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Hydration Properties of the Molecular Chaperone α-Crystallin in the Bovine Lens

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Abstract—Topographic studies of crystalline fractions from different morphological layers of the young adult bovine lens were conducted. Crystallin profiles were obtained for each lens layer, using thin-layer isoelectric focusing in polyacrylamide gel (IEF). Water soluble (WS) crystallins from the lens equator revealed a separation into HM (high molecular weight) α_L-, β_H-, β_L-, β_S-, and γ-crystallins. The nature of the water insoluble (WI) protein fraction in the separated lens layers reflected the aggregated state of α_L-, β_L-, β_S-, and γ-crystallins in different regions of the lens, concealed in the central cavity of the α-crystallin chaperone model. The IEF data demonstrate a possible chaperone-like function for α-crystallin in the nucleus and inner cortex of the lens, but not in the outer cortex. The water binding properties of bovine lens α-crystallin, calf skin collagen, and bovine serum albumin (BSA) were investigated with various techniques. The water adsorptive capacity was obtained in high vacuum desorption experiments volumetrically, and also gravimetrically in controlled atmosphere experiments. The NMR spin–echo technique was used to study the hydration of protein samples and to determine the spin–spin relaxation times (T_2) from the protons of water adsorbed on the proteins. Isolated bovine lenses were sectioned into 11-12 morphological layers (from anterior cortex through nucleus to posterior cortex). The water content in relation to dry weight of proteins was measured in individual morphological lens layers. During water vapor uptake at relative humidity $P/P_0 = 0.75$, α-crystallin did not adsorb water suggesting that hydrophobic regions of the protein are exposed to the aqueous solvent. At relative humidity $P/P_0 = 1.0$, the adsorption of water by α-crystallin was 17% with a single component decay character of spin echo ($T_2 = 3$ msec). Addition of water to α-crystallin to about 50% of its weight/weight in the protein sample showed $T_2 = 8$ msec with only one single component decay of the spin–echo signal. The single component decay character of the spin echo indicates water tightly bound by α-crystallin. Under a relative humidity $P/P_0 = 1.0$, collagen and BSA adsorbed, correspondingly, 19.3 and 28% of water and showed a two-component decay curve with T_2 about 5 and 40 msec. The findings demonstrate the presence of two water fractions in collagen and BSA which are separated in space. The IEF data suggest a tight binding of water with α-crystallin with similar distribution patterns in the lens layers. To conclude, it was found that α-crystallin can immobilize water to a greater extent than other proteins such as collagen and BSA. These results shed new light on structural properties of α-crystallin and its superhydration properties and have important implications for understanding the mechanism of the chaperone-like action of this protein in the lens and non-ocular tissues.

Key words: NMR spin–echo, bovine lens, chaperone, α-crystallin, isoelectric focusing, protein hydration, superhydration

The disciform eye lens continues to grow throughout life. The fresh fibers, developing by elongation from cubic anterior epithelial cells, are continually laid down in the equatorial zone. The older cells in the anterior and posterior cortex and supra-nuclear cortex gradually lose their nuclei and become more and more compressed in the center or nucleus of the lens. The water content in the nucleus is the lowest and in the cortex the highest of the lens. Consequently, the embryonic nucleus is the oldest part of the lens, as old as the animal itself [1]. The whole lens consists of about 65% water and 35% proteins. The lens has a high refractive index and high protein content, necessary for focusing light onto the retina. These structural proteins are called

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"crystallins", and they form most of the dry weight of the lens [2].

Transparency of the normal eye lens depends on the short range order that exists in the supramolecular organization, which results in interference effects [3-6]. Cataract formation or opacity, on the other hand, is enhanced by two cytoplasmic factors: 1) an increase in the size of the scattering units, i.e., crystallin aggregates [3], and 2) an increase in the refractive index difference between the scattering units and their environment [6, 7]. The conformational change during cataractogenesis leads to the formation of aggregates, which scatter light to produce opacification [2, 8]. For that reason, an aggregation process was proposed as the main contributor to cataractogenesis [9]. Another way to increase opacity is to increase the amplitude of the density fluctuations, which means an increase in the refractive index difference between the scattering unit (protein moieties) and its environment. The process by which such change in the refractive index difference can be brought about is called syneresis [10]. During syneresis, changes in secondary, tertiary, or quaternary structures of crystallins cause the release of water from the bound state (hydration layers) into the bulk. It must be emphasized, however, that this syneresis process is independent of aggregation during cataract formation [7]. Thus, it is important to know the hydration properties of the major structural proteins of the lens and how the hydration behavior may change with differing supramolecular organization of the lens crystallins [11].

In the lens, α-crystallin is one of the most abundant protein species of the crystallins, representing up to 35% of the total protein. It is also the largest, existing as an aggregate with an average molecular mass of around 800 kD [2]. α-Crystallin is composed of two homologous subunits, designated αA (acidic) and αB (basic), with molecular weights of 19,832 and 20,079 daltons, respectively. Because the X-ray crystallographic structure of α-crystallin has not yet been determined, several models have been proposed for the complex including a three-layered structure [12], a micellar structure [13], a combination of micellar and layer model [14], a rhombic dodecahedron model [15], and a cavity-like structure [16] similar to the structure of the chaperone GroEL [17]. The cavity-like structure is of superior interest because α-crystallin has demonstrated molecular chaperone activity, preventing the aggregation of denatured (or partially denatured) proteins [18]. This hypothesis known as Carver's model [16] represents more than just one among equals. The reason is that the structure and immunological determinants of small crystallins like β_S- and γ-crystallins, remain intact during the ageing process [19]. The latter crystallins of lower molecular weight sterically fit into the central cavity with a diameter of 53 Å [19]. Several authors have observed chaperone activity around 30°C and it steeply increased between 30 and 60°C, correlating with exposure of hydrophobic surfaces of the α-crystallin, as determined by a number of biophysical techniques [20-22]. It has been proposed that hydrophobic interactions play a major role in the chaperone activity of α-crystallin.

Our recent topographic studies of crystallin fractions from young adult bovine lens, using a frozen-sectioning technique followed by thin-layer isoelectric focusing in polyacrylamide gel, revealed that lenses do not have a homogeneous distribution of crystallins and water, although there are gradual differences in various morphological layers (from anterior cortex through nucleus to posterior cortex) [23]. Under nondenaturing conditions, the same study demonstrated chaperone activity of α-crystallin in separate lens layers, reflecting the aggregated state of α_L-, β_L-, β_S-, and γ-crystallins in the different regions of the lens, concealed in the central cavity of the α-crystallin chaperone model. To demonstrate this aggregated state of crystallins, the high-molecular-weight (HM) water-insoluble (WI) α-crystallin complex was without denaturation solubilized in 100% formamide [24]. In the present study using the NMR spin–echo technique, we demonstrate for the first time, besides chaperone function, the basic property of α-crystallin for tight binding of water at room temperature and the structure of water molecules in the subsequent morphological layers of the bovine lens.

MATERIALS AND METHODS

Hydration studies of proteins. Lyophilized bovine lens α-crystallin (C-4163) (type I), calf skin collagen (C-3511), and bovine serum albumin (BSA) (Fraction V powder, A-7906) were obtained from Sigma (USA). Water vapor uptake was measured gravimetrically. The lyophilized individual proteins were placed in weighing capsules and their dry weight was obtained by weighing. The samples were exposed for 4-5 days to a defined water vapor pressure by placing them in desiccators above saturated salt solutions, having a relative humidity P/P_0 = 0.75, 0.96, and 1.0 at 20°C. The hydration properties of the samples were measured using the NMR spin–echo technique [25]. To determine the spin–spin relaxation times (T_2) from the protons of water adsorbed on the proteins, the Carr–Purcell–Meiboom–Gill sequence of pulses was used [26, 27]. The accuracy of T_2 determinations was better than ±7% between different experiments (3-5 experiments). The measurements of T_2 were carried out using the NMR spin–echo instrument developed and manufactured at the Department of Biophysics, Lomonosov Moscow State University. The working frequency of the device is 16.5 MHz for protons. Operation of the device and acquisition and primary processing of the data were conducted using computer programs. The stability of working conditions was estimated using stan-

dard solutions of $CuSO_4$ in water. Measurements of hydration were conducted at room temperature.

Tissue processing and biochemical procedures. Bovine eyes were transported from a local abattoir under ice. The lenses were removed immediately and dissected free of surrounding tissues, keeping the lens capsule intact. The lenses were kept at −20°C until used. The age of the lenses was determined from the wet weights of whole lenses, according to the equation deduced by Hockwin et al. [28]. The frozen-sectioning technique utilized was described previously [29-31]. Using a trephine, the lenses were divided into equatorial ring and inner cylinder, the latter comprising 80% of the lens diameter. The remaining 20%, the equatorial ring, represents the outer equator (EQ). The inner cylinder was further divided into 11-12 layers, beginning at the anterior side. Measurements of layer dry weight and water content, and of the distribution of water-soluble (WS) and water-insoluble (WI) crystallins were published earlier [23]. Twelve fractions and the equatorial zone of the lens tissue were homogenized by hand in a glass Potter homogenizer and centrifuged in a Heraeus Christ Biofuge B at 11,630*g* for 60 min at 4°C to separate the WS and WI fractions. The sediment was then completely resuspended in distilled water and recentrifuged for 30 min at 4°C. This procedure was repeated four times until the Lowry test for proteins in the supernatant was negative and all WS-crystallins were thus removed. The final pellet was designated as the WI fraction. Supernatants and sediments of each layer were lyophilized, and the amounts of WS- and WI-crystallins determined by weight. The WI-crystallins of each layer were solubilized in 100% formamide, without disaggregation into smaller units, or dissolved in 7 M urea, immediately diluted to 3.5 M urea, and incubated for 15 min at room temperature. This procedure was carried out to avoid complete disaggregation of the crystallins of the WI fraction. In equilibrium with its ionized form ($NH_4^+COONH_2^-$), urea is eliminated from the sample by the action of the electric current. The samples were then immediately applied onto the plate.

The samples of WS- and WI-crystallins were separated by polyacrylamide thin-layer IEF (5% polyacrylamide cross-linked by 2.6% with bis-acrylamide). The Ampholine carrier ampholytes covered a pH range from 3.5-9.5 and consisted of 0.15 ml pH 3.5-5, 0.45 ml pH 5-7, and 0.4 ml pH 7-9. The gel was polymerized on the hydrophilic side of a Multiphor gel-bond film by photoactivation for at least 3 h at room temperature using two daylight fluorescent tubes. Up to 11-12 samples containing 400 μg of WS-crystallins in 20 μl 2% solution in distilled water, or 1.2 mg of WI-crystallins in 30 μl formamide, or in 3.5 M urea as a 4% solution, were applied onto 5 × 10 mm sample application papers, immediately after placing on the gel. The standard proteins used were Bio-Rad IEF Standards, broad p*I* calibration kit, pH 3-10 [23]. Papain was applied as an additional marker [32]. One electrode strip was soaked in 0.5 M acetic acid and the other in 0.5 M NaOH, and both were applied onto the gel at the anodic and cathodic side, respectively. The gel was then placed in a Multiphor electrophoresis instrument (LKB 2117-301) and operated at 4°C. The power unit (LKB 2197-301) parameters were preset to 1500 V, 20 mA, and 10 W. At the start, these parameters were 350 V, 20 mA, and 10 W. After 30 min, the sample application papers placed near the anode were removed to prevent distortions in the sample appearance after staining. At the end of the run (90 min), the voltage was 1300 V and the current 6-7 mA. The gel was fixed in 20% trichloroacetic acid for 30 min. The plate was washed in distilled water three times, 10 min each, and stained in 0.05% Serva Blue W in distilled water (Serva, 35053) for 30 min. Destaining was carried out with distilled water until the background became clear. The plate was placed on a leveling table, covered with a solution containing 7% gelatin (Merck, 4072), 5% glycerin (Merck, 4093.2500), and 0.02% sodium azide (Merck, 822335) at 48°C, and dried overnight at room temperature. The dried gel was photographed and used for densitometry in a Joyce-Loebl Chromoscan Mark 2. The reproducibility of the method including scanning was better than 2.5%.

RESULTS

Hydration properties of α-crystallin, calf skin collagen, and bovine serum albumin. NMR spin–echo technique was used to compare the hydration properties of bovine lens α-crystallin with the hydration behavior of individual proteins including the fibrillar protein collagen and the globular protein BSA. The NMR spin–echo technique enables assessment of the dynamic characteristics of water protons after the adsorption of water on the protein surface. The spin–spin relaxation time of protons (T_2) is used. At room temperature, the T_2 value for distilled water has a mean value of about 2-3 sec, whereas the T_2 value of protons typical for frozen water averages 10^{-5} sec. During the water vapor uptake experiment in the desiccator with a relative humidity of $P/P_0 = 0.75$, α-crystallin did not adsorb water, indicating that hydrophobic regions of the protein were exposed to the aqueous vapor. This observation demonstrates a tight packing of the protein that prevents the penetration of water molecules. In the lyophilized state, α-crystallin contains about 3% water (table), as measured gravimetrically. Such water protons have a T_2 relaxation time of about 10^{-2} msec (cannot be measured using the NMR spin–echo instrument). In a desiccator with a relative humidity of $P/P_0 = 0.96$, α-crystallin adsorbed an additional 5% of gravimetrically measured water, with spin–spin relaxation time of water protons $T_2 = 2$ msec, which corresponded to tightly bound water. At relative humidity $P/P_0 = 1.0$, the adsorption of water by α-crystallin was 17% and the

Relaxation characteristics of water protons of α-crystallin, collagen, and bovine serum albumin (BSA) after hydration

Sample	Relative humidity in desiccator, P/P_0	Water content in a sample, %	Time of spin–spin relaxation of proton, T_2, msec		Amount of water in 1 g of sample, mg	
			first component	second component	first component	second component
α-Crystallin	0.75	3.0 ± 0.2	$< 10^{-1}$	—	30 ± 2	—
α-Crystallin	0.96	8.0 ± 0.6	2.0 ± 0.2	—	80 ± 6	—
α-Crystallin	1.0	17.0 ± 1.2	3.0 ± 2.0	—	170 ± 12	—
BSA	1.0	28.1 ± 2.0*	5.0 ± 0.4	45.0 ± 3.2	240 ± 17	40.1 ± 2.8
Collagen	1.0	19.3 ± 1.4	4.0 ± 0.3	34.0 ± 2.4	165 ± 12	27.6 ± 1.9
α-Crystallin	water added	28.5 ± 2.0	8.0 ± 0.6	—	285 ± 20	—
BSA	water added	28.5 ± 2.0	8.0 ± 0.6	38.0 ± 2.7	200 ± 14	80.0 ± 5.6
Collagen	water added	28.5 ± 2.0	3.0 ± 0.2	148.0 ± 10.4	255 ± 18	30.0 ± 2.1
α-Crystallin	water added	33.0 ± 2.3	9.0 ± 0.6	—	330 ± 23	—
BSA	water added	33.0 ± 2.3	8.0 ± 0.6	35.0 ± 2.5	220 ± 15	110 ± 8
Collagen	water added	33.0 ± 2.3	5.0 ± 0.4	110 ± 8	300 ± 21	30.0 ± 2.1

Note: Results are expressed as mean ± S.D. for 3-5 experiments.
* $p < 0.001$ compared to the same relative humidity of α-crystallin.

spin–echo regression curve of the water protons had a single component character with T_2 = 3 msec. Such spin–spin relaxation times (T_2) are characteristic of tightly bound water protons.

The relaxation decay curves for individual samples of α-crystallin, BSA, and collagen proteins are presented in Figs. 1-3. The values of T_2 for BSA and collagen were compared to α-crystallin at 1.0 and at higher humidities to outline the differences in the hydration properties of the proteins (table). The data indicate a significant difference in adsorbed water between α-crystallin and BSA/collagen. The relaxation decay curve of the NMR amplitude signal of α-crystallin at 33% relative humidity has single component character (Fig. 1) and a time of the spin–spin relaxation of protons of 9.0 msec, which is characteristic of bound water protons. During the humidification of α-crystallin at a relative humidity of 100%, this protein adsorbs 17% water and the relaxation decay curve of the NMR spin–echo amplitudes has single component character and decays with T_2 = 3 msec (table), characteristic of the protons of tightly bound water. Under the same experimental conditions (relative humidity P/P_0 = 1.0), collagen and BSA adsorbed 19.3 and 28.1% water, respectively (table). These proteins showed a two-component decay curve with T_2 of about 5 msec for the fast component and about ~45 msec for the slow component (BSA) or about 4.0 msec for the fast component and 34 msec for the slow component (collagen protein). This shows the presence of two water fractions in collagen and BSA proteins, which are separated in space, otherwise the two water fractions would exhibit fast exchange of proton signals and a one-component decay curve would result. The following conditions should be satisfied for a reliable separation of relaxation decay curve of the NMR spin–echo amplitude into components: the spin–spin relaxation times of the protons (T_2) assigned to

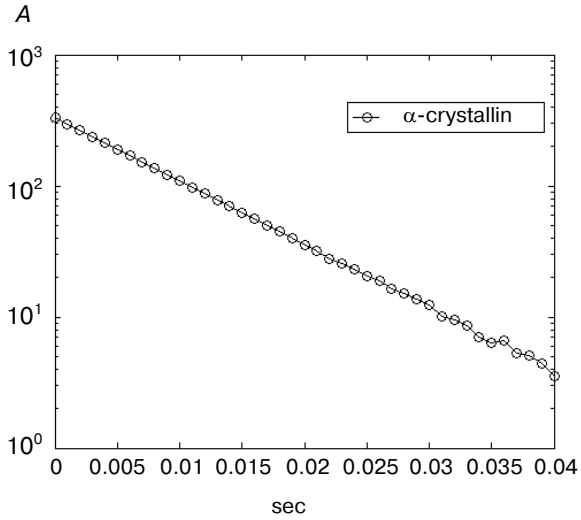

Fig. 1. Relaxation decay curve of the NMR amplitude signal characteristic of water protons sorbed on α-crystallin at 33% of relative humidity (T_2 = 9.0 msec). The plot uses a semi-logarithmic scale.

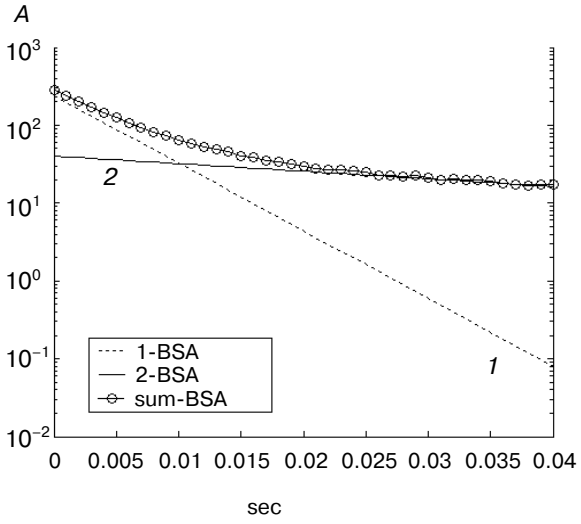

Fig. 2. Relaxation decay curve of the NMR amplitude signal of water protons sorbed on BSA at 28.1% relative humidity: *1*) first (fast) decay component, T_2 = 5 msec; *2*) second (slow) decay component, T_2 = 45 msec.

the decay components should differ by no less than 5- to 10-fold, and the relaxation decay amplitudes should differ no more than by 5- to 10-fold. Otherwise, the division of the relaxation decay curve into the components is insignificant.

During further addition of water to α-crystallin to 28.5 and 33%, its content in the sample and the times of spin–spin relaxation (T_2) of water protons are increased by 2- to 3-fold (table), that is to say water remains tightly bound up to its highest content in α-crystallin. Upon hydration, collagen showed two components of the spin–echo relaxation decay curve due to the fact that a fraction of protons are hydrated at the polar groups of collagen fibers with T_2 = 4 msec for this fraction of water and another fraction of water has T_2 = 34 msec. Protons of the latter fraction of water can be assigned to the capillary water which is accumulated in pores. Two components of the relaxation spin–echo decay observed in the fraction of protons sorbed on the polar groups of BSA at 28.1% relative humidity are separated in space due to the mosaic distribution of sorbed water and water molecules do not form a continuous layer on the protein surface.

The table documents that continued addition of water to α-crystallin up to 28.5 and 33% of the total sample leads practically to no increase (9.0 msec) of the spin–spin relaxation time of protons. The water remains tightly bound to α-crystallin even at the highest adsorption level. The results of the NMR spin–echo analysis indicate that α-crystallin can immobilize water to a greater extent than the other studied proteins, BSA and collagen. Besides, during the hydration of α-crystallin the water molecules uniformly occupy the protein surface in contrast to the non-continuous distribution of water during the hydration of collagen and BSA proteins.

Hydration properties of α-crystallin in different lens layers. The lens wet weights (LWW) of four fresh bovine lenses were determined with a mean LWW of 2.20 ± 0.04 g, corresponding to an age of 3.2 years. In the bovine lens divided into 12 layers, the anterior cortex (AC) is localized in layer 1, the anterior supranuclear layer (ASNL) at layers 2-4, the nucleus (N) at layers 5-7, the posterior supranuclear layer (PSNL) at 8-9, and the posterior cortex (PC) at 10-12.

The distribution of water and of the ratio of the dry weight to the lens wet weight content (H_2O, DW, and LWW in percent) in various sections from anterior cortex to posterior cortex and in the equatorial layers is presented in Fig. 4 including Fig. 4a of the earlier publication [23]. The water content in various layers of the lens was higher in cortical regions than in the nucleus. For the bovine lens, the dry weight (DW) content increased significantly from 20.0 to 41.0% going from AC to N (Fig. 4a). The ratio Q = H_2O/DW (mg/mg) was higher in anterior and posterior cortices as compared with nuclear regions (decreased significantly going from AC to PSNL, from 4.00 to 1.65 (Fig. 4c)).

The percentage of WS-crystallins decreased from 92.5 to 68.4%, going from AC to N, along with a distinct increase of the content of WI-crystallins (Fig. 4b). The ratio of WS- and WI-crystallins (R = WS/WI) decreased from 9.8 to 1.9, going from AC to the nuclear region (fractions 3-8), and thus demonstrated a U-shaped distribution of the crystallins (Fig. 4d) as was the case for Q

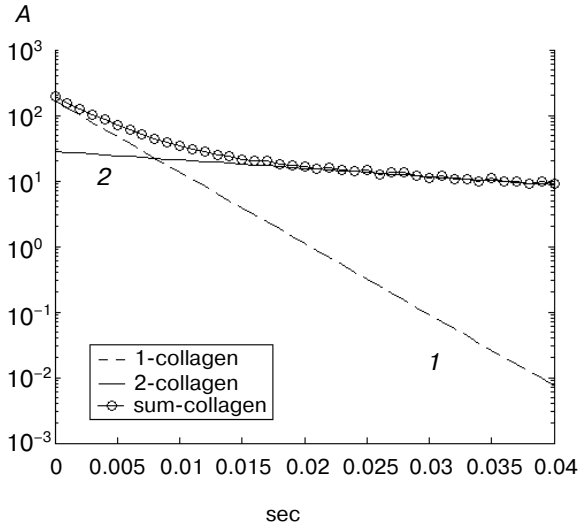

Fig. 3. Relaxation decay curve of the NMR amplitude signal of water protons sorbed on collagen at 19.3% of relative humidity: *1*) first (fast) decay component, T_2 = 4.0 msec; *2*) second (slow) decay component, T_2 = 27.6 msec.

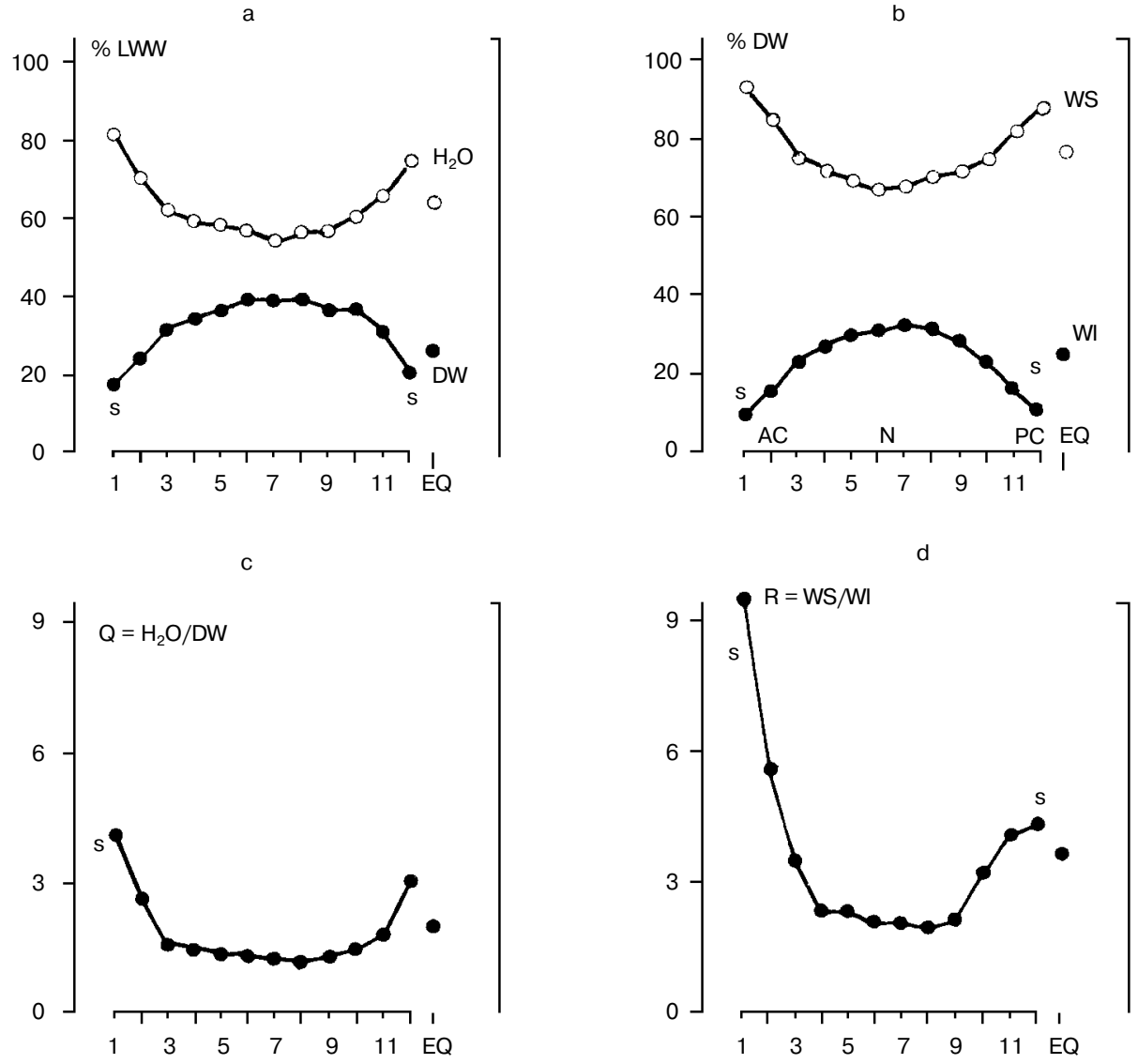

Fig. 4. Determination of: a) % H_2O, % DW (dry weight); b) % WS-crystallins, % WI-crystallins; c) quotient Q = H_2O/DW (mg/mg); d) ratio R = WS/WI of a bovine lens divided into 12 layers. S denotes a significant increase or decrease (layers 1-8, $n = 8$, $2p < 0.02$; layers 12-7, $n = 6$, $2p < 0.04$). LWW 2.20 g, age 3.2 years.

(Fig. 4c). The effects are significant for layers 1-8 from the AC and for layers 12-7 taken from the PC (Fig. 4, a-d), both in the direction of the nucleus.

The LWWs of a second set of fresh bovine lenses were determined at 1.874 ± 0.004 g, corresponding to an age of 1.3 years. Figure 5 shows the IEF patterns of young bovine WS-crystallins (1.3 years). Separation was accomplished into HM- (high molecular weight α-crystallin), α_L-, β_H-, β_L-, β_S-, and γ-crystallins. The α_L-crystallins comprised at least three native components, the β_H- and β_L-crystallins comprised six component groups, and β_S-crystallin comprised two single components. The γ-crystallins were separated into 11 different components. Densitometric scanning of the patterns of Fig. 5 gave the percent values as shown in Fig. 6 (a-f). The HM-crystallin (upper curve) concentration is higher in AC and PC than in N (Fig. 6a). This is also the case for α_L- and α_T-crystallins. The β_T-crystallins display a minimum in N (Fig. 6b), whereas the β_L-crystallins show either lower or higher concentrations in N (Fig. 6, c and d). The β_S-crystallin components show maxima in the anterior and posterior supranuclear layers (Fig. 6f), whereas the γ-crystallins and their components display clear maxima in N (Fig. 6, c and f).

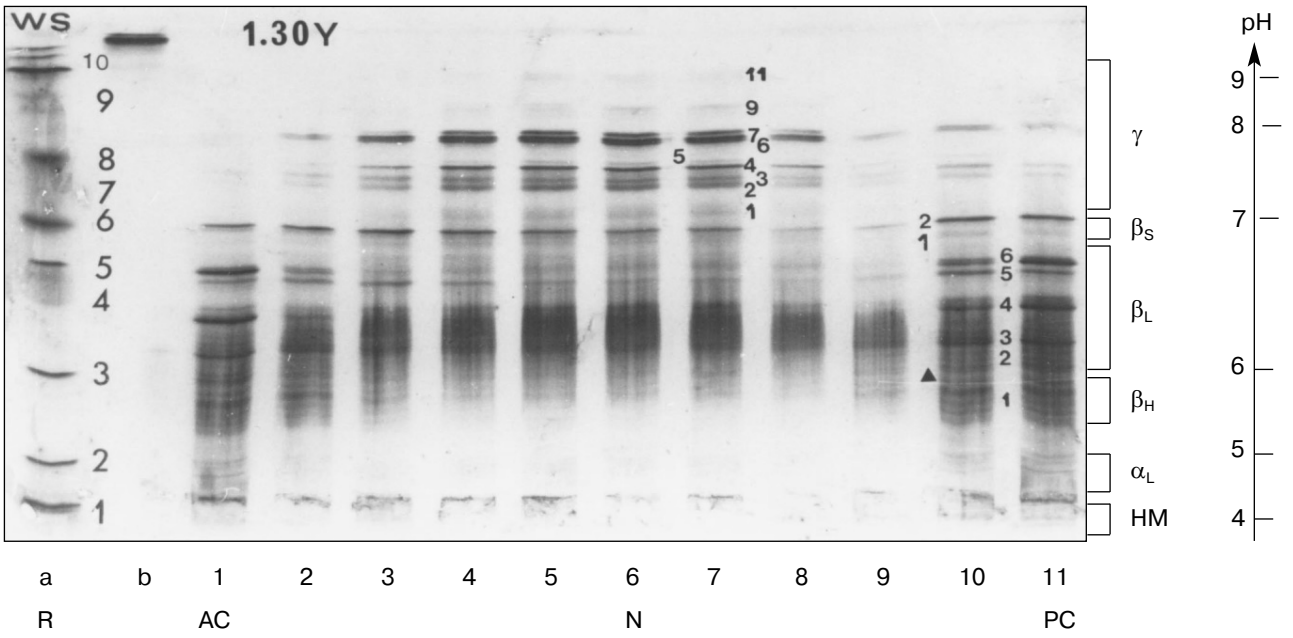

Fig. 5. Polyacrylamide thin-layer isoelectric focusing (IEF) of WS crystallins of the young bovine lens (LWW 1.874 g, age 1.3 years) divided into 12 layers (11 layers are presented) from anterior cortex (AC) through nucleus (N) to posterior cortex (PC). The WS-crystallins from the equatorial ring (EQ) were deleted. The crystallin samples (0.4 mg) are separated into HM-crystallins, α-crystallin of low molecular weight (MW) (α_L), β-crystallins of high MW (β_H) (component group No. 1), β_L-crystallins of low MW (β_L) (component groups No. 2-6), β-slow-crystallin (β_S) (2 components), and γ-crystallin (11 components). a) Bio-Rad reference proteins (R) from a broad calibration kit of pH 3-10. b) Papain as additional standard protein (p*I* = 9.8). The scale at the right shows the pH values measured at 4°C. An Ampholine gap (designated on gel) is noted between pH 5 and 6. L, low MW; H, high MW; T, total.

Demonstration of chaperone activity of α-crystallin in different lens layers. It is the aim of this section to prove the existence of chaperone activity of α-crystallin causing effective preservation of high- and low-molecular-weight β-crystallins (β_H, β_L, and β_S) and γ-crystallins in the WI material. The data were in part presented earlier [23]. When the WI-crystallins of layers 1-11 are solubilized in 100% formamide (a solvent with high protein structure-protecting properties), all WS-crystallins which were incorporated during the ageing process into the WI-moiety, remain concealed in the (HM + α_L)-crystallin complex. No components other than HM- and α_L-crystallins are visible after IEF (Fig. 7). For comparison, the WS crystallins of the equator (EQ) of the same lens are shown (Fig. 7). This demonstrates the chaperone activity of α-crystallin. However, when the same samples 1-11 of WI-crystallins are dissolved into urea, a partial breakdown of the WI-crystallin complex occurs by the action of urea. A simultaneous release of all WS-crystallins, like β_H-, β_L-, β_S-, and γ-crystallin, takes place and these crystallins are isofocused at apparently the same native pH-values, compared to those of Fig. 5. A similar appearance in the distribution of WS α-crystallins found in Fig. 6a is also valid for the WI α-crystallins after release from the WI-complex by 7 M urea. A partial breakdown of the aggregates of a lens was effected by urea with a simultaneous release of β_H-, β_L-, β_S-, and γ-crystallins at apparently native p*I* values (results not shown). At the anterior (AC) and posterior (PC) cortices, only very low concentrations of β- and γ-crystallins were incorporated into the WI complex, because of the young age of these layers. In the direction of N, more β- and γ-crystallins were incorporated into the WI complex. The same is valid for the WI crystallins from the equator, compared to the WS crystallins from the equator of the same lens. It appears that the β- and γ-crystallins are released from the α-crystallin complex in an unchanged form. Previously published results confirm that a significant age-related increase in several β- and γ-crystallins incorporated into α-crystallins exists in the patterns of WI fractions of the different layers of lenses from 2.2 to 5.9 years [23].

The distribution of α-crystallins in the lens layers coincides with the distribution of water content in the similar morphological lens regions (Figs. 4a, 4c, and 6a). This corresponds to the phenomenon of tight binding of water observed for α-crystallin by NMR spin–echo technique. The samples of proteins obtained during preparation of the lens sections were lyophilized and did not show

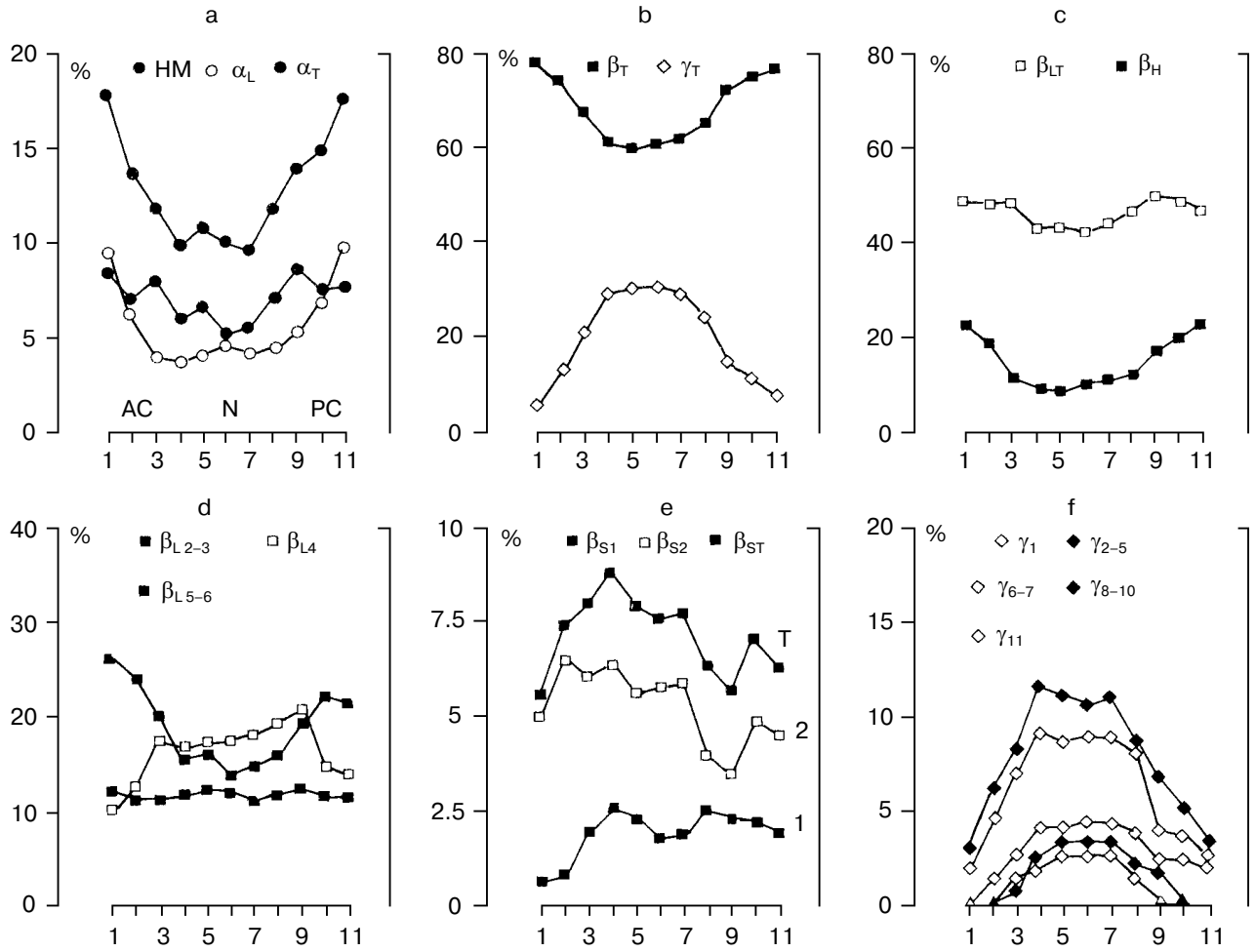

Fig. 6. Densitometric evaluation of the WS crystallin profiles from Fig. 5. All increases/decreases (layers 1-6, left (L); layers 6-11, right (R)) were significant ($n = 6$, DF = 4, $t > 2.825$, $2p < 0.048$; $n = 7$, DF = 5, $t > 3.195$, $2p < 0.024$; $n = 8$, DF = 6, $t > 3.200$, $2p < 0.019$) except for HM- (see upper curve, full dot symbol) (R), α_L-(L), β_{L4}-(L,R), $\beta_{L5\text{-}6}$-(L,R), and β_S-crystallins (L). d) $\beta_{L2\text{-}3}$ curve points are above $\beta_{L5\text{-}6}$ curve points; f) $\gamma_{2\text{-}5}$ curve points are above $\gamma_{8\text{-}10}$ curve points. For more information see the legend to Fig. 5.

different types of water in lens sections but only the water content parameters were matched with the distribution of α-crystallins in the various morphological lens regions (Fig. 4, a and c).

DISCUSSION

In the present study, NMR spin–echo experiments were used to examine at room temperature the hydration properties of individual proteins, i.e., samples of lens α-crystallin (a molecular chaperone), collagen, and BSA. The water binding properties and distribution of crystallins in individual lens layers were investigated by freeze-sectioning and IEF techniques. Macromolecules of α-crystallin humidified to 17% showed a single component decay of proton relaxation ($T_2 = 3$ msec), characteristic of tight binding of water. During the hydration of α-crystallin, a homogeneous distribution of water molecules over the protein surface is likely to be taking place. NMR spin–echo studies demonstrate the high hydration capacity of α-crystallin to bind water molecules. The sorbed water molecules are tightly bound to the polar groups of α-crystallin macromolecule. The hydration process of α-crystallin substantially differs from the hydration of collagen and BSA under the same conditions. During the hydration of collagen, a two-component spin–echo decay occurs due to the hydration of protons on the polar groups of collagen fibers (with $T_2 = 4$ msec), and to the appearance of another proton fraction with ($T_2 = 34$ msec) tentatively assigned to the capillary water probably sorbed in the pores of the protein [33].

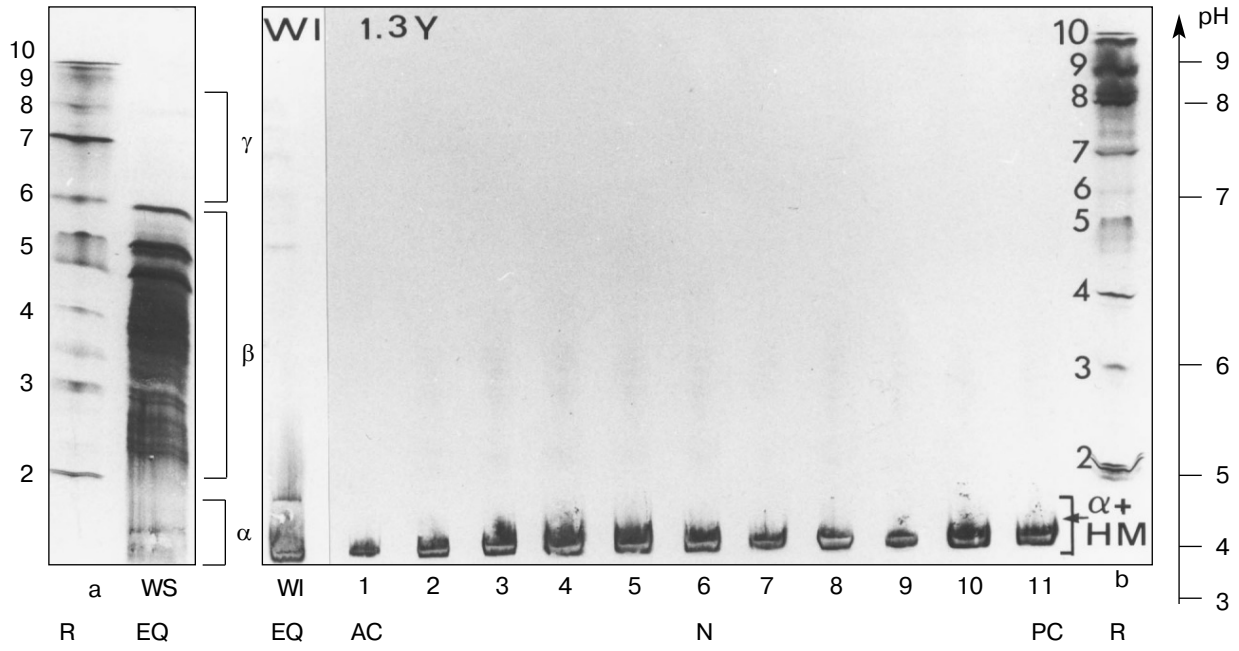

Fig. 7. IEF profiles of the WI-crystallins of a bovine lens (LWW = 1.874 g, age = 1.3 years) divided into the equatorial ring (EQ) and 11 layers by frozen-sectioning technique. WI-crystallin samples 1-11 from AC through N towards PC and also EQ WI (1.2 mg) were solubilized into 100% formamide. For comparison, EQ WS-crystallins (0.4 mg) from the same lens were applied. a, b) Reference proteins (R).

Two distinct components of the spin–echo decay are observed from the proton fractions that are adsorbed on the polar groups of BSA at 28.1% relative humidity. There are a fast (T_2 = 5 msec) and a slow component of the NMR spin–echo amplitude relaxation decay (T_2 = 45 msec), the latter corresponding to the relatively weakly bound water. These two proton populations are separated in space, possibly due to the mosaic deposition of adsorbed water at the surface of the macromolecule [34]. In contrast, the water in the cavity of α-crystallin is the least mobile.

Water molecules close to the biopolymer (protein) surface interact by H-bonding, their diffusion is slowed down, and their rotation is hindered. Bound water may have different degrees of freedom, from partial to full immobilization (superhydration of protein). Measurements of hydration of proteins usually deal with only two forms of water, freezable (free water) and non-freezable (bound water). Depending on the fitting of the relaxation curves, NMR spin–echo measurements can perceive one or up to three different forms of water. Stankiewicz et al. [35], Babizhayev et al. [25], and Racz et al. [36] fitted their T_2 relaxation of different lenses with a two-term exponential equation and therefore, they calculated relaxation times as well as concentrations of two component systems in the lens.

It is now claimed that the amplitude ratio of the bound and the free water fraction and the single character of the T_2 relaxation decay carries the most characteristic information as the index of superhydration of the protein in tissue structures. During cataract formation the weight of free water is raised by more than 25% [36]. The T_2 relaxation describes the average behavior of bound water. The motion of water protons bound to protein is relatively constrained exhibiting longer correlation (decay) time, hence shorter relaxation curve. A decrease in T_2 relaxation times means an increase in the strength of H-bonding that can occur when partially bound water becomes completely bound.

The relaxation times presented for α-crystallin versus BSA and collagen proteins in our study demonstrate the superhydration properties (tight binding of water) for α-crystallin molecular chaperone protein. With aging of the lens tissue the T_2 relaxation time decreases [25]. A shift from high to low T_2 is indicative of stronger H-bonding that occurs both when one proceeds from cortex to nucleus (younger to older from developmental point of view) as well as with an increase in the chronological age of the lens [37].

The hydration properties of α-crystallin are important for its physiological role as a molecular chaperone and for prevention of aggregation and denaturation of other proteins in the organic tissues [38].

Raman and Rao [20] have proposed that α-crystallin prevents aggregation of non-native structures due to its hydrophobic surfaces. Another study has proposed that the very hydrophobic regions around α_A 32-37, α_A 72-75, and α_B 28-34 that become solvent exposed with increasing temperature are, possibly, the hydrophobic surfaces involved in the chaperone activity [21]. Beside the hydrophobic interactions, which stabilize protein structure, water exposes another distinct mechanism of action to the protein conformation resulting from the competition of water molecules for the hydrogen bonds between CO- and NH-groups of the polypeptide chain [34]. The presence of water molecules is essential to promote the formation of hydrogen bonds during protein folding. During structuring of the protein conformation, the hydrophobic part of the molecular chaperone may be included in the cylindrical cavity of α-crystallin.

In the lyophilized state of α-crystallin, 3% of water still exists, which is probably trapped inside the central cylindrical cavity and is not evaporated during drying. The persistence of a small quantity of water in lyophilized protein macromolecules is important for the maintenance of their native structure. Conditions can be created in the cylindrical cavity of the molecular chaperone, where the water molecules are present up to ~3%; and precisely, tightly bound water may exist on the polar groups of C-terminal regions [13].

The data obtained document the tight binding of water by α-crystallin, and reveal a single water population. This is demonstrated by a single component decay curve for spin–echo at low hydration of α-crystallin, in contrast to BSA. The majority of water molecules of α-crystallin are most likely on the polar groups positioned near the circular entrance of the cavity [16].

Haff [24] fractionated water-insoluble proteins and found that formamide was a solvent endowed with high protecting properties to proteins. The proteins remained unchanged, because no denaturation was observed [24]. This is in contrast to the actions of urea, guanidine chloride, and SDS, which generate subunits.

The similar distribution of water with α-crystallin pattern revealed from microsectioning and the IEF data, respectively, suggests tight binding of water with α-crystallin in the bovine lens layers. It is interesting to compare the distribution of γ-crystallin and α-crystallin in the lens layers. Figure 6 reveals that the distribution of γ-crystallin is inverse to that of water (Fig. 4, a and c). α-Crystallin can immobilize water to a greater extent than γ-crystallins [11]. This water immobilization and binding may have to do with the openness or compactness of the quaternary structures of crystallins. The compact structure of γ- and β_L-crystallins is derived from having lower extent of aggregation in the case of β_L and monomeric form in γ. Thus, they bind and immobilize less water [11]. In accordance with a previous identification of the chaperone activity of α-crystallin in the lens layers [23], the IEF data suggest a possible chaperone-like function for α-crystallin in the nucleus of the lens (minimum of free water), but not in the outer cortex. The distribution of water and α-crystallin in the lens layers suggests that the chaperone-like activity of α-crystallin may play a greater role in the lens nucleus than in the outer cortex by preventing aggregation and oxidative insolubilization of low-molecular-weight crystallins and eventually lens enzymes. Such a change may initiate the increase in the refractive index difference in the lens layers between the protein moieties and their environment, which occurs both in normal development, ageing, and in cataract formation.

To conclude, we have found that α-crystallin can immobilize and bind water to a greater extent than other proteins tested, such as collagen and BSA. These results shed new light on the structural properties of α-crystallin and its superhydration properties and have important implications for understanding the mechanism of the chaperone-like activity of this protein in the lens and in non-ocular tissues.

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