# *ON THE BINDING OF CHITIN OLIGOSACCHARIDES TO LYSOZYME**

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In enzyme reactions, the binding of substrates to a specific site on the enzyme surface plays a major role in determining the specificity of the catalytic reaction. This phenomenon of binding is probably the least understood of the complex series of interactions involved in enzymic catalysis. In the Michaelis-Menten scheme of enzyme kinetics[1] the quantity $K_m$ is considered a measure of the binding process. It is desirable, however, to have independent methods of studying binding of substrates and inhibitors to enzymes. Such a study is possible if interaction between an enzyme and a substrate results in a perturbation that can be quantitatively determined. In this respect it is of interest that Hayashi *et al.*[2] have shown that lysozyme (E.C. 3.2.1.17) displays a red shift in its ultraviolet spectrum on interaction with a substrate (glycol chitin) or an inhibitor (N,N′-diacetyl chitobiose). The spectral red shift has been associated with a change in the environment of at least one tryptophanyl residue. From solvent perturbation measurements[2, 3] it has been proposed that such a red shift in a tryptophane spectrum is consistent with its removal from an exposed, solvent-accessible region to a less polar environment.

There is ample evidence for the occurrence of tryptophanyl residues at the binding site of lysozyme. The recent structure determination for the enzyme in the crystalline state[4] and the identification of the region of the enzyme to which inhibitors bind[5] by X-ray analysis methods have shown that three tryptophanes occur at the binding site. Chemical evidence[6–8] also confirms the presence of tryptophane residues in this region. It is thus a reasonable assumption that the tryptophane(s) responsible for the observed red shift,[2] on interaction of lysozyme with substrates and inhibitors, is intimately related to the binding site and that a study of this phenomenon should provide some insight into the binding process. In the present communication, we report the application of this spectral change for the quantitative measurement of binding equilibria of substrates and inhibitors to lysozyme.

From initial studies it became clear that the magnitude of the difference spectrum was related to the amount of inhibitor added to a constant amount of enzyme, and was in fact a measure of the concentration of enzyme-substrate complex formed. This observation provides us with a method for calculation of the binding constants for inhibitors and substrates to lysozyme.

*Materials and Methods.*—Lysozyme was purchased from Sigma Chemical Co. (lot #105B 8700). Chitin oligosaccharides were prepared by acid hydrolysis of chitin (California Corporation for Biochemical Research) followed by chromatography according to Rupley.[9] These saccharides were further purified by gel filtration.[10]

All difference spectra were measured in a Cary model 14 spectrophotometer, by the method of Williams *et al.*,[11] using a 0–0.1 absorbance slidewire assembly at 25°C ± 0.1°C. All enzyme concentrations were 0.36 mg/ml ($2.48 \times 10^{-5}$ *M*) and had an absorbance of 0.95 at 280 mμ. Substrate concentrations were varied from $5 \times 10^{-3}$

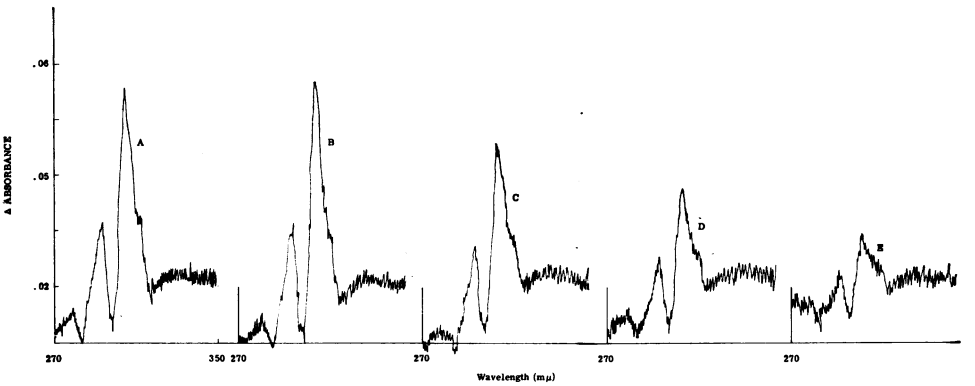


FIG. 1.—Difference spectra of lysozyme in the presence of chitotriose at pH 5.0, 25°C ± 0.1 showing maximum difference ($\Delta A$) at 293.5 m$\mu$. Lysozyme concentration was $2.48 \times 10^{-5}$ $M$. Chitotriose concentration was $2.56 \times 10^{-3}$ $M$ in ($A$), $1.28 \times 10^{-3}$ $M$ in ($B$), $4 \times 10^{-5}$ $M$ in ($C$). $2 \times 10^{-5}$ $M$ in ($D$), $1 \times 10^{-5}$ $M$ in ($E$).

$M$ to $1 \times 10^{-6}$ $M$. The phosphate buffers employed were constant in sodium ion concentration (0.1 $M$), and the total phosphate concentration was constant at 0.01 $M$. All pH measurements were made with a Sargent model DR pH meter at 25°C ± 0.1°C.

*Results and Discussion.*—An example of the difference spectrum obtained on interaction of lysozyme and chitotriose at pH 5.0 is shown in Figure 1$A$. The maximum difference was obtained at 293.5 m$\mu$. Figures 1$A$–$E$ show how the magnitude of the difference spectrum changed with decreasing chitotriose concentration while keeping the lysozyme concentration constant. This variation of peak height with substrate concentration amounts to a titration curve of the enzyme.

The region of high substrate concentration, where the magnitude of the difference spectrum remains constant, corresponds to saturation of the enzyme with substrate. The ratio of peak height ($\Delta A_0$) at saturation to the peak height ($\Delta A$) at some other substrate concentration is a measure of the amount of enzyme-substrate complex, $ES$, at that substrate concentration.

$\Delta A/\Delta A_0 = [ES]/[E^0]$, where $[E^0]$ is the total enzyme concentration, so that the concentration of $ES$ can be calculated. The amount of free substrate present can be estimated, since $[S] = [S^0] - [ES]$.

The following scheme was employed for calculation of the binding constant $K_s$.

$$E + S \rightleftharpoons ES$$

$$K_s = \frac{[E][S]}{[ES]}$$

$$\log K_s = \log \frac{[E]}{[ES]} + \log [S]$$

A plot of log $[S]$ versus log $[ES]/[E]$ gives a line with an intercept of $-\log K_s$ or $pK_s$. Such a plot for binding of chitotriose to lysozyme at pH 5.5 is portrayed in Figure 2 which shows that the slope equals one, indicating one molecule of chitotriose binding to the enzyme. The dissociation constant as determined from this

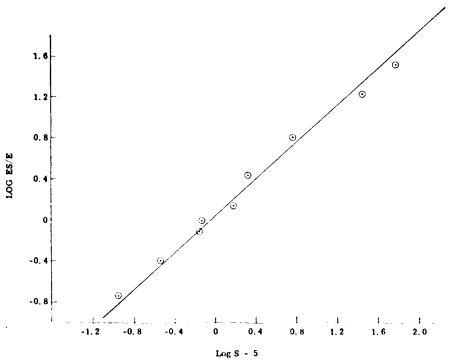


FIG. 2.—Plot of data obtained from difference spectra of lysozyme in the presence of chitotriose at pH 5.5. Lysozyme concentration was $2.48 \times 10^{-5}$ $M$. Chitotriose concentration varied from $2.56 \times 10^{-3}$ $M$ to $5 \times 10^{-6}$ $M$.

TABLE 1

BINDING CONSTANTS OF OLIGOSACCHARIDES TO LYSOZYME

| Oligosaccharide | $K_s$, pH 5.0, $\mu$ = 0.1 $M$ (dissociation) |
|---|---|
| N-Acetyl glucosamine | $4\text{–}6 \times 10^{-2}$ $M$ |
| Chitobiose | $1.75 \times 10^{-4}$ $M$ |
| Chitotriose ($\mu$ = 0.1 $M$) | $6.58 \times 10^{-6}$ $M$ |
| ($\mu$ = 0.2 $M$) | $1.08 \times 10^{-5}$ $M$ |
| ($\mu$ = 0.3 $M$) | $7.75 \times 10^{-6}$ $M$ |
| Chitotetraose | $9.45 \times 10^{-6}$ $M$ |
| Chitopentaose | $9.35 \times 10^{-6}$ $M$ |
| Chitohexaose | $6.15 \times 10^{-6}$ $M$ |

plot is $6.58 \times 10^{-6}$ $M$. Table 1 shows the results of similar determinations on a series of chitin oligosaccharides. The results show that the minimal structure necessary for good binding is that of the trisaccharide N,N′,N″-triacetylchitotriose.

Since chitotriose and the higher oligosaccharides are substrates for lysozyme, it was necessary to estimate the extent of any hydrolysis undergone while the dissociation constants were being determined. We have been able to show, by measurement of the increase of saccharide-reducing groups,[15] that less than 0.2 per cent hydrolysis of chitotriose occurred during the spectral determinations. For the tetrasaccharide approximately 1.0 per cent hydrolysis was observed. In contrast to this negligible degradation, the penta- and hexasaccharides were cleaved to the extent of 20 per cent and 50 per cent, respectively. From these results it is obvious that the dissociation constants for the penta- and hexasaccharides represent values obtained from mixtures of saccharides. It cannot be concluded, therefore, that the binding strength of the penta- and hexasaccharides is equal to that of the trisaccharide and the tetrasaccharide.

The effects of ionic strength on the binding process were determined at pH 5.0 using tri-N-acetyl chitotriose as a substrate, and the results which are shown in Table 1 indicate no significant changes on increasing the ionic strength of the buffers used.

The determinations of $K_s$ show that only one substrate molecule per lysozyme molecule is bound at that site which is responsible for the difference spectrum. This does not exclude the possibility of binding at other sites with no change in light absorption. Since the spectral change is believed to be due to a perturbation of the tryptophane spectrum, we conclude that the region of the lysozyme molecule, where the tryptophane(s) whose absorption spectrum has been perturbed occurs, includes only one binding site. Furthermore, the stoichiometry of the reaction with all substrates studied requires that this binding site which we are studying be the strongest binding site on the enzyme. Equilibrium dialysis studies to determine the total number of binding sites on the enzyme are currently under way.

The determination of the dissociation constant using chitotriose as a substrate

has been extended to include several pH values, and the results plotted as $-\log K_s$ ($pK_s$) versus pH, as recommended by Dixon,[12, 13] are shown in Figure 3. Dixon[12, 13] has given a theoretical treatment of the effects of pH on substrate (or inhibitor) binding to enzymes, and has devised a set of rules for their interpretation. By application of these rules to the data shown in Figure 3, we can say that two ionizable groups on the enzyme are perturbed by the presence of the trimer substrate. The $pK_a$ values involved are one of 4.2 for a group on the enzyme which is perturbed to a value 3.55 on the enzyme-substrate complex and a second group of $pK_a$ 5.8 on the enzyme which is changed to a value of 6.25 on the enzyme-substrate complex. The values observed suggest that the group of $pK_a$ 4.2 on the enzyme is probably a carboxyl side chain of aspartic or glutamic acids. The group of higher $pK_a$ value (5.8) is consistent with assignment to the imidazole group of histidine or possibly a carboxyl group of abnormally high $pK_a$. In this respect it is of interest that the structure of lysozyme in the crystalline state, as deduced by X-ray analysis,[4] shows that several aspartyl side chains occupy positions at the binding site of the enzyme, while the single histidine in the molecule occurs at a position far removed from the binding site. The titration data of Scheraga[14] on lysozyme indicate a carboxyl group of $pK_a$ 6.3, which is abnormally high. These results indicate that the second group on the enzyme ($pK_a$ = 5.8), which is perturbed on interaction with chitotriose, may also be a carboxyl group. However, a configuration change caused by substrate binding could also cause a change in $pK_a$ of a distant imidazole, as discussed below.

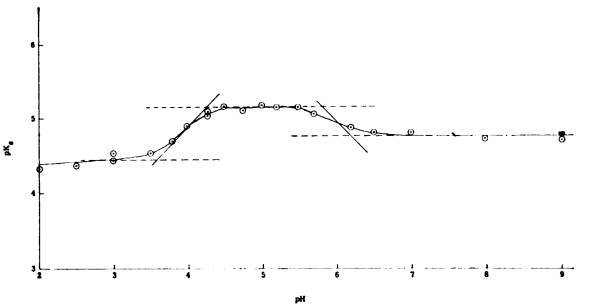

FIG. 3.—Plot of $-\log K_s$ ($pK_s$) for chitotriose and lysozyme with pH.

The theory of Dixon[12, 13] states that if one ionic form of an enzyme binds substrate more strongly than another, there will be proportionally more of this form present in the enzyme-substrate complex than in the free enzyme. This amounts to a change in the ionization constant for the ionizable group(s) involved. This does not require that the perturbed group(s) be actually involved in the binding process, but rather that the binding is affected by the ionization of these groups. It could be a result of configuration changes which involve ionizable groups some distance from the binding site. Further studies directed toward identification of the nature of the ionizable group of $pK_a$ 5.8 on lysozyme, which is disrupted on binding of the trisaccharide, are being carried out.

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