FLUORESCENCE QUENCHING AND CONFORMATIONAL CHANGES OF PROTEINS

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Summary. A fluorescence quenching technique for conformational studies of proteins is described. The dynamic quenching of fluorescein by iodide ions is sensitive to the molecular weight and the charge of proteins, and is suitable for following the conformational change of trypsin in the presence of a substrate.

Introduction. When a quencher is added to a solution of a fluorescent dye, the ratio of the fluorescence intensities with or without quencher is described by the relation :

$$\frac{F_o}{F} = \left(1 + K'(Q)\right) . \left(1 + \kappa \tau (Q)\right) \qquad (1)$$

where F_o and F are the fluorescence intensities without and with the quencher, K' is the equilibrium constant of the non fluorescent dye-quencher complex, κ is the rate of encounter by diffusion between the dye and the quencher, τ is the mean lifetime of the excited dye. The product $\kappa \tau$ is the well-known Stern-Volmer constant (K) (see fig. 1). The first term of relation (1) represents the " static quenching", the second " the dynamic or collisional quenching ".

Our intention was to follow the conformational changes of a protein by measuring the fluorescence quenching of a dye covalently bound to the protein. If a protein isomerizes by binding small molecules, the conformational change may induce variations of the quenching constants (see relation (1)). To label proteins, we have chosen fluorescein isothiocyanate (FITC), because the fluorescence quenching by iodide ions of fluorescein has already been studied, and is known to be of the dynamic type (1),(2),(3). The mechanism of fluorescence quenching of dyes in solution has been analyzed by Weller (4) Weber (5), (6) and Bowen (7).

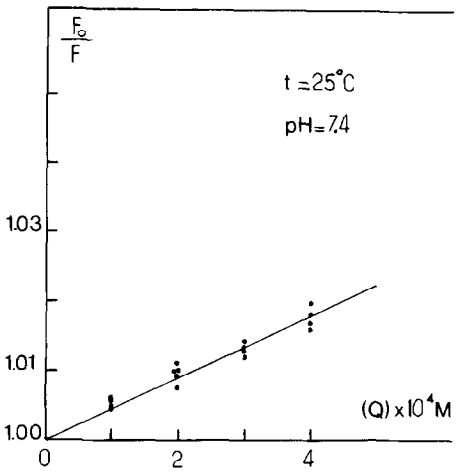

Figure 1 Stern-Volmer plot for trypsin-FITC quenched by iodide ions, (trypsin concentration 10^{-6} M).

First, assuming that the quenching of bound FITC fluorescence by iodide ions is also diffusional, we have followed the variation of the Stern-Volmer constant with the molecular weight of various proteins.

Second, with an enzyme, trypsin, we have shown differences between the Stern-Volmer constants when trypsin is in the presence of a low molecular weight substrate : N acetyl-1-tyrosine ethyl ester (ATEE), and in the absence of this substrate.

Material and methods. FITC was bound to glutathion, lysozyme, lactoglobulin A, lactoglobulin B, ovalbumin, bovine serum albumin and trypsin, using a method given by Nairn (8). Each protein was incubated in a 0.5 M carbonate bicarbonate buffer pH 8.2 with FITC at the same concentration as the protein. After one hour, the free FITC was eliminated by chromatography on Sephadex G 15 or G 25. For trypsin, 0.2 N butylamine was added to the buffer to protect trypsin against autolysis. The number of bound FITC was determined spectrophotometrically, using extinction coefficients given by Tietze (9). Fluorescence intensity and polarization were measured with an apparatus built by the authors. Decay time was measured with a T R W flash.

Results and discussion. Table I (10) gives the Stern-Volmer constants of free FITC and bound FITC. Except for trypsin, the Stern-Volmer constant decreases as the molecular weight increases. There is no simple relation between molecular weight and the Stern-Volmer constant, as one could expect if the rate of encounter between the FITC-protein complex and the quencher was dependent

TABLE I

	M.W.	$K(M^{-1})$
FITC	400	20
Glutathion-FITC	900	5.8
Lysozyme-FITC	17 000	5.0
Lactoglobulin A-FITC	35 000	4.3
Lactoglobulin B-FITC	35 000	4.3
Ovalbumin-FITC	40 000	3.9
Bovine Serum Albumin-FITC	70 000	3.9
Trypsin-FITC	23 500	43.0

on the molecular weight only. Other parameters can modify this rate : negative and positive charges carried by the protein can lower or increase it; steric hindrance and molecular shape may also influence it. The charge effect is particularly clear with trypsin which has numerous positive charges. To justify this interpretation of the high value of K obtained with trypsin, we have studied the state of interaction between trypsin and FITC. The following results indicate a very weak interaction :

1/ Decay times of free FITC and trypsin-FITC are the same : 4.5 ns $\pm$ 0.5 ns (pH 7.2 in phosphate buffer, μ = 0.1).

2/ Fluorimetric titration curves of free FITC and trypsin-FITC give the same pK 6.7 .

3/ Perrin's plot for trypsin-FITC by varying viscosity at constant temperature shows that there are at least two relaxation times for FITC (11). See fig. 2.

We can visualize the situation by assuming that the fluorescent group rotates freely around its covalent bound with the highly positively charged protein, which attracts the negatively charged quencher molecules. Similar effects have been described by Umberger and Lamer (1), Weller (4), Hodges (3) and Holmström and Tegner (12). About the quenching mechanism of trypsin FITC, we have observed the increase of fluorescence polarization (23 % to 27 %) and the decrease of lifetime (4.5 ns to 3.4 ns) in the

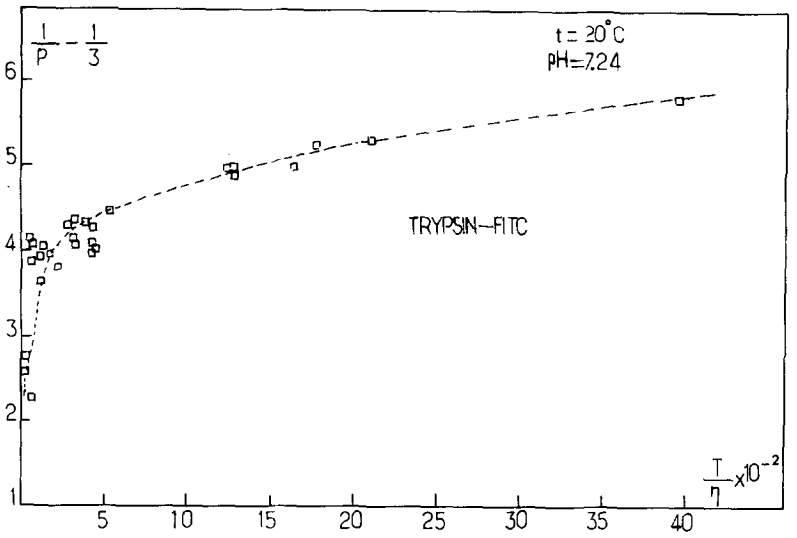

Figure 2 Perrin's plot for trypsin-FITC (concentration 10^{-6} M) in various buffer glycerol solutions at constant temperature (corrected curve).

presence of the quencher (iodide 10^{-4}M at pH 7.2 and 25°C). This result is in agreement with a diffusional quenching. The plot of the reciprocal of the polarization versus the ratio of the lifetime without quencher to the lifetime with quencher has not been represented because we have not precise measurements in the range of 10^{-6}M to 10^{-5}M iodide.

The quenching of trypsin FITC by iodide ions, being of the diffusional kind, is therefore suitable for following conformational changes induced by substrates.

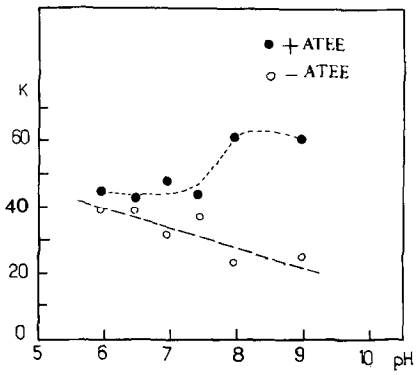

Figure 3 Stern-Volmer constants for trypsin-FITC (concentration 10^{-6} M), ATEE 7.10^{-3} M, 25°C.

In figure (3) are represented the Stern-Volmer constants at various pH with or without ATEE. We have verified that ATEE has no quenching effect on free and bound FITC. The more specific substrates of trypsin like BAEE (N benzoyl-1-arginine ethyl ester) have a quenching effect on free FITC. The variation of the Stern-Volmer constant follows the esterasic activity of trypsin and at the pH where hydrolysis is low, the constants are nearly the same. The esterasic activity on ATEE with and without FITC is the same. We can conclude that the variation of the quenching constant follows a conformational change of trypsin : the repartition charges around FITC can be affected and the encounter between iodide and fluorescein modified, by binding ATEE.

Conclusion . Such experiments can give information on the conformational changes of proteins. Recently Winkler (13) has described another technique of fluorescence quenching to get information on the binding sites of small molecules. Our results show that it is possible to distinguish a molecular population at low concentration (K_M ATEE = 5.10^{-2}) and that long range effects of small molecules can be observed because bound FITC has no interference with the active site of trypsin. The binding of ATEE causes the modification of the encounter between FITC and iodide.

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