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Red edge excitation shifts of crystallins and intact lenses

A study of segmental mobility and inter-protein interactions

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Abstract

The shift that occurs in the fluorescence emission wavelength upon changing the excitation wavelength towards the red edge of the absorption band is termed red edge excitation shift (REES). We have monitored the REES of intrinsic protein fluorescence of freshly isolated intact lenses, of individual crystallins in their native, denatured and photodamaged states and also of crystallin mixtures. The observed REES values for the lenses from different species are different suggesting that the mobilities and packing of the crystallins may vary with the species. Lens photodamage in all the cases resulted in an increase of REES. Denaturation of crystallins in solution reduces REES and renaturation restores it. Mixtures of α- and β-crystallins prepared either by directly mixing equimolar solutions or mixing them in 4 M urea followed by dialysis (reconstituting) gave similar REES values indicating the absence of any specific interactions in dilute solutions. Possible existence of induced alterations facilitating inter-crystallin interactions at high protein concentration is suggested.

Key words: Crystallin; Lens; Protein interaction; Red edge excitation shift

1. Introduction

The eye lens in its normal state is a clear, transparent and highly refractive cellular body. It is believed that intermolecular interactions and the organization of the lens components, largely crystallins, play a crucial role in its transparency, refractive properties and its ability to accommodate [1–4]. Hence any process that affects the physicochemical properties of crystallins and their interactions with themselves and with other components of the lens would be expected to affect its function and lead to cataract. Methods that monitor the properties of the intact lens, and compare them with the properties of the components would be of value in understanding the functional features of the supramolecular assembly in the lens.

Recently we have shown that the magnitude of red edge excitation shift (REES) of fluorescence can be used as a parameter in studying the photophysical and chemical properties of isolated, intact eye lenses [5]. The fluorescence decay rates and the wavelengths of maximum emission of a protein will display a dependence on the excitation wavelength if the protein matrix relaxes slowly around the increased dipole moment of the excited state [6–8]. If the spread of excitation energy is sufficient to excite all levels of ground state distributions, the Franck–Condon excited state distribution would be similar to the ground state distribution, and emission occurs at the mean. However, excitation on the red edge of the absorption band with quanta of lower energy photoselects a limited set of ground state configurations and the Franck–Condon excited state distribution would be different, resulting in emission which lacks the high energy components, and hence the emission spectrum is red shifted.

REES is normally observed at low temperatures or in very viscous and condensed phases. For example, the fluorophores indole and tryptophan display significant REES when they are dissolved in glycerol and glucose glass [9,10]. The fluorescence probe 2(*p*-toluidinylnaphthalene)-6-sulfonate shows no REES in ethyl alcohol but shows a REES value of about 7 nm when bound to proteins. REES seen here is indicative of restricted mobility around the probe binding site [11].

We extend the use of REES further in this paper and attempt to relate the properties of the lens with those of its constituent proteins, their homo- and hetero aggregates. We also compare the properties of whole eye lenses from different species.

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2. Materials and methods

2.1. Lenses and crystallins

Bovine lenses (from the local slaughter house), five-month-old hen lenses (from the local poultry farm) and five-month-old male Wistar-NIN rat lenses (from our animal house) were used. The lenses were freshly excised before the experiment, washed in Tris-HCl buffer (pH 7.0, 50 mM containing 100 mM NaCl and 1 mM EDTA) and kept on ice, protected from external light until the start of the experiment. All animal procedures were in accordance with the NIH Guide for the Care and Use of Animals in Research.

Crystallins from bovine and chick lenses were purified as described earlier [12]. The concentration of crystallins for fluorescence measurements were 1.4–1.5 mg/ml in 100 mM Tris-HCl pH 7.0 buffer.

2.2. Spectral measurements

Fluorescence measurements were carried out on the intact lens as described earlier [12], using a solid state accessory on a Hitachi model F-4000 steady-state spectrofluorimeter. The lens discal plane was kept at approximately 30° to the excitation beam and 60° to the emission beam. This configuration minimizes artifacts in fluorescence measurements. A drop of water was added at the bottom of the sample holder to increase the humidity, so as to prevent the drying of the lens during the experiment. The excitation and emission band pass values were 1.5 nm. In the solution state experiments, the solution was kept at constant temperature and under uniform stirring. All spectra were corrected for the lamp intensity distribution. The baseline spectra of the buffer solution were subtracted from the fluorescence spectra of crystallins, to remove the contributions of the Raman band which also varies with excitation wavelength. In all cases, the magnitude of REES was shown as the difference between emission maxima obtained for excitations at 292 nm and 308 nm, i.e. a $\Delta\lambda_{ex}$ of 16 nm. The REES values reported are good to ±1 nm.

The source lamp of the fluorimeter itself (150 W Xenon arc lamp) was used as the irradiation source in the photodamage experiments. Photodamage was monitored in the time drive mode, by following the emission intensity at 340 nm with 3 nm band pass.

3. Results and discussion

3.1. Isolated, intact lenses

We have chosen to excite the lenses and crystallins at 290 nm and above for two reasons: (i) the ambient photodamage occurring in the lens involves only radiation that has passed through the atmosphere and the cornea, namely wavelengths above 290 nm [13,14], and (ii) photodamage to the lens largely involves tryptophan (trp) residues as a primary chromophore, and the use of light above 290 nm selects trp residues, since the absorption bands of tyrosine and phenylalanine do not extend significantly to this wavelength.

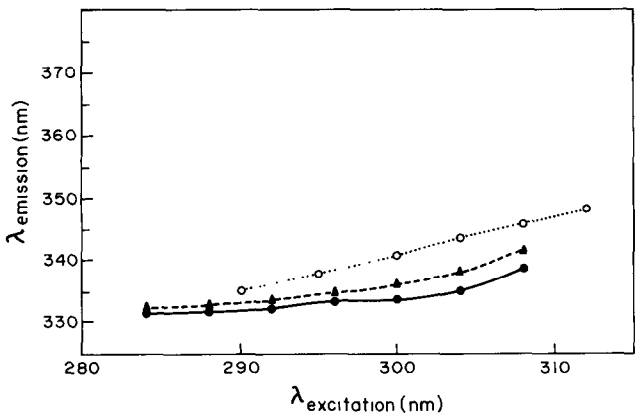

Fig. 1. Plots of emission wavelengths vs excitation wavelengths of freshly isolated lenses from: (····) rat; (---) bovine and (—) chick. Excitation and emission band pass were set at 1.5 nm each.

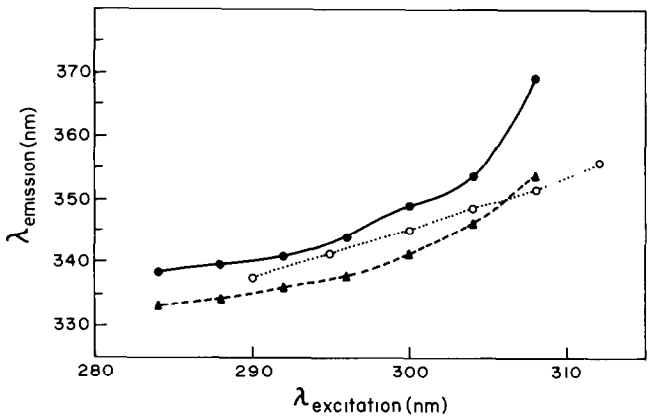

Fig. 2. Plots of emission wavelengths vs. excitation wavelength of photodamaged lenses from (····) rat; (---) bovine and (—) chick. Irradiation was done using the lamp source of the fluorimeter set at 300 nm, with 20 nm band pass for 1 h. Emission measurements were done with excitation and emission band pass set at 1.5 nm.

The ability to measure fluorescence emission and REES of intact lenses has allowed us to compare the environmental and mobility features of the trp residues in the lenses from different species. Fig. 5.1 and 2 shows the REES values of trp emission from rat, chick and bovine lenses freshly isolated from the organism and after photodamage upon irradiation with 300 nm light for 1 hour. Table 1 compares the emission maximum wavelengths and the REES values of these lenses. The differences seen amongst the band maximum values presumably reflect the different trp environments in the lenses of these species. The trp environment in the case of chick lenses appears to be the least polar, with a value of 332 nm while the rat lens offers a more polar environment (336 nm). Photodamage leads to a red shifting of the trp band maximum in all instances as observed earlier for rat lenses [5]. The red shift is as high as 9 nm in the case of chick lenses.

The magnitude of REES, that indicates restricted mobility around tryptophan residue, is larger for rat lenses (10 nm) compared to bovine (6 nm) and chick (7 nm) lenses. We believe that these differences reflect the differences in the packing features of the various lenses and their water contents.

The dual features of red shift of the emission maximum and increase in REES upon irradiation seems to be common to all lenses, as Figs. 1 and 2 and Table 1 show. The largest increase, both in the emission maximum and REES, occurs with the chick lens, suggesting a more dramatic change in the supramolecular organization in the chick lens upon photodamage. However, the reason

Table 1
Fluorescence maxima and REES values of isolated intact lenses

	Fresh		Irradiated[a]	
	Emission[b] maximum (nm)	REES (nm)	Emission[b] maximum (nm)	REES (nm)
Rat	336	10	340	15
Bovine	334	6	336	12
Chick	332	7	341	30

[a] At 300 nm, band pass 20 nm, 1 hour.
[b] Excitation at 292 nm.

for this large change in chick lenses compared to other lenses is not clear.

3.2. Isolated crystallins in solution

We next proceeded to study the REES of isolated and purified individual crystallins, in an effort to detect relationships, if any, between the behavior of the intact supramolecular assembly and of the major constituent proteins.

Table 2 lists the REES results obtained for purified individual crystallins from bovine and chick lenses. It can be seen that the magnitude of REES not only differs among the different crystallins, but differs for the same crystallin from two different species.

The REES of α-crystallin from bovine lens is 6 nm, while it is 11 nm for the same protein from the chick lens. This difference might be due to the different extents of hydration, differing subunit arrangements and probably different trp environments in the two molecules. Further indication for these differences if provided by the REES of the same protein in 4 M urea. It is known that under normal conditions, bovine α-crystallin is an aggregate of sedimentation coefficient value of 19.5 *s*, whereas is 4 M urea the major component has a sedimentation coefficient of 5.0 *s* or lower, depending on the protein concentration. Upon renaturation by dialyzing off the urea, the size of the reconstituted α-crystallin particle comes back to about 12.0 *s* [15]. In 4 M urea, bovine α-crystallin has a REES of 2 nm, while that from the chick lens with a less mobile trp environment, has a value of 6 nm. This difference, as well as the lower REES for α-crystallin in 4 M urea and its restoration upon renaturation suggests that REES can be used as a parameter to indicate packing properties. The same feature of REES for bovine β-crystallin lends support to this argument. It is also interesting to note that in all the cases, total denaturation in 7 M guanidinium chloride abolishes REES indicating a high degree of trp mobility (data not shown). Also, renaturation of the proteins by dialyzing off the denaturant restored the REES values of the native molecules.

The same features of absence of aggregation and the realization of equilibrium excited state distribution from the Franck–Condon excited state distribution in the subnanosecond range might explain the lower REES value of 3.3 nm, observed for γ-crystallin, which, unlike α- and β-crystallins, exists only in the monomeric state. There is no substantial change in REES of γ-crystallin upon denaturation in 4 M urea (Tabel 2).

The low value of 5 nm REES for chick δ-crystallin is probably related to its blue shifted emission (315 and 325 nm) as in the case of azurin [10]. Irradiation of crystallins for 1 h increases REES in all cases, but to different extents. This increase in REES could be due to the photoaggregation of crystallins and possible restriction of segmental mobility. The increased REES parallels that seen in the lens upon irradiation indicating that probably the same processes occurring in solution could occur in the lens.

Yet another interesting observation in that the REES value for aqueous solution of α-crystallin from chick lenses (11 nm) is larger than that observed for the intact lens (7 nm). The observation for aqueous solutions is consistent with the earlier reports that α-crystallin, in solution, forms large aggregates, three layered [16], micellar [17] or combination of the two [18]. But our intact lens data suggests a less restrictive environment. Purified α-crystallin in solution may form larger and tightly packed ensembles (unhindered intermolecular interactions), but in the lens, along with other crystallins and several cytosolic constituents, the nature and extent of packing might be different.

3.3. Mixtures of crystallins

The optical transparency of the lens, with its high

Table 2
Emission maxima and REES of crystallins from bovine and chick lenses

	Fresh		Irradiated	
	Emission maximum (nm)	REES (nm)	Emission maximum (nm)	REES (nm)
Bovine				
α, native	337	6	337	23
α, in 4 M urea	346	2	348	20
α, renatured	337	6	338	23
β, native	332	9	333	23
β, in 4 M urea	346	1	348	21
β, renatured	334	9	335	21
γ, native	329	3	precipitates	precipitates
γ, 4 M urea	328	4	330	8
Chicken				
α, native	337	11	339	27
α, in 4 M urea	346	6	347	15
β, native	333	6	334	13
δ, native	315	5	315	6
δ, native	325	5	326	6

[a] All parameters are the same as Table 1.

protein content, is explained on the basis of the supramolecular organization in which the dense packing of crystallins leads to short range ordering and spatial correlation of individual scatterers, providing for interference effects that would minimize turbidity [1,3,19,20]. One aspect that we have attempted to study here is whether the mixtures display REES values that would be the weighted average of those of the individual components. Any deviation from this weighted average value would be indicative of nonidial behavior, namely preferential interactions of the crystallins.

We thus proceeded to use REES as a parameter to monitor inter-crystallin interactions using mixtures of α-and β-crystallins. These mixtures were prepared either by directly mixing equimolar crystallin solutions or by mixing them together in 4 M urea followed by dialysis to remove urea, thus reconstituting the native mixture. The latter experiment was done since an earlier study on self-assembly of crystallins in vitro appeared to produce the correctly reassembled crystallins only when the associating crystallins are present together during the folding process [21]. In the above study, 7 M urea was used during reconstitution of native crystallins. However in our present study only 4 M urea was used since this was sufficient to dissociate the α-crystallin aggregate. In both the cases, equal amounts of crystallins are present in the mixture.

The REES of α- and β-crystallins mixed directly together was found to be 9 nm, and the same value was obtained when the mixture was reconstituted from 4 M urea. In both the cases the REES values are close, although not identical, to the weight average value (7.5 nm). These observations suggest that the crystallins, in dilute solutions, do not seem to have any detectable specific interactions.

It is also possible that inter-crystallin interaction, if any, might not result in any microenvironmental, reorintational-time alterations around the reporter fluorophore. In such case, interactions might not lead to observable changes in REES. However, NMR studies indicate that crystallins behave as independent globular proteins at low concentration and begin to show strong interactions only at concentrations as high as 16% of protein [22,23]. These reports support the argument that crystallins might not have any specific interaction in dilute solutions. More recently synchronous scan fluorescence study of crystallins [24] also suggested the absence of any observable interactions in dilute solutions.

Bettelheim and Zigler [25] have investigated the preferential interaction among lens crystallins by measuring accessibility of crystallins, in solid state, to ammonia gas. Comparison of sorptive and retentive capacities of self- and hetero-aggregates suggested preferred interactions among lens proteins. A similar study with HCl gas also lead to the same conclusion [26]. These observations, together with the suggestion (present study and [24]) that crystallins might not have any specific interactions in dilute solutions, may be taken to indicate the existence of induced alterations (as the molecular proximity increases) facilitating inter crystallin interactions at high concentrations.

Beaulieu et al. [23,27] have also suggested, from nuclear magnetic resonance dispersion (NMRD) profiles, that crystallins undergo a change in organization around 19% of protein concentration (v/v). Below this concentration they appear to behave as individual globular proteins. Above this concentration crystallins appear to interact to produce a distinctly different, three-dimensional organization [23].

It is becoming clear that crystallins behave as non-interactive independent globular proteins in dilute solutions, and as the concentration increases molecular proximity induces some alterations that appear to facilitate a preferred organization. However, the nature of inducible alterations remains to be understood. The present study, inter alia, suggests that REES could be used as a non-invasive tool to study both intermolecular interactions in solution, and lattice interactions that might exist in condensed phase assemblies such as the eye lens.

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References

[1] Benedek, G.B. (1971) Appl. Optics 10, 459–473.
[2] Tardieu, A. and Delaye M. (1988) Biophys. Biophys. Chem. 17, 47–70.
[3] Trokel, S.L. (1962) Invest. Ophthalmol. 1, 493–501.
[4] Vérétout, F., Delaye, M. and Tardieu, A (1989) J. Mol. Biol. 205, 713–718.
[5] Rao, C.M., Rao, S.C. and Rao, P.B. (1989) Photochem. Photobiol. 50, 399–402.
[6] Galley, M.C. and Purkey, R.M. (1970) Proc. Natl. Acad. Sci. USA 67, 1116–1121.
[7] Rubinov, A.N. and Tomin V.I. (1970) Opt. Spectrosk. (USSR) 29, 1082–1089.
[8] Lakowicz, J.R. (1983) Principles of Fluorescence Spectroscopy, Plenum Press, New York.
[9] Demchenko, A.P. and Ladokhin, A.S. (1988) Eur. Biophys. J. 15, 369–379.
[10] Demchenko, A.P. (1988) Eur. Biophys. J. 16, 121–129.
[11] Demchenko, A.P. (1982) Biophys. Chem. 15, 101–109.
[12] Rao, C.M., Balasubramanian, D. and Chakrabarti, B. (1987) Photochem. Photobiol. 46, 511–515.
[13] Sliney, D.H. (1986) Invest. Ophthalmol. Vis. Sci. 27, 781–790
[14] Pitts, D.G., Cullen, A.P. and Hacker, P.D. (1977) Invest. Ophthalmol. Vis. Sci. 16, 932–937.
[15] Siezen, R.J. and Bindels J.G. (1982) Exp. Eye Res. 34, 969–983.
[16] Tardieu, A., Laporte, D., Licinio, P., Krop, B. and Delaye, M. (1986) J. Mol. Biol. 192, 711–724.
[17] Augusteyn, R.C. and Koretz, J.F. (1987) FEBS Lett. 222, 1–5.
[18] Walsh, M.T., Sen, A.C. and Chakrabarti B. (1991) J. Biol. Chem. 266, 20079–20084.
[19] Bettelheim, F.A. and Siew, E.L. (1983) Biophys. J. 41, 29–33.

[20] Delaye, M. and Tardieu, A. (1983) Nature 302, 415–417.
[21] Bloemendal, H., Zweers, A. and Walters, H. (1975) Nature 255, 426–427.
[22] Koenig, S.H., Beauliie, C.F., Brown, R.D. and Spiller, M. (1990) Biophys. J. 57, 461–469.
[23] Beaulieu, C.F., Clark, J.I., Brown, R.D., Spiller, M. and Koenig, S.H. (1988) Magn. Reson. Med., 8, 45–47.
[24] Rao, C.M. (1991) Biochem. Biophys. Res. Commun. 176, 1351–1357.
[25] Bettelheim, F.A. and Zigler Jr., J.S. (1988) Exp. Eye Res. 47, 227–236.
[26] Bettelheim, F.A., Bove, A. and Dollan, R. (1991) Exp. Eye Res. 52, 269–276.
[27] Beaulieu, C.F., Brown, R.D., Clark, J.I., Spiller, M. and Koenig, S.H. (1989) Magn. Reson. Med. 10, 362–372.