Structural Changes in the Lens Proteins of Hereditary Cataracts Monitored by Raman Spectroscopy†

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ABSTRACT: Raman spectra have been measured for the lenses from cac-strain mice. These mice possess a hereditary defect and provide lenses at various stages of opacification. The Raman spectra of normal mouse lenses have been obtained also for comparison purposes. The amide I and III bands appear in very similar positions in the Raman spectra of cataractous and normal lenses, suggesting that the peptide backbone of main lens proteins does not undergo a major conformational change upon lens opacification. However, lens opacification causes significant changes in the intensity ratio of the tyrosine doublet near 840 cm^{-1} and in that of the Raman bands at 881 and 760 cm^{-1} due to tryptophan residues. These changes could be observed even in the incipient stage of hereditary cataract and became more pronounced with cataract development. These observations indicate that in the course of lens opacification some tyrosine residues undergo a change in their hydrogen-bonding environment and some buried tryptophan residues became exposed. In addition, the present Raman spectroscopic study provides insight into the 2SH → S–S conversion in lens proteins. It was found that the conversion proceeded at a faster rate in a hereditary cataractous lens than in a normal lens; however, this difference was fairly small at the early stage of cataract development. Importantly, the 2SH → S–S conversion was accelerated after nuclear cataract formation. These observations support the hypothesis that the formation of S–S linkages is not a predominant factor for initiating lens opacification. Probably the formation of S–S linkages plays an important role in stabilizing the protein aggregates which are the cause of lens opacification. The intensity of the SH stretching mode (2579 cm^{-1}) was very weak or absent in the Raman spectrum of a well-developed cataractous lens, suggesting that most sulfhydryl groups form disulfide bonds. Moreover, the fact that this occurs without major conformational changes of peptide backbones implies that most cysteine residues in lens crystallins are accessible to solvent or are clustered closely together.

The causes and processes of lens opacification have been extensively investigated from the biochemical and morphological standpoints (Cotlier, 1981). Biochemical investigations have revealed that the accumulation of polyol in a lens plays a key role in the formation of diabetic and galactosemic cataracts (van Heyningen, 1959; Kinoshita, 1974; Varma et al., 1977), and a possible cataractogenic factor in a cac-strain mouse lens is an endogenous inhibitor of Na–K-ATPase (Fukui et al., 1978). These cataracts have been recognized as an osmotic type of cataracts in which an osmotic overhydration initiates a series of physicochemical events that ultimately lead to the formation of cataracts.

The main dry components of lens fiber are three lens proteins named α-, β-, and γ-crystallins, and these undergo various changes during cataract formation. The most prominent physicochemical event bringing about lens opacification is the formation of protein aggregates (Spector, 1962; Jedziniak et al., 1973; Tanaka & Benedek, 1975). Although it is generally recognized that the amino acid residues involved in the protein aggregation are either aromatic amino acids or sulfhydryl groups of cysteine residues, or both, the detailed mechanisms of the formation of protein aggregates are still unknown. Thus, it is now important to probe the properties of the aromatic amino acid and cysteinyl residues of crystallins upon lens opacification.

Raman spectroscopy is an excellent nondestructive structural probe, at the molecular level, for constituents in biological materials. Raman spectroscopy of an intact ocular lens gives the vibrational spectrum of main lens proteins in situ. Thus, by measuring the Raman spectrum of an intact lens, it is possible to monitor the secondary structure of lens proteins (Yu & East, 1975; Schachar & Solin, 1975; Yu et al., 1977; Kuck et al., 1976) and the microenvironments of protein side groups such as tryptophan, tyrosine, and sulfhydryl (Yu & East, 1975; Yu et al., 1977; East et al., 1978; Askren et al., 1979; Ozaki et al., 1982; Kuck et al., 1982). The possibility of the clinical application of Raman spectroscopy in lens diseases was discussed by Mathies & Yu (1978), Mizuno et al. (1982), and Yu et al. (1982). Recently, in addition, Raman spectroscopy has been used by Thomas & Schepler (1980) and Ozaki et al. (1982) to study lens opacification, and the latter authors suggested that upon opacification changes occur in the microenvironment of some tyrosine residues (Ozaki et al., 1982).

In the present paper, we report a more detailed Raman spectroscopic study of cataract formation using cac-strain mice which possess a hereditary defect in the lens (Iwata & Kinoshita, 1971). For a cac-strain mouse, a cataract appears in the nuclear portion of the lens approximately 3 weeks after birth, and the nuclear opacity is completed within a few weeks. Therefore, Raman measurements of cac-strain mouse lenses at the various stages of opacification enable us to probe the molecular structural changes of the lens proteins during cataract formation.

Experimental Procedures

Materials. Cataractous lenses were obtained from cac-strain mice, and the normal lenses of ICR-strain mice were used as controls. The cac-strain mice were generously provided by Professor S. Iwata (Meijo University). The animals were kept in an air-conditioned room and fed ad lib. Both cac- and ICR-strain mice opened their eyes on the 14th day after birth, when the lens of a mutant animal was still perfectly transparent. A "pin head" opacity began to develop in a lens nucleus around 3 weeks after birth. The nuclear opacity of the lens was clearly noticed when the cac-strain mouse was about 4 weeks old. The nuclear cataract was almost completed about 5 weeks after birth. Mice were killed just before the

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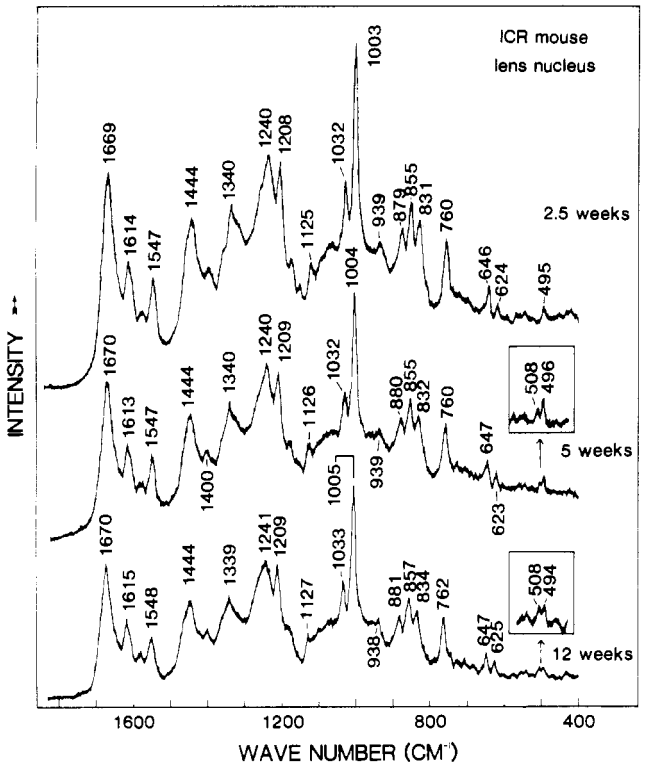

FIGURE 1: Raman spectra of a lens nucleus of the ICR-strain mice (2.5, 5, and 12 weeks old). Instrumental conditions: excitation wavelength, 488.0 nm; laser power, 180 mW; spectral slit width, 8 cm^{-1}; time constant, 4.0 s.

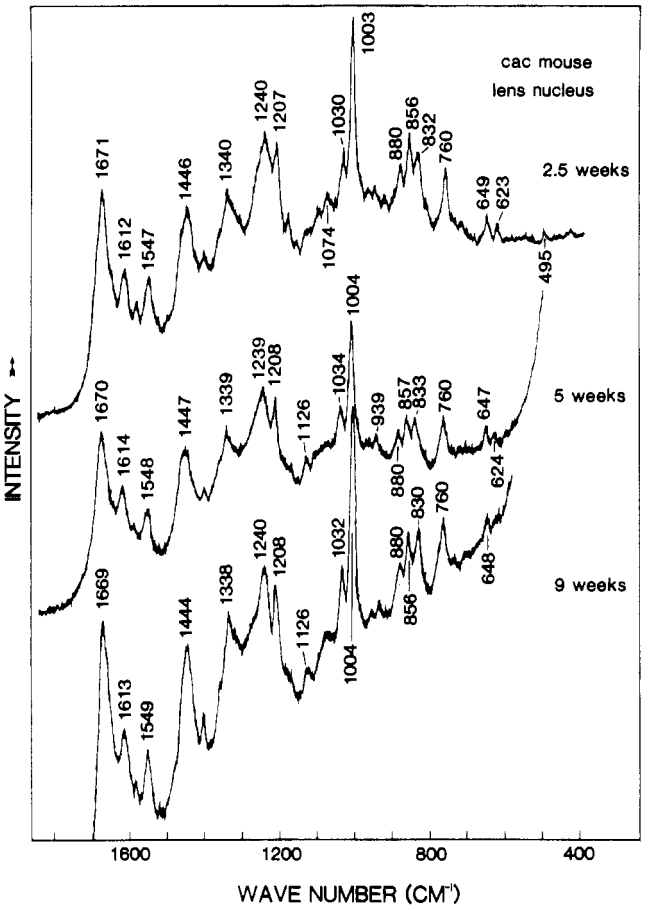

FIGURE 2: Raman spectra of a lens nucleus of the cac-strain mice (2.5, 5, and 9 weeks old). Instrumental conditions: excitation wavelength, 488.0 nm; laser power, 180 mW (2.5 week old mouse) or 270 mW (5 and 9 week old mice); spectral slit width, 8 cm^{-1} (2.5 week old mouse) or 9.5 cm^{-1} (5 and 9 week old mice); time constant, 4.0 s.

enucleation of the eyeball. The lens was removed from the eyeball by a posterior approach and then transferred into a cuvette cell. The lens was placed on the cell bottom and covered with 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. Raman measurements were made by using a JEOL 400 D laser Raman spectrophotometer equipped with an HTV R943-02 photomultiplier tube. The excitation wavelength was 488.0 nm from a Spectra-Physics Model 164 Ar laser. The power at the sample was about 180 mW for the normal lenses and typically 270 mW for the cataractous lenses, and 8–9.5 cm^{-1} spectral slit width resolution was employed. Peak frequencies were calibrated by measuring the spectrum of indene and were believed to be accurate to ±1 cm^{-1} for well-resolved bands. The lens was illuminated by the laser beam from the bottom of the cell, and the scattered light was collected at 90° to the incident beam. A narrow black tape slit was stuck on the front of the cell to collect selectively the Raman scattering from the nuclear portion of the lens. In the case of the cataractous lens, the laser beam was focused on the opaque nucleus. Raman spectra were repeated at least twice and showed good reproducibility. The laser power was varied from 130 to 360 mW at the sample; no spectral changes occurred for powers less than at least 360 mW. No changes in Raman scattering intensities and frequencies could be detected during 4 h of laser irradiation (270 mW).

Results

Figure 1 shows the Raman spectra of a normal lens nucleus of the ICR-strain mice (2.5, 5, and 12 weeks old), and the spectra are generally similar to those of a lens nucleus of other mammals (Yu & East, 1975; Schachar & Solin, 1975; Yu et al., 1977; Kuck & Yu, 1978; Thomas & Schepler, 1980; Mizuno et al., 1982; Ozaki et al., 1982). The amide I band at 1670 cm^{-1} and the amide III band at 1240 cm^{-1} show that the lens proteins of the ICR-strain mouse are mostly in the antiparallel β-sheet conformation (Yu & East, 1975; Schachar & Solin, 1975; Thomas & Schepler, 1980). The Raman spectra of a normal lens nucleus did not show an appreciable change with aging in the region of 550–1700 cm^{-1}. However, a small but significant change was observed in the region of 500–550 cm^{-1} where an S–S stretching mode is expected. The S–S stretching mode was not observed in the Raman spectrum of a normal lens nucleus at 2.5 weeks. However, a weak band due to the S–S stretching mode was found at 510 cm^{-1} in the spectrum of the lens nucleus at 5 weeks, and the intensity of the band increased gradually with age.

Raman spectra of lens nuclei from the cac-strain mice (2.5, 5, and 9 weeks old) are shown in Figure 2. The Raman spectra of the lens nuclei are almost identical, at 2.5 weeks, for the ICR-strain and cac-strain mice. The Raman spectra of cac-strain mouse lens nuclei changed little with aging in the 550–700- and 900–1700-cm^{-1} regions. Although an intensity change was expected in the region of 500–550 cm^{-1}, the rising background due to the intense Rayleigh scattering made the detection of the putative S–S stretching mode impossible for the cac-strain mouse lens nuclei. However, the normal lens nucleus and the cataractous lens nucleus showed clearly differential effects in the 700–900-cm^{-1} region of the Raman spectra upon aging.

Figure 3 displays an enlargement of the 650–950-cm^{-1} spectral region of the cac-strain mouse lens nuclei. The in-

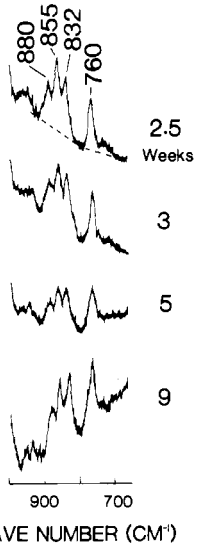

FIGURE 3: Raman spectra of cac-strain mouse lens nuclei in the region of 650–950 cm^{-1}. The experimental conditions were the same as those in Figure 2.

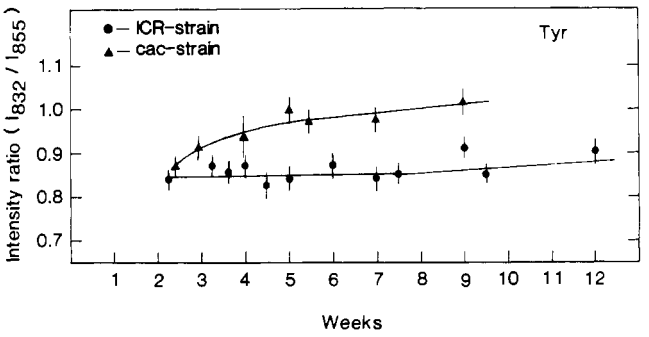

FIGURE 4: Changes of the intensity ratio of the tyrosine doublet (I_{832}:I_{855}) vs. age. (●) ICR-strain mouse; (▲) cac-strain mouse. Raman spectra were measured for lenses from two or three mice of the same age for the experimental data shown in Figures 4, 5, and 7.

tensity ratio of the doublet near 840 cm^{-1} due to tyrosine residues changes with aging. The band at 853 cm^{-1} is stronger than that at 831 cm^{-1} in the spectrum of a 2.5 week old lens nucleus while in the spectrum of a 5 week old lens nucleus it is of equal intensity. Moreover, the intensity ratio of the bands at 760 and 880 cm^{-1} (I_{880}:I_{760}), arising from tryptophan residues, shows an appreciable change with age. These intensity changes for tyrosine and tryptophan bands were rather small but reproducible.

Figure 4 plots the tyrosine intensity ratio as a function of age. The ratio was calculated by comparing peak heights, and base lines were estimated as shown in Figure 3. For normal lens nuclei, the relative intensity of the doublet (I_{853}:I_{831}) was constant at 1:0.85, while for the cac-strain mouse lens nuclei, the ratio changed gradually from 1:0.85 to 1:1 with increasing age. Importantly, the intensity change could be detected even at the incipient stage of hereditary cataract formation. After the fifth week, when the nuclear cataract was well developed, the intensity ratio was nearly constant.

The intensity ratio of the two tryptophan bands at 760 and 880 cm^{-1} (I_{880}:I_{760}) as a function of age is shown in Figure 5. The ratio was almost constant in the course of the normal mouse's growth. However, the corresponding ratio for the cac-strain mouse changes from 1:0.85 to 1:0.65 upon aging. As in the case of the tyrosine doublet, some scatter is observed in the relative intensity data for 4 week old cac-strain mouse lenses. The scatter indicates that individual difference is fairly

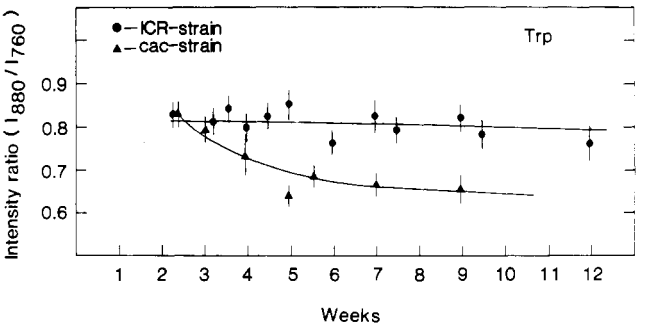

FIGURE 5: Changes of the intensity ratio of the bands at 760 and 880 cm^{-1} (I_{880}:I_{760}) vs. age. (●) ICR-strain mouse; (▲) cac-strain mouse.

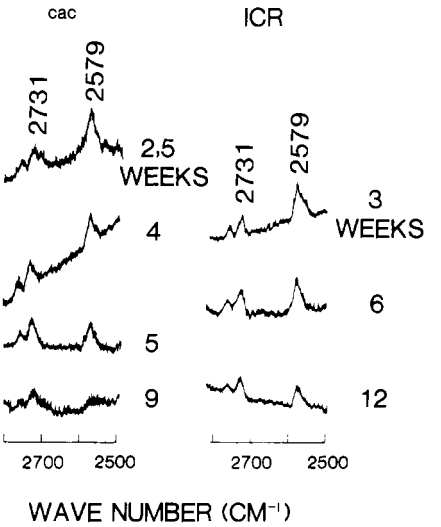

FIGURE 6: Aging effects on the intensity of the SH stretching mode of cysteine residues.

large among 4 week old mice. The intensity ratio became almost constant after the nuclear cataract was developed sufficiently.

Figure 6 shows the effects of age on the intensity of the cysteines' SH stretching mode located at 2579 cm^{-1}. The shoulder near 2560 cm^{-1} can also be assigned to the SH stretching mode. These assignments for the SH stretching modes were confirmed by measuring Raman spectra of a lens immersed in D_2O buffer solution. A new band with a shoulder appeared at 1875 cm^{-1} upon exchanging H_2O and D_2O. In H_2O buffer, the intensity-invariant band at 2731 cm^{-1} was used as an intensity standard. The effects of aging were clearly observed in the intensity of the SH stretching mode for both the normal controls and cataract mutants. However, the intensity decrease of the SH stretching mode proceeded faster in a cac-strain mouse lens nucleus than in an ICR-strain mouse lens nucleus. Figure 7 exhibits the intensity changes of the SH stretching mode (2579-cm^{-1} band) for both cac- and ICR-strain mice. For the ICR-strain mice, the intensity of the SH stretching mode dropped to approximately 40% of the initial value as an ICR-strain mouse aged from 16 days to 12 weeks. A corresponding intensity increase of the S–S stretching mode was observed as shown in Figure 1. The present result for 2SH → S–S conversion with aging is in good agreement with those previously reported for other rodents (East et al., 1978; Askren et al., 1979; Kuck et al., 1982). Although at the incipient stage of hereditary cataract formation there was no significant difference in the rate of SH decrease between the cac-strain mouse lens nuclei and the ICR-strain mouse lens nuclei, the rate observed for the cac-strain mouse lens nuclei seems to become gradually faster as

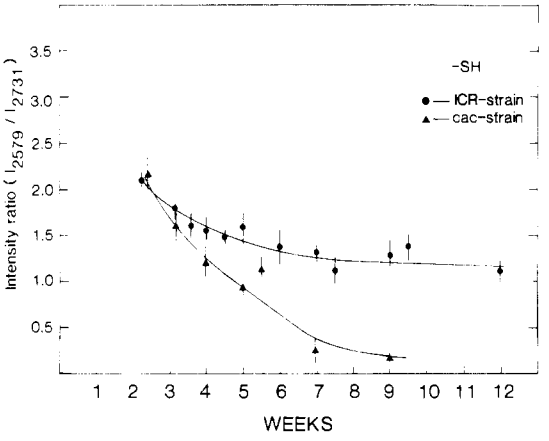

FIGURE 7: Changes of the intensity ratio of the bands at 2579 and 2731 cm^{-1} vs. age. (●) ICR-strain mouse; (▲) cac-strain mouse.

the cataract developed (Figure 7). The decrease of SH content in the cac-strain lens nuclei is caused by two effects, aging and lens opacification. Particularly striking is an extreme decrease of the SH stretching mode in a 7 week old cac-strain mouse lens nucleus. At 9 weeks, the SH stretching mode was so weak that it was difficult to quantitate the relative intensity of the SH stretching mode.

Discussion

Secondary Structure of Main Lens Proteins. The frequencies of amide I and amide III modes originating from a peptide backbone are the key indicators of conformational changes in a protein (Carey & Salares, 1980). These modes did not exhibit an appreciable shift between the Raman spectra of normal lens nuclei and those of hereditary cataractous lens nuclei, suggesting that no major conformational change of lens proteins occurs upon lens opacification. The same conclusion was reached in the Raman studies on lens opacification induced by a variety of methods (Yu & East, 1975; Thomas & Schepler, 1980; Ozaki et al., 1982). Therefore, it may be concluded that lens opacification is not associated with major conformational changes of protein secondary structure. Probably the secondary structure of lens proteins is little perturbed by environmental changes and external stimulations. In particular, the Raman data show that the process of protein aggregation, known to occur in the opaque lens (Spector et al., 1971; Jedziniak et al., 1972, 1973, 1975; Liem-The et al., 1975; Tanaka & Benedek, 1975; Roy & Spector, 1976; Takemoto & Azari, 1977; Spector & Roy, 1978; Giblin et al., 1978), is not accompanied by major conformational changes in the protein polypeptide changes. Instead, the protein aggregation probably causes microenvironmental changes of tyrosine and tryptophan residues as described below.

Tyrosine and Tryptophan Residues in Lens Proteins. In order to delineate the mechanism of lens opacification at the molecular level, it is especially important to monitor the microenvironments of tyrosine, tryptophan, and cysteine residues. The microenvironments of tyrosine, tryptophan, and cysteine residues of isolated α-, β-, and γ-crystallins have been investigated by circular dichroism and fluorescence spectroscopy (Liang & Chakrabarti, 1982; Andley et al., 1982), but very little is known about their situation in protein aggregates.

In our previous publication (Ozaki et al., 1982), we reported that the intensity ratio of the tyrosine doublet (I_{853}:I_{831}) was 1:0.85 for a normal lens nucleus whereas for a diabetic cataractous lens nucleus the corresponding intensity ratio was 1:1. The present results shown in Figures 3 and 4 are highly analogous to the results for the diabetic rat lens nucleus, suggesting that a similar change in the microenvironment of some tyrosine residues occurs also in the course of hereditary cataract formation. Moreover, the relative intensity change of the tyrosine doublet could be observed in the incipient stage of hereditary cataract formation, suggesting that the environmental change of tyrosine residues proceeds simultaneously with aggregation of lens proteins.

It was demonstrated by Siamwiza et al. (1975) that the tyrosine doublet is very sensitive to the nature of hydrogen bonding or the state of ionization of the phenolic hydroxyl group. If the OH group of a tyrosine residue is strongly bound to a negative acceptor, the doublet intensity ratio is ca. 0.5:1. For the tyrosyl residue forming a moderately strong hydrogen bond to H_2O, the ratio is ca. 1:0.8, and if the OH group functions as a strong hydrogen-bond acceptor, the ratio is ca. 1:0.4. Since the intensity ratio of the tyrosine doublet contains contributions from several tyrosine residues of lens proteins, it is rather difficult to identify which kind of environmental change occurs during the formation of a hereditary cataract. On the basis of the above empirical rule, however, the present result that the intensity ratio changed from 1:0.85 to 1:1 can be explained by a few possibilities. One possibility is that some tyrosine residues which form a hydrogen bond with H_2O prior to aggregation are captured by strong hydrogen-bond acceptors in the aggregate.

Kitagawa et al. (1979) have proposed that the intensity of a Raman feature found at 880 cm^{-1} can be used as a probe of tryptophan environment. When a tryptophan residue is buried, the relative intensity of the band at 880 cm^{-1} is fairly strong whereas an exposed tryptophan gives a weak feature at 880 cm^{-1} (Kitagawa et al., 1979). Thus, the decrease of the relative intensity of the band at 880 cm^{-1} (Figures 3 and 5) suggests that some buried tryptophan residues of the lens proteins become exposed during the course of lens opacification. However, the intensity change is rather small, suggesting that only a few tryptophan residues undergo an environmental change. Since the change could be detected in the fairly early stage of a hereditary cataract, it may be correlated with the formation of lens protein aggregates as in the case of tyrosine residues.

The possibility of the involvement of tyrosine residues in the protein aggregation process was suggested by Jedziniak et al. (1973), who observed that the tyrosine content in high molecular weight protein produced by the protein aggregation in a cataractous human lens was lower than that found for lower molecular weight soluble lens proteins. Recently Garcia-Castineiras et al. (1978) suggested that the formation of aggregates involves degradation products of tyrosine and tryptophan. They were able to isolate 3,3′-bityrosine and anthranilic acid as constituents of the proteolytic hydrolysate from human cataractous lenses (Garcia-Castineiras et al., 1978). However, in the present Raman spectra of cataractous lens nuclei, no specific Raman bands which could be assigned to bityrosine or anthranilic acid groups were observed. In addition, the marked intensity decrease of tyrosine and tryptophan bands was not observed under the present experimental conditions except for the relative intensity changes in the region of 700–900 cm^{-1}. Thus, the Raman spectra of hereditary cataractous lens nuclei gave no evidence for the formation of a large number of bityrosine and anthranilic acid groups in lens proteins, although the present observation does not exclude the existence of a small number of bityrosine and anthranilic acid groups.

Sulfhydryl Groups and the Formation of S–S Linkages. Evidence for the conversion of SH groups to S–S bonds in lens

proteins has been accumulated by many investigators (Dische & Zil, 1951; Pirie, 1968; Testa et al., 1968; Clark et al., 1969; Epstein & Kinoshita, 1970; Harding, 1970, 1973; Satoh, 1972; Kinoshita & Merola, 1973; Srivastava & Beutler, 1973; Takemoto & Azari, 1976; Harding & Dilley, 1976; Anderson & Spector, 1978). It has been considered that the formation of the S–S linkage would bring about less soluble, higher molecular weight products in the lens. However, a question has still remained about the role of 2SH → S–S conversion in cataractogenesis; there has been no agreement on whether or not the formation of the S–S linkage is a predominant factor bringing about lens opacification.

The present Raman spectroscopic study on the SH stretching mode during the formation of the hereditary cataract may give us a clue to the above question. It is particularly noteworthy that the intensity change of the SH mode exhibited a time dependency which was quite different from that of the tyrosine and tryptophan bands. As shown in Figures 4 and 5, the intensity ratio of the tyrosine doublet and that of the tryptophan bands changed appreciably even in the early stage of hereditary cataract formation, and after the fifth week, the intensity ratios were almost constant. On the other hand, in the early stage of the hereditary cataract, the difference in the rate of intensity decrease of the SH stretching mode was rather small between a cac-strain mouse lens nucleus and an ICR-strain mouse lens nucleus (Figures 6 and 7). It should be noted that approximately 40% of the sulfhydryl groups were still intact even in a 5 week old lens nucleus where the cataract is already developed considerably. After the nuclear cataract was already developed, the cac-strain lens nucleus showed a much faster rate of intensity decrease of the SH feature than the ICR-strain mouse lens nucleus. Thus, the changes in the intensity ratio of the tyrosine doublet and that of the tryptophan bands proceed simultaneously with the cataract formation whereas the intensity decrease of the SH stretching mode does not, which means that the formation of S–S bonds in the lens proteins is considerably delayed. This strongly suggests that the 2SH → S–S conversion is not a predominant factor to initiate the hereditary cataract but a process which is accelerated by protein aggregation. It is therefore unlikely that protein aggregation is brought about by the 2SH → S–S conversion. Presumably, the formation of S–S linkages plays an important role in stabilizing the protein aggregates.

It is also noted that the intensity of the SH stretching mode is very weak or almost missing in the Raman spectrum of a well-developed cataractous lens. Thus, most sulfhydryl groups form disulfide bonds, although the formation of the inter- or intramolecular S–S linkages appears to have little effect on the conformation of the peptide backbone. Thus, it may be concluded that almost all cysteine residues in the lens crystallins are accessible to solvent or are clustered closely together. This conclusion is consistent with the result of an X-ray crystallographic study of bovine γ-crystallin, which showed that five of six cysteine residues lie in the same domain and three are clustered closely together (Blundell et al., 1981). The same study also indicated that Cys-74 and Cys-18 can form an intramolecular disulfide bridge without radically affecting the overall conformation of the molecule, and four cysteine residues (Cys-15, Cys-18, Cys-23, and Cys-105) are accessible to solvent.

Acknowledgments

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Registry No. L-Tyrosine, 60-18-4; L-tryptophan, 73-22-3.

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Histone Synthesis by Lymphocytes in G_0 and G_1†

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ABSTRACT: Peripheral blood lymphocytes are a naturally occurring population of G_0 cells which can be activated in vitro to grow and divide. Upon activation with phytohemagglutinin (PHA), they enter G_1 and, after a 24-h lag, begin DNA replication (S phase). Using radioisotope labeling and gel electrophoresis of acid-soluble chromatin proteins, we investigated histone synthesis in G_0, G_1, and S phase cultures of human and pig lymphocytes. In G_0 and G_1 cultures, which have less than 0.1% S phase cells, all five histones are synthesized and are incorporated into chromatin in equimolar amounts. In G_0 lymphocytes histone synthesis accounts for at least 6% of nuclear protein radioactivity, and the rate of synthesis is about 2-3% of that of S phase lymphocytes. In contrast to histone synthesis by S phase cultures, G_0 and G_1 histone synthesis was completely resistant to treatment with hydroxyurea.

Although it is now well established that most histone synthesis is coupled to DNA replication [reviewed in Hohmann (1981)], evidence is accumulating which shows that some histone synthesis, especially of H1, occurs outside of the S phase of the cell cycle. This evidence has most often come from studies which involved detection of newly synthesized histones or their mRNA in established mammalian cell lines in G_1, obtained by synchronization or G_1 arrest (Curley et al., 1972; Melli et al., 1977; Tarnowka et al., 1978; Pehrson & Cole, 1982). However, there have been conflicting results, often with the same cell lines (Rickles et al., 1982; Marashi et al., 1982), and the G_1 synthesis observed in these cell lines has often been ascribed to the relatively large numbers (from 2 to 25%) of S phase cells which usually persist in such preparations (Rickles et al., 1982; Delegeane & Lee, 1982).

Circulating peripheral blood lymphocytes are a naturally occurring population of G_0 cells which can be activated in vitro by plant lectins to grow and divide [reviewed in Ling & Kay (1975); Hume & Weidemann, 1980]. During the course of studies on the synthesis of nuclear proteins by unstimulated and phytohemagglutinin- (PHA-) activated human and pig lymphocytes, we observed significant incorporation of labeled amino acids into histones of G_0 and G_1 phase lymphocyte cultures. We present here evidence that there is incorporation of all five newly synthesized histones into chromatin of G_0 and G_1 lymphocytes which is not due to contaminating S phase cells and which is resistant to hydroxyurea.

Materials and Methods

Lymphocyte Cultures. Human and pig lymphocytes were isolated from EDTA[1] anticoagulated or defibrinated blood by density gradient centrifugation on Ficoll-Isopaque as described by Boyum (1976) and washed and suspended in RPMI 1640 medium as previously described (Dauphinais & Waithe, 1977). The cells were incubated at 37 °C for 16-22 h before the start of the experiment to allow them to recover from the isolation procedure. Of the nucleated cells in the cultures, 80-90% were lymphocytes, and the rest were approximately equal numbers of granulocytes and monocytes (neither of which survive or divide in the cultures). Pure (over 99%) human T lymphocytes, prepared by rosette formation with neuraminidase-treated sheep red blood cells (Weiner et al., 1973), were used in some experiments and gave the same results. The cells were cultured at a concentration of 2×10^6 mononuclear cells/mL in 50 mL or conical-tip disposable culture tubes or in flat-

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate.

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