Accessibilities of the Sulfhydryl Groups of Native and Photooxidized Lens Crystallins: A Fluorescence Lifetime and Quenching Study†

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ABSTRACT: Fluorescence lifetime and acrylamide quenching studies on the *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS)-labeled sulfhydryl groups of bovine lens α-, β_H-, and γ-crystallins were carried out to characterize the microenvironment of the sulfhydryls and changes produced by singlet oxygen mediated photooxidation. For the untreated proteins, the lifetimes of the major decay component of the fluorescence-labeled crystallins were 15.2, 14.4, and 13.0 ns, and the quenching rate constant, k_q, values were 16.6×10^7, 26.9×10^7, and 32.7×10^7 M^{-1} s^{-1} for α-, β_H-, and γ-crystallins, respectively. The results indicate that as the polarity of the sulfhydryl site increased (i.e., its lifetime decreased), its accessibility to collisional quenching by acrylamide also increased. The minor decay component of the fluorescence label was not significantly quenched by acrylamide for all three classes of crystallins. When the proteins were irradiated in the presence of methylene blue, in a system generating singlet oxygen, the k_q value for acrylamide quenching of the major decay component of α-crystallin decreased to zero, while its lifetime decreased to 6 ns. Neither the lifetime nor the k_q of α-crystallin recovered completely in the presence of the singlet oxygen quencher sodium azide. Light-induced binding of the photosensitizer methylene blue to the crystallins was observed by absorption spectroscopy. The bound photosensitizer partially quenches the fluorescence lifetime of the *N*-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (AEDANS) label in irradiated α-crystallin. Further decrease in the lifetime occurs as a result of the singlet oxygen mediated conformational change. The results suggest that the fluorescence lifetime of the AEDANS is fully quenched in the irradiated α-crystallin and there is no further quenching by acrylamide. An increase in the fraction of the minor component of β_H-crystallin which was inaccessible to acrylamide quenching was observed after irradiation. There was no effect of irradiation on the k_q for acrylamide quenching of the major component of the decay of AEDANS bound to β_H- or γ-crystallins. Static quenching was found to contribute significantly to the steady-state quenching plots of the polar sulfhydryl sites of irradiated α-crystallin and of untreated and irradiated β_H- and γ-crystallins, but it had no detectable role in the case of untreated α-crystallin. Fluorescence anisotropy of the AEDANS label bound to the crystallins was higher in the irradiated crystallins as compared with the controls.

Oxidatively modified lens crystallins accumulate in the central region of human lenses during aging and cataractogenesis (van Heyningen, 1971; Harding, 1972; Zigman, 1977; Lerman, 1980; Zigler & Goosey, 1981). The characteristic changes that occur in these proteins include protein cross-linking, formation of high molecular weight aggregates, exposure of buried thiols, disulfide cross-linking, loss of tryptophan fluorescence, and formation of non-tryptophan products such as *N*-formylkynurenine (*N*-FK)[1] or related species. The latter compounds are well-known photosensitizers (Nilsson et al., 1973) and in the presence of light and oxygen can generate one or more active species of oxygen: singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2) (Walrant et al., 1975; Foote, 1976; Grossweiner, 1984), and may play a role in causing protein aggregation and opacification of the lens. The possibility that light and photosensitizers have an important role in human cataractogenesis is an active research area (Lerman, 1980; Goosey et al., 1980; Borkman et al., 1978; Dillon & Spector, 1980; Zigman, 1977; Zigler & Goosey, 1981; Andley et al., 1984, 1985a,b; Andley & Chylack, 1986; Andley & Chapman, 1986; Andley, 1987).

In model systems, the action of light and photosensitizers has been found to generate changes somewhat similar to those seen in old and cataractous human lenses. Irradiation of an aerobic solution of α-crystallin with near-UV light (300 nm) causes tryptophan photooxidation, increased non-tryptophan fluorescence, cross-linking to dimers (Borkman et al., 1981; Fujimori, 1982), and a change in the tertiary structure of the protein (Andley et al., 1984). It was found that *N*-FK (formed by tryptophan oxidation by a photodynamic process) generates H_2O_2 in the system and causes a change in the microenvironment of sulfhydryl groups to a more exposed environment (Andley & Chylack, 1986). In a methylene blue sensitized photooxidation system, singlet oxygen causes cross-linking of the lens crystallin polypeptides (Goosey et al., 1980; Zigler & Goosey, 1981). Fluorescence and circular dichroism measurements have recently shown changes in tertiary structure of α-, β_H-, and γ-crystallins (Andley & Chapman, 1986) and a loss of activity of several key enzymes by methylene blue sensitized photooxidation (Jedziniak et al., 1986). Alterations in microenvironments of tryptophan and cysteine

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[1] Abbreviations: BCA, bicinchoninic acid; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; MB, methylene blue; MIANS, 6-(4-maleimidoanilino)naphthalene-2-sulfonic acid; *N*-FK, *N*-formylkynurenine; SDS, sodium dodecyl sulfate; AEDANS, *N*-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine.

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residues of the proteins and a decrease in sulfhydryl content have been reported (Andley et al., 1985b; Andley & Chapman, 1986). Depending on the microenvironment of these residues in α-, β_H-, and γ-crystallin, the nature of the tertiary structure change was different for each protein. In calf lens α-crystallin, a multisubunit protein of molecular weight 8×10^5, there are two main types of subunits (A and B chains with molecular weight 20×10^3) in the ratio 3:1 (Siezen et al., 1978; Bloemendal, 1981). The 30 A chains each have 1 cysteine residue at position 131 whereas the B chains have none. The sulfhydryl groups of the cysteines in the aggregate of calf α-crystallin are probably in different environments: surface, hydrophobic, and buried (Andley et al., 1982). The β-crystallins are a family of oligomeric proteins [molecular weight $(4-18) \times 10^4$] in which the principal subunit (βBp) has two sulfhydryls at positions 39 and 66, which are likely to be accessible to solvent and could, therefore, form intermolecular disulfide bonds under oxidizing conditions (Siezen, 1981). The monomeric γ-crystallins of molecular weight 20×10^3 are rich in sulfhydryl groups; the primary gene products contain seven (γ_{II}) or six (γ_{III} and γ_{IV}) cysteines (Blundell et al., 1983). The cysteines are found in well-defined environments, as determined by X-ray diffraction analysis of γ_{II}-crystallin (Blundell et al., 1981).

In the present study, we have further investigated the conformational changes of α-, β_H-, and γ-crystallins in a dye-sensitized photooxidation system. Fluorescence quenching studies on 1,5-IAEDANS-labeled sulfhydryl groups of untreated and singlet oxygen treated proteins have been done to determine the change in the exposure of these residues. The fluorescence label is selective for sulfhydryls and has a relatively long lifetime (Hudson & Weber, 1973) for performing quenching studies conveniently (Tao & Cho, 1979). The neutral, highly efficient quencher acrylamide has been used as the fluorescence quencher, since it does not denature proteins even when present in molar quantities (Eftink & Ghiron, 1981). Lifetime quenching measurements of the IAEDANS-labeled proteins were used to resolve the presence of multiple decay components; lifetime and intensity quenching studies were used to characterize the quenching process in terms of static and dynamic contributions.

Materials and Methods

Materials. Calf lens crystallins (α, β_H, β_L, and γ) were isolated and purified from calf cortices as described previously (Andley & Chylack, 1986). The purity of each crystallin was checked by SDS gel electrophoresis according to Laemmli (1971). β-Mercaptoethanol was added to reduce any disulfide bonds that might have generated in the isolation procedure. The reducing agent was removed by dialysis. Of the β-crystallins, only the data on β_H-crystallin are reported. 1,5-IAEDANS was obtained from Molecular Probes, Inc. (Eugene, OR). Methylene blue and sodium azide were obtained from Sigma, and acrylamide (ultra-high-purity electrophoresis grade) was obtained from Bio-Rad Laboratories.

Irradiation of Crystallins. The crystallin solutions in 0.01 M phosphate buffer, pH 7.8, were irradiated at 37 °C for 5 h in the presence of the photosensitizer methylene blue (50 μM) as described previously (Andley & Chapman, 1986). The singlet oxygen quencher NaN_3 (0.1 M), when used, was added to the protein solutions prior to irradiation. Controls included crystallins in the dark, crystallin and dye in the dark, and crystallin in the light with no added photosensitizer. All protein solutions were dialyzed with one change of buffer at 4 °C to remove sodium azide and unbound methylene blue prior to SH group labeling.

The amount of MB bound to the crystallins was determined by measuring the absorption spectra of the crystallin treated with 50 μM MB after dialysis of the dark and irradiated samples. The extinction coefficient (ϵ_{664}) of MB in buffer was determined to be 56 000 M^{-1} cm^{-1} at 664 nm. The amount of MB bound was calculated at the absorption maximum of MB for each crystallin. All absorption measurements were done by using a Perkin-Elmer Lambda 4B spectrophotometer.

In another set of experiments, the native crystallins were first labeled with IAEDANS and subsequently irradiated in the presence of MB, followed by dialysis to remove the photosensitizer. Under these conditions, lifetime and intensity measurements show that the IAEDANS label is completely destroyed, presumably by the action of singlet oxygen. Therefore, the crystallin solutions were first irradiated in the presence of MB and then labeled with IAEDANS, as described below.

SH Group Labeling. The crystallin solutions (control and irradiated) were incubated overnight with a 4-fold molar excess of 1,5-IAEDANS solution according to the method described previously (Andley & Chylack, 1986), except that a higher concentration of crystallins (2–3 mg/mL) was used to obtain sufficient intensity for acrylamide quenching studies. Due to the light sensitivity of IAEDANS solutions, the labeled proteins were kept in the dark. Excess 1,5-IAEDANS was removed by gel filtration through a Sephadex G-25 column. Protein concentration was determined according to Smith et al. (1985) using the BCA reagent (Pierce).

The degree of labeling of IAEDANS was determined as follows. The absorption of the control and irradiated samples of the labeled crystallins at 337 nm was determined. For the irradiated crystallins, the absorption of an unlabeled solution (due to *N*-FK or related species) was also determined at the same protein concentration; the latter was subtracted from the absorption of AEDANS-labeled irradiated crystallin. The difference (due to absorption of bound AEDANS alone) was used to calculate the concentration of the label using an extinction coefficient $\epsilon_{337} = 6100$ M^{-1} cm^{-1} (Hudson & Weber, 1973). The concentration of the protein was also determined. The degree of labeling was calculated by assuming a molecular weight of 800 000 for α-crystallin, 180 000 for β_H-crystallin, and 20 000 for γ-crystallin (Bloemendal, 1981). The degree of labeling for the control and irradiated crystallins is shown in Table I.

Fluorescence Lifetime and Quenching Measurements. Fluorescence lifetime measurements were carried out on an Ortec 9200 photon counting nanosecond fluorometer by the method of Tao and Cho (1979) as described previously (Andley & Chylack, 1986; Andley & Chapman, 1986). Excitation was selected by a Corning CS 7-54 glass filter, emission by a CS 3-69 filter. Under these conditions, the emission due to tryptophan photoproducts *N*-FK or related species generated by photooxidation of the crystallins (Andley et al., 1985a,b; Goosey et al., 1980) was found to be insignificant compared to that of the fluorescence label. The excitation polarizer was set at vertical. The emission polarizer was set at 54.7° from the vertical. The time profile of the excitation was taken at the same wavelengths as those for the fluorescence decay, after changing the emission filter to a 334-nm interference filter. Thus, the errors due to variations in the time response of the photomultiplier as a function of wavelength were minimized. The extent of light scattering for the filter combination was ~2% of the fluorescence, using a Ludox colloidal silica suspension. The concentration of the labeled proteins was 0.5–0.6 mg/mL, unless otherwise indi-

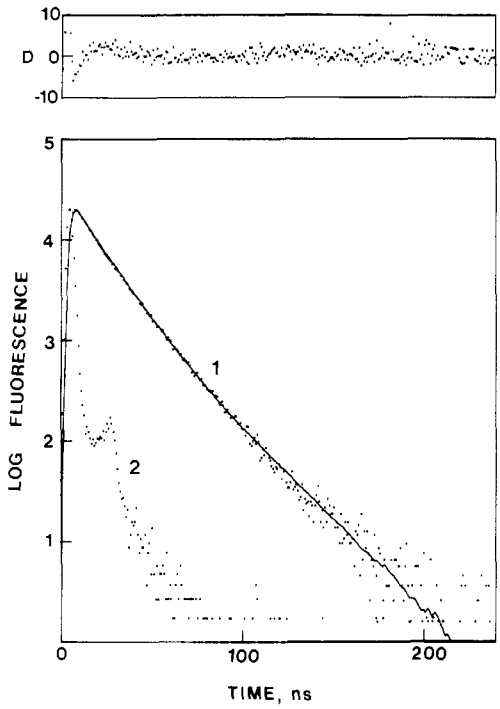

FIGURE 1: Fluorescence decay curve of AEDANS-labeled, irradiated β_H-crystallin (curve 1) and the lamp profile (curve 2). Dots are experimental points; solid lines are calculated decay curves obtained by the biexponential method of moments analysis (see Materials and Methods). The top panel shows the differences between the calculated and experimental decay curves.

cated. The data were analyzed by the method of moments procedure (Isenberg & Dyson, 1969) as described by Yguerabide (1972). Each decay curve was analyzed by one-, two-, and three-exponential analyses, and the quality of fit was judged by computing χ^2/N defined as

$$\chi^2/N = 1/N\sum_{i=1}^{N}[F_c(t_i) - F_e(t_i)]^2/F_e(t_i)$$

where N is the number of points and F_c and F_e are the calculated and the experimental fluorescence decay, respectively. For all the crystallins, two-exponential analysis gave clearly better fits than did single-exponential analysis, as judged by lower χ^2/N values at all concentrations of acrylamide. For decay curves reported as biexponential, three-exponential analyses yielded third components of negligible amplitudes. For irradiated β_H-crystallin, for example (Figure 1), two-exponential analyses yielded $A_1 = 0.83$, $\tau_1 = 11.3$ ns, $A_2 = 0.17$, and $\tau_2 = 20.9$ ns, while three-exponential analyses yielded $A_1 = 0.81$, $\tau_1 = 11.2$ ns, $A_2 = 0.19$, $\tau_2 = 20.6$ ns, $A_3 = 10^{-5}$, and $\tau_3 = 47.2$ ns, where A's and τ's are fractional amplitudes and lifetimes, respectively, of the three components. For decay curves in which the fraction of the minor component was ≤ 0.05, monoexponential analyses are reported in the absence and presence of various acrylamide concentrations in order to conclusively establish the significance of the second component.

Steady-state fluorescence measurements were carried out in the corrected mode using a Perkin-Elmer MPF 66 spectrofluorometer equipped with a professional 7300 series computer. Excitation for the AEDANS-labeled crystallins was at 340 nm, and the emission was monitored at the emission maximum for each protein. Fluorescence lifetime and steady-state quenching studies were performed by adding small volumes of 6 M acrylamide using a 25-μL Hamilton microsyringe. Appropriate corrections for dilution were made for the steady-state measurements (Lehrer & Leavis, 1978).

According to the theory of fluorescence quenching (Lehrer & Leavis, 1978; Eftink & Ghiron, 1981), the fluorescence lifetime of an emitter decreases in the presence of a quencher, due to the collisional quenching process, according to the Stern–Volmer equation:

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

where k_q is the bimolecular Stern–Volmer quenching rate constant and τ_0 and τ are the singlet lifetimes in the absence and presence of some quencher concentration, [Q], respectively. k_q approaches zero at low accessibilities and approaches the diffusion-control upper limit at high accessibilities.

The fluorescence intensity of an emitter is decreased by both the collisional and the static quenching processes (Eftink & Ghiron, 1976a; Lehrer & Leavis, 1978) according to

$$F_0/F = (1 + K_{sv}[Q])e^{V[Q]} \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of a quencher, respectively, at a quencher concentration [Q], K_{sv} is the Stern–Volmer quenching constant, and V is the static quenching parameter (Eftink & Ghiron, 1976 b). K_{sv} is related to k_q by $K_{sv} = \tau_0 k_q$.

The initial slope (IS) of a F_0/F vs [Q] plot is given by Tao and Cho (1979).

$$\left[\frac{d(F_0/F)}{d[Q]}\right]_{[Q]=0} = K_{sv} + V \quad (3)$$

In a system with multiple emission components, the intensity quenching equation is given by Lehrer and Leavis (1978):

$$F_0/F = \sum_{i=1}^{N}\left\{\frac{f_i}{(1 + K[Q])e^{V_i[Q]}}\right\}^{-1} \quad (4)$$

and the initial slope becomes

$$IS = \sum_{i=1}^{N} f_i(K_i + V_i) \quad (5)$$

where f_i is the fractional contribution from each emission component to the total emission, N is the total number of components, K_i is K_{sv}, and V_i is the V parameter for each component.

Only the collisional (dynamic) quenching process contributes to the lifetime measurements; therefore, a plot of $1/\tau$ vs [Q] should be linear as long as the measured lifetimes are truly representative of a single decay component (Tao & Cho, 1979).

Fluorescence anisotropy (r) is defined as

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \quad (6)$$

where $I_\parallel$ is the intensity of light when the excitation and emission polarizers are in a parallel orientation and $I_\perp$ is the intensity when the polarizers are in a perpendicular orientation (Weber, 1953). Fluorescence intensity readings on the 1,5-IAEDANS-labeled samples were taken with the excitation and emission polarizers in combinations of vertical (v) and horizontal (h) positions. These combinations are vv, vh, hh, and hv, where the order of the letters represents the positions of the excitation and emission polarizers, respectively (Bentley et al., 1985). Thus, $I_\parallel$ and $I_\perp$ (eq 6) correspond to fluorescence intensity measurements in the vv and vh settings, respectively.

Table I: Degree of Labeling and Emission Maxima of 1,5-IAEDANS-Labeled Lens Crystallins[a]

material	SH residues[b]	degree of labeling[c]	emission max (nm)
α, control	30	17.3	472
α, irrad	22	8.6	478
α, irrad, $+NaN_3$	30	15.8	478
β_H, control	14	7.5	479
β_H, irrad	7	1.0	479
γ, control	6	1.0	479
γ, irrad	2	0.4	479

[a] The crystallins were irradiated in the presence of MB prior to labeling with IAEDANS (see Materials and Methods for details). [b] The SH residues for the controls are from Bloemendal (1981) and Blundell et al. (1981). For the irradiated proteins, the total number of SH groups were determined in 6 M guanidine hydrochloride using Ellman's reagent (Palau & Daban, 1974). [c] The degree of labeling of 1,5-IAEDANS was determined as described under Materials and Methods.

To correct for instrumental artifacts, an instrument factor (G) was experimentally determined according to

$$G = I_{hv}/I_{hh} \tag{7}$$

and anisotropy was calculated by using

$$r = \frac{I_{vv} - I_{vh}(G)}{I_{vv} + 2I_{vh}(G)} \tag{8}$$

Results

Degree of Binding of IAEDANS. Table I shows the number of cysteine residues in the control and irradiated crystallins and their extent of labeling by the SH-specific fluorophore IAEDANS. The SH groups of the cysteines do not have equal reactivity with IAEDANS. In α-crystallin, only 17 out of 30 SH are accessible to IAEDANS labeling. It has been suggested that the IAEDANS-reactive SH's are in two different microenvironments—exposed and hydrophobic (Andley et al., 1982; Siezen et al., 1978). The remaining 13 SH's of control α-crystallin are inaccessible to the labeling agent. In irradiated α-crystallin, only approximately nine cysteines are accessible to labeling by IAEDANS; the remaining eight are oxidized by the action of $^1O_2$ (Andley & Chapman, 1986).

The major (40–45%) polypeptide, βBp, of β_H-crystallin contains two cysteines per polypeptide chain of molecular weight 26K (Siezen, 1981; Berbers et al., 1982). For the β_H aggregate of molecular weight 1.8×10^5, therefore, the total number of cysteines is likely to be $\sim$14. In the control β_H-crystallin, the degree of labeling of IAEDANS was determined to be 7.5 (Table I). It is not known if the two SH groups (at positions 39 and 66 in βBp) have the same reactivity toward IAEDANS. The exact labeling site on β_H-crystallin cannot be determined from this experiment, but it is likely that IAEDANS labeling occurs predominantly at one site. After irradiation of β_H-crystallin, 50% of the total SH's are oxidized. The degree of labeling in the irradiated β_H-crystallin is only 1.0 as compared to 7.8 in the the control, indicating that some of the oxidized SH's are not reactive toward IAEDANS. This result is due to the cross-linking of the protein by irradiation in the presence of MB, making some cysteines inaccessible or unreactive with IAEDANS.

Untreated γ-crystallin contains six or seven cysteine residues. On the basis of X-ray data on the subfraction γ_{II}-crystallin, the cysteine residue at position 15 has the highest (Wistow et al., 1983) solvent exposure (65 Å^2). The data in Table I show that in γ-crystallin, only one SH, probably cysteine-15, is predominantly reactive with IAEDANS. Very minor amounts of labeling may also occur at residues 22 (γ_{II}) and 41 (γ_{II}, γ_{III}, and γ_{IV}), having much lower solvent exposures, and thus lower reactivity with IAEDANS. In γ-crystallin irradiated in the presence of MB, there was only 0.4 IAEDANS-reactive cysteine per polypeptide of molecular weight 20K. Thus, as in the case of β_H-crystallin, there is a loss of IAEDANS labeling sites of γ-crystallin due to oxidation as well as cross-linking and conformational changes.

Table II: Fluorescence Lifetimes and Amplitudes of the Decay Components of AEDANS-Labeled α-Crystallin[a]

material	A_1	τ_1 (ns)	A_2	τ_2 (ns)	χ^2/N
α, dark	0.69	15.7	0.31	24.6	10
α, irrad	0.73	6.0	0.27	20.5	6
α, irrad, $+NaN_3$	0.70	8.7	0.30	21.1	7

[a] See Materials and Methods for experimental conditions. A_1 and A_2 are the amplitudes of the major and minor decay components, respectively; τ_1 and τ_2 are the corresponding lifetimes. All values were obtained by the biexponential method of moments analysis. χ^2/N is a parameter for judging the quality of fit of the calculated and experimental fluorescence decay.

Table III: Binding of MB to Lens Crystallins in Dark and Irradiated Samples[a]

material	bound MB[b]	absorption max (nm)
free MB (buffer)		664
α, dark	0.10	666
α, irrad	0.33	639
α, irrad, $+NaN_3$	0.32	644
β_H, dark	0.009	665
β_H, irrad	0.21	650
γ, dark	0	
γ, irrad	0.003	665

[a] The amount of bound MB was determined as described under Materials and Methods. [b] Moles of MB bound per mole of protein, assuming a molecular weight of 800 000 for α-crystallin, 180 000 for β_H-crystallin, and 20 000 for γ-crystallin, respectively (Bloemendal, 1981).

The emission maximum of AEDANS label bound to untreated α-crystallin was at 472 nm and shifted to 478 nm after irradiation in the presence of MB. When NaN_3 was included in the irradiation mixture, the emission maximum of the label was at 478 nm. The emission maxima for AEDANS bound to control β_H- and γ-crystallins were at 479 nm and were unaffected by irradiation in the presence of MB (Table I).

Lifetime and Lifetime Quenching. The analysis of the fluorescence decay parameters of AEDANS label bound to α-crystallin is summarized in Table II for a representative experiment. The lifetime of the major decay component was 15 ns in untreated α-crystallin and decreased to 6 ns in the irradiated α-crystallin. The lifetime of the major decay component for α-crystallin irradiated in the presence of the singlet oxygen quencher sodium azide was also determined and was about 9 ns. Thus, the singlet oxygen quencher only partially prevents the decrease in the lifetime of the bound AEDANS by irradiation in the presence of MB. This result suggests that light-induced binding of the photosensitizer MB to α-crystallin may itself cause a decrease in the lifetime of the AEDANS label. To test this hypothesis, the binding of MB to the crystallins in dark and irradiated samples was measured by absorption spectroscopy. When the crystallins were treated with MB in the dark, the MB could not be completely removed from α-crystallin by dialysis (Table III). This may be due to trapping of free MB, in the inaccessible sites of the polymeric protein, which was not released by dialysis. β_H- and γ-crystallin did not bind significant amounts of MB in the dark. Among the irradiated crystallins, α-crystallin showed the highest binding of MB. The binding of MB to α-crystallin

Table IV: Fluorescence Decay Parameters for Acrylamide Quenching of AEDANS-Labeled β_H-Crystallin[a]

	control				irradiated[b]			
acrylamide concn (M)	A_1/A_2	τ_1 (ns)	τ_2 (ns)	χ^2/N	A_1/A_2	τ_1 (ns)	τ_2 (ns)	χ^2/N
0	11.8	14.1	26.0	10	4.9	11.2	21.1	9
0.1	7.3	8.3	21.6	3	10.1	8.0	21.8	11
0.2	8.1	6.3	20.4	4	10.1	6.4	20.6	10
0.29	9.0	5.8	20.1	10	11.5	5.7	20.5	16
0.38	11.5	5.5	21.0	11	10.1	4.8	18.2	14
0.46	11.5	5.0	20.2	13	9.0	4.2	17.2	10

[a] A_1 and A_2 are the amplitudes of the major and minor decay components, respectively; τ_1 and τ_2 are the corresponding lifetimes. χ^2/N is a parameter for judging the quality of fit of the calculated and experimental fluorescence decay. See Table VI for k_q values. [b] The protein was irradiated in the presence of the photosensitizer MB (see Materials and Methods).

Table V: Fluorescence Decay Parameters for Acrylamide Quenching of AEDANS-Labeled γ-Crystallin[a]

	biexponential analysis				monoexponential analysis		
acrylamide concn (M)	A_1/A_2	τ_1 (ns)	τ_2 (ns)	χ^2/N	A	τ (ns)	χ^2/N
			Control				
0	11.5	13.4	20.0	6	1.0	13.7	10
0.02	5.3	10.2	18.7	5	1.0	12.4	7
0.05	11.4	9.1	19.6	5	1.0	10.9	10
0.09	15.7	8.1	20.2	5	1.0	9.7	15
0.19	19.0	6.3	19.5	8	1.0	8.1	30
0.34	24.0	5.0	19.3	10	1.0	6.9	51
0.46	24.0	4.3	18.0	10	1.0	6.3	62
			Irradiated[b]				
0	6.1	11.6	20.2	6	1.0	13.8	10
0.02	9.0	11.0	20.9	7	1.0	12.6	13
0.06	10.1	9.2	20.1	11	1.0	11.1	20
0.11	10.1	7.7	19.2	9	1.0	9.9	29
0.16	11.5	6.8	18.7	9	1.0	9.1	38
0.25	15.7	5.8	19.7	10	1.0	8.2	57
0.34	13.3	4.9	18.1	10	1.0	7.6	72
0.43	15.7	4.4	17.6	8	1.0	7.2	86

[a] A_1 and A_2 are the amplitudes of the major and minor decay components, respectively; τ_1 and τ_2 are the corresponding lifetimes. χ^2/N is a parameter for judging the quality of fit of the calculated and experimental fluorescence decay. See Table VI for k_q values. [b] The protein was irradiated in the presence of the photosensitizer MB (see Materials and Methods).

could not be prevented by irradiation in the presence of the $^1O_2$ quencher sodium azide. Thus, the lack of recovery by sodium azide of the fluorescence lifetime of the AEDANS moiety bound to α-crystallin (Table II) is likely to be due to quenching by bound MB.

The addition of acrylamide decreased the lifetime of the AEDANS attached to the crystallins in accordance with the Stern–Volmer law. The fluorescence decay curves of AEDANS-labeled control and irradiated α-, β_H-, and γ-crystallins were obtained at various concentrations (0–0.5 M) of acrylamide. The lifetimes and amplitudes of the decay components were obtained for each curve by using the method of moments analysis. The analyses showed that the decay curves of control or irradiated α-crystallin are well represented by the sum of two exponentials at all concentrations of acrylamide. The lifetimes of the components were plotted according to the Stern–Volmer equation (eq 1) as shown in Figure 2. The plots were linear for both the major and minor components in the case of control and irradiated α-crystallin, suggesting that each component can be represented by one exponential (Tao & Cho, 1979). The Stern–Volmer quenching constant, k_q, is the slope of the acrylamide quenching plot in each case, and its values for the various IAEDANS-labeled crystallins are shown in Table VI. It can be seen from the table that the major component of IAEDANS-labeled control α-crystallin (k_q = 16.6 × 10^7 M^{-1} s^{-1}) is 15 times more accessible to quenching by acrylamide than the minor component, which is nearly inaccessible. Irradiation of α-crystallin in the presence of methylene blue results in a decrease of k_q to zero. If the irradiation of α-crystallin was done in the presence of sodium azide, the k_q value for the major component of the AEDANS-labeled protein is the same as for the control, within experimental error (Figure 2; Table II).

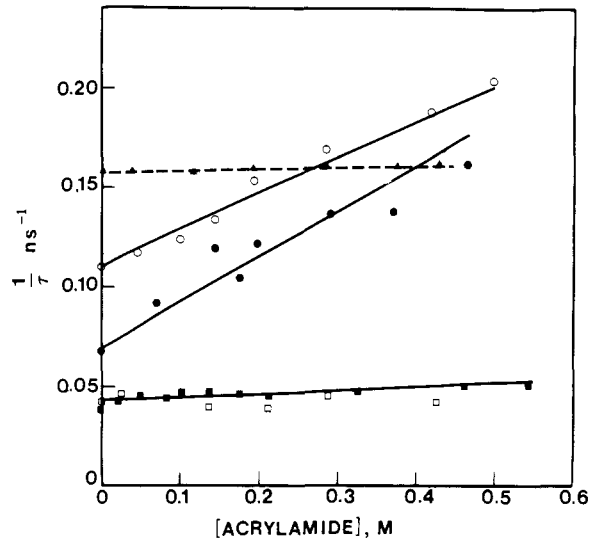

FIGURE 2: Stern–Volmer plots for AEDANS-labeled α-crystallin. Major decay component of control α-crystallin (●); minor decay component of AEDANS-labeled control α-crystallin (■); major decay component of irradiated α-crystallin (▲); minor decay component of irradiated α-crystallin (□); major decay component of α-crystallin irradiated in the presence of 0.1 M sodium azide (○). See Table VI for k_q values.

The fraction of the minor component for β_H- and γ-crystallins was very small. Representative biexponential analyses for untreated and irradiated β_H- and γ-crystallin are shown in Tables IV and V. For untreated β_H-crystallin, the fraction

Table VI: Lifetimes (τ), Amplitudes (A), and Lifetime Quenching Constants (k_q) for AEDANS-Labeled Control and Irradiated Crystallins[a]

material	A_1	τ_1 (ns)	k_{q1} ($\times 10^{-7}$ M^{-1} s^{-1})	A_2	τ_2 (ns)	k_{q2} ($\times 10^{-7}$ M^{-1} s^{-1})
α, control	0.67 ± 0.12	15.2 ± 0.5	16.6 ± 3.6	0.33 ± 0.09	24.2 ± 1.7	1.0 ± 0.4
α, irrad	0.73 ± 0.10	5.8 ± 1.1	0	0.27 ± 0.03	21.5 ± 0.7	0
α, irrad, + NaN_3	0.67 ± 0.03	9.3 ± 0.7	11.6 ± 3.2	0.33 ± 0.03	21.5 ± 0.5	0
β_H, control	0.95 ± 0.02	14.4 ± 0.1	26.9 ± 2.4	0.05 ± 0.02	28.2 ± 2.2	0
β_H, irrad	0.85 ± 0.02	11.2 ± 0.9	26.3 ± 1.6	0.16 ± 0.03	21.1 ± 1.8	0
γ, control	0.92 ± 0.08	13.0 ± 0.9	32.7 ± 1.5	0.08 ± 0.08	27.6 ± 8.9	1.0 ± 0.5
γ, irrad	0.86 ± 0.10	11.8 ± 0.1	33.6 ± 2.7	0.14 ± 0.05	19.8 ± 0.9	1.1 ± 0.5

[a] All values were obtained from the biexponential methods of moments analysis of the lifetime quenching data. k_q was calculated as the slope of the Stern–Volmer plot (eq 1), using linear regression analysis. Mean and standard deviations are calcualted from four sets of experiments.

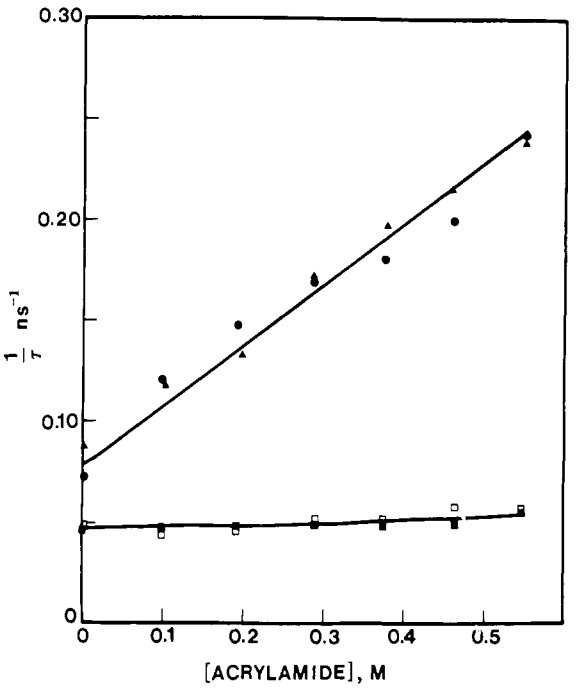

FIGURE 3: Stern–Volmer plots for AEDANS-labeled β_H-crystallin. Major decay component of control β_H-crystallin (●); minor decay component of control β_H-crystallin (■); major decay component of irradiated β_H-crystallin (▲); minor component of irradiated β_H-crystallin (□).

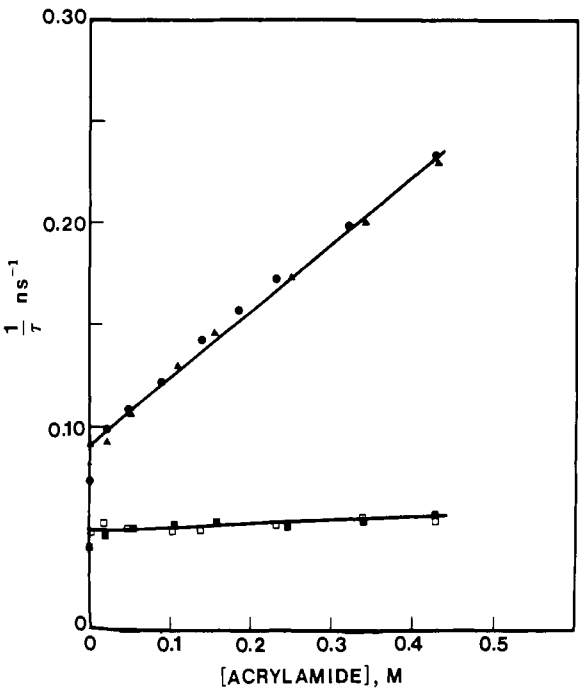

FIGURE 4: Stern–Volmer plots for AEDANS-labeled γ-crystallin. Major decay component of control γ-crystallin (●); minor decay component of control γ-crystallin (■); major decay component of irradiated γ-crystallin (▲); minor decay component of irradiated γ-crystallin (□).

of the minor component is an average of approximately 0.05 in the absence of acrylamide, and it increased to 0.16 in the irradiated β_H-crystallin. χ^2/N values were used to judge the quality of the biexponential fit to the experimental decay curves. For control β_H-crystallin, at zero acrylamide concentration, biexponential analysis gave $\chi_2/N = 10$, and monoexponential analysis (not shown) gave $\chi^2/N = 13.9$. As the acrylamide concentration increased to 0.46 M, χ^2/N for biexponential analysis was 13 (Table IV), but its value increased to 122 for monoexponential analysis, indicating that the significance of the second component increases at high concentrations of acrylamide. In irradiated β_H-crystallin, biexponential analysis gave $\chi^2/N = 9$ at zero acrylamide, and it increased to 10 at 0.46 M acrylamide. Monoexponential analysis for irradiated β_H-crystallin (not shown) was inadequate for describing the decay of the AEDANS label, with $\chi^2/N = 16$ at zero acrylamide and increasing to 96 at 0.46 M acrylamide.

Figure 3 shows the Stern–Volmer quenching plot for the major and minor decay components of AEDANS-labeled β_H-crystallin. The scatter and apparent curvature of the data points from a linear approximation are likely to be due to additional effects of acrylamide other than lifetime quenching. For example, binding or conformational effects of acrylamide on the protein may account for some increase in A_1/A_2 ratios with increasing acrylamide concentration (Table IV). The fit of the closed circles to a linear function using linear regression analysis yields a k_q value of 26.9×10^7 M^{-1} s^{-1} for the major decay component of AEDANS-label control β_H-crystallin. The major component of AEDANS-labeled control β_H-crystallin was more susceptible to quenching by acrylamide than that of AEDANS-labeled α-crystallin (Figure 2). The minor component of the decay of AEDANS-labeled β_H-crystallin was not quenched by acrylamide. k_q for the major component of AEDANS-labeled irradiated β_H-crystallin was also determined (Figure 3, Table VI) and found to be nearly identical with that of the control.

The lifetime quenching data for AEDANS-labeled γ-crystallin (Figure 4) were analyzed in a manner similar to those for α- and β_H-crystallins. At zero acrylamide concentration, the minor component of untreated γ-crystallin was negligible (Table VI), and it increased to 0.13 in the irradiated γ-crystallin. The results of mono- and biexponential analyses for the decay curves of control and irradiated AEDANS-labeled γ-crystallin at various acrylamide concentrations are shown in Table V. The χ^2/N values in the last column are ≤ 10 for biexponential analyses, indicating that the fit is excellent. Furthermore, there is a trend toward increasing χ^2/N as the acrylamide concentration increased. Monoexponetial analysis for the same samples yields higher χ^2/N values. Thus, χ^2/N increased from 10 to 62 for monoexponential analysis of the decay curves, as the acrylamide concentration increased

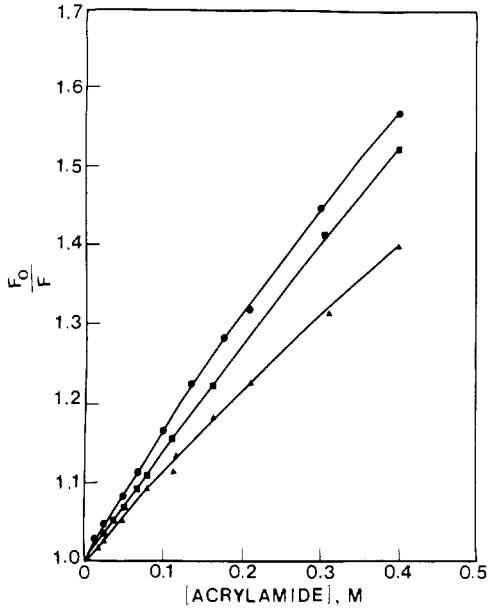

FIGURE 5: Steady-state fluorescence intensity quenching curves for AEDANS-labeled α-crystallins. Control α-crystallin (●); irradiated α-crystallin (▲); α-crystallin irradiated with NaN_3 (■).

Table VII: Initial Slopes (IS) of Stern–Volmer Steady-State Quenching Plots and Fluorescence Anisotropy for AEDANS-Labeled Control and Irradiated Crystallins[a]

material	IS (M^{-1})	anisotropy[b]
α, untreated	1.60	0.166
α, irrad	1.20	0.182
α, irrad (+NaN_3)	1.51	0.166
β_H, untreated	4.26	0.120
β_H, irrad	4.26	0.158
γ, untreated	6.66	0.083
γ, irrad	5.60	0.098

[a] All values were obtained from steady-state fluorescence measurements; uncertainty in the initial slopes of steady-state Stern–Volmer quenching plots was estimated to be ±0.1 M^{-1}. [b] Uncertainty in the anisotropy measurement was ±0.002.

from 0 to 0.46 M, indicating that monoexponential analysis is inadequate to descibe the decay curves of irradiated γ-crystallin. The fact that the fractional amplitudes (or A_1/A_2 ratios) are not constant at different concentrations of acrylamide (Table V) indicates that there may be additional effects of acrylamide on protein conformation as discussed earlier for β_H-crystallin. This is also reflected in the Stern–Volmer quenching plot for the major component of AEDANS-labeled untreated γ-crystallin (Figure 4). The plot cannot be approximated as linear unless the first data points are given very low statistical weight. As in the case of α- and β_H-crystallin, the major component of AEDANS-labeled γ-crystallin is preferentially quenched by acrylamide. The magnitude of k_q is very low for the minor component as compared to 32.7×10^7 M^{-1} s^{-1} for the major component of AEDANS-labeled γ-crystallin (Table VI). Moreover, there is no effect of irradiation on the k_q value of AEDANS-labeled γ-crystallin.

Steady-State Quenching. The fluorescence intensity quenching plots (F_0/F vs [Q]) for AEDANS-labeled control and irradiated α-crystallin are shown in Figure 5. The initial slope of the intensity quenching plot decreased from 1.6 M^{-1} for the control to 1.2 M^{-1} for the irradiated α-crystallin (Table VII). α-Crystallin irradiated in the presence of sodium azide gave an initial slope of 1.4 M^{-1} (Table VII). This result shows that although τ_1 and τ_2 for AEDANS-labeled irradiated α-crystallin did not change with the addition of acrylamide (Figure 2), F_0/F shows a positive slope for the same experiment (Figure 5). This disagreement between the steady-state and the lifetime quenching experiment can be explained on the basis of static quenching of the AEDANS labeling site (see below). Figure 6 shows the intensity quenching curves for AEDANS-labeled control and irradiated β_H-crystallin. No change in the initial slope was observed, which remained at a value of 4.3 M^{-1} for both curves. The intensity quenching curves of AEDANS-labeled control and irradiated γ-crystallin are shown in Figure 7. The initial slope decreased from 6.7 M^{-1} for the control to 5.6 M^{-1} for the irradiated γ-crystallin. The nonlinearity of the intensity quenching plots in Figures 5–7 can be attributed to heterogeneity in the fluorescence emission (Eftink & Ghiron, 1976a). As evident from the lifetime quenching experiments, the AEDANS label is bound in two different environments, of vastly different accessibilities. For a fluorophore in a protein having quenching constants ($K_i + V_i$, eq 5) differing by a factor of 4 or more, the plots of F_0/F vs [Q] appear downward-curving (Eftink & Ghiron, 1981). The intensity quenching measurements were repeated at three concentrations of the labeled protein varying by an order of magnitude; the initial slopes were found to be independent of the concentration of the labeled protein.

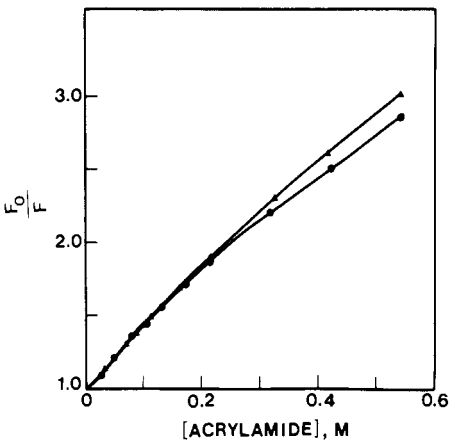

FIGURE 6: Steady-state fluorescence intensity quenching curves for AEDANS-labeled β_H-crystallin. Control β_H-crystallin (●); irradiated β_H-crystallin (▲).

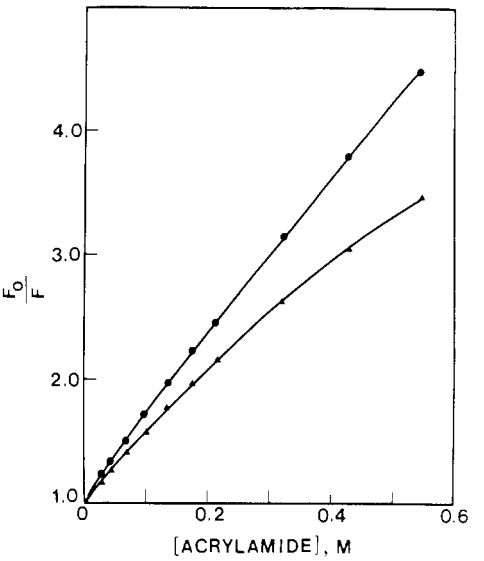

FIGURE 7: Steady-state fluorescence intensity quenching curves for AEDANS-labeled γ-crystallin. Control γ-crystallin (●); irradiated γ-crystallin (▲).

The intensity quenching plots of the AEDANS-labeled crystallins represent contributions from both static and dynamic quenching. Eftink and Ghiron (1976a, 1981) have

interpreted the static quenching parameter V (eq 2–5) as an active volume element surrounding the excited fluorophore, within which the emission is quenched instantaneously by the quencher molecule which may exist randomly near the fluorophore after photon absorption. Thus, static quenching requires the formation of a ground-state complex between fluorophore and quencher, whereas dynamic quenching requires collision between quencher and the excited fluorophore and is diffusion dependent. The intensity quenching plot of AEDANS-labeled irradiated α-crystallin shows a positive slope even though the collisional quenching constant k_q was zero. It is likely that static quenching of the highly polar sulfhydryl site can occur. The initial slope contains contributions according to

$$IS = f_1(K_1 + V_1) + f_2(K_2 + V_2)$$

where "1" and "2" are the major and minor components, respectively. f_1K_1 and f_2K_2 can be estimated by using the values of lifetime and the quenching constant k_q (Table VI). For irradiated α-crystallin

$$f_1K_1 = f_1\tau_{01}k_{q1} = 0$$

since k_{q1} is zero. Similarly, f_2K_2 is zero, since k_{q2} for the minor decay component was zero. f_2/V_2 is likely to be negligible, since V_2, the static quenching parameter for a hydrophobic site, is likely to be very small (Eftink & Ghiron, 1976a). Thus $IS \sim f_1V_1$ or $1.2 = 0.7V_1$ or $V_1 = 1.6\ M^{-1}$. Thus, V_1 for the major component of irradiated α-crystallin is nearly 65% of that of indole in water (Eftink & Ghiron, 1976). By use of similar arguments and eq 5, V_1 was zero, within exprimental error, for native α-crystallin. The presence of static quenching for the highly polar suflhydryl site of irradiated α-crystallin indicates that the AEDANS label is not completely enveloped by the protein fabric, so that the acrylamide molecules can be bound to this site.

As shown below, the static quenching parameter was smaller for AEDANS-labeled β_H-crystallin than for the AEDANS-labeled γ-crystallin. For AEDANS-labeled untreated β_H-crystallin

$$f_1K_1 = f_1\tau_{01}k_{q1} = (0.95)(14.4 \times 10^{-9}\ s)(26.9 \times 10^7\ M^{-1}\ s^{-1}) = 3.7\ M^{-1}$$

f_2K_2 is nearly zero and can be ignored, since lifetime studies showed that the minor component was not quenched by acrylamide. Thus

$$IS \sim f_1K_1 + f_1V_1 = 3.7\ M^{-1} + 0.97V_1 \text{ or } V_1 = (4.26 - 3.7)/0.95 = 0.59\ M^{-1}$$

Similarly, for irradiated β_H-crystallin, $V_1 = 1.4\ M^{-1}$. For AEDANS-labeled control γ-crystallin

$$V_1 = 6.66\ M^{-1} - (13.0 \times 10^{-9}\ s)(32.7 \times 10^7\ M^{-1}\ s^{-1}) = 2.2\ M^{-1}$$

and for AEDANS-labeled irradiated γ-crystallin

$$V_1 = 5.6\ M^{-1} - (11.8 \times 10^{-9}\ s)(33.6 \times 10^7\ M^{-1}\ s^{-1}) = 1.6\ M^{-1}$$

The above results are in agreement with Eftink and Ghiron (1976a), who suggested that a more exposed site is more susceptible to static quenching but steric factor can also play an important role. Thus, the decrease in the initial slope of the intensity quenching plot of γ-crystallin by irradiation might simply be due to a decrease in the extent of static quenching. In β_H-crystallin, there is less static quenching of the AEDANS label than in γ-crystallin, indicating the presence of steric shielding by the segements of the protein side chains (Eftink & Ghiron, 1976). This picture is consistent with the oligomeric nature of the protein.

Fluorescence Anisotropy. Fluorescence anisotropy of AEDANS-labeled control and irradiated α-, β_H-, and γ-crystallins was determined, and their values are shown in Table VII. For the untreated crystallins, the anisotropy value decreased as the lifetime of the major component increased. An increase in the anisotropy of the AEDANS label bound to the irradiated crystallins was observed as compared with the respective controls.

Discussion

It has been reported that the fluorescence lifetime and yield of 1,5-IAEDANS vary inversely with the polarity of the surrounding medium (Hudson & Weber, 1973). If one assumes a homogeneous solvent environment around the IAEDANS labeling site of the crystallins, the lifetime of the label reflects the polarity around the labeling site and the exposure of the site to the solvent (Tao & Cho, 1979; Leavis et al., 1984). The results of the present study show that in the untreated crystallins, the accessibility of the AEDANS-labeled sulfhydryl groups to acrylamide quenching, as measured by k_q, increases as the lifetime of the major component of the fluorescence decay decreases. Thus, the lifetime of the major decay component decreases in the order $\alpha > \beta_H > \gamma$, and the magnitude of k_q decreases in the order $\gamma > \beta_H > \alpha$. Furthermore, our results indicate that k_q values for the untreated crystallins are a more sensitive indicator of the changes in environment than the τ values. Thus, the difference in the AEDANS lifetime between α- and β_H-crystallin is $\sim$5%, whereas k_q differs by nearly 45%. Similarly, a previous study using MIANS (lifetime $\sim$3–4 ns) showed no significant difference in the lifetime of the probe bound to β_H- and γ-crystallin (Andley et al., 1982). Table VI shows that when the long- lifetime sulfhydryl label IAEDANS is used, there is a measurable difference between the lifetime of the major decay components of untreated β_H- and γ-crystallins; this result is reinforced by the 21% higher value of k_q for γ-crystallin as compared to β_H-crystallin.

Since calf α-crystallin contains a single cysteine residue (Cys-131), one can safely infer that the AEDANS label is attached at this site in the control and irradiated protein. The fluorescence decay of the untreated α-crystallin appears to be well characterized by two components: the major component has values of τ and k_q that correspond to an exposed environment, which is accessible to acrylamide quenching, and a minor component with τ and k_q values corresponding to a hydrophobic environment, which is inaccessible to acrylamide quenching. As reported earlier (Andley & Chylack, 1986), the presence of two decay components of IAEDANS-labeled control or irradiated α-crystallin is due to two different classes of the accessible SH groups of the 30 A chains in the aggregate of α-crystallin subunits.

In α-crystallin samples treated with MB, binding of the photosensitizer to the protein is noted both in the dark and in irradiated samples (Table II). It has been suggested that the binding of photosensitizers to proteins may be an important factor in determining the efficiency of the photoprocesses (Amagasa, 1981; Jori & Spikes, 1981). The extent of binding increased by irradiation, and the absorption maximum of MB shifted from 666 nm in the dark to 639 nm in the irradiated α-crystallin, suggesting a change in the binding site for MB by photooxidation of the protein. Binding of MB also occurs when irradiation of α-crystallin was done in the presence of the $^1O_2$ quencher sodium azide. It has been shown that azide prevents cross-linking and the loss of sulfhydryl content of the

crystallins by irradiation in the presence of MB (Andley & Chapman, 1986). However, azide does not prevent the binding of MB to α-crystallin or the decrease in the lifetime of the major component of α-crystallin (Table III). The latter was ~9 ns in α-crystallin irradiated in the presence of azide, as compared to ~15 ns in the untreated protein and 6 ns in the irradiated protein. The results suggest that the decrease of the lifetime from 15 ns for the control to 9 ns when irradiation was done in the presence of azide is due to the presence of bound MB. In α-crystallin irradiated with MB but no azide, further quenching of the lifetime to 6 ns occurs, due to the effects of irradiation on the protein structure, as a result of the generation of singlet oxygen. In a previous study on the effect of 300-nm irradiation on the conformation of α-crystallin in the absence of added photosensitizers, the lifetime changes of the AEDANS label were different from the present results. The lifetime of the label decreased from 15.7 to 10.2 ns by 300-nm irradiation (Andley & Chylack, 1986). Since the mechanism of photodamage by 300-nm irradiation was mainly via type I reactions (Foote, 1976), involving O_2^- and H_2O_2, whereas irradiation in the presence of MB mainly follows type II photochemistry, generating $^1O_2$, it is not surprising that the effect on the conformation of the protein as indicated by the lifetime of the AEDANS label is different under the two conditions.

The change in k_q values of the major component of AEDANS-labeled α-crystallin by irradiation is considered next. In α-crystallin samples irradiated in the presence of MB but no azide, the lifetime of the label was fully quenched to 6 ns; thus, no further quenching by acrylamide is seen, resulting in a k_q value of zero, within experimental error (Figure 2, Table VI). The loss of quenching by acrylamide in irradiated α-crystallin is due to (a) bound MB and (b) cross-linking of the protein. When azide was included in the reaction mixture for irradiation, the k_q value was nearly the same as the control (Table VI).

Photobinding of MB also occurs in β_H-crystallin but does not appear to quench the lifetime of IAEDANS as much as in the case of α-crystallin. The results in Table VI show that for β_H- and γ-crystallins, k_q is not affected by singlet oxygen treatment, even though the total sulfhydryl content as well as the degree of labeling of AEDANS is less.

The minor decay component in control and irradiated β_H-crystallin is inaccessible to acrylamide quenching. Its origin in the control sample of β_H is likely to be either a minor AEDANS labeling site or a minor amount of supraaggregated protein. The latter has been found in purified solutions of β_H-crystallin (Siezen et al., 1986). It has also been reported by the same authors that the hexameric aggregates of β_H-crystallin (M_r 1.8×10^5) dissociate almost completely to smaller aggregates (dimers and trimers) of β_L-crystallin. The fraction of the minor decay component of AEDANS-labeled β_H-crystallin was found to be 42%, in a previous study (Andley et al., 1982) performed at one-tenth the protein concentration of the present study. The difference in the amount of the minor component observed in the two studies could result from either the extent of supraaggregate formation or the difference in dissociation to β_L-crystallin, or both. Heterogeneity in the data on β_H-crystallin can also arise from the heterogeneous population of polypeptides found in this protein (M_r 24K, 26K, 27.5K, 30K, and 32K) and their tendency to form aggregates of varying size (Li, 1978; Bindels et al., 1981). Slight differences in their tertiary structures can lead to heterogeneity in the fluorescence decay curves. The two studies, however, show a remarkable consistency in the k_q values for the major component of AEDANS decay in β_H-crystallin: 2.7×10^8 M^{-1} s^{-1} (Andley et al., 1982) and 26.9×10^7 M^{-1} s^{-1} (present study) (Table VI).

Although the k_q for the major component of AEDANS-labeled β_H-crystallin remained unaffected by irradiation in the presence of MB, we observed an increase (from 5% to 16%) of the fraction of the minor component in the irradiated β_H-crystallin. As discussed above, this increase can be due to the increase in the supraaggregation of the protein by irradiation. Cross-linking as well as conformational changes of β_H-crystallin under these conditions of irradiation (Andley & Chapman, 1986) can be expected to cause an increase in larger aggregates of the proteins, which remain soluble. As in the case of α-crystallin, the microenvironment of the sulfhydryls which give rise to the minor component of β_H-crystallin is likely to be hydrophobic; these sulfhydryl groups can be labeled by AEDANS but are inaccessible to quenching by acrylamide.

In untreated γ-crystallin, the minor decay component of the fluorescence decay of AEDANS is negligible. In the irradiated γ-crystallin, the fraction of minor component increases but is inaccessible to acrylamide quenching. The minor decay component can arise from a minor labeling site such as a cysteine residue(s) having limited solvent accessibility and reactivity with IAEDANS.

Polarization or anisotropy measurements reveal the average angular displacement of the fluorophore which occurs between absorption and subsequent emission of a photon (Lakowicz, 1983a). This angular displacement is dependent upon the rate and extent of rotational diffusion during the lifetime of the excited state. In dilute nonviscous solutions, the anisotropy is primarily determined by the rotational motion of the fluorophore. For labeled proteins, these motions, are dependent upon the size, shape, and extent of protein aggregation, among other factors. The increase in anisotropy of α-, β_H-, and γ-crystallins by irradiation in the presence of MB reveals a decrease in the rotational diffusion of the fluorophore, probably as a result of the increase in molecular size and rigidity of the proteins after cross-linking and aggregation.

It should be pointed out that in the interpretation of the lifetime results for the untreated crystallins, we have assumed that the binding site of AEDANS is like homogeneous solution conditions. Thus, the decrease in lifetime indicates an increase in the polarity of the binding site. Irradiation in the presence of MB generates cross-linked polymeric forms of the crystallins in which the binding site may not be a homogeneous solvent environment. A polar but rigid binding site of AEDANS would allow a long lifetime of the label due to the absence of solvent reorientation (Lakowicz, 1983a). The increase in fluorescence anisotropy of the crystallins indeed suggests an increase in rigidity of the proteins by irradiation. Therefore, it is likely that the increase in polarity of the sulfhydryl groups of the crystallins by irradiation is greater than that indicated by our lifetime data (Andley & Chapman, 1986; present study).

The k_q values observed in the present study are well below the reported k_q (Eftink & Ghiron, 1984) for the acrylamide quenching of tryptophan in solution (5.9×10^9 M^{-1} s^{-1}). Although acrylamide is a highly efficient quencher for the fluorescence quenching of tryptophan, its efficiency may be less for another fluorophore (Eftink & Ghiron, 1981) such as the AEDANS moiety. The k_q values for acrylamide quenching of the AEDANS-labeled lens crystallins fall within the same range of values as those reported for the acrylamide quenching of the tryptophan residues of some proteins (Eftink

& Ghiron, 1984) and for the AEDANS-labeled sulfhydryl sites of actin (Tao & Cho, 1979) and troponin (Leavis et al., 1984) but are slightly lower than the oxygen quenching (efficiency unity) of porphyrin fluorescence from iron-free myoglobin (Gratton et al., 1984; Jameson et al., 1984).

In conclusion, the present results provide further evidence for singlet oxygen mediated alterations in protein structure of the lens crystallins. We have previously documented that dye-sensitized photooxidation results in loss of some oxidation-sensitive residues and alterations in the microenvironment of tryptophan and cysteines (Andley et al., 1985a,b; Andley & Chapman, 1986). These changes accompany the modification of the near-ultraviolet circular dichroism spectra (Andley & Chapman, 1986) and cross-linking (Goosey et al., 1980) of the lens crystallins in this photodynamic system. Changes in histidine and methionine also occur in proteins modified by the photodynamic action of dyes such as MB, riboflavin, and *N*-formylkynurenine (Dillon, 1984; Nilsson et al., 1973). The results of the present study on sulfhydryl groups of the lens crystallins have clearly demonstrated that (1) there is a quantitative loss of AEDANS labeling sites due to photooxidation as well as structural changes of the lens crystallins. (2) Permanent photobinding of the dye sensitizer, MB, to the crystallins occurs. The photobinding cannot be prevented by the singlet oxygen quencher, sodium azide. (3) Loss of collisional quenching by acrylamide in irradiated α-crystallin occurs due to structural change as well as energy transfer from bound AEDANS label to bound MB. (4) There is an increase in the proportion of AEDANS labeling sites, which are inaccessible to collisional quenching by acrylamide, after irradiation of β_H- and γ-crystallins. (5) Nonlinear lifetime quenching plots and changes in relative proportions of the decay components by the addition of acrylamide suggest conformational effects of the quencher (Lakowicz, 1983b). (6) The AEDANS labeling sites of singlet oxygen modified α-crystallin, and the native and singlet oxygen modified β_H- and γ-crystallins, are highly susceptible to static quenching, as expected for polar sites (Eftink & Ghiron, 1981).

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Effect of Differences in Optical Properties of Intermediate Oxygenated Species of Hemoglobin A_0 on Adair Constant Determination†

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ABSTRACT: Careful evaluation of the so-called isosbestic properties of oxygenated and deoxygenated hemoglobin spectra demonstrates that the spectral changes are not strictly linear with respect to the degree of saturation. In order to quantify the extent of nonlinearity, optical measurements of O_2 binding to human hemoglobin were made at different wavelengths in the Soret region approaching the presumed isosbestic point. The results indicate that the extinction coefficient of intermediate oxygenated hemoglobin is 1% less than that of the fully oxygenated hemoglobin, with a resulting 3% (±0.15%) nonlinearity effect on measurements taken at the peak of the oxygenated hemoglobin spectrum (414 nm). The lack of isosbestic conditions allows one to investigate the functional properties of the oxygenated intermediates directly. The small difference in the absorbance of different oxygenated species has practically no influence on the determination of Adair constants at wavelengths removed from the critical isosbestic region.

Spectrophotometric techniques have been used extensively over the past few decades in the analysis of ligand binding to hemoglobin and are a major source of information about the functional properties of the molecule. A basic assumption of these techniques is the linear relationship between O_2 binding and the concomitant optical density changes. Each intermediate ligated species is assumed to have spectral properties based simply upon a weighted contribution of filled and empty binding sites. A key question, pointed out long ago by Gibson (1959), concerns the validity of this assumption.

Analysis of the wavelength dependence of spectrally determined O_2 binding constants of hemoglobin (Hb)[1] (Enoki & Tyuma, 1964; Mills et al., 1976; Imaizumi et al., 1978; Imai, 1982; Gill et al., 1987) has shown that within experimental error no significant alteration in the constants is observed, suggesting the validity of the linearity between spectral changes and degree of saturation. On the other hand, early studies on horse Hb found a discrepancy between spectral measurements of the O_2 binding curve and direct measurements made by gasometric techniques (Rifkind & Lumry, 1967). Possible differences in the optical properties of the two types of Hb chains have also been suggested (Nasuda-Kouyama et al., 1983). Furthermore, recent work on the kinetics of CO binding to human Hb required at least three spectrally distinguishable species for deconvolution of the data (Hofrichter et al., 1985).

Since interpretation of the cooperative properties of hemoglobin relies to a large extent on O_2 binding curves gained by precision spectral data, we wish to quantify the possible optical nonlinearity for O_2 binding. In this study, we determine the degree of optical nonlinearity for O_2 binding to human HbA_0 and its effect on resolution of the Adair constants. Interest in such analysis also comes from the possibility of investigating the binding properties of the oxygenated intermediates directly if the ligated species have different optical properties.

Experimental Procedures

Solution Conditions. Hb samples, isolated by the standard procedures of Williams and Tsay (1973) and stored in deionized water, were reduced for 5 h at 4 °C with the enzymic reducing system of Hayashi et al. (1973). The final solution conditions of 2 mM heme, 20 mM IHP, 0.1 M HEPES, 0.1 M NaCl, and 1 mM Na_2EDTA, 25 °C, pH 7.0, were obtained by mixing the appropriate buffer solution with Hb in deionized water. These conditions were chosen for two reasons: (1) The entire O_2 binding curve can be measured rapidly, largely due to the negative heterotropic effect of IHP. (2) These conditions have been found in a recent study (Di Cera et al., 1987a) to result in a highly stable environment for HbA_0, minimizing both metHb formation (less than 1% during the time frame of an O_2 binding curve) and possible contamination effects. The IHP was used in excess saturating amounts to avoid possible complications in the spectral measurements due to release of this effector during oxygenation, even though such effects are minimal (Imaizumi et al., 1978).

Differential O_2 Binding Curve Measurements. Oxygen binding curves were obtained spectrophotometrically by a

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[1] Abbreviations: Hb, hemoglobin; metHb, methemoglobin; oxyHb, oxygenated hemoglobin; deoxyHb, deoxygenated hemoglobin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; IHP, inositol hexaphosphate.

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