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Structure and Stability of γ-Crystallins: Tryptophan, Tyrosine, and Cysteine Accessibility†

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ABSTRACT: The solute perturbation techniques of fluorescence of tryptophan (Trp) and dye-labeled thiol groups of cysteine as well as phosphorescence of tyrosine (Tyr) were utilized to obtain information on the relative solvent exposure and accessibility of these residues in γ-crystallins. Both acrylamide and iodide quenchers were used to evaluate the quenching parameters in terms of accessibility and charge characteristics of the proteins. Stern–Volmer plots reveal the presence of more than one class of Trp residues in γ-III and γ-IV, and these residues in γ-II are least accessible compared to the other two. Both steady-state and lifetime quenching studies of the dye-labeled fluorescence indicate that distinct differences also exist among these crystallins in cysteine (Cys) accessibilities. All three proteins, γ-II, γ-III, and γ-IV, show two distinct lifetime components of the dye-labeled Cys residues. Both components of γ-II undergo dynamic quenching, whereas only the major component of the other two crystallins is affected by the quenchers. Addition of acrylamide causes a decrease in Tyr phosphorescence of γ-III and γ-IV, but no change in the emission of γ-II. The decrease is attributed to the formation of a nonemittive ground-state complex between the acrylamide and Tyr of the proteins; the association constant, K_a, calculated from the emission data, has been considered as a measure of Tyr accessibility. K_a values indicate that Tyr residues in γ-III are most exposed and accessible compared to those in the other two proteins. Results of quenching by iodide ion reveal significant differences in the surface charge of the proteins. This study demonstrates that despite the high degree of sequence homology and similarity in the secondary structure of these proteins, differences exist in the arrangements and microenvironments of Trp, Tyr, and Cys residues, causing significant variation in tertiary structure and charge characteristics. These specific molecular features may be primarily responsible for their remarkable denaturation and cryoprecipitation behavior and photoinduced aggregation.

The α-, β-, and γ-crystallins are the water-soluble proteins of the mammalian lens. The γ-crystallins are low molecular weight (M_r 20 000–21 000), monomeric proteins consisting of several gene products, the major ones being γ-II, γ-III, and γ-IV, with more than 75% sequence homology (Harding & Dilley, 1976; Bloemendal, 1982; Schoenmakers et al., 1984). The essential refractive and accommodative properties of the human eye are derived largely through the ordered distribution of these crystallins (de Jong, 1981). In a properly functioning lens, the crystallins must maintain their short-range ordering (Delaye & Tardieu, 1983). During aging and cataractogenesis, the human lens proteins undergo a number of changes, in-

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cluding increased protein aggregation (Jedziniak et al., 1975), increased 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). In protein–protein aggregation, both disulfide (Dische & Zil, 1951) and non-disulfide (Buckingham, 1972; Kramps et al., 1978; Fujimori, 1982) bonds were identified. Photooxidation of Trp by near-UV light or in the presence of a photosensitizer yields pigmented and fluorescent oxidation products (Andley et al., 1984; Bose et al., 1985, 1986; Chakrabarti et al., 1986; Mandal et al., 1986; Chakrabarti & Mandal, 1987), some of which are present in the mature lens (Lerman, 1980; Goosey et al., 1980). The formation of dityrosine in the cataractous lens has also been reported (Garcia-Castineiras et al., 1978). 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 mostly Trp, Cys, or Tyr residues (Kuck et al., 1982; Liang & Chakrabarti, 1982; Messmer & Chakrabarti, 1988). In addition to the large number of aromatic amino acid residues present in all crystallins (Harding & Dilley, 1976; Bloemendal, 1982), γ-crystallins are also rich in Cys residues (Björk, 1970; Croft, 1972, 1973; Blundell et al., 1983).

Investigation of these proteins was facilitated by the introduction of X-ray studies (Blundell et al., 1981). γ-Crystallins have closely related amino acid sequences (Croft, 1972), and the three-dimensional folding of the polypeptide backbones, as manifested in detailed X-ray analysis (Blundell et al., 1981; Chirgadze et al., 1981; Wistow et al., 1983; Summers et al., 1984), is reported to be similar. Furthermore, these authors have suggested that these proteins have a remarkably symmetrical structure that confers stability. However, in a series of studies of γ-crystallins in solution, we have shown that the microenvironments of Trp, Tyr, and Cys residues differ markedly (Mandal et al., 1985, 1987a) and that they have different stabilities toward chemical denaturants, pH, and proteolytic enzymes (Mandal et al., 1987b). γ-Crystallins not only differ from α- and β-crystallins in cryoprecipitation (Siezen et al., 1985) and photoinduced changes (Chakrabarti et al., 1986; Mandal et al., 1988; Kono et al., 1988), but variations also exist among the γ fractions in susceptibility to cryoprecipitation and ease and extent of photoaggregation.

In this study, we used solute perturbation techniques (Lehrer & Leavis, 1978; Eftink & Ghiron, 1981) of the emission properties (fluorescence and phosphorescence) of Trp, Tyr, and fluorescent dye labeled thiol (labeled with fluorescent probe) groups of Cys residues of γ-crystallins to obtain information on the relative solvent exposure and accessibility of these residues. The decrease in phosphorescence intensity of these proteins upon addition of acrylamide, as observed in this study, is attributed to the formation of a nonemittive ground-state complex between acrylamide and Tyr residues; the value of the association constant, calculated from the data (see Materials and Methods), has been considered a measure of Tyr accessibility. We, for the first time, successfully utilized phosphorescence spectroscopy to obtain information on Tyr accessibility, and the method is particularly suitable for proteins containing both Tyr and Trp residues. Fluorescence quenching studies would be unsuitable for these proteins because Trp emission overshadows the fluorescence of Tyr when both residues are present. The sulfhydryl groups were covalently labeled with the fluorescent probe *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS).[1] For the labeled thiol group fluorescence, we measured both steady-state and dynamic (lifetime) quenching to obtain more precise information about the accessibility of the fluorophore bound to the proteins. Because the accessibility of fluorophore to a quencher depends upon the polarity and steric environment of sites (Lehrer & Leavis, 1978), we used both neutral (acrylamide) and ionic (I^-) quencher to evaluate the quenching parameters in terms of accessibility and charge characteristics of the proteins in the vicinity of the emitters. In the present report, we show that the γ fractions differ considerably in accessibility of their Trp, Tyr, and Cys residues to the quenchers used.

Materials and Methods

Preparation of Crystallins. γ-Crystallins were routinely isolated from 1–2-week-old calf lenses as described previously (Mandal et al., 1985, 1987a,b). The nuclear (20–30%) portion of the lens was extracted after removal of the cortex (70–80% of lens volume) in 50 mM phosphate buffer (pH 6.7). γ-Crystallins were isolated by exclusion chromatography of the nuclear extract on Sephadex G-75 at room temperature. The mixture of γ-crystallins thus obtained was dialyzed against 0.2 M sodium acetate buffer (pH 5.0) and separated into fractions I, II, III, and IV on sulfopropyl (SP)-Sephadex C-50. The purity of individual fractions was checked by ion-exchange high-performance liquid chromatography (HPLC) on Synchropak CM 300. Fractions III and IV consisted of subfractions IIIa and IIIb (approximate ratio 1:2) and IVa and IVc (approximate ratio 3:1), respectively, but no further separation or purification was attempted. Protein solutions were dialyzed against 20 mM phosphate buffer (pH 7.0) at 4 °C for all spectroscopic measurements. Protein concentrations were determined by using a specific absorbance value ($A_{1cm}^{0.1\%}$) of 2.1 at 280 nm.

Chemicals. Potassium iodide (Sigma), acrylamide (Bio-Rad), and 1,5-IAEDANS (Molecular Probe) were used as supplied without further purification.

IAEDANS Labeling. γ-Crystallins (1 mg/mL) were incubated with a 10 M excess of IAEDANS at 4 °C overnight in 20 mM phosphate buffer, pH 7.0. Excess (unlabeled) dye was removed by extensive dialysis. The IAEDANS-labeled protein was used for steady-state and lifetime quenching measurements.

Emission Measurements. Fluorescence measurements were recorded at 22 °C on a Perkin-Elmer MPF-44F spectrofluorometer. Fluorescence quenching was carried out by monitoring the change in intensity at the emission maximum. The spectral band-pass was 4 nm for all measurements.

Phosphorescence spectra were measured on the same spectrofluorometer equipped with a phosphorescence attachment. The spectral band-pass for these measurements was 8 nm, to give a high phosphorescence intensity, convenient for the quenching study. Protein samples for phosphorescence measurement were placed in a capillary tube of 3-mm internal diameter. Phosphorescence was recorded at 77 K using a chopper rotating at 2000 rpm to cut off short-lived signals (fluorescence and stray light). For measurement of phosphorescence, the protein solutions (0.1 mg/mL) in phosphate buffer were diluted (1:1 v/v) with ethylene glycol, resulting in half the original concentration. This solvent mixture provided a good transparent glass at 77 K for all phosphorescence

[1] Abbreviations: 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; MIANS, 6-(4-maleimidoanilino)-naphthalene-2-sulfonic acid; SP, sulfopropyl; HPLC, high-performance liquid chromatography.

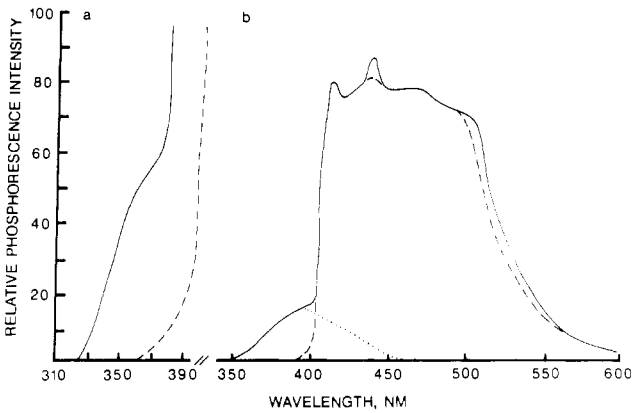

FIGURE 1: (a) Phosphorescence emission of γ-III crystallin excited at 275 (—) and 300 (---) nm. This representative figure indicates the way we measured acrylamide quenching of Tyr phosphorescence of all three γ fractions. Tyr phosphorescence quenching was monitored at 360 nm where Trp phosphorescence (λ_{ex} 300 nm) was absent. The sensitivity scale was more than 3-fold higher than the spectrum in (b). (b) Phosphorescence emission spectrum at 77 K of γ-III crystallin excited at 275 (—) and 300 (---) nm. The phosphorescence spectrum (λ_{ex} 300 nm) was normalized at 470 nm with the spectrum excited at 275 nm. The Tyr spectrum (···) was resolved by subtracting the normalized spectrum from the total protein spectrum. This resolved spectrum indicates that at 360 nm there is no contribution of Trp emission.

measurements. The solutions used for phosphorescence measurement were dialyzed to remove free quencher molecules. Following dialysis, CD and fluorescence spectra of the samples were measured. In all cases, the spectra remain unchanged, indicating that neither glycol nor the liquid nitrogen temperature affects the native property of the protein.

Photoselection Method for Separating Tyr and Trp Phosphorescence. When excited below 290 nm, all crystallins show similar (but not identical) phosphorescence spectra due to Trp and Tyr. The spectrum extends from 340 to 650 nm with a shoulder near 395 nm, presumably due to Tyr, and several peaks between 400 and 550 nm originate from Trp transitions. The phosphorescence spectra of γ-III crystallin at 77 K are representative (Figure 1). To resolve this spectrum into Tyr and Trp phosphorescence, the two spectra were obtained by exciting at 275 and 300 nm; the former is composed of both Tyr and Trp (both absorb at 275 nm), and the latter is due entirely to Trp (Tyr does not absorb at 300 nm). The two spectra were then normalized at 470 nm to obtain the emission due to Tyr (Andley & Chakrabarti, 1982). We chose 360 nm to monitor Tyr emission. This resolved spectrum shows that at 360 nm the emission is entirely due to Tyr and Trp makes no contribution.

Lifetime Measurements and Decay Analysis. Fluorescence lifetime measurements were carried out at 22 °C on a modified Ortec 9200 nanosecond fluorometer by the methods of Tao and Cho (1979). Fluorescence decay analyses were performed on a PDP-11/44 computer using the method of moments (Isenberg & Dyson, 1969).

Quenching Measurements. Acrylamide (neutral quencher) and I^- (anionic quencher) were used to quench the intrinsic fluorescence of Trp residues as well as the probe fluorescence used for the Cys residues. All data for ionic quenchers were obtained at constant ionic strength by using KCl. For I^- as a quencher, $Na_2S_2O_3$ was used to keep the iodide reduced. Otherwise, I_2 is formed, which is reactive and can penetrate into the protein's nonpolar, hydrophobic region (Lakowicz, 1983). For the neutral quencher acrylamide, it was not necessary to add KCl. Because acrylamide has a molar extinction coefficient of 0.23 at 295 nm and 9.86 at 275 nm, it attenuates the exciting light intensity, and a correction for this screening effect was suggested (Parker, 1968). Accordingly, we obtained corrected fluorescence intensity (F_{cor}) from the observed value (F_{obsd}) by using the equation $F_{cor} = F_{obsd}\, 10^{\Delta A/2}$ where $\Delta A/2$ is the increase in absorbance at the center of the cuvette caused by the addition of acrylamide.

Data Analysis. Quenching studies were analyzed according to the Stern–Volmer relationship (eq 1) by plotting F_0/F vs [Q], where F_0 and F are the emission intensity in the absence and presence of a quencher, respectively. In the presence of a quencher, the intensity of an emitter is decreased by both collisional and static quenching (Lehrer & Leavis, 1978), and accordingly, the Stern–Volmer relationship is expressed as

$$F_0/F = (1 + K_{sv}[Q])e^{V[Q]} \quad (1)$$

K_{sv} is the Stern–Volmer (Stern & Volmer, 1919) quenching constant, and V is the static quenching parameter. Eftink and Ghiron (1976a) introduced the factor $e^{V[Q]}$ to take into account the static quenching contributions. The contribution of static quenching often gives an upward curving on a simple Stern–Volmer plot of F_0/F vs [Q]. Although the presence of multiple components causes the plot to curve downward, lifetime studies done in parallel with intensity measurements can separate the static quenching contribution (Lakowicz & Weber, 1973; Eftink & Ghiron, 1976a,b). The collisional quenching process decreases the emission lifetime by

$$1/\tau = 1/\tau_0 + k_q[Q] \quad (2)$$

where τ_0 and τ are the lifetimes in the absence and presence of a quencher, respectively, and k_q is the bimolecular Stern–Volmer quenching constant that can serve as a quantitative measure for the accessibility of the emitter (Andley et al., 1982). k_q approaches zero at low accessibilities and is related to K_{sv} by

$$K_{sv} = k_q\tau_0 \quad (3)$$

k_q can be calculated directly from this relation. Since Trp fluorescence lifetimes of the individual γ-crystallins were not available, the quantum yield values measured previously (Mandal et al., 1985) were used to obtain the bimolecular quenching rate constant as follows.

The quantum yield (ϕ) of fluorescence is related to lifetime (τ_0) by

$$\phi = k_f\tau_0 \quad (4)$$

where k_f is the radiative rate constant for fluorescence. From eq 3 and 4

$$k_q = K_{sv}k_f/\phi \quad (5)$$

In relation to k_q of the protein Trp and free Trp, eq 5 can be written as

$$k_q^P/k_q^{Trp} = (K_{sv}^P/K_{sv}^{Trp})(k_f^P/k_f^{Trp})(\phi^{Trp}/\phi^P) \quad (6)$$

where the superscripts P and Trp represent protein-bound and free Trp, respectively. Since the ratio k_f^P/k_f^{Trp} is close to unity, k_q^P was calculated from the measured values of k_q^{Trp}, K_{sv}^{Trp} of free Trp (Phillips et al., 1986), and ϕ of γ-crystallins (Mandal et al., 1985). The value of 0.20 for ϕ of free Trp was used for this calculation (Teale & Weber, 1957; Kronman & Holmes, 1971).

For bimolecular rate constants of IAEDANS-labeled thiols, however, we used the lifetime values measured in this study. No attempts were made to measure the bimolecular rate constant of Tyr phosphorescence quenching.

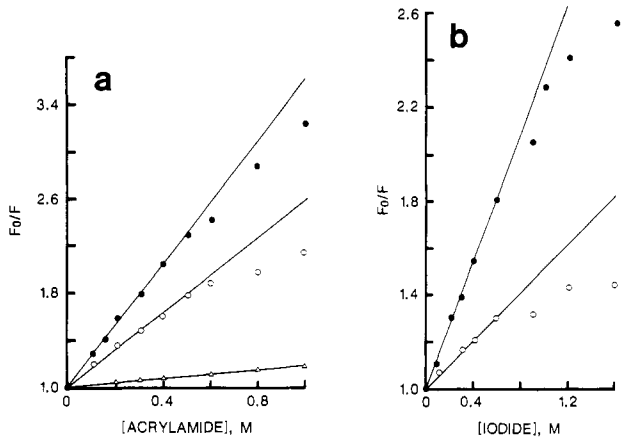

FIGURE 2: Stern–Volmer plots. Protein concentration, 0.1 mg/mL, in 20 mM phosphate buffer, pH 7.0; cell path length, 3 mm; λ_{ex}, 295 nm. (a) Acrylamide quenching of Trp fluorescence of γ-II (△), γ-III (○), and γ-IV (●) crystallins. (b) Iodide quenching of Trp fluorescence of γ-III (○) and γ-IV (●) crystallins.

The accessibility of an emitter to a quencher can also be calculated by a modified Stern–Volmer equation (Lehrer, 1971):

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a} \quad (7)$$

where $\Delta F = F_0 - F$, f_a is the fraction of the fluorophore accessible to the quencher, and K_a is the average quenching of all accessible Trp residues (both buried and exposed). A plot of $F_0/\Delta F$ vs $1/[Q]$ allows calculation of the value of f_a from the intercept equal to $1/f_a$.

Data Analysis for Phosphorescence Quenching at 77 K. Since the possibility of collisional quenching can be safely excluded at 77 K, any quenching, if it occurs, results from the formation of a nonemitting, ground-state complex between emitter and quencher. In that case, the dependence of the emission intensity upon quencher concentration can be derived by considering the association constant for the complex formation, which was shown to be identical with the Stern–Volmer dynamic quenching constant (Lakowicz, 1983). The association constant, K_a, is given by

$$K_a = [e\text{–}q]/[e][q] \quad (8)$$

where [e–q] is the concentration of the complex and [q] and [e] are the concentrations of the quencher and uncomplexed emitter, respectively. If the complexed species is nonphosphorescent, the fraction of the phosphorescence intensity that remains is given by I/I_0, where I_0 is the phosphorescence intensity in the absence of the quencher and I is the emission of the uncomplexed emitter. Recalling that the total concentration of the emitter, $[e]_0$, before addition of the quencher can be written as

$$[e]_0 = [e] + [e\text{–}q] \quad (9)$$

Combining eq 8 and 9 yields

$$K_a = \frac{[e]_0 - [e]}{[e][q]} = \frac{[e]_0}{[e][q]} - \frac{1}{[q]} \quad (10)$$

Since the emission intensities are proportional to their concentration

$$I_0/I = 1 + K_a[q] \quad (11)$$

Thus, the plot of I_0/I vs [q] will yield the value of K_a identical with the Stern–Volmer constant, K_{sv}.

RESULTS

Steady-State Quenching Studies. Figure 2 shows the Stern–Volmer plot for acrylamide and iodide quenching of Trp fluorescence intensity of γ fractions, γ-II, γ-III, and γ-IV. At acrylamide concentrations <0.8 M, all the plots are reasonably linear, but at concentrations >0.8 M, downward curvatures are apparent for both γ-III and γ-IV. However, the plot for γ-II is linear at all acrylamide concentrations (Figure 2a). Stern–Volmer constants, K_{sv}, evaluated from the slope of the linear part of the plot, and the values for both acrylamide and iodide quenching of γ fractions are given in Table I. Because the γ-fraction lifetimes are not available, the bimolecular quenching rate constants, k_q, were determined by using eq 6. Accessibility parameters, f_a, as measured by using the modified Stern–Volmer plot, are also included in Table I. The order of Trp accessibility to acrylamide is γ-IV > γ-III > γ-II.

Table I: Stern–Volmer Quenching Constant (K_{sv}), Bimolecular Quenching Constant (k_q), and Accessibility for Acrylamide and Iodide Quenching of Trp Fluorescence (f_a) of γ-Crystallin Fractions

	K_{sv} (M^{-1})		$k_q \times 10^{-8}$ (M^{-1} s^{-1})		f_a	
fraction	acrylamide	iodide	acrylamide	iodide	acrylamide	iodide
γ-II	0.17	0	1.80	0	0.34	0
γ-III	1.64	0.46	11.00	3.14	0.62	0.22
γ-IV	2.66	1.38	15.50	8.17	0.83	0.66

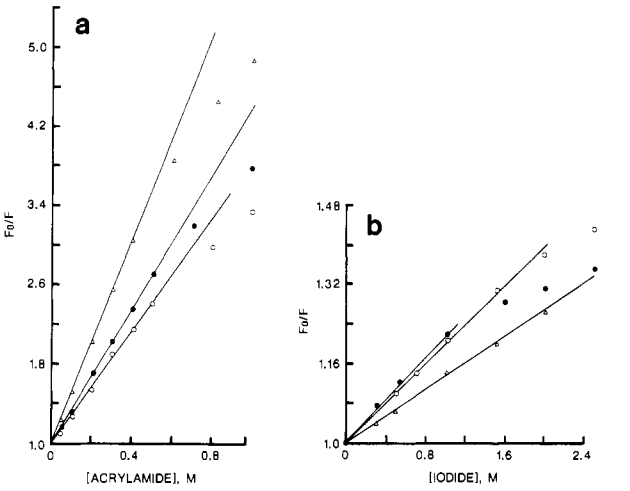

FIGURE 3: Change in fluorescence intensity was monitored at 500 nm; λ_{ex}, 370 nm. Other conditions were the same as in Figure 2. (a) Acrylamide quenching of IAEDANS-labeled γ-II (△), γ-III (○), and γ-IV (●) crystallins. (b) Iodide quenching of IAEDANS-labeled γ-II (△), γ-III (○), and γ-IV (●) crystallins.

When iodide ion was used as a quencher, only γ-III and γ-IV emission was quenched (Figure 2b); no quenching of γ-II fluorescence was observed. In this case also, the plots for γ-III and γ-IV deviate downward from a straight line, and the deviation can be ascribed to the presence of multiple emissive components. For both γ-III and γ-IV, and curvature begins at an iodide concentration of ~0.6 M. As Figure 2b indicates, the iodide quenching of Trp fluorescence is much more efficient in γ-IV than in γ-III. The K_{sv} value for γ-IV is 3 times as large as that for γ-III (Table I). The k_q values, obtained by using eq 6, indicate that iodide as a quencher for Trp fluorescence is much more effective for γ-IV than for γ-III.

Figure 3a shows the intensity quenching plot (F_0/F vs [Q]) for 1,5-IAEDANS-labeled γ fractions, using acrylamide as the quencher. No upward curvature was observed, indicating the absence of static quenching. However, downward curvature was obtained at a certain concentration range for each γ fraction. The Stern–Volmer quenching constants, K_{sv}, and the k_q values, as determined by using the equation $K_{sv} = k_q\tau_0$, are given in Table II. The order of acrylamide quenching

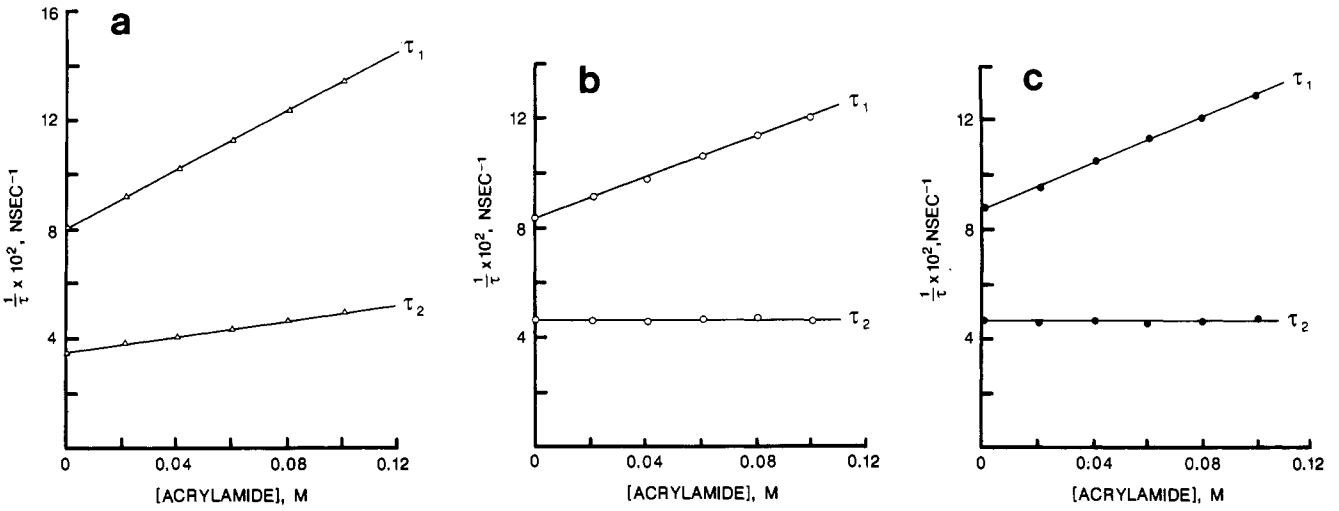

FIGURE 4: Stern–Volmer plots for acrylamide quenching of lifetimes of IAEDANS-labeled (a) γ-II, (b) γ-III, and (c) γ-IV crystallins.

Table II: Stern–Volmer Quenching Constant (K_{sv}) and Bimolecular Quenching Constant (k_q) for Acrylamide Quenching of IAEDANS-Labeled γ Fractions

fraction	K_{sv} (M^{-1})		$k_q \times 10^{-8}$ (M^{-1} s^{-1})	
	acrylamide	iodide	acrylamide	iodide
IAEDANS-γ-II	5.00	0.14	3.66	0.11
IAEDANS-γ-III	2.86	0.20	2.04	0.17
IAEDANS-γ-IV	3.33	0.21	2.30	0.18

Table III: Fluorescence Decay Parameters of 1,5-IAEDANS-Labeled γ Fractions

fraction	A_1	τ_1 (ns)	A_2	τ_2 (ns)
IAEDANS-γ-II	0.97	12.39	0.03	27.18
IAEDANS-γ-III	0.87	12.04	0.13	22.08
IAEDANS-γ-IV	0.83	11.31	0.17	21.33

efficiency for labeled thiols of γ fractions is γ-II > γ-IV > γ-III.

The fluorescence intensity quenching of IAEDANS-labeled γ fractions by iodide is shown in Figure 3b. Because the fluorescence intensity did not change considerably upon addition of a low concentration of iodide, we extended the range to 2.4 M. No curvature was detected for labeled γ-II over this wide concentration range, but a downward curvature occurs in both γ-III and γ-IV; in γ-III, at 2.0 M iodide concentration and in γ-IV at ~1.2 M. Both K_{sv} and k_q values of γ-II were lowest, compared to the other two proteins, when iodide was used as a quencher (Table II).

No significant change was observed in either the far-UV or the near-UV CD upon labeling with IAEDANS (data not included), indicating that the conformation of the dye-labeled protein remains the same as that of the unreacted one. This is supported by the fact that the interaction of the dye does not cause any change of the tryptophan fluorescence of the protein (Mandal et al., 1987b).

Lifetime Measurements. The fluorescence decay parameters of IAEDANS-labeled γ fractions are shown in Table III. The method of moments yielded a major decay component of lifetime ~12 ns and a minor decay component of lifetime 21–28 ns for IAEDANS-labeled γ-crystallins.

Lifetime Quenching. The fluorescence decay curves of IAEDANS-labeled γ fractions at various acrylamide concentrations were obtained. Use of higher acrylamide concentrations was avoided because of significant decrease in lifetime in the quenching process. The quencher concentration was kept in the range 0–0.1 M. Analysis by the method of moments showed that the decay curve can be represented by the sum of two exponentials at all acrylamide concentrations. The lifetimes of the two components were obtained and plotted (Figure 4) by the Stern–Volmer equation (eq 2). The lifetimes of only the major component of γ-III and γ-IV fractions are quenched by the collisional process; the minor one remains virtually unchanged. In γ-II, however, both components undergo dynamic quenching. The plots for both components resulted in good linearity, indicating the pure exponential nature of each component. The k_q values of the major and minor components, obtained from the slopes of the Stern–Volmer plots, are shown in Table IV.

Table IV: Dynamic Quenching Rate Constants for 1,5-IAEDANS-Labeled γ-Crystallin Fractions

fraction	$k_q \times 10^{-8}$ (M^{-1} s^{-1})	
	major component	minor component
IAEDANS-γ-II	5.45	1.45
IAEDANS-γ-III	3.75	
IAEDANS-γ-IV	4.25	

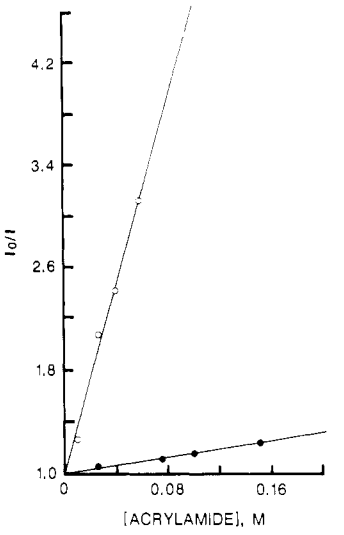

FIGURE 5: Acrylamide quenching of Tyr phosphorescence of γ-III (○) and γ-IV (●) crystallins. Protein concentration, 0.5 mg/mL; cell diameter, 3 mm; λ_{ex}, 275 nm. Phosphorescence intensity change upon addition of quencher was monitored at 360 nm.

Figure 5 shows the acrylamide quenching of Tyr phosphorescence intensity of γ fractions. Using the molar extinction coefficient value of 9.86 for acrylamide at 275 nm, we calculated the corrected phosphorescence from the equation $F_{cor} = F_{obsd} 10^{\Delta A/2}$. The quenching in this case is very likely

due to formation of a ground-state complex between acrylamide and Tyr residues. Cowgill (1976) demonstrated the existence of a nonfluorescent complex between the phenolic OH and amides. No such decrease in phosphorescence intensity was observed when iodide was used as a quencher, indicating the inability of iodide ion to form such a complex even though the Tyr residues in γ-III appear to be surface exposed and readily accessible (Figure 5). No significant decrease in phosphorescence intensity of γ-II was observed. For γ-III and γ-IV, the intensity plot, I_0/I vs [Q], resulted in a straight line, indicating the existence of a single class of Tyr in these proteins. The association constant, K_a, calculated from the slope was found to be 35 M^{-1} for γ-III and 1.55 M^{-1} for γ-IV.

Although the samples after phosphorescence measurements gave identical CD spectra with that of the untreated one, the conformation of the protein may change at liquid nitrogen temperature when the phosphorescence was measured. Unfortunately, we are not equipped to perform CD measurements at 77 K to verify this possibility. However, in our phosphorescence studies, three γ-crystallins behaved differently (Figure 5). Had there been any denaturation (or conformational change), we would expect similar quenching behavior as we observed the fluorescence behavior of these proteins in 6 M guanidine hydrochloride (Mandal et al., 1987b). Furthermore, the possibility of gross conformational change on denaturation can also be ruled out by the fact that many proteins have almost similar phosphorescence spectra at 77 K and at room temperature (Konev, 1967). Even the CD spectrum of many proteins remains unchanged at liquid nitrogen temperature (Strickland, 1974).

Discussion

We have previously described the positioning of Trp and Cys residues in γ-crystallins in qualitative terms, such as "exposed", "buried", and "partially exposed" (Mandal et al., 1985, 1987a). The present quenching studies have provided a kinetic yardstick for surveying the topography not only of Trp and Cys residues but also of Tyr residues much more quantitatively. The degree of exposure and accessibility, measured by the magnitude of k_q and f_a, respectively, differs significantly among the crystallins. We will first discuss the results of individual residues on which emission quenching studies have been performed and then evaluate them in terms of overall molecular features and relative stability of each crystallin.

Trp Fluorescence Quenching. In agreement with our previous report (Mandal et al., 1985), both acrylamide and iodide quenching data indicate that Trp residues are more exposed in γ-IV crystallin than in γ-III and γ-II. Accessibilities (f_a values) of Trp residues to quenchers calculated from the modified Stern–Volmer equation are also higher in γ-IV and γ-III than in γ-II. However, for acrylamide, both the k_q and the f_a values are considerably larger (Table I) than for iodide quencher. The difference can be attributed to the fact that acrylamide is a polar, uncharged compound that has been shown to quench the fluorescence of indole derivatives predominantly by a collisional process (Eftink & Ghiron, 1976a). In protein studies, this probe has been shown to have the ability to quench any excited Trp residues that it collides with, regardless of whether the residue is located on the surface or in the apolar interior of a protein (Eftink & Ghiron, 1976b). However, because of iodide's large ionic volume, hydrophilic nature, and negative charge, the ion's penetration toward buried Trp residues is expected to be very low (Lehrer, 1971; Lehrer & Leavis, 1978). Even the surface charge of the protein, particularly in the vicinity of the emitter, affects the quenching rate constant values considerably when an ionic quencher is used (Lehrer, 1971).

Stern–Volmer plots (Figure 1) reveal the presence of more than one class of Trp residues in γ-III and γ-IV crystallins; the existence of multiple components (Lehrer & Leavis, 1978; Tao & Cho, 1979) is evident from the downward curvature of the plots of these two proteins. Absence of upward curvature in the case of all three proteins suggests that the static quenching parameter, V, is small. For acrylamide quenching, the perfectly linear Stern–Volmer plot of γ-II indicates the uniformity of the microenvironments of Trp residues in this protein. X-ray crystallography (Blundell et al., 1981; Wistow et al., 1983) shows that all four Trp residues are buried in the hydrophobic interior of γ-II. This is also in agreement with our reported low values of quantum yield and the position of emission maxima of Trp fluorescence of γ-II (Mandal et al., 1985). Iodide ion is completely ineffective in decreasing the Trp emission in γ-II, but it has considerable effect in the other two proteins, particularly γ-IV. Despite the similarity in number (four) of Trp residues in all γ-crystallins (Croft, 1972) and predicted homology in three-dimensional structure (Chirgadze et al., 1981), it is evident that the positions of Trp residues in γ-III and γ-IV differ considerably from γ-II. Detailed X-ray studies of γ-III and γ-IV will reveal this fact more precisely.

Quenching of IAEDANS-Labeled Cys Residues. Bovine γ-crystallins have six (γ-III and γ-IV) and seven (γ-II) Cys residues, more than the average for a protein of about 175 amino acid residues (Fahey et al., 1977). More than one class of Cys residues is reactive to the fluorescent probe MIANS (Mandal et al., 1987a), similar to IAEDANS, and one inaccessible class may exist in all of them. Thus, quenching studies may not provide accessibility information about all the thiol groups. This is particularly true for Cys-32 and Cys-78 of γ-II, which are completely inaccessible to any of the reagents used (Wistow et al., 1983; Summers et al., 1984). Nevertheless, the quenching data obtained in this study are in excellent agreement with our earlier studies (Mandal et al., 1987a,b). For example, the number of residues accessible to MIANS (similar to IAEDANS) is least in γ-III and most in γ-II; γ-IV is intermediate (Mandal et al., 1987a). Since acrylamide has the ability to quench the excited emitter regardless of whether the residue is located on the surface or in the interior of the protein, polar or apolar (Eftink & Ghiron, 1976b), the k_q values (Table II) follow the same order, namely, γ-II > γ-IV > γ-III. However, for iodide quenching, the situation is very different. Because of the inability of the iodide ion to penetrate into the hydrophobic interior of the protein, only the solvent-exposed thiols were accessible to this quencher. Cys-15 has the highest solvent exposure, whereas residues 22 (absent in γ-III and γ-IV) and 41 are only partially exposed (Wistow et al., 1983; Mandal et al., 1987a). The unusually low values of k_q for iodide quenching compared with those for acrylamide quenching (Table II) indicate that the thiols that are present in the hydrophobic interior and have reacted with IAEDANS are readily accessible to acrylamide but not to iodide ions. The small difference in k_q among the crystallins very likely reflects variations in the charge characteristics in the protein surface and in the vicinity of the labeled thiols (Lehrer, 1971).

Interpretation of steady-state emission and quenching studies is not always straightforward, because in most cases there are several sites, and the data reflect a composite of the steric environments of each emitter. Furthermore, in steady-state quenching, two processes, one collisional and one static, con-

tribute to the quenching. On the other hand, in the lifetime quenching studies, the degree to which a fluorophore is exposed to quenchers in the medium can be discussed in quantitative terms (Tao & Cho, 1979; Andley et al., 1982). This is evident from the results of dynamic quenching. The decay curves of all crystallins consisted of more than one component and were satisfactorily represented by the sum of two exponentials at all acrylamide concentrations (Figure 4). The definite existence of two lifetimes of IAEDANS-labeled γ-crystallins and the difference in dynamic quenching behavior validate either or both for the following reasons: (1) lifetime and dynamic quenching studies provide information (not obtainable from steady-state emission) more precisely about the microenvironments of the labeled probe (Tao & Cho, 1979; Lehrer et al., 1981), and (2) the existence of more than one conformer of the probe itself could yield more than one lifetime value (Andley & Chakrabarti, 1981; Andley et al., 1982).

In the case of γ-II, both major and minor components are quenched by acrylamide (Figure 4a and Table IV), whereas the minor components of γ-III and γ-IV remain virtually unaffected (Figure 4a,b). There are two possible reasons for this difference: either (1) the IAEDANS-reactive SH in γ-II is greater (assuming similar reactivity to that of MIANS) than in γ-III or γ-IV (Mandal et al., 1987a), and possibly both components of the additional reactive thiols are accessible to the quencher; or (2) the observed differences in tertiary structure of γ-II from the other two crystallins (Mandal et al., 1985, 1987a,b) and in photoaggregation properties (Mandal et al., 1988; Kono et al., 1988) are well reflected in this differential quenching behavior.

Tyr Phosphorescence Quenching. Difference (pH 11.0–7.0) absorption spectra of these crystallins indicated that the number of alkali-titratable Tyr residues of γ-III is larger than of γ-II and γ-IV (Mandal et al., 1985). This may suggest that the microenvironments and accessibility of these residues of γ-III are different from those of γ-II and γ-IV. The Tyr phosphorescence quenching studies have indeed revealed that the ease of complex formation is maximum for γ-III (K_a is highest), which, in turn, indicates that the accessibilities of the Tyr residues in γ-III are significantly higher than the other two (Figure 5). As a matter of fact, these residues in γ-II are practically inaccessible to acrylamide. This is in agreement with the results of Borkman and Phillips (1985), who demonstrated the high efficiency of Tyr to Trp energy transfer in γ-II; Trp in γ-II is completely buried in the hydrophobic interior of this protein (Wistow et al., 1983; Mandal et al., 1985) as well as being least accessible to the quenchers (this study), and most of the Tyr in γ-II is located within the Forster distance for an efficient energy transfer (Wistow et al., 1983; Borkman & Phillips, 1985). Results of photochemically induced dynamic nuclear polarization NMR measurements have also indicated that, except for Tyr-165 and Tyr-62, most of the Tyr residues of γ-II are buried in the protein core (Garner et al., 1984).

In all cases, acrylamide appears to be a more efficient quencher than iodide. This may not be due solely to the restricted permeability of the ion to the inner core of the protein. Cys residues in γ-II are more exposed and accessible than those of the other two proteins, and accordingly, both steady-state and lifetime quenching constants are higher for IAEDANS-labeled γ-II than for the other two crystallins. The fact that the iodide quenching constant for γ-II is lower than for γ-III and γ-IV suggests strongly that ion permeability is not the cause of the difference but rather the surface charge of γ-II, particularly in the vicinity of the emitter. Thus, it appears that these proteins differ considerably in their charge characteristics.

In summary, the present study has revealed the specific differences in the arrangements of Trp, Tyr, and Cys residues that are responsible for the observed variations in tertiary structure and stability of these crystallins. The quenching parameters demonstrate quantitatively that the Trp and Tyr residues of γ-II are less accessible than the other two. We predict that arrangements and microenvironments of both Trp and Tyr residues of γ-III and γ-IV will be found not to be similar to γ-II even though their three-dimensional structures (secondary) were suggested to be homologous (Chirgadze et al., 1981). Tyr in γ-III and Trp in γ-IV are particularly expected to differ most from those of γ-II. We do not anticipate a major difference in the positioning of Cys residues of γ-III and γ-IV. The subtle variations observed in the accessibilities among these crystallins are most likely due to the fact that their tertiary folding is not similar to each other because of the specific difference in the positioning and orientations of the Trp and Tyr residues. This is supported by the fact that both steady-state emission and dynamic quenching kinetics of the dye-labeled thiols are not significantly different from each other. The lack of fluorescence energy transfer from Trp to MIANS-labeled thiols compared to the highly efficient transfer in the other two proteins (Mandal et al., 1987b) can be explained by their relative positioning of their Trp rather than their Cys residues. High-resolution X-ray crystallographic studies of γ-III and γ-IV are not yet available in support of these results. Nevertheless, our solution studies have provided enough evidence to conclude that these specific molecular features of individual γ-crystallins are primarily responsible for their remarkable denaturation (Horwitz et al., 1977; Mandal et al., 1987b) and cryoprecipitation (Garner et al., 1981; Lerman et al., 1966, 1983; Siezen et al., 1985) behavior and photoinduced aggregation (Chakrabarti et al., 1986).

The cause of cataract has been attributed to the denaturation and aggregation of lens proteins to form high molecular weight aggregates, soluble and insoluble, of sufficient size to scatter light (Dische & Zil, 1951; Benedek, 1971; Harding & Dilley, 1976; Spector et al., 1979). γ-Crystallins were found to be the major proteins associated with these aggregates (Garner et al., 1981), and this pathological involvement of γ-crystallins in cataractogenesis was suggested to be associated with changes in native tertiary structure (Garner et al., 1984). Further investigations are obviously needed to ascertain this hypothesis.

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