Spectroscopic Investigations of Bovine Lens Crystallins. 1. Circular Dichroism and Intrinsic Fluorescence†

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ABSTRACT: Circular dichroism (CD) and intrinsic fluorescence measurements have been carried out to study the secondary and tertiary structure of the lens proteins α-, β_H-, β_L-, and γ-crystallins. CD spectra of crystallins in the far-ultraviolet (far-UV) region, typical of the β conformation of a protein, show some variation in β content, which may not have any significant biological correlation. In addition, β_H- and γ-crystallins show a band at 235 nm which probably originates from an interaction between a peptide amide transition and a transition of aromatic amino acid residues. Near-UV CD spectra (250–310 nm) of these proteins, which arise mostly from aromatic amino acid residues, differ considerably from each other. The differences cannot be attributed to variations in the content of aromatic amino acid residues; rather, they reflect the extent of the interactions between these residues (μ–μ coupling) and, thus, their difference in tertiary structure. Attempts have been made to assign the CD bands, as much as possible, on the basis of absorption and pH-dependent CD spectra. There is no significant contribution of disulfide bonds to the near-UV CD, as indicated by the lack of difference in the dichroic behavior between dithiothreitol-reduced and untreated samples. Near-UV CD spectra further indicate that tryptophan residues in γ-crystallin are mostly buried in a hydrophobic environment, whereas in α-crystallin, they are least buried; similarly, tyrosine residues in both β-crystallins are in more polar environments than are α- and γ-crystallins. Emission maxima and quantum yield values of the fluorescence of lens crystallins suggest that exposed tryptophan residues in these proteins are in the order $\alpha > \beta_H \simeq \beta_L > \gamma$, which is consistent with near-UV CD results. Because of efficient energy transfer from tyrosine to tryptophan in these proteins, tyrosine fluorescence can be observed only when it is measured in guanidine hydrochloride (Gdn·HCl). Unlike emission maxima, values of the quantum yields in Gdn·HCl are not uniform, indicating the sensitivity of tryptophan fluorescence to the residual three-dimensional structure or to the persisting interaction between tryptophan residues and other side chains even after denaturation.

The water-soluble proteins of the mammalian lens can be divided into three main classes, α-, β-, and γ-crystallins, based on their chemical characteristics such as molecular weight and electrophoretic mobility. β-Crystallins can further be separated into two components of differing molecular size, β_H (high) and β_L (low), by gel chromatography (Bloemendal & Herbrink, 1974). Human lens crystallins undergo a number of changes during aging and cataractogenesis, including increased protein aggregation (Jedziniak et al., 1975) and formation of insoluble proteins (Mach, 1963; Satoh, 1972), increased pigmentation in the nucleus (Pirie, 1968; Zigman, 1971), production of blue fluorescence (Satoh et al., 1973; Augusteyn, 1975), and increased near-UV absorption (Fujimori, 1978). The aggregation is stabilized either by disulfide bonds formed by oxidation of sulfhydryl groups (Dische & Zil, 1951) or by unknown nondisulfide cross-links (Buckingham, 1972; Kramps et al., 1978; Fujimori, 1982). Photooxidation of tryptophan by near-UV light yields pigmented and fluorescent oxidation products, some of which are present in the mature lens (Lerman, 1980; Goosey et al., 1980). Although the nature of many of the age- and cataract-related changes is still unknown, it is apparent that the amino acids involved in the chemical modification and aggregation are either aromatic amino acids or sulfhydryl groups of the cystinyl residues of the protein, or both. It has also become evident that the transparency of lens proteins, their main optical feature, is closely related to their molecular spatial arrangement (secondary and tertiary structure) which in turn is determined by their primary structure (Lerman, 1972; Bloemendal, 1977).

The primary structure of the lens crystallins has been investigated in detail (Harding & Dilley, 1976), but very little is known about their three-dimensional structure, the interactions between different segments of the specific chains of amino acids, and the orientation of specific reactive amino acids and groups susceptible to chemical change. Although some circular dichroism (CD) studies (Croft, 1972; Li, 1974; Li & Spector, 1974; Horwitz, 1976; Horwitz et al., 1977; Zigler et al., 1980) have been reported, systematic studies and detailed analyses are lacking.

In the present paper, we have undertaken a systematic investigation of the structural aspects of lens crystallins by using various physicochemical techniques. We report here the far- and near-UV CD and intrinsic fluorescence measurements of bovine lens crystallins, analysis of the results in terms of their secondary and tertiary structure, and assignment, as far as possible, of the contributions of aromatic amino acids and disulfide bonds to the near-UV CD bands. The microenvironments of tryptophan and tyrosine in different crystallins and the interaction between these two chromophores and disulfide have been assessed primarily from intrinsic protein fluorescence measurements. In the next paper (Andley et al., 1982), we report the results of using extrinsic fluorescent probes to determine the polar and nonpolar characteristics of different crystallins and to examine the reactivity of the sulfhydryl groups present in these proteins.

Materials and Methods

Lens crystallins were isolated from bovine eyes by gel filtration as previously described (Liang & Chakrabarti, 1981), except that Bio-Gel A5m was replaced by Sephacryl S-200 superfine gel (Pharmacia Co.). This agarose gel gave slightly

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better separation of lens crystallins. The protein solutions were made by dissolving lyophilized crystallins in the tris(hydroxymethyl)aminomethane (Tris) buffer; their concentrations were determined by the Lowry method (Lowry et al., 1951). Proteins were denatured by diluting protein samples in buffer with concentrated guanidine hydrochloride (Gdn·HCl) to make a final concentration of 6 M.

Absorption measurements were performed in a Cary 15 spectrophotometer. Difference spectra were obtained by subtracting spectra at pH 8.0 from those at pH 11.5.

CD spectra were measured in a Cary 60 spectropolarimeter with CD attachment. Quartz cells of 1-cm path length were used in the near-UV region (250–310 nm) and 0.1 or 0.05 cm in the far-UV region. Two or three measurements were made for each sample, and excellent reproducibility was obtained at 5 nm/min with a pen period of 1 s. The spectra were corrected for the base line obtained with buffer solvent. The CD intensity was calibrated with an aqueous solution of *d*-10-camphorsulfonic acid (Cassim & Yang, 1969). Molar ellipticity values per residue were computed by using 130 as the mean residue weight; the values were expressed as deg·cm^2 dmol^{-1}. Difference spectra were obtained by measuring two samples of the same concentration at pH 8.0 and 11.5 and then subtracting one from the other at every 5 nm of wavelength.

Fluorescence measurements were performed in a Perkin-Elmer MPF 44A spectrofluorometer equipped with a differential corrected spectral unit. Protein fluorescence was obtained by excitation at 270–295 nm.

Quantum yields (ϕ) were obtained by excitation at 280 or 295 nm, by using the method of Parker & Rees (1960)

$$\frac{A_P}{A_R} = \frac{\phi_P}{\phi_R}\frac{D_P}{D_R} \quad (1)$$

where A is the area under the fluorescence spectrum and D is the optical density at the exciting wavelength. The subscripts P and R refer to protein and reference substance, respectively. The value of 0.20 for ϕ_{Trp} was used (Teale & Weber, 1957; Kronman & Holmes, 1971). The protein solutions used in measuring quantum yields were made so that they had an optical density at the exciting wavelength close to that of the reference solution of tryptophan. The protein solutions used for this measurement had an optical density of less than 0.1.

Tyrosine quantum yields cannot be measured directly from the spectra. They can be calculated from the protein and tryptophan quantum yields (Kronman & Holmes, 1971). The equation used is

$$\phi_{Tyr} = \phi'_{Tyr}[1 + (m/n)(\epsilon_{Trp}/\epsilon_{Tyr})] \quad (2)$$

where m and n are the number of tryptophan and tyrosine residues per protein molecule, respectively, $\epsilon_{Trp}/\epsilon_{Tyr}$ is the ratio of extinction coefficients for the free amino acid, ϕ'_{Tyr} is estimated from $\phi'_{Tyr} = \phi_P - \phi'_{Trp}$, and ϕ'_{Trp} is estimated from the amplitude (F) and area (A) of the emission spectra according to

$$^{(F/A)}\lambda_{ex=280nm}/^{(F/A)}\lambda_{ex=295nm} = \phi'_{Trp}/\phi_{Trp} \quad (3)$$

Results

Circular Dichroism and Absorption Spectra. CD spectra for the four crystallins in the far- and the near-UV regions are shown in Figure 1. In the far-UV region, all four crystallins give a band at 216 nm, which is characteristic of the β conformation. The amount of β conformation can be estimated by using poly(L-lysine) as a standard (Carver et al., 1966); values of 50%, 55%, 64%, and 45% were obtained for α-, β_H-, β_L-, and γ-crystallins, respectively. These values agree with those reported in the literature (Croft, 1972; Li & Spector, 1974). None of these crystallins contain a significant amount of α-helical structure. In addition to the 216-nm band, another negative band was observed for β_H-crystallin at 235 nm, and two more bands (one negative band at 245 nm and one positive band at 235 nm) for γ-crystallin. The appearance of a 235-nm band in β_H- and γ-crystallins (this study), in nuclear α-crystallin (Li, 1974), and in dogfish lens γ-crystallins (Jones & Lerman, 1971) seems to originate from the same transition (see Discussion). In the near-UV region (250–310 nm), lens crystallins give CD spectra with their individual characteristic features.

Table I: Aromatic Amino Acid and Cystinyl Composition of Crystallins[a,b]

amino acid	α	β_H	β_L	γ
Tyr	32	41	41	76 (91)[c]
Trp	12	45	38	16 (20)
Phe	77	48	43	48 (59)
$^1/_2$-Cys	3	17	9	33 (39)

[a] Data were compiled from a review article (Harding & Dilley, 1976). [b] Expressed as amino acids/1000 residues. [c] Recalculated from the data of Kabasawa & Kinoshita (1974).

Dithiothreitol has been used to reduce the disulfide bond in proteins (Cleland, 1964; Breslow, 1970). When it was used in lens crystallins, none of the crystallins showed any change in CD above 250 nm, indicating that the disulfide bond contributes no measurable CD in this region.

When the pH was brought to 11.5, the near-UV CD of all crystallins underwent a change similar to that of poly(L-lysine) (Beychok & Fasman, 1964)—a new band at 250 nm and decreased intensity of the 270-nm band. However, in the crystallins, the negative band between 290 and 300 nm was replaced by a positive band. The difference CD and absorption spectra are shown in Figure 1. Qualitatively they all look very similar, two positive bands (at 250 and 290–300 nm) and a negative band at 270 nm. The positive band at the higher wavelength appears to be due to vibronic structure. In the presence of 6 M Gdn·HCl, the near-UV CD intensity for all crystallins was reduced almost to base line. Urea (8 M) is less effective in denaturing crystallins, especially γ-crystallin whose near-UV CD decreases only slightly.

The absorption spectra in the near-UV region are shown in Figure 2. The band shape for each crystallin seems to reflect the aromatic amino acid composition (Table I). Only α-crystallin, which has the largest amount of phenylalanine among the crystallins, gives the vibronic structure between 250 and 270 nm that is typical for phenylalanine.

Fluorescence. In the native form, emission maxima (excited at 285 nm) of all crystallins were shifted to a wavelength shorter than that of free tryptophan. γ-Crystallin shows a maximum blue shift to 329 nm and α-crystallin the least shift to 335 nm (Table II). In the presence of 6 M Gdn·HCl, emission maxima of all crystallins shift to a longer wavelength (close to 350 nm). When excited at a lower wavelength (270 nm), a shoulder at 305 nm was observed for all denatured crystallins (Figure 3a), with α- and γ-crystallins giving a more prominent shoulder than the other two. The tyrosine band was not visible in the native crystallins. Using a technique of exciting first at 280 nm, where both tyrosine and tryptophan are excited, and then at 295 nm, where only tryptophan is excited, and normalizing one emission spectrum above 370 nm to the other, we obtained a difference spectrum that was contributed by tyrosine (Figure 3b).

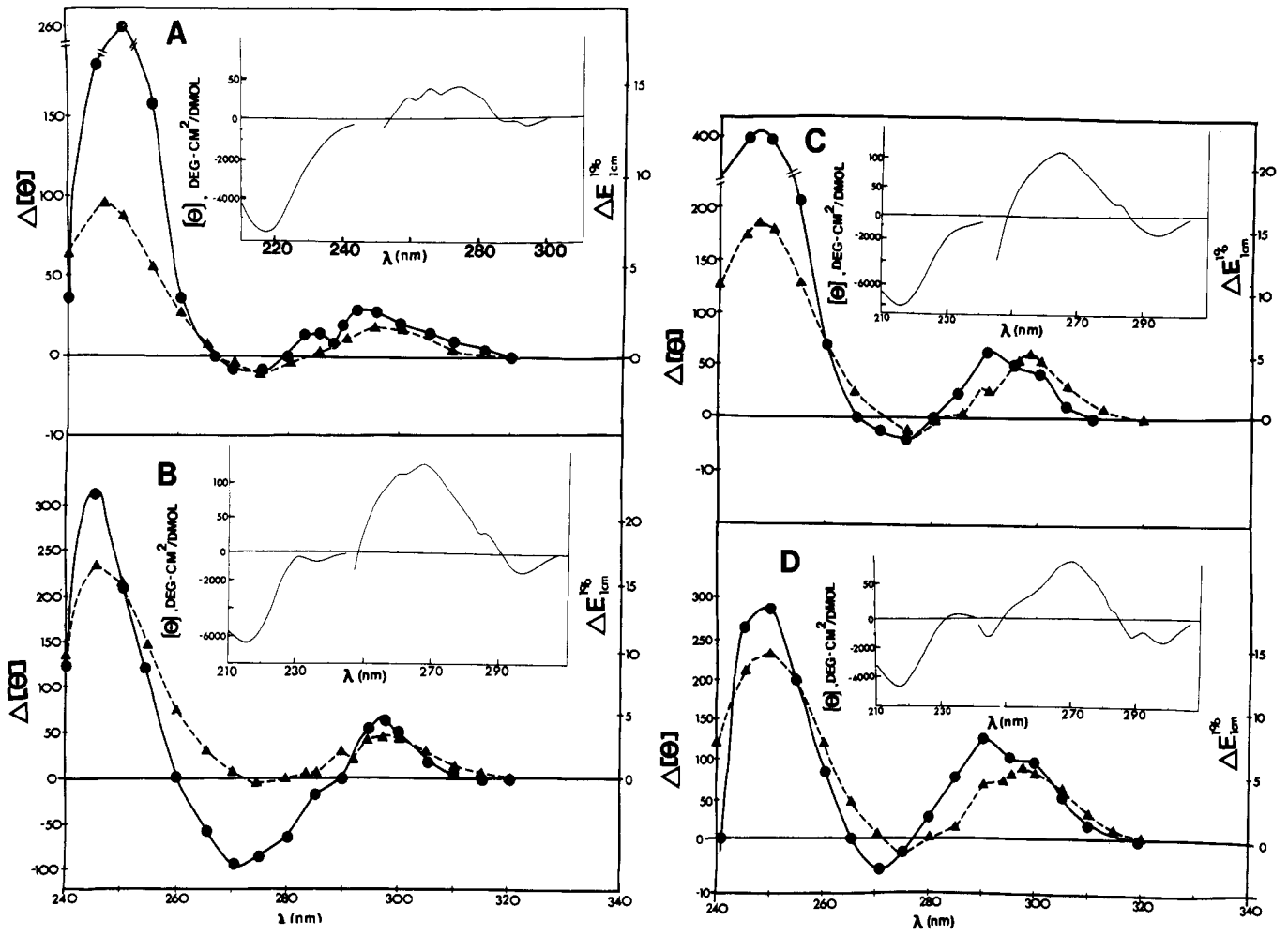

FIGURE 1: Difference CD spectra (circles) and difference absorption spectra (triangles), in the near-UV region, obtained by subtracting spectra at pH 11.5 from those at pH 8.0. (Inset) Near- and far-UV CD spectra of various crystallins in 0.05 M Tris, pH 8.0. The cell path length was 10 mm in the near-UV region and 0.5 mm in the far-UV region. (A) α-Crystallin; (B) β_H-crystallin; (C) β_L-crystallin; (D) γ-crystallin.

Table II: Protein, Tryptophan, and Tyrosine Quantum Yields of Bovine Lens Crystallins

	native [a]			denatured [b]		
crystallin	ϕ_P (λ_{max}) [c]	ϕ_{Trp} (λ_{max}) [d]	ϕ_{Tyr}	ϕ_P (λ_{max}) [c]	ϕ_{Trp} (λ_{max}) [d]	ϕ_{Tyr}
α	0.164 (335) [e]	0.186 (336)	<0.001	0.113 (348)	0.113 (350)	0.060
β_H	0.101 (332)	0.102 (334)	0.022	0.128 (350)	0.128 (351)	0.032
β_L	0.107 (332)	0.106 (334)	0.052	0.118 (350)	0.121 (351)	0.019
γ	0.070 (329)	0.077 (329)	<0.001	0.106 (349)	0.142 (351)	<0.001

[a] In 0.01 M Tris buffer, pH 7.5. [b] Denaturation in 6 M Gdn·HCl. [c] Excitation at 280 nm. [d] Excitation at 295 nm. [e] The values of quantum yields and emission maxima are averages obtained from two measurements. The deviation of λ_{max} is ±1 nm and of ϕ about ±5%.

The quantum yield and emission maxima for native and denatured crystallins are given in Table II. The emission maximum changes only a little at the two different exciting wavelengths, 280 and 295 nm, for both the native and the denatured proteins. The quantum yields obtained by exciting at 280 nm are termed "protein quantum yields", and they are contributed by both tryptophan and tyrosine. The quantum yields obtained by exciting at 295 nm are contributed mostly by tryptophan. The tyrosine quantum yields calculated from tryptophan and protein quantum yields are also listed in Table II. They were calculated from eq 3, the ratio of tryptophan to tyrosine from Table I, and a value of 4 for the ratio $\epsilon_{Trp}/\epsilon_{Tyr}$ (Weber & Young, 1964). The three quantum yields obtained for the crystallins are comparable with those for many other proteins (Kronman & Holmes, 1971). The tryptophan quantum yields exhibit wide variation among native α-, β-, and γ-crystallins. The range of values narrows upon denaturation. The tyrosine quantum yields for crystallins are much lower than that for the free amino acid, which is 0.21 (Teale & Weber, 1957).

Discussion

The far-UV CD spectra indicate that the secondary structures of all the crystallins are apparently very similar; variation in the content of the β conformation may not be significant for any biological correlation. However, dissimilarities may exist in the arrangement of the β chain such as parallel or antiparallel, but the available theories (Pysh, 1966; Woody, 1969) to distinguish between the two from CD data obtained with a commercial spectropolarimeter are still far from satisfactory. Vacuum ultraviolet CD (Liang et al., 1979) has been successful in this regard. An interesting difference is the existence of a band at 235 nm for both β_H- and γ-crystallins; nuclear α-crystallin shows a band at the same position when the lens is mature (Li, 1974). The absorption profile of the nuclear α-crystallin, shown by Li (1974), strongly suggests

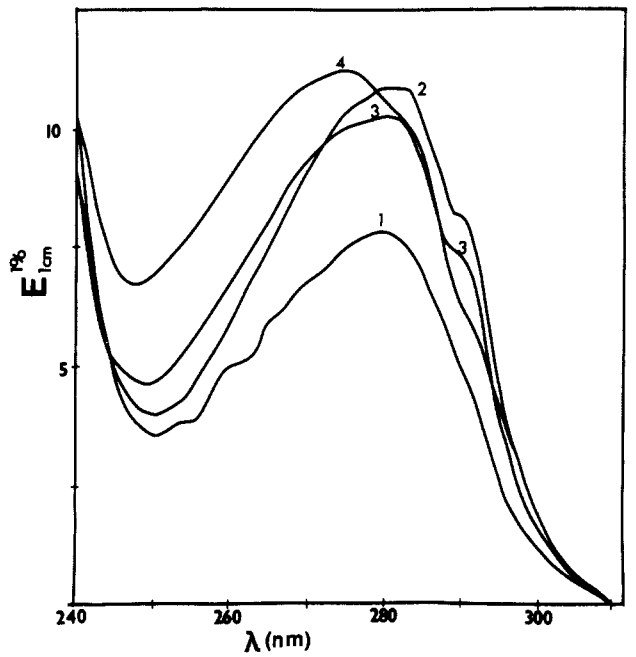

FIGURE 2: Absorption spectra in the near-UV region of various crystallins in Tris buffer solutions, pH 8.0, expressed in terms of the absorptivity of a 1% protein solution in a cell of 1-cm path length. (1) α-Crystallin; (2) β_H-crystallin; (3) β_L-crystallin; (4) γ-crystallin.

that it is due to an n–π* amide transition rather than to any extraneous chromophore as suggested by the author. It is very likely that the 235-nm band originates from an interaction between the peptide amide transition and a transition of aromatic amino acid residues (Kahn, 1979). The possibility of such an interaction with the ^1L_a transition of tyrosine is stronger in the sense that the ^1L_a band is located in the far-UV region (230–235 nm); the band is shifted toward red at alkaline pH. The argument may have further support from the fact that the CD band at 235 nm disappears when the proteins are denatured by alkaline pH or guanidine hydrochloride. However, the interaction occurs only when some specific difference exists in the peptide backbone structures of the crystallins. Alternatively, some changes in the secondary structure due to maturation (aging) of the lens can cause this interaction, as observed in the nuclear α-crystallin (Li, 1974).

The near-UV CD spectra of the crystallins, on the other hand, are not very similar to each other (Horwitz, 1976; Horwitz et al., 1977; Zigler et al., 1980). The chromophores that give rise to near-UV CD bands in proteins are tryptophan, tyrosine, phenylalanine, and disulfide bonds (Strickland, 1974). The CD intensities, obviously, depend upon the extent of interactions (μ–μ coupling) of aromatic rings with each other and the amide backbone, the rigidity of the protein, the number of aromatic residues, and the geometry of the disulfide bridge. In the absence of any effect of the reducing agent dithiothreitol on the near-UV CD of the crystallins, the contribution of disulfide bonds to the dichroic properties of the crystallins can be considered negligible. Although this makes the interpretation a little less complicated, the overlapping of CD bands arising from different aromatic acid residues makes the analysis as difficult as for many other proteins.

For the convenience of interpretation of the CD results, let us first examine the near-UV CD absorption spectra of these proteins. For each crystallin, the absorption intensity around 270 nm represents the content of tryptophan and tyrosine, and intensity around 290 nm, the tryptophan content alone. The position of the band maximum is determined by the ratio of tyrosine to tryptophan, and as a result, the λ_{max} of γ-crystallins is at a lower wavelength than that of the others because of its higher tyrosine content (Table I). The spectrum of α-crystallin, below 280 nm, is structured, which is expected from its high phenylalanine content. This is also reflected in the near-UV CD of α-crystallin.

The long-wavelength CD minima around 295 nm of all crystallins are quite distinct and can be safely assigned to the 0–0 ^1L_b tryptophan band (Strickland et al., 1969; Strickland, 1974). The red shift of the bands in the CD spectra from the absorption wavelength (290 nm) implies that most of the tryptophan residues of all crystallins are buried (Kahn, 1979). If, indeed, the shift is a measure of the percentage of buried tryptophans, γ-crystallin, whose CD minimum is at 298 nm, has the maximum amount of the residue buried, and α-crystallin (292 nm) has the least. The coincidence may be fortuitous, but the fluorescence results (discussed later) also indicate the same situation.

Both α- and γ-crystallins show a second CD minimum in the lower wavelength with a separation of 950 and 1304 cm^{-1}, respectively, from their 0–0 ^1L_b tryptophan band. For α-

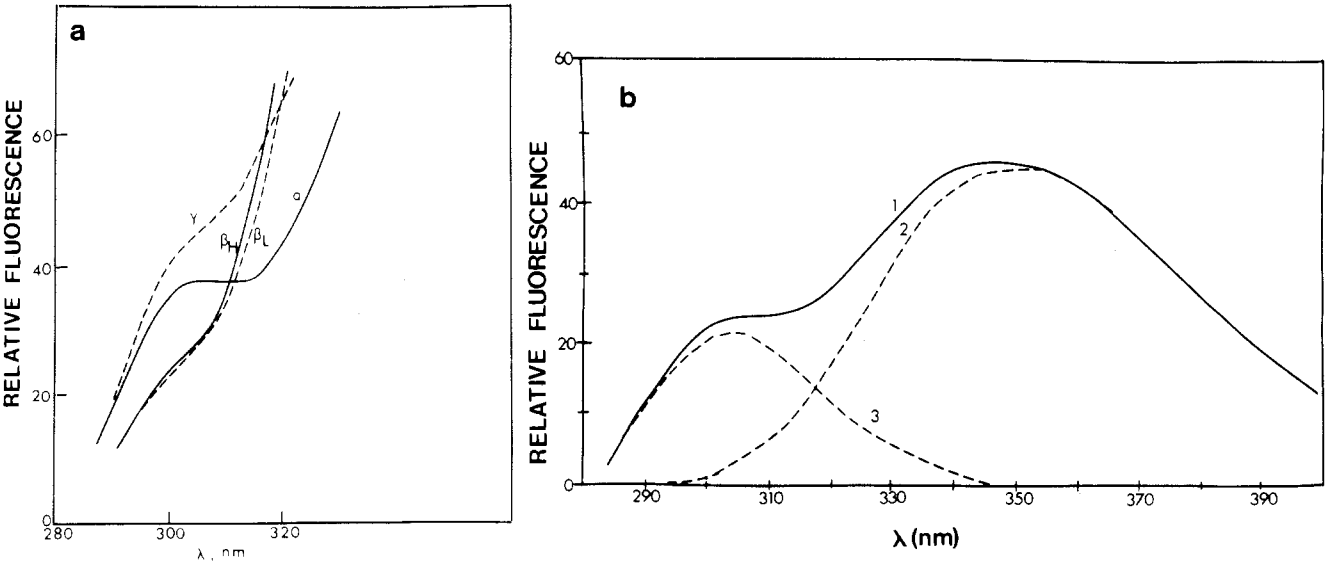

FIGURE 3: (a) Tyrosine fluorescence of various crystallins in the denatured form; λ_{ex} = 270 nm. (b) Resolution of tyrosine fluorescence from tryptophan fluorescence in denatured α-crystallin. Emission spectrum 1 was excited at 280 nm; spectrum 2, at 295 nm with normalization above 360 nm. Spectrum 3, the difference between spectra 1 and 2, represents tyrosine fluorescence.

crystallin, 0 + 950 cm^{-1} roughly corresponds to the vibrational progression of the tryptophan band, but for γ-crystallin, it is very likely that the 288-nm CD minimum arises from the superimposed bands of tryphophan and tyrosine. Because of the presence of a large amount of tyrosine in γ-crystallin, the contribution of tyrosine to the CD can be large (although this may not be necessarily true). The near-UV CD of α-crystallin is the least complicated of the crystallins. It is apparent that negative dichroism of all these proteins is contributed by a ^1L_b tryptophan transition, whereas the positive CD is a clear mixture of ^1L_a tryptophan, ^1L_b tyrosine, and phenylalanine bands.

Both absorption and CD difference spectra show vibrational structures of tyrosine ^1L_b bands; the 0–0 CD bands of β_L- and γ-crystallins are shifted more toward the red than those of α- and β_H-crystallins. This is also true for the ^1L_a tyrosine bands of these proteins. From the pH-dependent spectra, it is apparent that both of the β-crystallins show their ^1L_b tyrosine bands near 286 nm; α- and γ-crystallins show theirs around 281 and 283 nm, respectively. On the basis of the position of the tyrosine bands, it can be inferred, as before, that most of the tyrosine residues in β-crystallin are buried as compared with α- and γ-crystallins.

Except for α-crystallin, the ^1L_a tryptophan in these proteins shows considerable dichroism; β_L-crystallin is less structured than β_H- and γ-crystallins, indicating the predominance of ^1L_a tryptophan bands, which are generally broad and structureless (Strickland, 1974). The CD maxima of β_H- and β_L-crystallins around 266 nm can be attributed to the ^1L_a bands of tryptophan, which are relatively stronger than for the others (Strickland, 1974). The CD maximum of γ-crystallin at 271 nm is likely superimposed bands of ^1L_a tryptophan and ^1L_b tyrosine. The vibrational structure of α-crystallin CD in the lower wavelength region largely corresponds to its absorption spectrum. The 272-nm band can be assigned to 0 + 800 cm^{-1} 0–0 ^1L_b tyrosine and 266 nm to ^1L_a tryptophan; the broad tryptophan bands appear structured probably because of weak negative vibrational bands of phenylalanine (Strickland et al., 1969).

The fluorescence of all crystallins is predominantly due to tryptophan. The position of the λ_{max} of the native crystallins might indicate that most of the tryptophan residues of γ-crystallin are buried in the hydrophobic interior of the protein and that in α-crystallin relatively the least number of the residues are buried. This view is in general agreement with Borkman & Lerman (1978) although the emission maxima of α- and γ-crystallins of their preparation are somewhat different from ours. Weinryb & Steiner (1970) suggested that the quantum yields of the exposed fluorophore should be less than those in the hydrophobic interior of the protein. Although neither the emission maxima nor the quantum yields can be a simple function of the degree of exposure of tryptophan residues (Kronman & Holmes, 1971), the λ_{max} and ϕ values obtained for crystallins are consistent enough to suggest that exposed tryptophans in these proteins are in the order $\alpha > \beta_H \simeq \beta_L > \gamma$. Furthermore, it has been observed that the denaturation process is much slower in γ-crystallin than in the others. The failure of Horwitz et al. (1977) to observe a change in the near-UV CD of γ-crystallin with urea denaturation is likely due to the same reason, as pointed out by Borkman & Lerman (1978).

For many proteins, it has been possible to obtain a shoulder for tyrosine emission at 305 nm when the excitation wavelength is chosen around 270–275 nm (Andley & Chakrabarti, 1982). For the crystallins, no such shoulder can be observed in the native state. This may indicate that the tyrosine-to-tryptophan energy transfer is more efficient in lens proteins than in many other proteins. Upon denaturation, the efficiency is obviously less, making the tyrosine emission quite visible. Konev (1967) argued that the screening effect rather than the energy transfer is responsible for the disappearance of the tyrosine emission in most of the tryprophan-containing protein fluorescence in the native state. But a Forster resonance transfer mechanism of tyrosine → tryptophan fits well with the experimental results of many proteins (Kronman & Holmes, 1971). Phosphorescence measurements are often helpful in demonstrating such energy transfer (Andley & Chakrabarti, 1982).

Low values of tyrosine quantum yields, particularly of α- and β-crystallins, are not uncommon with proteins containing tryptophan. The fluorescence of tyrosine in these proteins is weak but detectable in the presence of guanidine hydrochloride. Cowgill (1976) studied a number of low molecular weight tyrosyl derivatives and peptides and reviewed the possible quenching effect of functional groups, side chains, tyrosinates, and hydrogen-bonded phenolic hydroxyl groups of the tyrosine molecule. Transfer of the excited-state energy from tyrosyl to tryptophan can also be suggested to explain the low quantum yields, but this would seem of lesser importance when there is a lack of apparent relationship between the magnitude of tyrosyl quantum yield and the transfer efficiency calculated for many proteins (Kronman & Holmes, 1971). Nevertheless, the large increase in the quantum yield of tyrosine after denaturation, in contrast to other proteins (Kronman & Holmes, 1971), suggests that the quenching by peptide bonds in these proteins may be of minor significance.

The apparent nonuniformity of the values of the tryptophan quantum yields in guanidine hydrochloride among the proteins indicates the sensitivity of tryptophan fluorescence to the residual three-dimensional structure or to the persisting interaction between tryptophan and other side chains even after denaturation. Because of the presence of a large number of disulfide bonds in the crystallins, the possibility of such residual structure is not unlikely.

The present study has revealed the subtle differences that exist among these proteins in their tertiary structure and in the microenvironments of the aromatic acid residues. In model studies, any cataractous changes in these properties brought about by sugar molecules (Liang & Chakrabarti, 1981), ultraviolet radiation (Fujimori, 1978), or Ca^{2+} (Fein et al., 1979) can be better understood when the normal lens proteins are well characterized. The vulnerability of the tryptophan molecule to photochemical oxidation (Borkman & Lerman, 1978) will largely depend on their location and microenvironments. Similarly, the formation of a nondisulfide linkage involving amino acid side chains (Lerman, 1980) or possible bityrosine formation (Garcia-Castineiras et al., 1978) in the mature lens will also be dependent on these factors.

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