 50 mM Hepes buffer, pH 7.6, containing 200 mM KCl. The homogenate was centrifuged at 27,000 x g for 30 min. and the pellet was discarded. The low molecular weight fraction containing the gamma crystallins was obtained by gel permeation chromatography using a Sephacryl 200 column (2.5 x 100 cm). The individual gamma crystallins were separated by ion exchange chromatography (10). A portion of the samples was dialyzed against distilled water and lyophylized. The remainder was stored at 4°C until used.

Dilute solution of the isolated gamma II crystallin was dialyzed against three changes of distilled water at 4°C for 48 hours. The dilute solution was also concentrated to about 30 % (w/V) by vacuum distillation maintaining the temperature of the residue at 30°C.

Lyophylized samples were prepared for isoelectric focusing by redissolving in distilled water or in 200 mM sodium acetate buffer, pH 5.0, or in 75 mM Tris-HCl buffer, pH 9.0. The samples were centrifuged to remove protein that did not go into solution. Isoelectric focusing was done on a Pharmacia Fast Electrophoresis Apparatus with IEF gel, pH 3 to 9 for 500 aVh. Circular dichroism spectra were taken on a JASCO J-500C Spectropolarimeter. The pathlength was 1 cm for the near UV and 1 mm for the far UV range.

Samples for hydration studies were prepared by diluting the stock solution (30 %) to the desired concentrations using distilled water at pH 7.0.

Hydration studies were performed by a combination of differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). Five to ten mg samples were hermetically sealed into preweighed aluminum sample pans. An empty sealed pan served as reference. The freezable water content of the sample was obtained in a DSC apparatus (DuPont 990; DuPont, Wilmington,DE) first by cooling the sample to -30°C, maintaining it at this temperature for 30 minutes and finally heating it to 30°C at a programmed rate of 3°C/min. The DSC curves obtained, differential heat flow versus temperature, were integrated to obtain the heat used to melt the frozen water (11,12). From this the freezable water content of the sample was calculated.

The total water of the sample was obtained in the TGA part of the instrument. The sample pan was punctured and the weight loss was recorded on a Cahn balance while the sample was heated from 30 to 105°C (12). The non-freezable water content was obtained from the difference between the total and freezable water content and recalculated as % of the total water.

Denaturation studies were run in the DSC apparatus on hermetically sealed samples between 30°C and 100°C.

RESULTS AND DISCUSSION

The results of the isoelectric focusing of freshly prepared and lyophylized gamma II crystallin are shown in Figure 1. Fresh preparations of gamma II crystallin focus on these gels as a single very basic species. Samples that have been lyophylized, regardless of whether they are redissolved in distilled water or buffer, appear as several species. Some of the new species are more basic than the sample that was not lyophylized and some are more acidic. These results indicate that the surface charge of the gamma II crystallin is changed after lyophylization. This can be due to formation of several discrete aggregated forms, or to changes in conformation states.

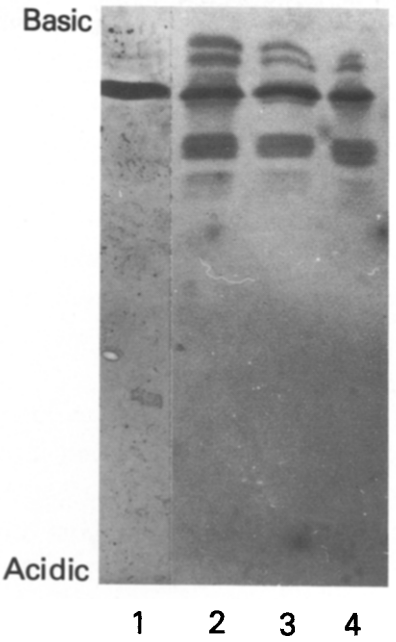

Figure 1. Isoelectric focusing of gamma II crystallin (1) before and (2,3,4) after lyophylization. The lyophylized samples were redissolved (1 mg/mL) in (2) distilled water, (3) 200 mM sodium acetate buffer, pH 5.0,and (4) 75 mM Tris-HCl buffer, pH 9.0.

The hydration properties of the two gamma II crystallin preparations are given in Figure 2. The hydration parameter calculated here is given in terms of the non-freezable water

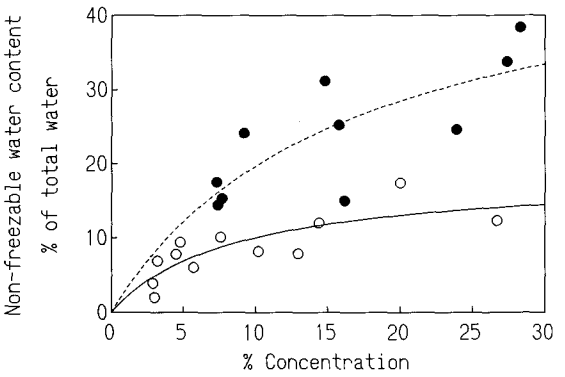

Figure 2. Non-freezable water content (as % of total water) of gamma II crystallin O———O non-lyophylized and ●------● prepared by lyophylization as a function of concentration.

content percent of the total water content. The non-freezable water content can be identified with the bound or partially bound water that is incapable to undergo first order transition. Its absolute value depends on the method of investigation (NMR, DSC-TGA, near IR etc.) (13). However, the concentration dependence shows the same trend irrespective of the method employed. The lyophylized gamma II crystallin sample shows much higher bound water content than the not lyophylized sample. It is also apparent that the spread of the experimental points in this sample is much greater than in its counterpart.

One could interpret this result that the gamma II crystallin underwent some aggregation process during lyophylization and the aggregates can trap more water in the interstices in the form of non-freezable (immobilized) water. We have seen similar enhanced non-freezable water content upon polymerization when the hydration properties of G- and F-actins were compared (14). It is interesting to note that the low molecular weight (LMW) fraction of crystallins which contains the gamma fractions beside others, shows similar hydration behavior to that of the non-lyophylized gamma II sample. The non-freezable water content of LMW crystallin is only slightly higher than that of the non-lyophylized gamma II (15). This happened inspite the fact that the LMW crystallin was prepared by lyophilization. Therefore, it seems that the presence of other proteins beside gamma II prevents the self aggregation of this cryoprotein. This is also evident from the solubility behavior of LMW preparation which dissolves in buffer easily and does not show high molecular weight residues in SDS-gel electrophoresis.

An indication of what may have happened during lyophylization of the gamma II crystallin can be gained from the denaturation scans given in Figure 3. The upper DSC scan curve in Fig.3 is that of the non-lyophylized gamma II sample. The lower DSC scan represents the lyophylized sample. The main difference can be seen in the 80-95°C range. The non-lyophylized sample in this range undergoes a slight denaturation that consumes 113 kcal/mol endothermic heat. In contrast, the lyophylized sample, although exhibiting a few fluctuations in the DSC scan in this range, has a heat consumption (the integrated are under the curve) that corresponds only to 62 kcal/mol. These heats of denaturation are of the same magnitude as reported by Sen et al. (16) albeit in different buffer and hence at somewhat different temperatures. The decrease in the endothermic heat implies that the lyophylized

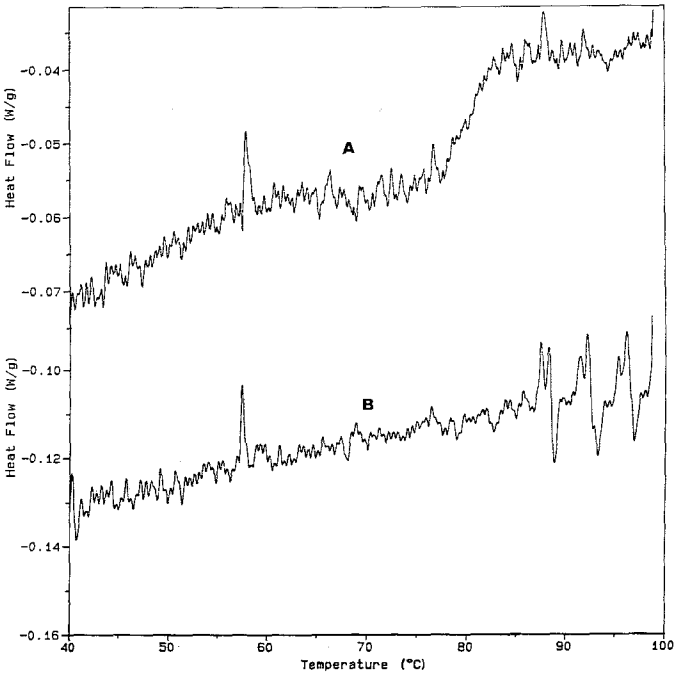

Figure 3. Differential calorimetric scans during denaturation of gamma II crystallin samples between 30 and 100°C. A. non-lyophylized sample; B. lyophylized sample.

sample is already denatured somewhat. This interpretation gains strength if we take into account that when partially denatured gamma II crystallin samples are scanned the second time around by DSC the resultant integrated area between 80-and 95°C yields approximately the same heat 80-97 kcal/mol for both samples.. Thus, the partially denatured gamma II crystallin suffered conformational changes that may be comparable in magnitude to that of the lyophylized gamma II crystallin.

No conformational change in the secondary structure upon lyophylization can be seen in the CD spectra of the two preparations. The near UV and the far UV spectra of the two samples exhibited no significant differences.

The importance of these observations are two fold: For one it implies that the more purified a gamma II preparation is, the more prone it is to suffer conformational changes due to temperature and concentration gradients; second one must reevaluate the data obtained in the literature on gamma II crystallins especially those that have been lyophylized.

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