The Selective Quenching of Tryptophan Fluorescence In Proteins By Iodide Ion: Lysozyme in The Presence and Absence of Substrate*

S. S. Lehrer

Retina Foundation, Institute of Biological and Medical Sciences,
Department of Muscle Research, Boston, Massachusetts 02114

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The fluorescence spectrum of the indole chromophore of tryptophan has been shown to be markedly dependent upon the polarity of the environment (Van Duuren, 1961; Walker, Bednar, and Lumry, 1966). Thus, the fluorescence spectrum of tryptophan containing proteins reflects the variety of environments in which the individual tryptophan residues are located within the protein. Information about the environment of certain tryptophan residues can be obtained by a technique which may be called "fluorescence perturbation" and is analogous to the method of solvent perturbation in absorption spectroscopy (Laskowski, 1966). The technique involves the study of the fluorescence difference spectrum produced by a selective perturbation. The resulting protein difference spectrum indicates the fluorescence changes only of those tryptophan residues that have been affected by the perturbation. This technique has recently been used in the case of lysozyme where partial separation of the fluorescence contribution of tryptophan residues in the substrate binding site has been obtained (Lehrer and Fasman, 1967). The difference spectra obtained by varying the pH were attributable to specific internal quenching by certain ionizable groups in the protein molecule. In this work, the technique is used to study the selective quenching of tryptophan fluorescence in

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lysozyme by the addition of iodide ion as an external quenching agent. Differences in accessibility of tryptophan residues to this external quenching agent occur as a result of pH changes and substrate binding.

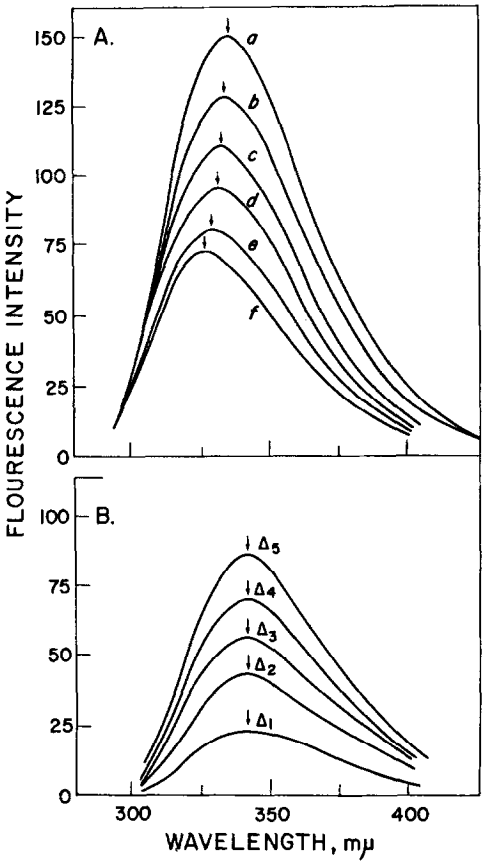

Figure 1. A, Fluorescence spectra of lysozyme at pH 2.1, $\mu = 0.18$, in the presence of KI: a, initial spectrum; b, 0.02M KI; c, 0.06M KI; d, 0.10M KI; e, 0.18M KI; f, a-Δ_5. B, Difference spectra; Δ_1 = a-b; Δ_2 = a-c; Δ_3 = a-d; Δ_4 = a-e; Δ_5 = extrapolated to infinite iodide concentration. Lysozyme concentration, 0.004%; excitation at 280 mμ.

From the fluorescence spectra of lysozyme at pH 2 shown in Fig. 1A, it would appear that the protein fluorescence is quenched non-uniformly by the addition of KI because of the spectral shifts that occur along with the quenching. The difference spectra shown in Fig. 1B, however, indicate that the fluorescence is indeed quenched uniformly. The difference spectra indi-

cate that the fluorescence of lysozyme can be separated into two contributions; a contribution from residues exposed to solvent and accessible to iodide ion, and a contribution from inaccessible residues. The accessible residues appear, as expected, to be in a more polar environment than the inaccessible residues because the quenched fluorescence spectrum is situated at higher wavelengths. The fluorescence of the inaccessible residues (unquenched), curve f, and of the accessible residues (quenched), curve Δ_5, was obtained by extrapolation. From the relative areas under the fluorescence spectra it is seen that more than half of the tryptophan fluorescence in lysozyme at pH 2 is quenched. It is not possible from these data alone to calculate the exact number of exposed tryptophan residues. One can, however, make an estimate if a value for the average quantum yield of an exposed tryptophan, q_e, is taken to be equal to values found for tryptophan in peptides. The extreme values range from $q_e = 0.06$ for small peptides (Cowgill, 1963) to $q_e = 0.12$ for poly-α-amino acids (Fasman, Bodenheimer, and Pesce, 1966). The number of exposed residues, n_e, is then obtained from $n_t \Delta Q_p = n_e q_e$ where $n_t = 6$, is the total number of tryptophan residues in lysozyme and ΔQ_p is the protein quantum yield change associated with the quenching. Using the above extreme values for q_e, the relative area under the curve Δ_5 and the earlier measured quantum yield associated with the area under curve a (Lehrer and Fasman, 1966), the number of exposed tryptophans is estimated to be 3 ± 1. This estimation is consistent with the X-ray data (Blake, Mair, North, Phillips, and Sarma, 1967) and solvent perturbation data (Laskowski, 1966) which indicates a large degree of exposure of the tryptophan residues.

The quenching by foreign dissolved molecules in general will depend upon the accessibility of the indole ring of a particular tryptophan residue and its quantum yield or fluorescence lifetime. The greater the lifetime, the greater will be the possible degree of quenching. The accessibility will depend upon steric conditions and charged groups (Holmström and Tegner, 1966) around the tryptophan residue. Thus, the negative charge of an ionized

carboxyl group located near a tryptophan will decrease the degree of quenching of tryptophan fluorescence by iodide ion. This has been shown preliminarily by obtaining the Stern-Volmer quenching constant for various model tryptophan compounds and poly-α-amino acids. In one outstanding example the Stern-Volmer quenching constant for tryptophan in copoly(L-glu, L-trp) by KI at pH 7 was almost an order of magnitude smaller than in copoly(L-lys, L-trp) at pH 7 even though the quantum yield was the same in the two copolymers. The main difference may be assumed to be the influence of the negatively charged carboxyl groups of the glutamate copolymer and positively charged amino groups of the lysine copolymer on the accessibility of the negatively charged iodide ion for tryptophan. The effect of pH and the presence of saturating amounts of substrate on the quenching of lysozyme are presented in Table. I.

Table I

QUENCHING OF LYSOZYME AND LYSOZYME-triNAG[1] FLUORESCENCE BY 0.18M IODIDE[a]

System	pH[b]	Quencher	λmax(mμ) of Initial Spectrum	λmax(mμ) of Difference Spectrum	% Quenching[c]
Lysozyme	7.4	CsI,KI	338±1	350±3	20
	5.4	KI	338±1	350±3	25
	2.1	CsI,KI	338±2	342±2	47
Lysozyme + 0.07% triNAG	7.4	CsI	328±2	335±3	12
	5.4	KI	322±2	325±3	7
	2.1	KI	328±2	332±3	19

[a]Ionic strength kept constant at 0.18 with NaCl. [b]pH maintained by 0.1M HEPES buffer, succinate buffer, or HCl, respectively. Quantum yield in 0.18M iodide compared to initial quantum yield. Lysozyme concentration, 0.004%.

The data in Table I indicate that quenching is always less in the complex than in the free enzyme at the same pH. In addition, the λmax of the

[1]triNAG = tri-N-acetyl-D-glucosamine.

quenched tryptophan residues (given by the difference spectrum) is lower in the complex than in the free enzyme. These data indicate that upon binding substrate, tryptophan residues become less accessible for quenching and that some of the accessible residues are "located" in environments slightly less polar than an aqueous one.[2] The lower λmax of the complex in absence of iodide was shown earlier to be due to a change in the environment of tryptophan residues in the binding site (Lehrer and Fasman, 1967; Lehrer and Fasman, 1966; Shinitzky, et al., 1966). From these data it appears that tryptophan residues in the binding site become less accessible to iodide quenching. The increase in quenching with decreasing pH for both systems is probably due to electrostatic effects between the increased positive change on the protein and the iodide ion.

The spectra were obtained with an Aminco spectrofluorometer and all data were normalized to equal instrumental conditions and sample absorbances which were approximately 0.1 in a 1 cm cell at the exciting wavelength, 280 mμ. Corrections were made for the loss of exciting radiation due to absorption by the added iodide. No correction was necessary for loss of fluorescence since iodide ion does not absorb above 300 mμ. Corrections for variation of instrumental sensitivity with wavelength were found to be unnecessary since correction factors determined as outlined previously (Lehrer and Fasman, 1965) indicated that they were constant within $\pm$ 10% over the wavelength range of the fluorescence spectra obtained. Relative quantum yields were determined by measuring areas under the fluorescence spectra. Enzyme activity determinations of lysozyme using chitin and a modified Morgan-Elson assay (Reissing, Strominger, and Leloir, 1955) indicated little loss of activity in the presence of 0.2M KI. From the data of Rupley et al. and Dahlquist et al. and the concentration of enzyme and triNAG used in Table I, it is calculated that the degree of saturation is at least 95% over the pH range studied..

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[2]Indole compounds fluoresce maximally in the range 325-335-350 mμ in dioxane, ethanol, and water, respectively, (Van Duuren, 1961).

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