Stopped-Flow Fluorescence Studies on Saccharide Binding to Lysozyme

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The binding of the β -1-4-linked trimer of *N*-acetyl-D-glucosamine to hen egg-white lysozyme was studied by rapid-reaction-kinetic methods with tryptophyl fluorescence observation of the transients. It was found that discrete segments of the fluorescence-difference spectrum from this reaction were perturbed at different time-points during the binding process. The results were interpreted as the formation of the initial complex, the fast phase of the reaction, perturbing the environment of tryptophan-62 and a subsequent and slower rearrangement of the initial complex perturbing the environment of tryptophan-108. At pH 4.4, the release of protons from aspartate-101 occurred during the rearrangement step of the binding reaction. A model for the reaction is presented (E, enzyme; L, ligand):

The association of this ligand with lysozyme may be visualized in three-dimensional terms as initial complex-formation across the top of the active-site cleft followed by a diving motion of the ligand into the cleft.

Kinetic studies on the binding of specific ligands to enzymes have in many cases shown that a single bimolecular step (eqn. 1) is an inadequate description of the mechanism and that often an additional step such as a unimolecular isomerization of the enzyme–ligand complex (eqn. 2) is required to accommodate the kinetic data (Gutfreund, 1971):

Such reactions are usually monitored in rapid-reaction equipment by some spectral perturbation of either enzyme or ligand upon complex-formation. But a limitation on kinetic studies is that although they may reveal the sequence of events in ligand binding and in favourable cases implicate the role of an isomerization of an enzyme–substrate complex in the catalytic mechanism (Halford *et al.*, 1969), the spectrophotometric probes are not generally open to a molecular interpretation and the kinetics by themselves provide no information about the nature of the isomerization. The isomerization could be a conformational change

in either the enzyme, the ligand or both, or it could be a relocation of the ligand from one binding site to another. One would also like to know which sections of the molecules move and at what rate. Alternative techniques for the study of enzyme–ligand combinations have included X-ray crystallography and n.m.r. (nuclear magnetic resonance), but both of these also suffer from limitations. The crystallographic structure of an enzyme–ligand complex supplies a wealth of data about binding interactions and conformational changes but nothing as yet of kinetic significance. However n.m.r. methods yield in principle both structural and dynamic parameters (Sykes & Scott, 1972) but the analysis of n.m.r. spectra in terms of eqn. (2) is difficult and may require supplementary data from orthodox kinetic experiments (Baldo *et al.*, 1975). Thus despite the attention that has been given to ligand-binding reactions on account of their importance in enzyme mechanisms, drug–receptor interactions and suchlike, very few adequate descriptions of the binding of a ligand to an enzyme, including both molecular and kinetic terms, have yet been given.

However, for an enzyme such as hen egg-white lysozyme (EC 3.2.1.17), about which considerable

knowledge of the protein structure and the properties of individual side chains has already been accumulated (Imoto *et al.*, 1972a; Moore & Osserman, 1974), a re-investigation of the kinetics of ligand binding might now be pertinent because the structural information on this enzyme can be used to provide a molecular interpretation of the kinetic events. The present paper describes some experiments on the binding of the β -1-4-linked trimer of *N*-acetyl-D-glucosamine (GlcNAc β 1-4GlcNAc β 1-4GlcNAc) to lysozyme. The active site of lysozyme contains six subsites labelled A-F, each capable of binding one *N*-acetylglucosamine residue (Blake *et al.*, 1967b) with the hydrolysis of a polymer occurring between subsites D and E (Rupley & Gates, 1967). The GlcNAc trimer is hydrolysed by lysozyme but with an extremely low turnover number, for it has been shown by a variety of techniques to bind almost exclusively as a non-productive complex in subsites A-B-C (references in Imoto *et al.*, 1972a). The crystallographic studies have revealed the pattern of bonds between the enzyme and the ligand in these subsites (Blake *et al.*, 1967b). Consequently experiments on the binding of the GlcNAc trimer to lysozyme are of interest with regard to the elucidation of specificity and recognition processes rather than catalysis.

Two techniques have been used here to monitor the binding of GlcNAc trimer to lysozyme: protein fluorescence and proton uptake/release. Lerher & Fasman (1967) found that the binding of GlcNAc trimer to lysozyme was accompanied by an enhancement of fluorescence above pH 5.7 and by a quench at lower pH values. The bulk (>80%) of the fluorescence of lysozyme arises from two out of the six tryptophan residues in the protein (Imoto *et al.*, 1972b; Teichberg & Sharon, 1970): the fluorescent tryptophans are at positions 62 and 108 and both are located in the active-site cleft (Blake *et al.*, 1967a). In addition, the pK_a values of two carboxylates within the active site alter on the formation of the complex with the GlcNAc trimer that of aspartate-101 from 4.3 to 3.5 and that of glutamate-35 from 6.0 to 6.4 (Rupley *et al.*, 1967; Parsons & Raftery, 1972; Banerjee & Rupley, 1973). Thus the binding of GlcNAc trimer to lysozyme results in proton release from the enzyme at pH 4 and proton uptake at pH 6.

Temperature-jump and stopped-flow experiments on GlcNAc trimer binding to lysozyme have been carried out by Chipman & Schimmel (1968), Holler *et al.* (1969, 1970) and Pecht *et al.* (1970). The first conclusion of the compatibility of the kinetics with eqn. (1) was subsequently revised in favour of eqn. (2). However, all of these studies were done at pH values above 6.0. On account of the pH-dependent dimerization of lysozyme seen at high protein concentrations such as those used in n.m.r. experiments (Studebaker *et al.*, 1971), correlations between the

transient n.m.r. data and results from transient kinetic experiments can only be drawn among studies at lower pH values. Consequently the experiments in the present paper were initiated to study by rapid-reaction methods the binding of GlcNAc trimer to lysozyme at lower pH values, with the view to comparing the kinetics against the results from n.m.r. experiments on the binding of other saccharides (Sykes, 1969; Sykes & Scott, 1972; Baldo *et al.*, 1975). Additional experiments were later carried out that confirmed and extended the conclusions of previous workers and moreover provided a molecular model for this reaction in terms of perturbations at individual side-chains within the different steps of the ligand-binding mechanism.

Materials and Methods

Materials

Hen egg-white lysozyme was obtained from PL Biochemicals, Milwaukee, Wis., U.S.A. (lot no. 267-6, $3\times$ crystallized). Stock solutions of enzyme were as a routine dialysed against 0.1 M-KCl before experiments to remove acetate present in this preparation. Concentrations of lysozyme were determined by absorbance with an ϵ_{280} $3.65\times 10^4\text{ M}^{-1}\cdot\text{cm}^{-1}$ (Imoto *et al.*, 1973). The GlcNAc trimer, a gift from Dr. J. Baldo, was prepared by the method of Rupley (1964) with the additional purification step of Sephadex G-15 filtration as described by Baldo *et al.* (1975). All other chemicals were of analytical grade and were used without further purification.

Fluorescence experiments were carried out in solutions of 0.01 M-buffer-0.10 M-KCl with one of the following buffers: citrate-citric acid (both 5 mM) for pH 4.4, 0.01 M-pyrophosphate adjusted to pH 5.9 with H_3PO_4 , 0.01 M-Tris adjusted to pH 7.4 with HCl. To check for specific buffer effects, the pyrophosphate-phosphate buffer system was also used in experiments at pH 4.4 and 7.4. Results were identical with those obtained with the citrate and the Tris buffers at these pH values. Proton release from lysozyme on the binding of GlcNAc trimer was measured in solutions of 0.20 mM-2,4-dinitrophenol-0.10 M-KCl, pH 4.4, by monitoring the protonation of 2,4-dinitrophenylate at 365 nm. These solutions contained no additional buffer and were titrated directly to pH 4.4 with 0.1 M-HCl. The absence of any interaction between lysozyme and 2,4-dinitrophenol was demonstrated by Baldo *et al.* (1975).

Experimental methods

Fluorescence emission spectra were recorded at room temperature ($22\pm 1^\circ\text{C}$) in the 1 cm cuvette of a Farrand Foci spectrofluorimeter with 5 nm band widths on both excitation and emission beams. Excitation was at 290 nm. The spectra were uncorrected

though the contributions to the emission from buffer and saccharide were both negligible. For each set of experimental conditions, emission spectra were taken between 300 and 450nm first on lysozyme (3 μ M) alone and then repeated after the addition of GlcNAc trimer to a concentration of 0.5mM. This ligand concentration is sufficient for 98% saturation of the enzyme. Equilibrium dissociation constants of GlcNAc trimer from lysozyme were taken from Banerjee & Rupley (1973): at 22°C, $K_0 = 10 \mu$ M at both pH4.4 and 7.4, and 6 μ M at pH5.9.

Two stopped-flow instruments were used for experiments at room temperature: the split beam spectrophotometer (reaction cell of light-path 1cm, dead-time 3ms; Gutfreund, 1972) and the differential stopped-flow fluorimeter (light-path 0.2cm, dead-time 1ms) of Bagshaw *et al.* (1972). The latter was operated as in Bagshaw *et al.* (1972), but with a different optical arrangement as described below. For each experiment, the apparatus was calibrated by measuring the fluorescence from the same concentration of lysozyme alone in the same buffer as used in the subsequent experiment on GlcNAc trimer binding; this value was defined as 100% fluorescence. Excitation was with monochromatic light at 290nm with a band pass of 10nm and the 90° emission detected through filters placed in front of the observation photomultiplier. For most experiments, a combination of Schott Jena (Mainz, West Germany) filter types WG 335 and UG 11 were used; this provides a band pass of >50% transmission between 325 and 380nm and is called the 'band-pass filter'. Interference filters from Balzers (Liechtenstein) were occasionally used for a better wavelength discrimination; one with λ_{max} 323nm, 17% peak transmission and a 28nm band width and another at λ_{max} 369nm, 26% peak transmission and an 11nm band width. To narrow the band passes, the interference filters were used in conjunction with Schott Jena filters types WG 320 and UG 11 for work at 323nm and types WG 345 and UG 11 at 369nm. On account of their low percentage transmission, the interference filters caused a marked decrease in the signal-to-noise ratio.

Temperature-jump studies in the absorption mode were done with the instrument and by the methods described (Halford, 1972; Baldo *et al.*, 1975). Starting from 18°C, a temperature-jump of 4°C was achieved with a heating-time of 10 μ s. The apparatus was modified for fluorescence observations by placing an E.M.I. 9526B photomultiplier at 90° to the excitation beam. Excitation of lysozyme fluorescence was with monochromatic light at the 292nm mercury line and the emission detected through the 'band-pass filter'. The signal from the fluorescence photomultiplier was referenced against that from the transmission photomultiplier with the light before the latter being attenuated with a neutral density filter.

Within the time-period that mixtures of GlcNAc trimer and lysozyme were studied (up to 1h in temperature-jump experiments), negligible hydrolysis of the trimer would have taken place (Rupley & Gates, 1967). Transients from both stopped-flow and temperature-jump experiments were recorded on a Tektronics storage oscilloscope, photographed and whenever possible analysed conventionally after projection on to graph paper. Reaction records that could not be fitted within the signal-to-noise limits by a single exponential were analysed by computer using a non-linear least-squares procedure (from W. D. Hull, Harvard University) which generated the best fit to the experimental data with the sum of two exponentials. Each data point in Figs. 2, 4, 6 and 9 is the average of three rate constants determined from separate trials of one reaction mixture.

Of the two kinetic methods, the stopped-flow technique was the preferred method since larger signals were obtained from the overall approach to equilibrium than from a small perturbation to a system at equilibrium. Moreover the stopped-flow method yielded unambiguous data about the spectral characteristics of intermediates that was not available from temperature-jump experiments. However, this project required the frequent measurements of rate constants at about 250s⁻¹. Although such rate constants could readily be measured on the stopped-flow fluorimeter which had a dead-time of 1ms and had been shown to complete >99% of the mixing within that time interval (Bagshaw *et al.*, 1974), nearly 50% of the reaction record would be lost within the 3ms dead-time of the stopped-flow absorption spectrophotometer. The latter was therefore measured more accurately in the temperature-jump apparatus.

Kinetic methods

The kinetics of the binding of GlcNAc trimer to lysozyme are compatible with mechanism (2) (Holler *et al.*, 1970; Pecht *et al.*, 1970). The kinetic equations for this system have been collected from the literature and are presented below (Eigen & de Maeyer, 1963; Gutfreund, 1972). It is assumed throughout that the bimolecular step is much faster than the unimolecular step. In temperature-jump experiments, the perturbation to the equilibrium in eqn. (2) would generate two relaxations and the dependencies of their reciprocal relaxation times on the concentration of reactants are given by:

$$\frac{1}{\tau_1} = k_{-1} + k_{+1}([\bar{E}] + [\bar{L}]) \quad (3)$$

and

$$\frac{1}{\tau_2} = k_{-2} + \frac{k_{+2}}{1 + K_1/([\bar{E}] + [\bar{L}])} \quad (4)$$

where $K_1 = k_{-1}/k_{+1}$, the dissociation constant from the initial step, and $([\bar{E}] + [\bar{L}])$ the sum of the free

concentrations of enzyme and ligand. The concentration of free reactants may be evaluated from the total concentrations and the equilibrium dissociation constant (K_0). Since:

$$K_0 = K_1 \cdot k_{-2} / (k_{-2} + k_{+2}) \quad (5)$$

the value of the equilibrium dissociation constant (between all complexes and the free reactants) provides a check on the values for the rate constants determined from the concentration-dependencies of the reciprocal relaxation times.

For stopped-flow experiments on the approach to equilibrium after mixing the reactants together, the trace of the concentration of EL against time would be biphasic, containing a rapid rise due to EL formation from free E and L and a later component caused by the conversion of EL into EL*. The apparent first-order rate constant from the rapid phase of EL formation (k_a) is given by:

$$k_a = k_{-1} + k_{+1}[L] \quad (6)$$

and that for the slow phase (k_b), equal to the rate of formation of EL*, by:

$$k_b = k_{-2} + \frac{k_{+2}}{1 + K_1/[L]} \quad (7)$$

where the symbols are defined as above. The derivation of eqns. (6) and (7) assume that the concentration of ligand is in a large excess over enzyme.

In the experiments described below, the assumption that the bimolecular step in eqn. (2) is very much faster than the unimolecular step (i.e. $\tau_1 \ll \tau_2$ or $k_a \gg k_b$) is not strictly valid. Under conditions where both steps were observed and over the concentration range examined here, k_a is often just a factor of five larger than k_b . One consequence of the similarity of these rates is that there should be an early lag phase in EL* production during the build-up of EL at the beginning of the reaction (Gutfreund, 1955). An analogue computer representation of EL* production with the rate constants derived from Fig. 9 at $[L] = 0.30 \text{ mM}$ did show the expected lag phase, but the deviation of this curve from the best fit with a single exponential never exceeded 10% of the total signal and thus any lag phases that might have existed would have been hidden within the experimental noise. The quality of the experimental data does not warrant a more detailed kinetic analysis than that given here. Moreover, the data has been analysed only in terms of mechanism (2) and although some alternative schemes for ligand binding may be discounted on account of the concentration-dependencies of reciprocal relaxation times or apparent rate constants (Halford, 1972), others are kinetically indistinguishable from mechanism (2) on these criteria alone (Viale, 1971).

Results and Discussion

Fluorescence perturbations at pH 4.4

Holler *et al.* (1970) and Pecht *et al.* (1970) described stopped-flow and temperature-jump experiments on the enhancement of lysozyme fluorescence due to GlcNAc trimer binding at pH 6.3 and 8.0 respectively. Both groups found a single relaxation whose rate depended on the concentration of reactants in the hyperbolic manner of eqn. (4) and the data were therefore analysed in terms of the unimolecular process in the two-step binding mechanism (eqn. 2). The binding of GlcNAc trimer to lysozyme at lower pH values produces a quenching of the protein fluorescence and it was decided to compare the kinetic parameters from the quenching of fluorescence with those from the enhancement. Consequently, stopped-flow experiments have been done here on the binding of GlcNAc trimer to lysozyme at pH 4.4 with fluorescence observations of the transients utilizing in this instance the 'band-pass filter' on the fluorescence emission.

The reaction was studied at a constant enzyme concentration of $15 \mu\text{M}$ but with various concentrations of GlcNAc trimer between $25 \mu\text{M}$ and 2.0 mM . Stopped-flow records of the fluorescence quench during the approach to equilibrium after mixing enzyme and two different concentrations of ligand are shown in Fig. 1. At concentrations of trimer below 0.3 mM , the progress curves of the reaction at any single ligand concentration were biphasic (Fig. 1a). Similar biphasic traces were obtained by fluorescence observation of temperature-jump experiments on the same system. The relative amplitudes of the fast and the slow phases in the stopped-flow fluorescence records were found to depend on the concentration of GlcNAc trimer, the proportion of the slow phase falling from 60% of the total signal at 0.05 mM GlcNAc trimer to 15% at 0.20 mM GlcNAc trimer and to a barely detectable value at still higher concentrations. The stopped-flow record of the reaction at 0.60 mM GlcNAc trimer (Fig. 1b) appeared monophasic: a second phase could not be detected in records of this reaction taken over a longer time-base.

Apparent rate constants (k_a) were calculated from the fast phase of the reaction at the lower concentrations of the trimer and the monophasic reaction at higher concentrations. The dependence of the values of k_a on the ligand concentration was linear over the concentration range tested and is shown in Fig. 2. The experiments could not be extended to still higher concentrations of GlcNAc trimer because the proportion of the reaction then completed within the stopped-flow dead-time became too high and the relaxation amplitudes in temperature-jump experiments too small to be analysed.

The kinetics of GlcNAc trimer binding to lysozyme at pH 4.4 cannot be interpreted in the same manner

Fig. 1. Fluorescence records at pH 4.4

The stopped-flow fluorimeter was used to monitor the quenching of fluorescence in the reaction of lysozyme with GlcNAc trimer in 0.01 M-citrate-0.10 M-KCl, pH 4.4. Excitation was at 290 nm and the emission observed through the 'band-pass filter'. Concentrations were 50 μ M-GlcNAc trimer in trace (a), 0.60 mM-GlcNAc trimer in trace (b) and 15 μ M-lysozyme in both. In each photograph, two reaction records are shown at the same sensitivity but with the sweep speeds as indicated.

as the other experiments at the higher pH values. If the quenching of lysozyme fluorescence from trimer binding occurred during the isomerization of the initial complex in mechanism (2), monophasic reactions with a hyperbolic concentration-dependency of their apparent rate constants should have been observed (see eqn. 7). However, the linear relationship between the values of k_a and the concentration of trimer can still be accommodated on mechanism (2), provided that the fluorescence of lysozyme is quenched under these conditions on the initial bimolecular step. If the kinetic data refers to the bimolecular step, then the linear concentration-dependency in eqn. (6) should be obeyed. From the slope and the intercept of the line in Fig. 2, values for k_{+1} and k_{-1} were obtained as $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and 125 s^{-1} respectively. These yield a value of 83 μ M for the initial dissociation constant, K_1 , as compared with 10 μ M for the overall dissociation constant (K_0). The fact that the fluorescence records were biphasic at low concentrations of GlcNAc trimer and monophasic at higher concentrations confirms that the quenching of fluorescence occurs between E and EL

Fig. 2. Concentration-dependency of fluorescence data at pH 4.4

Apparent rate constants (k_a) were evaluated in stopped-flow records of GlcNAc trimer binding to lysozyme at pH 4.4 from the fast phase of fluorescence quenching at low concentrations of ligand and the monophasic reaction at higher concentrations and are plotted against the concentration of the trimer. The line drawn is the theoretical line for eqn. (6) with $k_{+1} = 1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-1} = 125 \text{ s}^{-1}$.

whereas EL and EL* have the same degree of quenched fluorescence: when $[L] = K_1 > K_0$, only 50% saturation of the enzyme would be achieved in the initial equilibrium between the free reactants and the EL complex but further saturation, governed by the value of K_0 , would occur at the slower rate of the isomerization. Alternatively, when $[L] \gg K_1$, the saturation of the enzyme would be complete within the initial equilibration. If the fluorescence of EL and EL* were not the same, then the biphasicity of the traces would have been observed at all concentrations of the trimer.

Proton release at pH 4.4

The formation of the complex between lysozyme and GlcNAc trimer can also be monitored by the detection of proton uptake/release on ligand binding. Temperature-jump experiments were done on equilibrium mixtures of lysozyme and trimer at pH 4.4 in the presence of 2,4-dinitrophenol over a range of concentration of both enzyme (0.1–0.3 mM) and ligand (0.05–1.2 mM). A representative relaxation is shown in Fig. 3. At all reactant concentrations tested, only one relaxation was detected and this could be described by a single exponential ($1/\tau$). The relationship between the values of $1/\tau$ and the concentrations of free reactants ($[E] + [L]$) is given in Fig. 4. The hyperbolic concentration-dependency is compatible with the reaction mechanism in eqn. (2) and the data were fitted to eqn. (4) with the following kinetic parameters: $K_1 = 83 \mu\text{M}$, $k_{+2} = 220 \text{ s}^{-1}$ and $k_{-2} = 30 \text{ s}^{-1}$.

Fig. 3. Proton release at pH 4.4

The temperature-jump apparatus was used to measure the increase in absorbancy at 365 nm in the relaxation of an equilibrium containing 0.20 mM-lysozyme and 0.30 mM-GlcNAc trimer in 0.20 mM-2,4-dinitrophenol-0.10 M-KCl, pH 4.4.

These parameters obey the relationship in eqn. (5) between the overall dissociation constant and the individual rate constants. In the stopped-flow experiments on the same system, only one transient was observed (with an apparent rate constant equal to the reciprocal relaxation time at the same reactant concentrations) and by back-extrapolation of the observed transient through the instrumental dead-time, it was found that the change in absorbance with this process originated from the base-line. Hence the protons that are liberated in this reaction are released during the isomerization for otherwise biphasic traces would have been observed in the stopped-flow records. [The absence of a second phase from the temperature-jump records could in itself be due to a number of causes (see Thusius, 1972).]

Experiments on the kinetics of GlcNAc trimer binding to lysozyme at pH 4.4 have thus resulted in different profiles of the apparent rate constants or reciprocal relaxation times against the concentrations, depending on whether the reactions were monitored by fluorescence quenching or proton release. However, the two sets of data can readily be accommodated by one mechanism (eqn. 2) provided that the signals arise from different steps. Both sets of data are compatible with the same value for K_1 . If protons are released at pH 4.4 only during the conversion of EL into EL*, the experimental result of a single relaxation with a hyperbolic concentration-dependency is in accord with theoretical expectations. From the data of Holler *et al.* (1970), the uptake of protons during trimer binding at pH 6.3 also occurs in the second step. The kinetics of proton uptake at pH 6.3 are qualitatively similar to those of proton release at pH 4.4 even though the proton perturbations are from different carboxylates within the active site (Parsons & Raftery, 1972; Banerjee & Rupley, 1973).

Fig. 4. Concentration-dependency of proton-release data at pH 4.4

Reciprocal relaxation-times ($1/\tau$) were evaluated from temperature-jump records of the relaxations of lysozyme and GlcNAc trimer with 2,4-dinitrophenol at pH 4.4 and are plotted against the concentration of free reactants ($(E) + (L)$). The line drawn is the theoretical curve for eqn. (4) with $K_1 = 83 \mu\text{M}$, $k_{+2} = 220 \text{ s}^{-1}$ and $k_{-2} = 30 \text{ s}^{-1}$.

Fluorescence perturbations at pH 7.4

The scheme for the binding of GlcNAc trimer to lysozyme at pH 4.4 appears to be identical with that determined in experiments at higher pH values. But at pH 4.4, the fluorescence of the protein was quenched on the bimolecular step (Fig. 2) whereas it was reported that the enhancement of fluorescence caused by ligand binding at pH 6.3 or 8.0 took place during the unimolecular step (Holler *et al.*, 1970; Pecht *et al.*, 1970). Therefore it was decided to re-examine the kinetics of the fluorescence enhancement in experiments at pH 7.4.

The binding of the trimer to lysozyme at pH 7.4 was studied in the stopped-flow fluorimeter over a range of GlcNAc trimer concentrations (25 μM to 2.0 mM) but at a constant concentration of enzyme (15 μM). The 'band-pass filter' was again used across the fluorescence emission. A typical stopped-flow trace of the fluorescence enhancement is given in Fig. 5. Apparent rate constants were evaluated from the observed transients and their dependency on the concentration of trimer plotted in Fig. 6. A hyperbolic relationship was obtained. These kinetics are characteristic of unimolecular rearrangement in mechanism (2) and can be accommodated to eqn. (7) with the following parameters: $K_1 = 170 \mu\text{M}$, $k_{+2} = 235 \text{ s}^{-1}$ and $k_{-2} = 15 \text{ s}^{-1}$. This set of parameters also fulfils the demands of eqn. (5). Over the entire range of GlcNAc trimer concentrations tested, the stopped-flow records were monophasic and back-extrapolated to the 100% fluorescence mark of free enzyme at zero time. Hence

Fig. 5. Fluorescence record at pH 7.4

The stopped-flow fluorimeter was used to monitor the enhancement of fluorescence in the reaction of $15\text{ }\mu\text{M}$ -lysozyme with $50\text{ }\mu\text{M}$ -GlcNAc trimer in 0.01 M -Tris- 0.10 M -KCl, pH 7.4. Excitation was at 290 nm and the emission observed through the 'band-pass filter'. Two reaction records are superimposed.

Fig. 6. Concentration-dependency of fluorescence data at pH 7.4

Apparent rate constants (k_b) were evaluated in stopped-flow records of the fluorescence enhancement from GlcNAc trimer binding to lysozyme at pH 7.4 and are plotted against the concentration of the trimer. The line drawn is the theoretical curve for eqn. (7) with $K_1 = 170\text{ }\mu\text{M}$, $k_{+2} = 235\text{ s}^{-1}$ and $k_{-2} = 15\text{ s}^{-1}$.

the enhancement of lysozyme fluorescence from the binding of trimer at pH 7.4 may be solely assigned to the conversion of EL into EL*. Thus the data and conclusions of the previous workers are confirmed in experiments run here in parallel with those in which different results were obtained under different experimental conditions.

Fluorescence spectra

It remains to determine why the quenching of the fluorescence of lysozyme on GlcNAc trimer binding

Fig. 7. Fluorescence-difference spectra

Fluorescence-emission spectra were recorded with excitation at 290 nm on both $3.0\text{ }\mu\text{M}$ -lysozyme and $3.0\text{ }\mu\text{M}$ -lysozyme with 0.50 mM -GlcNAc trimer and the former was subtracted from the latter. Difference spectra traces (a), (b) and (c) refer to experiments at pH 7.4, 5.9 and 4.4 respectively.

at pH 4.4 happens within the first step of the binding mechanism whereas the enhancement seen at pH 7.4 occurs in the second step. An explanation of this behaviour was sought by a detailed examination of fluorescence-emission spectra of lysozyme and the lysozyme-trimer complex. At all pH values below 8.0, the binding of GlcNAc trimer to lysozyme causes a blue shift in the peak of the fluorescence emission from 340 to about 330 nm (Shinitzky *et al.*, 1966). However, the difference between the intensities of the fluorescence emission from lysozyme and the lysozyme-GlcNAc trimer complex at their relative peak wavelengths alters as a function of pH, with the complex displaying the higher intensity above pH 5.7 and the free enzyme at lower pH values (Lerher & Fasman, 1967). The difference-emission spectra between the complex and the free enzyme were recorded under the experimental conditions used in these kinetic experiments (Fig. 7). The spectra are slightly different from those of Lerher & Fasman (1967), probably due to the higher excitation wavelength used here. The difference spectra reflect both the wavelength and the intensity perturbations caused by ligand binding and their variations with pH. But the spectra at the three pH values are qualitatively similar in that all three contain a positive limb of fluorescence enhancement at about 320 nm and a negative limb of fluorescence quenching at or above 360 nm .

I propose that the positive limb in the difference-emission spectra between the complex and the free enzyme can be assigned predominantly to an enhancement in the fluorescence of tryptophan-108 of lysozyme and the negative limb to a quenching of tryptophan-62, for the following reasons. First, Imoto *et al.* (1972b) showed by chemical-modification

Fig. 8. Fluorescence records at pH 5.9

The stopped-flow fluorimeter was used to monitor the perturbations of fluorescence in the reaction of $10\mu\text{M}$ -lysozyme with 0.25 mM -GlcNAc trimer in 0.01 M -pyrophosphate-phosphate- 0.10 M -KCl, pH 5.9. Two reaction records are superimposed in each photograph. Excitation was at 290 nm and the emission observed through interference filters; trace (a) at 369 nm for the fluorescence quench; trace (b), at 323 nm for the fluorescence enhancement.

techniques that over 80% of the fluorescence of lysozyme originated from tryptophan-62 and -108. Of these, tryptophan-62 is completely exposed to the solvent whereas tryptophan-108 is partially buried within the apolar interior of the protein (Lee & Richards, 1971). Increasing polarity of the solvent causes a red shift in the emission of indole chromophores (Van Duuren, 1963) and thus the fluorescence from tryptophan-62 would be expected at a longer wavelength than that from tryptophan-108. Secondly, iodide selectively quenches the long wavelength emission of lysozyme (Lerher, 1971) presumably through a collisional quenching mechanism dependent on the accessibility of the different tryptophans to solvent, and thus the long wavelength fluorescence is most likely due to tryptophan-62. The suggestion of Lerher (1971) that the effect of iodide was on tryptophan-108 was made before the discovery of the high quantum yield of tryptophan-62 (Imoto *et al.*, 1972b). Thirdly, in human leukemic lysozyme, a structurally homologous protein to the hen egg-white enzyme (Blake & Swan, 1971), a tyrosine residue has replaced

the tryptophan in the location equivalent to position 62 (Canfield *et al.*, 1971). The difference fluorescence spectrum between the complex of the human enzyme with GlcNAc trimer and the free human enzyme exhibits a positive limb at low wavelengths that is quantitatively similar to that of the hen egg-white enzyme but the negative limb at longer wavelengths is greatly decreased in amplitude and is independent of pH below pH 5.5 (Teichberg *et al.*, 1972; Mulvey *et al.*, 1973).

The observations of fluorescence perturbations in the stopped-flow experiments described above were made with the 'band-pass filter' that transmitted light between 325 and 380 nm . Examination of the fluorescence difference spectra in Fig. 7 between these wavelengths reveals that at pH 7.4 the amplitude of the negative limb is insignificant compared with that of the positive limb and thus the stopped-flow data must refer primarily to the latter enhancement. Conversely at pH 4.4 the insignificance of the positive limb compared with the negative would result in the quenching process being the major contributor to the stopped-flow records. With this spectral discrimination and the above proposals for the distinction between the two tryptophan residues, it is now possible to assign the fluorescence quenching seen in the bimolecular step of GlcNAc trimer binding at pH 4.4 (Fig. 2) to tryptophan-62 and the fluorescence enhancement seen on the subsequent rearrangement of the enzyme-ligand complex at pH 7.4 (Fig. 6) to tryptophan-108.

Fluorescence perturbations at pH 5.9

To test this model for GlcNAc trimer binding to lysozyme, a stopped-flow experiment was carried out at pH 5.9 where the amplitudes of the two limbs in the difference spectrum are approximately equal (Fig. 7). In this experiment, a fixed enzyme concentration ($10\mu\text{M}$) was mixed with a range of ligand concentrations ($15\mu\text{M}$ to 0.40 mM). At each ligand concentration, the reaction was studied twice by using interference filters to analyse both the fluorescence quenching at 369 nm and the fluorescence enhancement at 323 nm . Two stopped-flow traces from one reaction mixture are shown in Fig. 8. In this and every other pair of reactions at one ligand concentration, it was found that the rate of the fluorescence quenching (Fig. 8a) was considerably faster than that of the enhancement (Fig. 8b). The concentration dependencies of the apparent rate constants from both processes were examined and the results reported in Fig. 9.

The kinetics of the quenching reaction observed at pH 5.9 with the 369 nm filter was qualitatively similar to the quenching observed at pH 4.4 (Figs. 1 and 2); the reaction records were again biphasic at low concentrations of trimer and monophasic at higher

Fig. 9. Concentration-dependencies of fluorescence data at pH 5.9

(a) With observation at 369 nm, apparent rate constants (k_a) were evaluated in stopped-flow records of GlcNAc trimer binding to lysozyme at pH 5.9 from the first phase of fluorescence quenching at low concentrations of ligand and the monophasic reaction at high concentrations and are plotted against the concentration of the trimer. The line drawn is the theoretical line for eqn. (6) with $k_{+1} = 1.75 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-1} = 140 \text{ s}^{-1}$. (b) With observation at 323 nm, apparent rate constants (k_b) were evaluated in stopped-flow records of GlcNAc trimer binding to lysozyme at pH 5.9 from the fluorescence enhancement and are plotted against the concentration of the trimer. The line drawn is the theoretical curve for eqn. (7) with $K_1 = 80 \mu\text{M}$, $k_{+2} = 115 \text{ s}^{-1}$ and $k_{-2} = 10 \text{ s}^{-1}$.

concentrations. The apparent rate constants for the quench were evaluated from the fast phase at low ligand concentrations and the single phase at higher concentrations and varied linearly with the concentration of GlcNAc trimer (Fig. 9a). This kinetic behaviour is compatible with the bimolecular component of the two-step mechanism (eqn. 2) and was analysed on eqn. (6) to yield values for the rate constants: $k_{+1} = 1.75 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{-1} = 140 \text{ s}^{-1}$ from which $K_1 = 80 \mu\text{M}$. Likewise the kinetics of the enhancement of fluorescence observed at pH 5.9 with the 323 nm filter was qualitatively similar to the enhancement observed at pH 7.4 (Figs. 5 and 6): the reaction records appeared monophasic at all the concentrations of GlcNAc trimer tested. The apparent rate constants from the fluorescence enhancement varied in a hyperbolic manner with the concentration of the

trimer (Fig. 9b) which is compatible with the unimolecular process in the mechanism of ligand binding (eqn. 2). The concentration-dependency was fitted to the appropriate relationship (eqn. 7) with the kinetic parameters: $K_1 = 80 \mu\text{M}$, $k_{+2} = 115 \text{ s}^{-1}$ and $k_{-2} = 10 \text{ s}^{-1}$. The data from both sets of experiments at pH 5.9 could be accommodated to a single value for K_1 and to the value of K_0 (eqn. 5) determined in equilibrium measurements as $6 \mu\text{M}$.

Thus in the formation of the complex between lysozyme and GlcNAc trimer the quenching of the protein fluorescence at wavelengths above 360 nm occurs before the enhancement of protein fluorescence at wavelengths below 330 nm. This much is unambiguous (Fig. 8) though the assignment of the signals to individual side chains is not proven as such. Energy transfer between the fluorescent tryptophan side chains of lysozyme has been suggested by Imoto *et al.* (1972b) and the validation of this proposal would prevent the assignment of fluorescence perturbations to individual tryptophan residues. However, it is impossible for the perturbation of the long-wavelength fluorescence to originate from a tryptophan residue with short wavelength emission and then be transferred to another tryptophan with long-wavelength emission because, in the kinetic experiments on GlcNAc trimer binding described above, the long-wavelength fluorescence was perturbed before that at the short wavelength.

Conclusions

The interpretation given to the experiments described above may be summarized by:

In n.m.r. experiments on the binding of GlcNAc dimer to lysozyme, Baldo *et al.* (1975) studied the alterations in the chemical shift and the line width of the peak from the *N*-acetyl methyl protons on the reducing saccharide in the presence of the enzyme. The n.m.r. data were interpreted in terms of the two-step model for ligand binding (eqn. 2) and it was concluded that the chemical shift of the ligand in the first complex was not altered beyond experimental error from that of the free ligand whereas the subsequent rearrangement of the enzyme-GlcNAc dimer complex caused a large upfield shift. The upfield shift of the *N*-acetyl methyl protons is considered to be caused by the shielding effect of the indole ring of

Fig. 10. Active-site cleft of lysozyme

A model of the substrate binding site of hen egg-white lysozyme is shown with the permission of Professor D. C. Phillips (Blake *et al.*, 1967*b*). The bound substrate shown here is the hexasaccharide, GlcNAc hexamer: the saccharide residues bound at subsites A, B and C occupy the location of GlcNAc trimer when bound to the protein crystal whereas those in subsites D, E and F were placed there by model building. In the experiments described in the present paper, subsites D-F would not be occupied by ligand.

tryptophan-108 when the ligand is juxtaposed against the face of the ring (Sykes, 1969). Therefore the n.m.r. data on GlcNAc dimer binding show that the ligand approaches tryptophan-108 at an equivalent stage in the binding mechanism to the step in which the fluorescence of tryptophan-108 is perturbed by GlcNAc trimer binding. Although the GlcNAc dimer is a suitable ligand for n.m.r. studies on account of the fast exchange between free and bound forms, it is unsuitable for the type of kinetic experiments on ligand binding in this paper because its reaction is too fast to measure with stopped-flow instrumentation though some temperature-jump experiments on GlcNAc dimer binding were described by Baldo *et al.* (1975).

In the three-dimensional structure of lysozyme (Blake *et al.*, 1967a), tryptophan-62 is located at the top edge of the active-site cleft above subsite B whereas tryptophan-108 is partially buried underneath subsites C and D (Fig. 10). Hence it is possible to visualize in molecular terms the association of the GlcNAc trimer at subsites A-B-C of lysozyme. The first stage of the binding affects the environment of tryptophan-62 but not that of tryptophan-108 and thus presumably takes place across the top of the active-site cleft. Subsequently the ligand could dive into the cleft and only then perturb the fluorescence of tryptophan-108; glutamate-35, which is near tryptophan-108, undergoes the perturbation to its pK_a value at the same stage. The possibility of tryptophan-108 rising towards the ligand is contrary to the crystallographic data (Blake *et al.*, 1967b). However, tryptophan-62 was found by the structure determinations to be located in a different position in the enzyme-ligand complex from that in the free enzyme. It is conceivable that the absence of any detectable perturbation to tryptophan-62 during the rearrangement of the initial complex could be due to the formation of the hydrogen bond between this residue and GlcNAc trimer in the initial complex which would remain intact if tryptophan-62 followed the ligand into the cleft. The hydrogen bonds between aspartate-101 and the saccharides in subsites A and B (Blake *et al.*, 1967b), which presumably generate the pK_a shift in this carboxylate, could not be formed until the second stage of the reaction, as indicated by the proton-release experiments. An interesting feature of this mechanism is that the rearrangement of the complex at pH 4.4 is associated with a standard free-energy change of only -4.9 kJ/mol even though it involves the formation of many of the specific interactions between lysozyme and the GlcNAc trimer.

There exist some minor divergences between the rate constants measured for GlcNAc trimer binding in this work and those derived in previous studies. For instance, values for k_{+2} of 115 s^{-1} and 220 s^{-1} were obtained here at pH 5.9 and 7.4 respectively (Figs. 9b and 6) whereas Holler *et al.* (1969, 1970)

evaluated k_{+2} at 240 s^{-1} and 400 s^{-1} in experiments at pH 6.3 and 7.0. These differences are probably due to the variations in experimental conditions such as ionic strength and temperature. A more significant discrepancy in this work is the value obtained for k_{+1} at $1.5 \times 10^6\text{ M}^{-1}\cdot\text{s}^{-1}$ though second-order rate constants for enzyme-ligand combinations are normally diffusion-controlled and of the order of $5 \times 10^7\text{ M}^{-1}\cdot\text{s}^{-1}$ (Gutfreund, 1971). One possibility is that there is a range of initial complexes, only one of which quenches the fluorescence of tryptophan-62 and proceeds through to the final complex.

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