Spectroscopic Investigations of Bovine Lens Crystallins. 2. Fluorescent Probes for Polar–Apolar Nature and Sulfhydryl Group Accessibility†

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ABSTRACT: The hydrophobic fluorescent probe 6-(*p*-toluidinyl)naphthalene-2-sulfonate, when bound to α-crystallin, shows significantly higher emission than that of the dye in water or bound to the other two crystallins, β and γ. The dissociation constant for the binding is 30.2×10^{-6} M at pH 7. The fluorescence intensity of α-crystallin-bound probe decreases considerably in the presence of guanidine hydrochloride, which is known to disaggregate the subunits of the crystallin. Results indicate that α-crystallin has a large number of hydrophobic sites and that these nonpolar regions are formed upon the association of the subunits to the protein. On the basis of the reactivity of 5,5′-dithiobis(2-nitrobenzoic acid) and the fluorescent sulfhydryl probe 6-(4′-maleimidylanilino)-naphthalene-2-sulfonic acid, it appears that α- and γ-crystallins have three different classes of sulfhydryl groups, two of them accessible and the other inaccessible to the reagents employed. All sulfhydryl groups of β-crystallin, on the other hand, are readily accessible to these reagents and can be represented by one class only. The total sulfhydryl groups in the crystallins were estimated in guanidine hydrochloride by using 5,5′-dithiobis(2-nitrobenzoic acid). Accessible classes of sulfhydryl groups of α-crystallin are in a more nonpolar region than in the other two crystallins, indicated by the blue shift of the fluorescence maxima of the probe (415 and 420 nm of α-crystallin compared with 433 nm of β- and γ-crystallins). This is consistent with the results of steady-state quenching by acrylamide and lifetime measurements. Lifetime quenching studies of *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine-labeled β-crystallin reveal that only the major component, 10.2 ns, is quenched by acrylamide, with a quenching constant (k_q) of 7.7×10^8 M^{-1} s^{-1}, while the minor one, 19.6 ns, is inaccessible to the quencher.

In the preceding paper (Liang & Chakrabarti, 1982), we described the conformational aspects, particularly the tertiary structure, of lens crystallins as manifested in the circular dichroism and intrinsic fluorescence measurements. Lens proteins aggregate by either noncovalent or covalent interactions between them (Harding & Dilley, 1976) during aging and cataractogenesis. Noncovalent interactions between proteins, in many cases, are related to the polar and nonpolar characteristics of the protein (Cassel, 1966; Hofmann et al., 1978). Major covalent aggregations of lens proteins are due to oxidation of sulfhydryl groups forming the disulfide linkage (Dische & Zil, 1951). The reactivity of the sulfhydryl groups is of particular interest because the disulfide bond formation is related to the accessibility of the groups to the oxidant. Such studies are limited to α-crystallin only (Spector & Zorn, 1967; Siezen et al., 1978).

In the present paper, we report on studies of the polar and nonpolar nature of the various crystallins as well as the reactivity of the various classes of sulfhydryl groups of these proteins, by using the fluorescence technique which, to our knowledge, has never been employed for such studies of lens crystallins. Extrinsic fluorescent probes such as 8-anilino-1-naphthalenesulfonate (ANS)[1] or TNS have been successfully utilized to determine the overall hydrophobic or hydrophilic nature of a protein molecule (Radda, 1971; Andley et al., 1981; Andley & Chakrabarti, 1981; Liang & Chakrabarti, 1981a). In the present study, we used TNS, a hydrophobic fluorescent probe, for this purpose. For the sulfhydryl group reactivity studies, the fluorescent analogue of maleimide, MIANS, which binds covalently to sulfhydryl groups (Haugland, 1975; Gupte & Lane, 1979) has been used mostly. The probe offers the advantage of convenient monitoring and ease of detection due to the sensitivity of the fluorescence method over chemical modification with the reagents 4,4′-dithiopyridine, DTNB, iodoacetamide, and *N*-ethylmaleimide. However, for comparison and convenience of measurement, in some cases we have also used DTNB and 1,5-IAEDANS labeling. Results of the study reveal the polar–nonpolar characteristic of the different crystallins and the accessibility and environments of the sulfhydryl groups of the proteins from the kinetics of binding, quenching by acrylamide, and lifetime measurements.

Materials and Methods

Preparation of Crystallins. Bovine lens crystallins were isolated and separated into α, β_H, β_L, and γ fractions as described previously (Liang & Chakrabarti, 1981b, 1982). In our preliminary study, we did not find any significant difference between β_H and β_L as regards their SH-group reactivity, and hence, the results of only β_H, referred to as β, are presented here. The protein solutions were stored in the dark at 0–4 °C. Protein concentration was determined according to the method of Lowry et al. (1951).

TNS Fluorescence Measurements. TNS (Kodak Chemical Co.) solutions were made in Tris buffer. A small volume of TNS stock solution was added to the protein solutions. Emission and excitation spectra of TNS were obtained by using a Perkin-Elmer MPF-44A spectrofluorometer. Fluorescence of TNS was excited at 365 nm and measured between 400 and 600 nm. The dissociation constant was determined by the method of Zierler (1977).

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; TNS, 6-(*p*-toluidinyl)naphthalene-2-sulfonate; MIANS, 6-(4′-maleimidylanilino)naphthalene-2-sulfonic acid; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Gdn·HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)-aminomethane.

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MIANS Labeling and Fluorescence Measurements. MIANS (Molecular Probes, Inc.) was freshly prepared as a 1 mM stock solution in 10 mM Tris buffer, pH 7.4. The actual concentration of the solutions was determined according to the method of Gupte & Lane (1979), assuming an extinction coefficient of $\epsilon_{322} = 20\,000\ M^{-1}\ cm^{-1}$. The fluorescence intensity of MIANS as a function of time was monitored at the emission maximum of the probe complexed with different crystallins (420 or 430 nm) with an excitation wavelength of 328 nm. Low concentrations of MIANS were used (OD_{322} less than 0.1) to avoid inner filter effects.

The reaction of MIANS with the crystallins was terminated by adding a 3–5-fold molar excess of 2-mercaptoethanol to stop alkylation. The excess MIANS–2-mercaptoethanol complex was removed by dialysis, and the optical density of the dialyzates at 322 nm was used to determine the amount of MIANS bound to the protein. The crystallins were denatured by 6 M Gdn·HCl. The total SH groups in the crystallins were estimated in 6 M Gdn·HCl at pH 7.4 with DTNB, by using an extinction coefficient of $13.9 \times 10^3\ M^{-1}\ cm^{-1}$ at 412 nm for the released thionitrobenzoate anion (Palau & Daban, 1974). For labeling with IAEDANS, we incubated the crystallins (0.1 mg/mL) with a 5-fold molar excess of the label at 4 °C for 12 h in 10 mM Tris-HCl buffer, pH 7.4. The excess reagent was removed by gel filtration through a Sephadex G-25 column.

Fluorescence Lifetime Measurements. Fluorescence lifetime measurements were carried out on an Ortec 9200 photon counting nanosecond fluorometer (Andley & Chakrabarti, 1981). The data were analyzed by the method of moments procedure (Isenberg & Dyson, 1969), as described by Yguerabide (1972). All studies were carried out at 20 °C.

Both steady-state and dynamic quenching studies were done by adding different amounts of a concentrated stock solution of acrylamide (ultra-high-purity grade), used as a quencher, to the complex of crystallin with MIANS after removal of excess probe by dialysis. MIANS or IAEDANS (Molecular Probe, Inc.) was used for the quenching experiments, depending upon the convenience of the probes. Data were generally analyzed according to the following theory.

In the presence of a quencher, the fluorescence intensity of an emitter is decreased by both the collisional and the static quenching processes (Lehrer & Leavis, 1978) according to

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

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, at a quencher concentration ([Q]), K_{sv} is the Stern–Volmer (Stern & Volmer, 1919) quenching constant, and V is the static quenching parameter. The collisional quenching process decreases the fluorescence lifetime by

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

where τ_0 and τ are the singlet lifetimes in the absence and presence of a quencher, respectively, and k_q is the bimolecular Stern–Volmer quenching constant, which can serve as a quantitative measure for the accessibility of the emitter; k_q approaches zero at low accessibilities and is related to K_{sv} by $K_{sv} = \tau_0 k_q$.

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

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

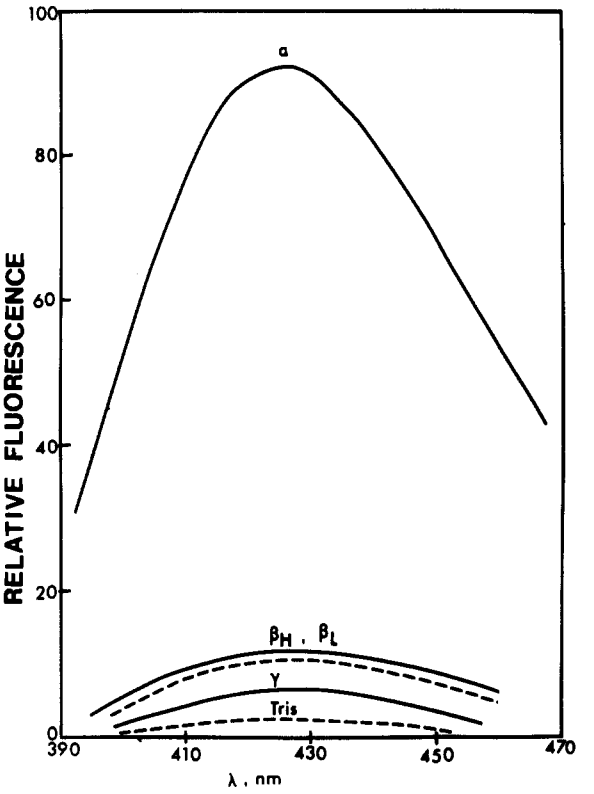

FIGURE 1: TNS fluorescence in various crystallins. Proteins (0.06 mg/mL) were dissolved in 50 mM Tris-HCl buffer, pH 7.4. TNS concentration, 14 μM; λ_{ex}, 365 nm.

For multiple emission components, the initial slope (IS) becomes

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

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.

It should be noted that for intensity quenching measurements, the presence of static quenching causes the F_0/F vs. [Q] plot to curve upward from linearity, while the presence of multiple components causes the plot to curve downward. The shape of the curve, thus, can provide some qualitative information regarding the static quenching parameter, V, and the presence of one or more emitting components.

Results

Interaction of TNS with the Crystallins. The hydrophobic fluorescent probe TNS was found to interact only with α-crystallin (Figure 1) with a significant enhancement of the fluorescence intensity and blue shift of the emission maximum (from 520 to 430 nm) in Tris buffer, pH 7.0. The dissociation constant (K_D) of TNS with α-crystallin was determined to be 30.2 ± 0.6 μM at pH 7.0. In the presence of β- and α-crystallin, TNS showed only slight enhancement of its fluorescence intensity in Tris buffer. There was very little increase in the fluorescence intensity of α-crystallin-bound TNS when the protein was prior treated with 6 M Gdn·HCl (not shown). It should be noted that Gdn·HCl disaggregates α-crystallin to its monomeric subunits (Harding & Dilley, 1976).

Reaction of MIANS with Crystallins. The addition of MIANS to 0.1 mg/mL α-crystallin in 10 mM Tris-HCl, pH 7.4, buffer resulted in a time- and concentration-dependent increase in the fluorescence intensity at 420 nm, indicating

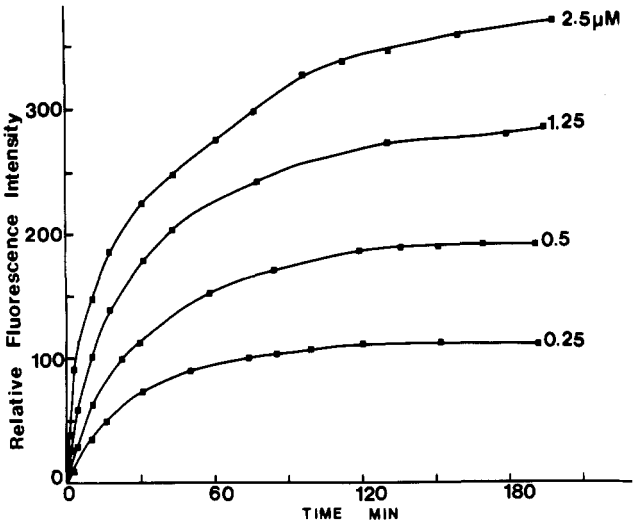

FIGURE 2: Time-dependent increase in the fluorescence intensity of MIANS with α-crystallin at varying concentrations of MIANS. α-Crystallin (0.1 mg/mL) was incubated in 10 mM Tris-HCl buffer, pH 7.4, at 20 °C before the addition of MIANS at the concentrations (micromolar) shown. The increase in fluorescence intensity at 420 nm (λ_{ex}, 328 nm) was monitored as a function of time.

that MIANS was reacting with the protein (Figure 2). In the presence of 0.25 μM MIANS, the fluorescence intensity reached a plateau within 120 min, indicating that the reaction of MIANS with α-crystallin was complete. At MIANS concentrations between 0.25 and 2 μM, the time required to reach the plateau increased with increasing concentrations of the probe. At concentrations of 2.5 μM or greater, the fluorescence intensity continued to increase at 420 nm, indicating that the reaction of MIANS with α-crystallin was incomplete. At the higher concentration range, the increase in fluorescence intensity exhibited a slower phase, which suggests that the MIANS molecules are bound to a set of slower reacting sulfhydryl groups. The emission maximum of the MIANS–α-crystallin complex is at 415 nm at low concentrations of MIANS (0.2–2.0 μM). At higher concentrations (2–10 μM), the emission maximum shifts to 420 nm.

The reaction between β-crystallin and MIANS was also stoichiometric with increasing fluorescence intensity and with the time required to reach a plateau with increasing MIANS concentration. However, at all concentration ranges, a plateau was observed after about 180 min, even at lower protein to dye ratios. Furthermore, no change in the emission maximum of the MIANS–β-crystallin complex was observed at different concentrations. The emission maximum was 432 nm at all MIANS concentrations. The amount of MIANS bound was saturated at about 1.6 mol per 20000 g of β-crystallin protein.

In the case of γ-crystallin, the reaction with MIANS plateaus after 30 min when the concentration is 0.25 μM, and the time required to reach the plateau increases with increasing concentration of MIANS. At higher concentrations (2 μM or more) the fluorescence level does not reach a plateau even after 200 min, indicating that the reaction is incomplete. This observation is very similar to that of α-crystallin and again suggests that a second set of sulfhydryl groups is reacting with MIANS at a slower rate. The emission maximum of MIANS with γ-crystallin was at 432 nm at low concentrations (0.25–2 μM) of the probe and shifted to 433 nm at higher concentrations (2.5–5 μM).

The reaction between MIANS and all the crystallins was stoichiometric with increasing number of moles of the probe bound with increasing concentration. At low concentrations of MIANS, the fluorescence intensity of the MIANS–crystallin complexes was proportional to the number of moles of MIANS bound.

Table I: Fluorescence Properties of MIANS in α-, β-, and γ-Crystallins in 10 mM Tris-HCl Buffer, pH 7.4

material (0.1 mg/mL)	emission max, λ (nm)	no. of SH groups reacting[a]	total SH in 6 M Gdn·HCl with DTNB[a]
α-crystallin	415 (0.25–2 μM MIANS)	14–16/ 800 000 g of protein	27
	420 (2.5–10 μM MIANS)		
β-crystallin	432 (at all MIANS concn ranges)	1.6/20 000 g of protein	1.7
γ-crystallin	432 (0.25–2 μM MIANS)	0.68/ 20 000 g of protein	1.6
	433 (2.5–10 μM MIANS)		

[a] Based on a molecular weight of 800 000 for α-crystallin (Siezen et al., 1978) and 20 000 for β- and γ-crystallins (Kabasawa et al., 1974).

Table II: Fluorescence Decay Parameters of MIANS-Labeled Crystallins[a]

material	A_1	τ_1 (ns)	A_2	τ_2 (ns)	χ^2/N
MIANS–α-crystallin	0.91	4.99	0.092	13.57	28.2
MIANS–β-crystallin	0.93	3.62	0.071	11.74	31.1
MIANS–γ-crystallin	0.95	3.62	0.049	12.14	26.2

[a] See Materials and Methods for experimental conditions. A_1 and A_2 are the amplitude coefficients of the major and minor decay components, respectively; τ_1 and τ_2 are the corresponding lifetimes. All values were obtained from the two-exponential method of moment analysis. χ^2/N is a parameter for judging the quality of fit defined as $1/N\Sigma_{i=1}^{N}[F_c(t_i) - F_e(t_i)]^2$ where N is the number of points and F_c and F_e are the calculated and the experimental fluorescence decays, respectively.

Table I summarizes the fluorescence properties of MIANS in the three different crystallins from bovine lens. When the crystallins were denatured in the presence of 6 M Gdn·HCl, the fluorescence intensity of MIANS reached a plateau within 20 min, and a 2–3-fold enhancement of the fluorescence intensity was observed. In the presence of the denaturant, the emission maximum of the MIANS–crystallin complex shifted to the red (from 415 to 441 nm for α-crystallin, 432 to 440 nm for β-crystallin, and 432 to 439 nm for γ-crystallin). The total SH groups in the different crystallins in 6 M Gdn·HCl, as quantified with DTNB, are shown in Table I.

Lifetime Measurements. Table II shows the fluorescence decay parameters of MIANS-labeled crystallins. The fluorescence decay curves for all three MIANS-labeled crystallins were plotted on a semilogarithmic scale and found to deviate from linearity. The decay was assumed to be composed of two components and was analyzed by the method of moments (Isenberg & Dyson, 1969). The fit was good, as the deviation between the experimental and calculated curves was small and random. MIANS–α-crystallin yields a major decay component of lifetime 4.99 ns that accounted for 91% of the total fluorescence and a minor decay component of lifetime 13.57 ns accounting for the remaining 9%. For MIANS-labeled β-crystallin, the lifetimes of major and minor components were 3.62 (93%) and 11.74 (7%), and for γ the values were 3.62 (95%) and 12.14 (5%), respectively.

Steady-State Quenching. Figure 3 shows the effect of the quencher acrylamide on the fluorescence intensity of MIANS

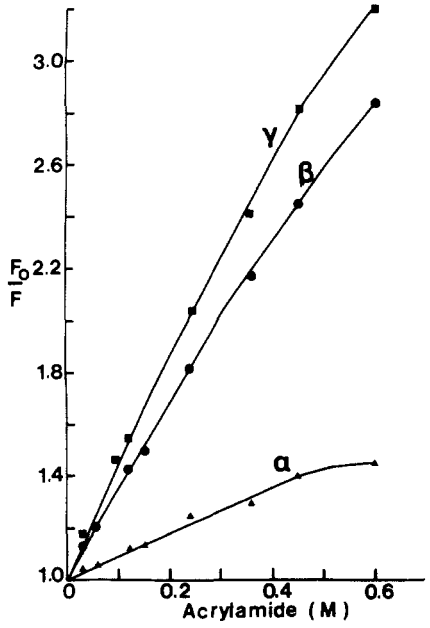

FIGURE 3: Steady-state fluorescence intensity quenching curves for MIANS–crystallin complexes: MIANS–α-crystallin (▲); MIANS–β-crystallin (●); MIANS–γ-crystallin (■). λ_{ex} = 328 nm; λ_{em} = 420 nm for MIANS–α-crystallin and 430 nm for MIANS–β- and MIANS–γ-crystallins. Crystallin concentrations, 0.1 mg/mL.

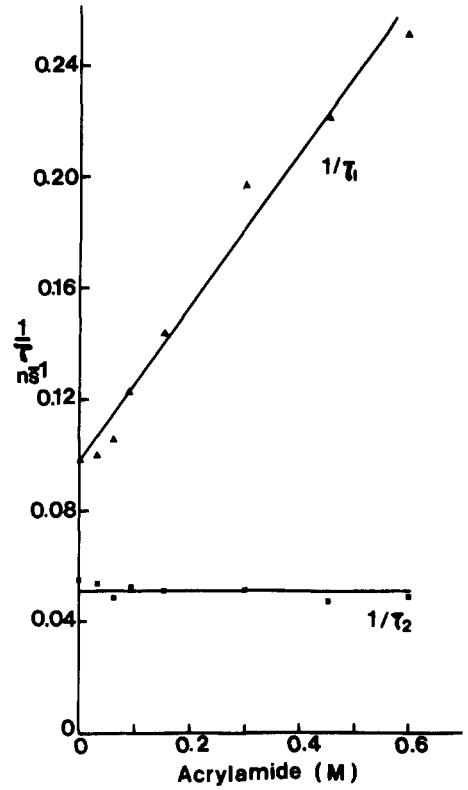

FIGURE 4: Stern–Volmer plots for the major component (τ_1) of AEDANS–β-crystallin (▲) and the minor component (τ_2) of AEDANS–β-crystallin (■). k_q, the fluorescence quenching rate constant for the major component (τ_1) of AEDANS–β-crystallin, was determined to be 7.7 × 10^8 M^{-1} s^{-1}.

in the three crystallins. The Stern–Volmer plot (F_0/F vs. [Q]) shows that both static and dynamic (collisional) processes may contribute to the quenching of the fluorescence intensity of the MIANS–α-crystallin complex. However, no upward curvature was observed for any of the crystallins, indicating that the static quenching parameter (V) is small. Instead, downward curvature was apparent for all curves, suggesting the presence of multiple emission components. The initial slopes of the curves were determined to be 1.00 M^{-1} for MIANS–α-crystallin, 3.0 M^{-1} for MIANS–β-crystallin, and 4.40 M^{-1} for MIANS–γ-crystallin.

Lifetime Quenching. In our attempt to measure the collisional quenching, we encountered few problems. Although the intensities of MIANS–crystallin complexes are high enough for steady-state measurements, the lifetimes of the emissions are low for quenching studies. AEDANS–crystallins have higher lifetime values, but the fluorescence intensity of crystallin–dye complexes, except β, is very low. Thus, the fluorescence decay values of AEDANS–β-crystallin at various acrylamide concentrations were obtained. 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 according to the Stern–Volmer equation (Figure 4). It can be seen that the lifetime of only the major component (58%) is quenched by the collisional process while the minor one (42%) remains essentially constant. The quenching constant (k_q) of the major component was determined to be 7.7 × 10^8 M^{-1} s^{-1}.

Discussion

Proteins are known to bind TNS to sites that are hydrophobic in nature (McClure & Edelman, 1966). The fluorescence yield of TNS, when bound to α-crystallin, is significantly higher than that of the dye in water or bound to the other two crystallins. Therefore, the results indicate that α-crystallin contains a large number of hydrophobic sites. The large difference in nonpolar characteristics between α-crystallin and other crystallins may also be envisaged from their amino acid composition and partial sequencing studies (Harding & Dilley, 1976). Of all crystallins, α has been studied the most. The protein is found as an aggregation product of its subunits held together by hydrophobic linkages; with age, it undergoes further high molecular weight aggregation, presumably noncovalent in nature (Bloemendal, 1977). Proteins with a large number of hydrophobic sites tend to aggregate more by noncovalent interchain interactions (Cassel, 1966; Hofmann et al., 1978). It appears that the subunit of α-crystallin, when it aggregates, forms clusters of hydrophobic domains along the chain. This is supported by the fact that, on dissociation of the subunits by Gdn·HCl, the fluorescence intensity of bound TNS, observed for native crystallin, decreases considerably.

Siezen et al. (1978) suggested the presence of three different classes of sulfhydryl groups in α-crystallin, and class III was shown to be inaccessible to any reagent. MIANS appears to react with both class I, surface-exposed SH groups, and class II, which are in a hydrophobic environment of the protein. MIANS is known to react quickly with SH groups located in a nonpolar region (Gupte & Lane, 1979). The emission maximum at 415 nm of bound dye at the lower concentration of α-crystallin suggests that the probe initially reacts with the SH groups located in the hydrophobic environments (class II); at higher concentration, the maximum shifts to 420 nm when MIANS reacts with surface-exposed (class I) sulfhydryl groups. The number (Table I) and percentage of reactive SH groups in α-crystallin agree reasonably with the earlier reports (Spector & Zorn, 1967; Siezen et al., 1978). The microenvironment around the fluorescent dye labeled SH groups of α-crystallin is more nonpolar compared with that of the other two crystallins, as indicated by the emission maxima of MIANS (Table I). This is also compatible with the results of TNS fluorescence. It is interesting to note that the tryptophan

residues in α-crystallin, on the other hand, are located in more polar environments than in the other two crystallins (Liang & Chakrabarti, 1982). This is not surprising because sequence studies show that tryptophan and cysteine residues in α-crystallin are separated by a considerable distance (Bloemendal, 1977).

Details of SH group reactivity of β- and γ-crystallins have not yet been reported. From Table I, it appears that β-crystallin has one class of SH group and that almost all of them are readily accessible to the reagents. γ-Crystallin, on the other hand, contains two classes of reactive SH groups and a third inaccessible one. Although the emission maxima of MIANS, when it reacts with two sets of SH groups of γ-crystallin, are almost in the same position, the difference in kinetics clearly indicates the presence of two distinct classes.

The fluorescence yield and, hence, lifetime of a fluorescent-labeled probe increase with decreasing polarity (Hudson & Weber, 1973). Thus, it is evident from the positions of the emission maxima (Table I) and the lifetime values (Table II) that the reactive SH groups of α-crystallin are in a less polar environment than are those of β- and γ-crystallins. On the other hand, β- and γ-crystallins have similar lifetime values and similar emission maxima, indicating that the microenvironments of the SH groups of these two proteins do not differ significantly from each other.

Accessibility of fluorescent dye labeled sulfhydryl groups to a quencher depends upon the polarity and steric environment of sites (Lehrer & Leavis, 1978). The steady-state quenching studies show that the values for the initial slope are smallest in the case of α-crystallin, indicating that the acrylamide molecule is least accessible to the bound probe. This is again expected, from the fact that the SH groups of α-crystallin are located in more nonpolar environments than those of the other two crystallins.

The interpretation of the 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, contribute to the quenching of fluorescence intensity. 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). The results of our limited study of lifetime quenching of AEDANS–β-crystallin are interesting; the minor component remained virtually unaffected by the quencher, indicating the inaccessibility of the acrylamide molecule to the emitter. Kinetic studies with MIANS showed that there may be only one class of SH groups present in β-crystallin. The existence of two lifetimes of both MIANS- and AEDANS-labeled β-crystallin and the difference in lifetime quenching behavior of the latter can be due to various reasons: (a) lifetime and dynamic quenching studies provide more precisely the difference (not detectable by steady-state emission study) in the microenvironments around the labeled probe (Tao & Cho, 1979; Lehrer et al., 1981); (b) the existence of more than one conformer of the probe itself could yield two lifetime values (Andley & Chakrabarti, 1981). Further studies of all crystallins with suitable probes will undoubtedly provide more precise information regarding the accessibility of the fluorophore bound to the proteins.

In conclusion, the results indicate the possible nature and ease of noncovalent and covalent aggregation of lens crystallins during aging and cataract formation. It appears that α-crystallin will tend to aggregate more by noncovalent interactions whereas β- and γ-crystallins are likely to form a large number of disulfide bonds. At present, there is little experimental evidence to support this hypothesis. However, it has been shown that disulfide bonds are not involved in maintaining the aggregate of α-crystallin structure (Spector & Zorn, 1967). The conversion of water-soluble crystallins to high molecular aggregates, which occurs upon aging, may proceed to the stage where these aggregates become insoluble (albuminoid) or at least large enough to cause opacification (Manski et al., 1968; Waley, 1969; Benedek, 1971; Spector, 1972; Spector et al., 1974; Liem-The & Hoenders, 1974). Both nondisulfide and disulfide cross-links may be involved in the high molecular aggregates of α-crystallin (Siezen et al., 1979; Bindels et al., 1978; Jedziniak et al., 1973). In the consideration of normal and cataractous lens, the relative content of free SH groups and disulfide of lens proteins may be an important variable (Harding & Dilley, 1976; Anderson & Spector, 1978). The ease and extent of disulfide formation in different crystallins will obviously be determined by the number, location, and environments of the thiol groups. During cataractogenesis, however, conformational change of these proteins may occur; as a result, the environments of the SH groups would alter (Harding, 1969; Hess et al., 1981). Because of the high sensitivity of the fluorescence technique, further studies of normal and cataractous lens by this method are likely to yield detailed information regarding those changes.

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Reversal of T4 RNA Ligase†

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ABSTRACT: Unexpected products detected in oligoribonucleotide synthesis reactions catalyzed by T4 RNA ligase are shown to be a result of a partial reversal of the enzyme reaction. A transfer assay for the reversal of the third step in the RNA ligase reaction mechanism and an exchange assay for the reversal of both the second and third steps are described. Reversal is confirmed by the formation of the expected covalent intermediates, adenylylated donor and adenylyl ligase, from a reaction containing 5′-AMP, unadenylylated ligase, and the tetranucleotide $(Ap)_3Cp$. In the reverse reaction, RNA ligase shows a strong preference for hydrolysis of the 3′-terminal phosphodiester bonds of oligoribonucleotides which terminate in a 3′-phosphate. Several strategies are discussed to minimize the effects of reversal in the enzymatic synthesis of oligoribonucleotides.

T4 RNA ligase catalyzes the ATP-dependent formation of a 3′→5′ phosphodiester bond between an oligonucleotide acceptor with a 3′-hydroxyl and an oligonucleotide donor with a 5′-phosphate (Uhlenbeck & Gumport, 1981). This reaction has proven useful in the synthesis of both ribo (Ohtsuka et al., 1980) and deoxyribo (Gumport et al., 1980) oligonucleotides of defined sequence as well as the extension of the 3′ (England et al., 1980) and 5′ (Stahl et al., 1980) termini of natural RNA molecules. In analogy with the ATP-dependent DNA ligases (Kornberg, 1980), the RNA ligase reaction mechanism can be dissected into three distinct steps involving two known covalent intermediates.

$$\text{ATP} + \text{E} \rightleftarrows \text{E–pA} + \text{PP}_i \quad (1)$$

$$\text{E–pA} + \text{pX}_n \rightleftarrows \text{E[A-5′pp5′-X}_n\text{]} \quad (2)$$

$$\text{E[A-5′pp5′-X}_n\text{]} + \text{Y}_m \rightleftarrows \text{Y}_m\text{pX}_n + \text{pA} + \text{E} \quad (3)$$

The first step is the reaction of ATP with RNA ligase to form a covalent adenylylated enzyme intermediate with the release of pyrophosphate. The adenylylated protein is stable at neutral pH, and this step can be reversed by the addition of pyrophosphate (Cranston et al., 1974). The second step involves the transfer of the 5′-AMP from adenylyl ligase to the 5′-phosphate of the donor, forming an intermediate with a 5′–5′ phosphoanhydride bond (Kaufmann & Littauer, 1974). Adenylylated donors isolated from reactions or chemically synthesized are reactive in the last step of the reaction in the absence of ATP, further suggesting their role as intermediates in the mechanism (Sninsky et al., 1976). In the third step, the 5′–5′ phosphoanhydride bond is broken, and the donor forms a 3′→5′ phosphodiester bond with the acceptor, and AMP is released (Sugino et al., 1977).

In this work, we will demonstrate the reversibility of the second and third steps of this reaction. This study was prompted by the detection of anomalous products in oligonucleotide synthesis reactions. Several lines of evidence are presented indicating that the rapid reversal of RNA ligase reactions can occur under synthetic conditions. Assays for the reverse reaction are developed, and several procedures for minimizing its effect are examined.

Materials and Methods

T4 RNA ligase was purified by the procedure of Moseman-McCoy et al. (1979) and was essentially homogeneous with a specific activity of 2100 units/mg. Analysis of RNA ligase on sodium dodecyl sulfate ($NaDodSO_4$)–urea–polyacrylamide gels was carried out by the procedure of Matsudaira & Burgess (1978). Venom 5′-nucleotidase, creatine phosphinokinase, and adenylate kinase were purchased from Sigma Chemical Co.

[5′-^{32}P]AMP was prepared in two steps by first synthesizing [5′-^{32}P]pAp from 3′-AMP and [γ-^{32}P]ATP with poly-

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