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Raman Spectroscopic Study of Age-Related Structural Changes in the Lens Proteins of an Intact Mouse Lens†

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ABSTRACT: Age-related structural changes in the lens proteins of a normal mouse lens have been monitored in situ by laser Raman spectroscopy. The Raman spectrum of an ICR-strain mouse lens nucleus showed virtually no change in the 550–850- and 900–1800-cm^{-1} regions as the mouse aged. Lens aging, however, did cause a significant intensity decrease of the Raman band at 880 cm^{-1} due to tryptophan residues, and the intensity decrease seems to be stepwise. This observation implies that a microenvironmental change of tryptophan residues takes place twice at different places of the lens proteins during normal aging. Particularly striking is that the intensity decrease of the band at 880 cm^{-1} proceeds in parallel with that of the Raman band at 2579 cm^{-1} due to a SH stretching mode for the first 4 months. Thus, the first microenvironmental change of tryptophan residues seems to be correlated with the formation of S–S bonds. In contrast to tryptophan residues, no evidence was observed of a microenvironmental change in tyrosine residues. In this respect, the structural changes of lens proteins in aging are sharply distinct from those in lens opacification, in which tyrosine as well as tryptophan residues undergo microenvironmental changes [Itoh, K., Ozaki, Y., Mizuno, A., & Iriyama, K. (1983) *Biochemistry 22*, 1773-1778]. The relative intensity of the band at 3390 cm^{-1} due to an OH stretching mode of lens water fell rapidly for the first 4 months and then decreased very gradually. The observation clearly exhibits the process of lens dehydration. The age-dependent profile of the relative intensity of the OH stretching mode is similar to that of the SH stretching mode, implying that lens dehydration is also related to the 2SH → S–S conversion.

The predominant dry components of a mammalian lens are three structural proteins called α-, β-, and γ-crystallins, and their combined weight accounts for approximately 33% of the total weight of the lens (Cotlier, 1981). A disorder in the physicochemical arrangement of the lens proteins brings about lens aging or opacification. The well-known phenomena occurring to the lens proteins during such aging and opacification are lens dehydration or hydration, the formation of protein aggregates, and the chemical modification of protein subgroups (Kinoshita, 1974; Harding & Dilley, 1976; Cotlier, 1981; Bettelheim & Siew, 1982). To understand lens aging or

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opacification, it is essential to elucidate the processess of these physicochemical events at the molecular level. However, in spite of extensive studies by many investigators the detailed mechanisms of the events have not yet been fully explained. For instance, there has long been dispute as to the role of protein subgroups such as cysteine, tryptophan, and tyrosine in the formation of protein aggregates (Kuck et al., 1982).

Raman spectroscopy holds a number of advantages for delineating the mechanism of lens aging or opacification at the molecular level (Yu & East, 1975; East et al., 1978; Askren et al., 1979; Thomas & Schepler, 1980; Kuck et al., 1982; Ozaki et al., 1982; Itoh et al., 1983). First, Raman spectroscopy enables us to study constituents in a lens under normal physiological conditions. Second, almost the entire vibrational spectral region is open to analysis for the aqueous lens; a variety of information about the lens proteins can be obtained simultaneously from a single scan of a Raman spectrum in the 400–3800-cm^{-1} region. The third advantage is that Raman spectral bands of high intensity are generally expected for the protein subgroups which have been considered to be involved in the aggregation and chemical modification of the lens proteins. In fact, when Raman spectra of cac-strain mouse lenses were measured at various stages of opacification, it was indicated in our previous paper (Itoh et al., 1983) that some tyrosine residues undergo a change in their hydrogen bonding environment and some buried tryptophan residues become exposed during cataract formation, and the formation of S–S linkages by sulfhydryl groups in the lens proteins is not a predominant factor for initiating lens opacification.

As part of a series of Raman spectroscopic studies on structural changes of the lens proteins, we have undertaken a systematic investigation of lens aging. Yu and his group (East et al., 1978; Askren et al., 1979; Kuck et al., 1982) reported detailed studies on the 2SH → S–S conversion in lens proteins using Raman spectroscopy. However, a full investigation of lens aging by this means has not yet been presented. Further insight into the mechanism involved can be gained by the understanding of different age-dependent changes in the lens proteins together with the formation of S–S linkages. Complete Raman spectra in the 400–3800-cm^{-1} region are reported in this paper for ICR-strain mouse lens nuclei at different ages. Thus, not only the structural changes of the lens proteins but also the process of lens dehydration can be examined. We have mainly studied a lens nucleus where the proteins retain their original amino acid composition. Thus, confining the study enabled us to focus the analysis on the postsynthetic modifications of preexisting proteins.

Of particular importance in this series is to compare the changes involved in lens aging with those relating to lens opacification. The present results are therefore discussed in this comparative context (Ozaki et al., 1982: Itoh et al., 1983).

Experimental Procedures

Materials. Normal lenses from ICR-strain mice (Charles River, Japan) were employed in the present experiments. The lens removed from the eyeball was immersed in a tris(hydroxymethyl)aminomethane (Tris) buffered balanced salt solution containing 5.5 mM glucose (Epstein & Kinoshita, 1970). The osmolarity of the solution was 291 mOsm.

Methods. The sample-handling technique and instrumentation for recording Raman spectra were described previously (Itoh et al., 1983). The laser beam was focused exclusively on the center of a nucleus. Peak heights were taken as reliable measures of band intensities, and their mean values from two or more spectral scans of the same sample were employed to calculate the intensity ratios. Base lines were estimated as

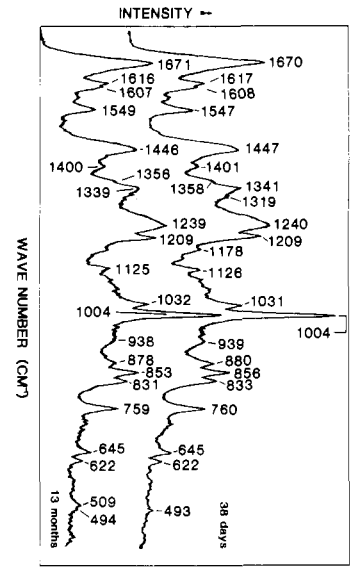

FIGURE 1: Raman spectra in the region of 400–1800 cm^{-1} of ICR-strain mouse lens nuclei (38 days and 13 months old). Instrumental conditions: excitation wavelength 488.0 nm; laser power 180 mW; spectral slit width 7 cm^{-1}; time constant 2 s; scan speed 50 cm^{-1}/min.

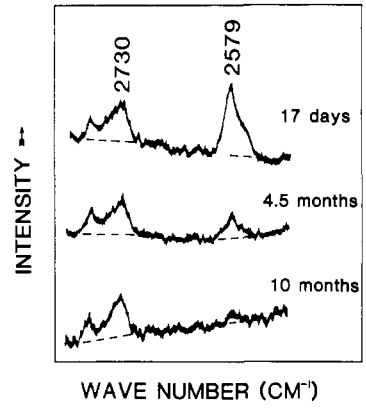

FIGURE 2: Raman spectra in the region of 2500–2800 cm^{-1} of ICR-strain mouse lens nuclei (17 days and 4.5 and 10 months old). Time constant 4 s; scan speed 25 cm^{-1}/min. Other instrumental conditions were the same as those in Figure 1.

shown in each figure. The laser power measured at the sample position was about 60 mW for the region of 2800–3800 cm^{-1}, or typically 180 mW for the rest. The temperature was measured by a thermocouple at the laser illuminating spot of the water-filled cell and found to be about 25 °C. Under these conditions lenses older than 18 days of age retained their transparency, and no changes in Raman scattering intensities or frequencies could be detected during at least 2 h of laser irradiation. Accordingly, it seemed very unlikely that denaturation of the lens proteins or any local disturbance of the protein–water concentration occurred during the Raman measurements. However, in the case of a 17-day-old lens there was observed a slight sign of annular opacification for about 30 min of laser irradiation (180 mW), and thus each partial spectrum shown in Figures 1–3 was recorded within 20 min by using a fresh lens.

Results

The Raman spectrum of a normal lens nucleus of an ICR-strain mouse can be divided into three spectral regions, the 400–1800-cm^{-1} region, the 2500–2800-cm^{-1} region, and the 2800–3800-cm^{-1} region as shown in Figures 1–3, respectively. No band was observed in the 1800–2500-cm^{-1} region. For the regions of 400–1800 and 2500–2800 cm^{-1}, Raman spectra have been reported for the normal lens of several species, and their

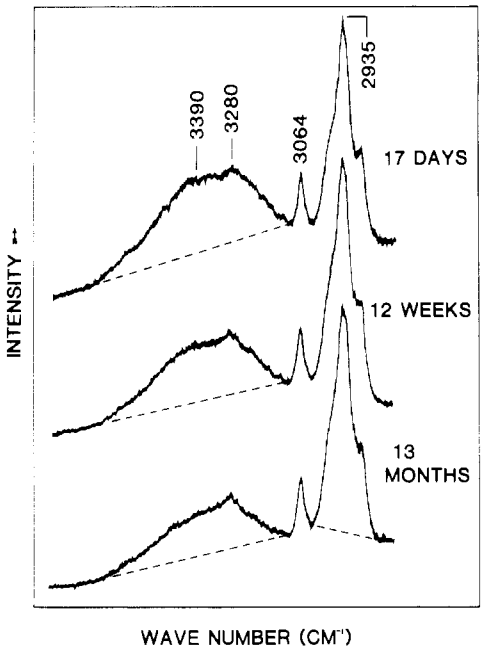

FIGURE 3: Raman spectra in the region of 2800–3800 cm^{-1} of ICR-strain mouse lens nuclei (17 days, 12 weeks, and 13 months old). Laser power 60 mW; time constant 0.8 s; scan speed 100 cm^{-1}/min. Other instrumental conditions were the same as those in Figure 1.

interpretations in terms of molecular composition and conformation have been well-established (Yu & East, 1975; Schachar & Solin, 1975; Yu et al., 1977; Kuck & Yu, 1978; Thomas & Schepler, 1980; Mizuno et al., 1982; Itoh et al., 1983). Thus, in descriptions of the results for those regions emphasis is mainly placed on age-dependent changes of Raman spectral features.

Vibrational Spectral Region from 400 to 1800 cm^{-1} (Figure 1). The amide I and III bands are observed at 1670 and 1240 cm^{-1}, respectively, in the Raman spectra shown in Figure 1. The vibrational frequencies and band shapes of these bands differ little between the two spectra, indicating that lens aging does not cause a major conformational change in the peptide backbone of main lens proteins. Raman features for the probe of the tryptophan or tyrosine microenvironments appear in the 700–900-cm^{-1} region. The intensity ratio of the tyrosine doublet near 840 cm^{-1} (I_{855}:I_{832}), which correlates with the strength of the hydrogen bond of the phenolic hydroxyl group (Siamwiza et al., 1975), did not show a change with aging. In contrast, a small but vital intensity change occurred on the environment-sensitive band at 880 cm^{-1} due to tryptophan residues. The detail of the 700–900-cm^{-1} region is described below. A S–S stretching mode of cystine residues in the lens proteins was expected in the 500–550-cm^{-1} region (Yu & East, 1975; Yu et al., 1977; East et al., 1978; Itoh et al., 1983). There was no hint of the existence of S–S bond in the spectrum of an ICR-strain mouse lens nucleus of 17 days old. However, a weak band due to the S–S stretching mode appeared at 510 cm^{-1} in the spectrum of the nucleus at 5 weeks (not shown), and the band intensity increased gradually as the mouse aged. Other prominent Raman bands in the spectra appeared at 622 (Phe), 644 (Tyr), 760 (Trp), 1004 (Phe), 1031 (Phe), 1209 (Tyr), 1447 (CH_2 deformation), and 1547 cm^{-1} (Trp). These Raman bands are known to be insensitive to environmental changes of protein subgroups.

Figure 4 gives age-dependent plots of the Raman intensity ratios, I_{644}:I_{622}, I_{760}:I_{1447}, and I_{1004}:I_{1447}. The intensity ratios I_{1031}:I_{1447}, I_{1209}:I_{1447}, and I_{1547}:I_{1447} were also measured for ICR-strain mouse lens nuclei at different ages (not shown).

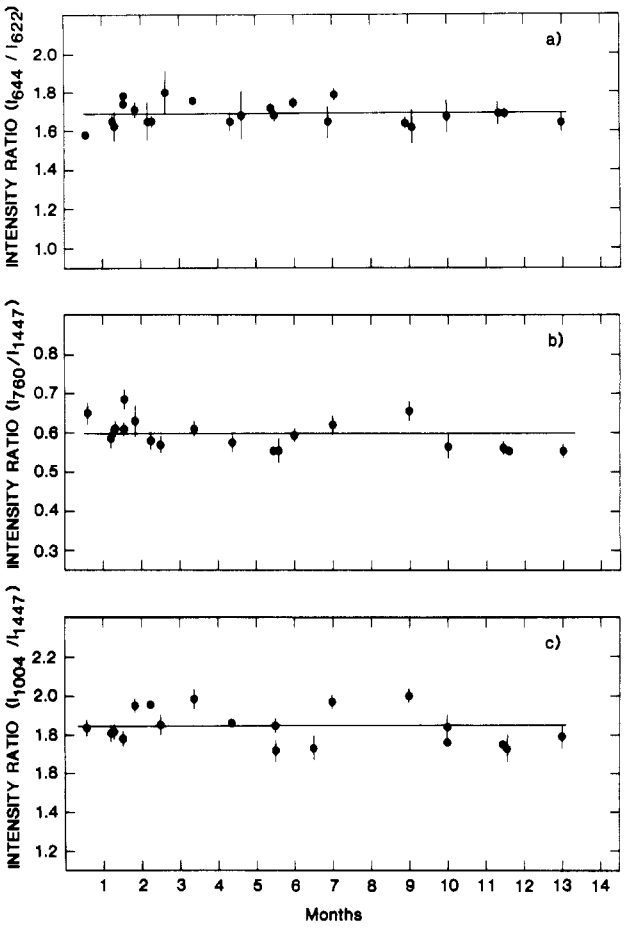

FIGURE 4: Plots of Raman intensity ratio vs. age, from the spectra of ICR-strain mouse lens nuclei. (a) Ratio of Raman peak heights at 644 and 622 cm^{-1} (I_{644}:I_{622}); (b) ratio of Raman peak heights at 760 and 1447 cm^{-1} (I_{760}:I_{1447}); (c) ratio of Raman peak heights at 1004 and 1447 cm^{-1} (I_{1004}:I_{1447}). Each point in Figures 4–7 represents the mean value of two spectral scans of the same sample.

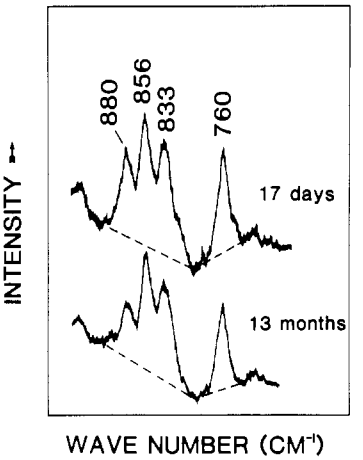

FIGURE 5: An enlargement of Raman spectra of ICR-strain mouse lens nuclei in the region of 650–950 cm^{-1} (17 days and 13 months old). The experimental conditions were the same as those in Figure 1.

Use of the 1447-cm^{-1} band as an intensity standard has been rationalized by Fasman et al. (1978). The plots in Figure 4 and those of other intensity ratios allow us to examine age-dependent variations of amino acid composition in the lens proteins. Very little variation was observed in any of the intensity ratios described above in the aging process.

In Figure 5 is presented an enlargement of the 650–950-cm^{-1} spectral region. The relative intensity of the Raman band at 880 cm^{-1} to that at 760 cm^{-1} clearly changes between the two

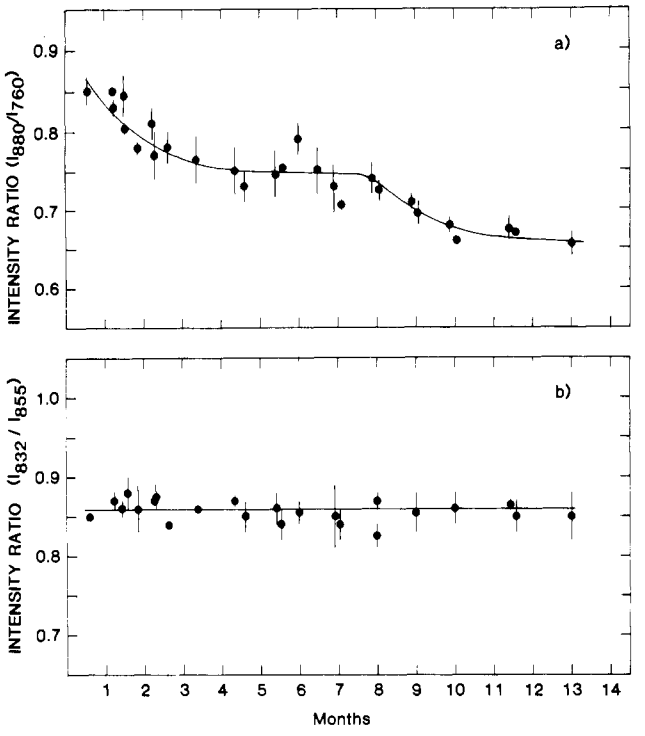

FIGURE 6: (a) Plot of the intensity ratio of the bands at 760 and 880 cm^{-1} (I_{880}:I_{760}) vs. age. (b) Plot of the intensity ratio of the tyrosine doublet (I_{855}:I_{832}) vs. age. The data were obtained for Raman spectra of ICR-strain mouse lens nuclei.

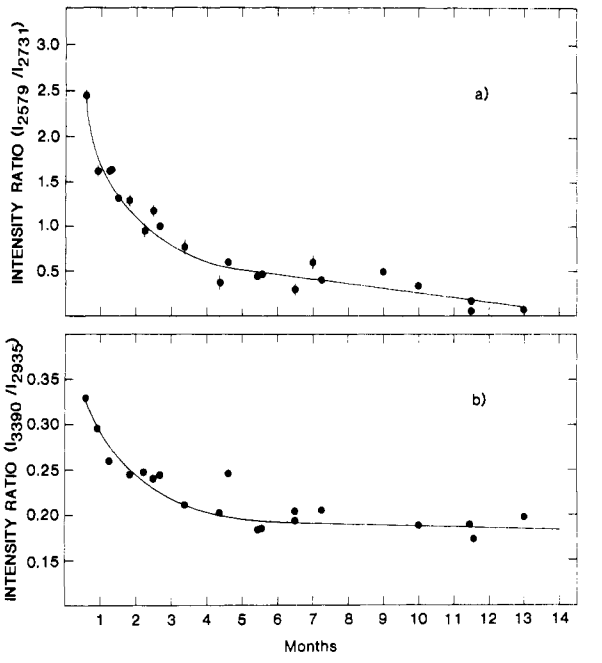

FIGURE 7: (a) Effect of aging on the intensity ratio of the Raman bands at 2579 (SH stretching mode) and 2731 cm^{-1}. (b) Effect of aging on the intensity ratio of the Raman bands at 3390 (OH stretching mode) and 2935 cm^{-1} (CH stretching mode). The data were obtained from Raman spectra of ICR-strain mouse lens nuclei.

spectra, whereas the intensity ratio of the tyrosine doublet near 840 cm^{-1} stays constant. Parts a and b of Figure 6, respectively, plots the intensity ratio of two tryptophan bands at 880 and 760 cm^{-1} (I_{880}:I_{760}) and that of the tyrosine doublet (I_{855}:I_{832}) as a function of age. The intensity ratio of the two bands at 880 and 760 cm^{-1} appears to show a stepwise decrease in the course of lens aging; the ratio changed rapidly from ca. 0.85:1 to ca. 0.75:1 for the first 4 months and then became almost constant until about 8 months after birth but after that decreased again up to ca. 0.66:1 (13 months after birth). In contrast, the intensity ratio of the tyrosine doublet was constant at about 1:0.86.

Vibrational Spectral Region from 2500 to 2800 cm^{-1} (Figure 2). The Raman band at 2579 cm^{-1} and the shoulder near 2560 cm^{-1} in Figure 2 can be assigned to SH stretching modes of cysteine residues (Yu & East, 1975; Itoh et al., 1983). The origin of the band at 2731 cm^{-1} is still unknown, although Askren et al. (1979) have suggested that the band may be a combination of the vibrations of protein side chain and backbone. Since the relative intensity of the bands at 2731 and 1447 cm^{-1} (CH_2 deformation) did not change with aging, we used the band at 2731 cm^{-1} as an internal standard. The intensity of the band at 2579 cm^{-1} decreased markedly as a lens aged (Figure 2). A corresponding intensity increase of the S–S stretching mode was observed as shown in Figure 1. Although Yu and his group (East et al., 1978; Askren et al., 1979; Kuck et al., 1982) have already reported the aging effect on the 2SH → S–S conversion in the lens proteins for several species, we reexamined the effect for an ICR-strain mouse lens nucleus in order to compare the age-dependent profile of the relative intensity of the SH stretching mode at 2579 cm^{-1} with those of other vibrational modes, especially the mode at 880 cm^{-1} due to tryptophan residues or that at 3390 cm^{-1} due to lens water.

Figure 7a exhibits the intensity ratio of the Raman bands at 2579 and 2731 cm^{-1} (I_{2579}:I_{2731}) for the ICR-strain mouse lens nuclei as a function of age. The relative intensity of the SH stretching mode showed a precipitous decrease for the first 4 months and then gradually approached zero. The profile in Figure 7a is essentially in good agreement with that previously reported for a CFW-strain mouse (Kuck et al., 1982). It is noted that the age-dependent profile for the SH stretching mode (Figure 7a) resembles the profile for the tryptophan mode (Figure 6) until 8 months after birth, although the latter shows another decrease afterward.

Vibrational Spectral Region from 2800 to 3800 cm^{-1} (Figure 3). The overlapped Raman bands at and near 2935 cm^{-1} and the weak band at 3064 cm^{-1} can be assigned to CH stretching modes of the lens proteins. The broad feature in the region of 3100–3800 cm^{-1} may be divided into two peaks; the first peak at approximately 3280 cm^{-1} probably consists of a band due to the overtone of an OH deformation mode of water included in a lens nucleus and a band due to an NH stretching mode of the lens proteins, while the second peak near 3390 cm^{-1} contains exclusively a band assigned to an OH stretching mode of lens water (Iriyama et al., 1983). Figure 3 clearly shows that the intensity ratio of the bands at 3390 and 2935 cm^{-1} (I_{3390}:I_{2935}) decreases with aging.

Figure 7b shows the aging effect on the intensity ratio of the bands at 3390 and 2935 cm^{-1}. The ratio changed from ca. 0.33 to ca. 0.17 as a mouse aged from 17 days to 13 months, indicating that the relative concentration of the lens proteins to lens water undergoes a dramatic change with age. Importantly, the profile in Figure 7b fairly resembles that for the SH stretching mode in Figure 7a.

Discussion

Lens Dehydration. A mammalian lens slowly increases in size as new lens fibers develop throughout life and older lens fibers in a nuclear portion become dehydrated (Cotlier, 1981). On the other hand, increased hydration is found in the process of various cataract formations (Kinoshita, 1974). Therefore, the relative concentration of lens water in a nucleus may be an indicator of the physiological condition of the lens. It was proposed in our previous communication (Iriyama et al., 1983) that the intensity ratio of the Raman bands at 3390 (OH

stretching mode of lens water) and 2935 cm^{-1} (CH stretching mode of the lens proteins) can be used as a practical probe for lens hydration. The present study reveals that a lens nucleus experiences rapid dehydration for the first 4 months and then lens dehydration continues very gradually with age (Figure 7b). The profile in the figure corresponds reasonably well with that obtained by gravimetric analysis (Kinoshita, 1974). On the assumption that the amount of lens proteins in a nucleus does not change with age, it can be roughly estimated from the plots in Figure 7b that the water content of an ICR-strain mouse lens nucleus drops to approximately 55% of the initial value as the mouse ages from 17 days to 5 months.

Relative Concentration and Secondary Structure of Main Lens Proteins. Lens proteins consist of water-soluble proteins (α-, β-, and γ-crystallins) and a water-insoluble protein (albuminoid) (Rink et al., 1982). Rodent water-insoluble protein derives largely from γ-crystallin (Lerman, 1969), and its quantity increases with age. The relative content of α-, β-, and γ-crystallins differs among species and moreover between a nucleus and a cortex (Rink et al., 1982). It is well-known that all changes of lens proteins in the nucleus can be ascribed to postsynthetic modifications. Thus, although some crystallins form protein aggregates in the course of aging, the total relative content of α-, β-, and γ-crystallins probably changes little in the lens nucleus.

A Raman spectrum of a normal lens reflects the relative concentration of α-, β-, and γ-crystallins (Yu & East, 1975; Schachar & Solin, 1975; East et al., 1978; Mizuno et al., 1982). On the basis of the change in the intensity ratio of the Raman bands at 624 (Phe) and 644 cm^{-1} (Tyr), East et al. (1978) suggested an increased proportion of α-crystallin and a decreased proportion of β-crystallin in the cortex of an aged rat lens. However, it is more appropriate to investigate the relative intensities of various Raman bands along with the intensity ratio of the bands at 624 and 644 cm^{-1} in order to confirm whether or not the relative concentration varies. Thus, besides the intensity ratio of these particular two bands, the relative intensities of the Raman bands at 760 (Trp), 1004 (Phe), 1031 (Phe), 1209 (Tyr), and 1547 cm^{-1} (Trp) were estimated in the present study for lens nuclei of different ages. These relative intensities did not exhibit an appreciable variation with aging, supporting the idea that the relative concentration of α-, β-, and γ-crystallins stays nearly the same in the nuclear portion.

The X-ray analysis of bovine γ-crystallin II (Blundell et al., 1981) and Raman spectra of mammalian lenses from several species (Yu & East, 1975; Schachar & Solin, 1975; Yu et al., 1977; Thomas & Schepler, 1980) established that their lens proteins have mainly antiparallel β-pleated sheet structure. Whether the formation of protein aggregates, generally found in the course of lens aging and opacification (Harding & Dilley, 1976; Cotlier, 1981; Bettelheim & Siew, 1982), involves a conformational change in peptide backbone of the lens proteins is now of keen interest. The results in Figure 1 show that the secondary structure of main lens proteins does not undergo a major conformational change with aging. The same conclusion was reached earlier by East et al. (1978) for a rat lens. It was also previously found that no major conformational change in peptide backbone of the lens proteins occurs during various cataract formations (Thomas & Schepler, 1980; Ozaki et al., 1982; Itoh et al., 1983) or thermal denaturation of a bovine lens (Yu & East, 1975). Consequently, the present result together with the previous results suggest that the secondary structure of lens proteins is little perturbed by the formation of the protein aggregates.

Microenvironments of Tyrosine and Tryptophan Residues in Lens Proteins. It has long been recognized that the formation of protein aggregates is mainly responsible for lens aging and opacification (Spector, 1962; Benedek, 1971; Jedziniak et al., 1973; Tanaka & Benedek, 1975; Harding & Dilley, 1976; Cotlier, 1981; Bettelheim & Siew, 1982). Numerous experimental studies have suggested that sulfhydryl groups of cysteine residues and/or aromatic amino acid residues such as tyrosine and tryptophan are concerned with the modification processes in the lens proteins. It is therefore highly significant to monitor the situational changes of protein side groups in the course of the lens aging under the physiological conditions.

The relative intensity of the 880-cm^{-1} band has been proposed as a practical probe for the microenvironment of tryptophan residues (Kitagawa et al., 1979). According to Kitagawa et al. (1979) a buried tryptophan gives a relatively intense feature at 880 cm^{-1} while an exposed tryptophan shows a weak band at the same position. In our previous paper (Itoh et al., 1983), we reported that the intensity ratio of the Raman bands at 880 and 760 cm^{-1} (I_{880}:I_{760}) changed from ca. 0.85:1 to ca. 0.65:1 as a cac-strain mouse aged from 16 days to 9 weeks and suggesting that some buried tryptophan residues become exposed in the course of hereditary cataract formation. The result in Figure 6a is similar to that for the cac-strain mouse lens, although the change seems to be stepwise in the former. Thus, it is probable that in ICR-strain mouse lens nuclei some tryptophan residues in a hydrophobic environment become accessible to water during the first 4 months and some other tryptophan residues undergo the same environmental change from 8 months to 13 months after birth. There may actually be two different mechanisms which bring about the microenvironmental change of these residues.

A change in a hydrogen bonding environment of a tyrosine residue can be monitored by a doublet feature near 840 cm^{-1} (Siamwiza et al., 1975). The intensity ratio of the bands at 855 and 832 cm^{-1} (I_{855}:I_{832}) for an ICR-strain mouse lens nucleus retained nearly the same value of 1:0.86 until at least 13 months of age. The observation suggests that lens aging does not cause a microenvironmental change on tyrosine residues. In other words, the formation of protein aggregates in the course of lens aging is not accomplished by a change in the hydrogen-bonding environment of tyrosine residues.

Conversion of Sulfhydryl Groups to Disulfide Linkages in Lens Proteins. γ-Crystallin with a high content of sulfhydryl groups is predominant in a rodent lens nucleus. It was shown that the intensity decrease of the SH stretching mode at 2579 cm^{-1} can be primarily ascribed to the postsynthetic conversion of sulfhydryl groups of γ-crystallin to S–S bonds (East et al., 1978; Askren et al., 1979). On the basis of Raman measurements of rodent lens nuclei at various ages, Kuck et al. (1982) further suggested that the 2SH → S–S conversion plays a highly important role in aging changes in the rodent lens nuclei and explained the rapid decrease in sulfhydryl group concentration with aging by the formation of protein aggregates mainly by γ-crystallin.

The result in Figure 7a confirms that the 2SH → S–S conversion proceeds very rapidly for the first several weeks as reported earlier (Kuck et al., 1982). The intensity of the SH stretching mode approached zero with age, and the mode was missing in the Raman spectrum of a 13-month-old mouse lens nucleus (Figure 2).

It is particularly notable that the age-dependent profile of the intensity of the SH stretching mode is similar to that of

the OH stretching mode (Figure 7). This similarity in profile between these two modes implies that the rapid decrease of the sulfhydryl groups, leading to the formation of protein aggregates, is related to lens dehydration. It seems likely that lens dehydration concentrates the proteins and thus makes the formation of disulfide bonds more feasible.

The intensity decrease of the tryptophan band at 880 cm^{-1} proceeded almost in parallel with that of the SH stretching mode for the first 4 months (Figures 6a and 7a). Therefore it appears that the first microenvironmental change occurs in some tryptophan residues upon the formation of protein aggregates by the sulfide linkages. However, the second microenvironmental change of tryptophan residues which appears to take place from 8 to 13 months after birth is probably independent of the formation of disulfide linkages since the 2SH → S–S conversion is almost completed within the first 4 months as is evident from Figure 7a.

Structural Changes of the Lens Proteins in Lens Aging and Lens Opacification. It was suggested that in the course of lens opacification tyrosine and tryptophan residues undergo microenvironmental changes and that these changes are involved in the formation of protein aggregates (Itoh et al., 1983). In contrast, only tryptophan residues seem to experience microenvironmental change during aging. Comparing the present experimental results for lens aging with the previous results for lens opacification leads us to the temporary conclusion that the microenvironmental change of tyrosine residues is specific for the latter and the formation of protein aggregates involving the microenvironmental change of tyrosine residues is deeply correlated with lens opacification. It is however, obvious that much remains to be explored in the role of tyrosine residues in lens opacification. The microenvironmental change of tryptophan residues is commonly observed in lens aging and lens opacification, although the mechanism of change might differ. The present investigation suggests that this change occurs twice stepwisely during the course of normal aging, the first change being in concert with the 2SH → S–S conversion. However, it is not clear whether the second change is involved in the process of a certain protein aggregation. If this is the case, there would be at least two kinds of mechanisms in the formation of protein aggregates in lens aging.

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