THE FLUORESCENCE OF LYSOZYME AND LYSOZYME SUBSTRATE COMPLEXES[1]

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The recent X-ray studies of lysozyme and lysozyme-inhibitor complexes have indicated that three of the six tryptophan residues are located in the region of the binding site (Johnson and Phillips, 1965). At least one tryptophan has been implicated as being involved in the active site (Hayashi et al., 1965). A study of the fluorescence of lysozyme and lysozyme substrate complexes was undertaken because of the known sensitivity of the emission of the indole chromophore to its environment. This study has indicated remarkable differences in the fluorescence behavior of the free and complexed enzyme and has provided information regarding interactions in the enzyme binding site in solution to complement the X-ray studies of the binding site in the crystal.

The emission spectra of the enzyme and various complexes (at enzyme saturation) at pH 7.5 are seen in Fig. 1. The respective

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areas correspond to the relative quantum yields. Comparison with the total fluorescence of L-tryptophan and assuming that Q(L-tryp) = 0.20 (Teale and Weber, 1957), produced the absolute quantum yield values shown in the figure. The values shown are protein

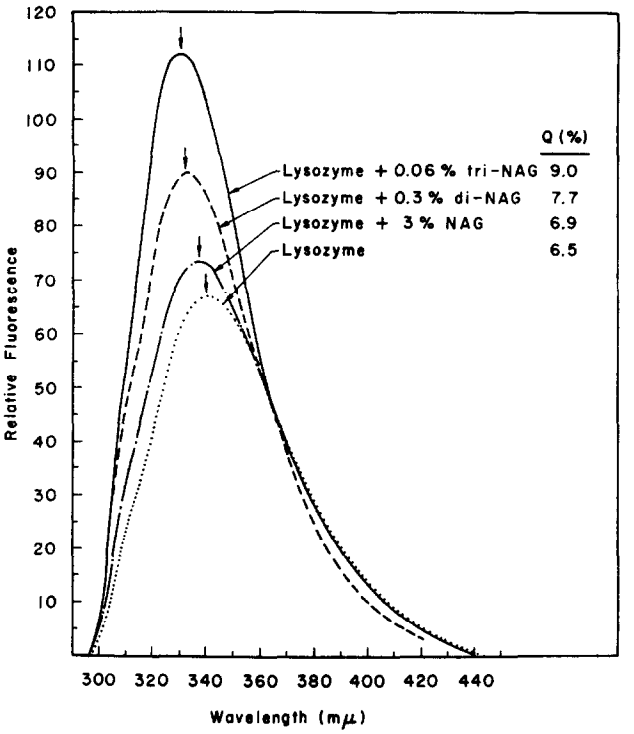

Fig. 1. Fluorescence Spectra of Lysozyme and Lysozyme Complexes. Conditions: pH 7.5, "Tris" buffer, 0.2M NaCl, 25°C, lysozyme concentration 0.005%. Corrected for variation of photomultiplier sensitivity and dispersion of monochromator of the Zeiss fluorometer.

quantum yields because they do not take into account tyrosine absorption. The corresponding quantum yields for tryptophan in lysozyme are approximately 10% higher. The simplest substrate is tri-N-acetyl-D-glucosamine (tri-NAG) while the dimer (di-NAG) and monomer (NAG) are competitive inhibitors (Rupley, 1964) and their respective binding constants decrease in this order. These three materials do not contribute to the absorption or fluorescence and

do not affect L-tryptophan fluorescence in aqueous solution.

The emission observed is essentially due to tryptophan, although a small shoulder in the 310 mμ region indicates a small tyrosine contribution, as expected. The quantum yield of the tryptophan emission in the enzyme is unusually low, compared to the 20% and higher values exhibited by many proteins (Teale, 1960). Therefore, most of the tryptophan fluorescence must be quenched in the native enzyme. The progressive enhancement and shift to lower wavelengths of the emission spectra produced by increasing the size of substrate or inhibitor is probably due to some group or groups that become progressively less available for quenching of one or more tryptophan residues in the complex.

Further evidence regarding this de-quenching effect was obtained from the pH dependence of fluorescence as shown in Fig. 2. These data were obtained by a fluorometric titration. That is, the fluorescence and the pH were measured directly in the

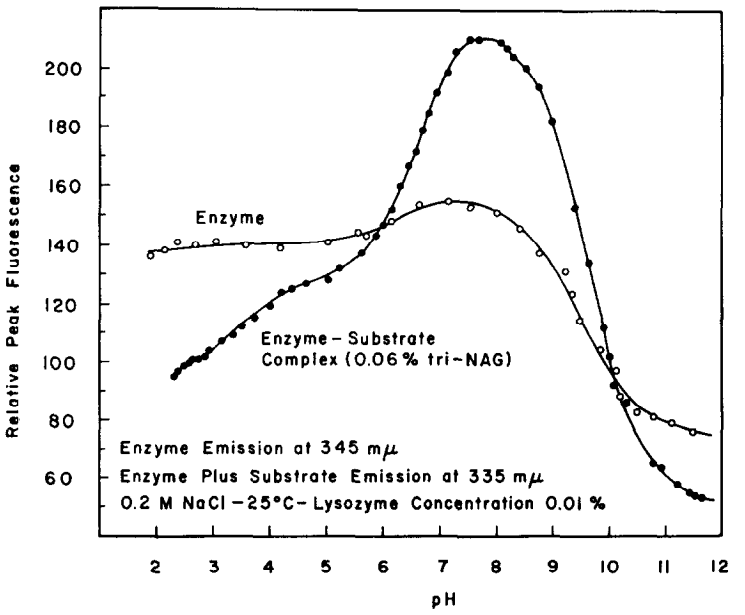

Fig. 2. Fluorescence vs. pH of Lysozyme and Lysozyme-tri NAG.

fluorometer as the solutions were titrated with 0.5M NaOH or 0.5M HCl. A micropipet was used and the solutions were stirred by nitrogen bubbling. The fluorescence changes were found to be reversible.

The enzyme fluorescence changes slightly with pH up to the basic region. The enzyme-tri-NAG complex, however, shows marked fluorescence changes between the pH regions 2.5 to 4.5 and 5.5 to 7.5. From this fluorescence titration, values of apparent dissociation constants, pK' = 3.5 and pK' = 6.5 can be assigned to the groups associated with the phenomena observed. A de-quenching of fluorescence occurs because of the removal of protons from these groups. The decreasing fluorescence for both the free and complexed enzyme in the basic pH range can be associated with a group of pK' = 9.6. This is probably due to quenching by energy transfer to ionized tyrosine (Cowgill, 1965), although amine quenching cannot be completely eliminated (Fasman et al., 1966). The first two groups appear to have similar pK' values reported for certain anomalous carboxyl groups, one with high pK' (6.3) and one or two of anomolously low pK' (3.3) (Donovan et al., 1960,1961). Studies on model systems (White, 1959; Cowgill, 1963; Fasman et al., 1966) have shown that unionized carboxyl groups quench tryptophan fluorescence. This strongly suggests that it is these anomolous carboxyls that interact with the tryptophan residues in the binding site and quench fluorescence.

The pH variation of the binding constant, K_a is shown in Fig. 3. These values were obtained by fluorometric titration of the native enzyme with tri-NAG and the values calculated by the following method. When the fluorescence is proportional to the concentration (absorbance < 0.1) it can be shown that where β is the degree of association,

$$\beta = \frac{F - F_E}{F_{ES} - F_E} \quad \text{and,} \quad K_a = \frac{\beta}{1 - \beta} \cdot \frac{1}{C_s} \quad \text{where,} \quad C_s = C_s^\circ - \beta C_E^\circ.$$

Here C_s is the free substrate, C_s° is the total substrate present, C_E° is the total enzyme present, F_E is the fluorescence of the uncomplexed enzyme, F_{ES} is the fluorescence of the enzyme-substrate complex and F is the fluorescence of the mixture. The titration was performed at wavelengths where the difference in $F_{ES} - F_E$ is large. The value of K_a was determined by first extrapolating a plot of $\frac{1}{F - F_E}$ versus $\frac{1}{C_s^\circ}$ to obtain $F_{ES} - F_E$ at $\frac{1}{C_s^\circ} = 0$, and then calculating K_a for the case $\beta = 1/2$, where $K_a(\beta = 1/2) = \frac{1}{C_s^\circ - 1/2C_E^\circ}$. A knowledge of the original concentration of enzyme and the concentration of added substrate at $\beta = 1/2$ enabled the calculation of K_a.

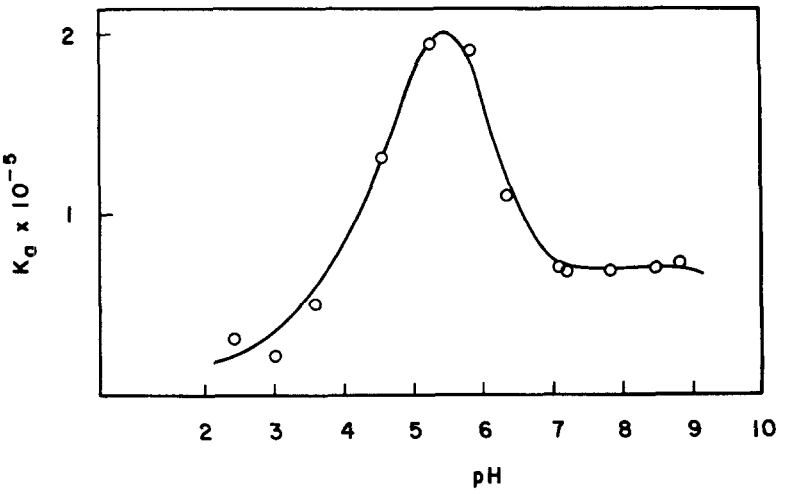

Fig. 3. Binding Constant of Lysozyme-tri NAG Complex vs. pH. Conditions: 0.2M NaCl, 25°C.

It is seen in Fig. 3 that there is optimum binding at pH 5.5 caused by the ionization of two groups of approximately pK' = 6.2 and pK' = 4.2. This binding curve approximately follows the activity versus pH curve determined by Rupley (1966). The group of pK' = 6.2 is probably the same group that is involved in the de-quenching of fluorescence seen above allowing for a possible

small shift in pK' by binding substrate. The pK' of 4.2 is approximately normal for carboxyl groups and seems to be somewhat higher than the pK' of the group that changes the fluorescence in the acid region.

From this data it is possible to construct a very schematic model to explain the binding and fluorescence behavior, involving tryptophan and three carboxyl groups in the binding region. More than one tryptophan may be involved in the change of fluorescence and binding, and different carboxyls may affect different tryptophans. The data require that the two anomolous carboxyl groups be **in** juxtaposition to tryptophan so that quenching by "contact" may occur. The carboxyl group with pK' = 6.3-6.5 is also involved in binding the substrate as is the group with pK' = 4.2. This latter group, however, is sufficiently removed from the tryptophan to affect the fluorescence.

Since the fluorescence changes with pH are much smaller for the free enzyme than for the complexed enzyme, the carboxyl groups must be more accessible for quenching the tryptophan in the latter. That is, the spatial relationships of the carboxyl groups relative to the tryptophan must change upon substrate binding.

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