# LABELLING OF THE CATALYTIC SITE OF LYSOZYME[1]

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SUMMARY

A method for direct measurements of the binding of N-acetyl glucosamine polymers to the catalytic site of lysozyme is described. The method utilizes the dye Biebrich scarlet for which the enzyme has a single binding site with a dissociation constant of 0.3 mM. The data indicate that the trimer and hexamer of N-acetyl glucosamine do not displace the dye from the unproductive binding site of lysozyme to which they both bind with a dissociation constant of about 10 μM. The two sugars competitively displace the dye from the catalytic site of the enzyme, allowing one to differentiate between productive and unproductive binding of these two substrates. Spectrophotometric determinations of the concentrations of the enzyme-dye complex in the presence of various concentrations of substrate gave productive binding dissociation constants for the trimer and hexamer of 20 mM and 5 μM respectively.

Crystallographic data of Phillips and co-workers (Blake, Johnson, Mair, North, Phillips and Sarma, 1967) show a cleft in the lysozyme molecule that can accommodate six pyranose rings of the substrate--N-acetylglucosamine (GlcNAc) polymers-- at sites designated A, B, C, D, E and F. Available experimental results (Blake, Johnson, Mair, North, Phillips and Sarma, 1967; Rupley, 1967; Sharon, 1967; Canfield, 1963; Jollès, Jauregui-Adell, Bernier and

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Jollès, 1963; Rupley and Gates, 1967; Rupley, Butler, Gerring, Hartdegen and Pecararo, 1967; Vernon, 1967; Blake, Mair, North, Phillips and Sarma, 1967) indicate that the predominant mode of binding of all GlcNAc polymers to lysozyme is an unproductive mode, to sites A-C of the enzyme, that is crystallographically observed for the trimer. A method for direct measurements of the binding of GlcNAc polymers in a productive mode, to sites D-F of the enzyme, is presented here. The method utilizes the reversible, competitive binding of the dye Biebrich Scarlet (BS) to the active site of the enzyme, and specifically to the active region of the enzyme molecule. Our measurements with BS suggest that the hexamer, which is the highest GlcNAc polymer that can fit into the cleft of the lysozyme molecule, binds in a productive mode to about the same extent that it and smaller polysaccharides bind in the unproductive mode.

The use of a chromophoric inhibitor in binding studies has had previous application in investigations of the individual steps in chymotrypsin-catalyzed reactions (Bernhard, Lee and Tashjian, 1966; Bernhard and Gutfreund, 1965; Brandt and Hess, 1966; Brandt, Himoe and Hess, 1967). The dye BS, which was known to be an inhibitor of chymotrypsin (Glazer, 1967), was observed to bind to lysozyme, and was subsequently found to be usable as a competitive inhibitor in the present experiments with lysozyme.

Equilibrium measurements of difference spectra between solutions of BS and solutions of BS plus excess enzyme permitted evaluations of the dissociation constant pertaining to the BS-lysozyme complex. A Hildebrand-Benesi plot (Benesi and Hildebrand, 1949) (Fig. 1) yielded for the dissociation constant of the dye-lysozyme complex the value $K_D/n = 0.3$ mM, where n is the number of binding sites. That n = 1 was determined by a Job plot (Job, 1928) (Fig. 1 inset) of absorbance at various ratios of initial dye ($D_0$) and enzyme ($E_0$) concentrations, with $D_0 + E_0$ = constant.

Evidence that BS is a competitive inhibitor in reactions catalyzed by lysozyme is given in Fig. 2, which shows Lineweaver-Burk plots (Lineweaver

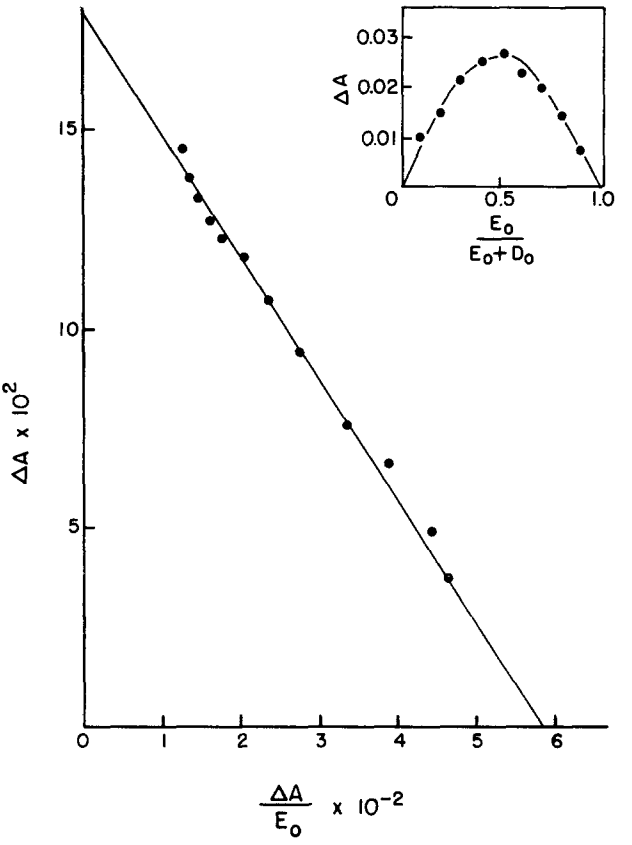


Fig. 1

Hildebrand-Benesi plot for determination of the dissociation constant of the complex between BS and lysozyme. $\Delta A$ represents the magnitude of the difference spectrum between enzyme-dye complex and dye, measured as difference in absorption at two wavelengths: $A_{565} - A_{500}$. (A typical difference spectrum is shown in Fig. 3, solid line.) The concentration of dye was held constant (0.025 mM) and was smaller than the concentration of enzyme (1.1 to 0.08 mM). Inset, Job plot of $\Delta A$ at various ratios of dye and enzyme concentrations, with $\overline{E_0 + D_0}$ = 0.1 mM.

These and all other spectrophotometric measurements were made with a Cary 14 automatic recording spectrophotometer at 25°. Experimental solutions were of pH 7.6, buffered with phosphate, and had ionic strength of 0.2. Egg white lysozyme, batches LYS D7CA and LY 71A, was obtained from Worthington. Stock solutions containing 70 mg of protein per ml of buffer solution were clarified by centrifugation. BS, purchased from K and K Laboratories, was recrystallized from N,N-dimethyl formamide-ether to yield a product about 75% pure as estimated by spectral measurements with use of an extinction coefficient of $\varepsilon_{max}$ = 30,000 $M^{-1}$ $cm^{-1}$ (Glazer, 1967). This preparation was found to show maximum light absorption at 505 mμ, and to follow Beer's law up to a concentration of about 0.1 mM. It was observed that unless the dye is adequately purified, an impurity with absorption properties similar to those of BS also binds to lysozyme.

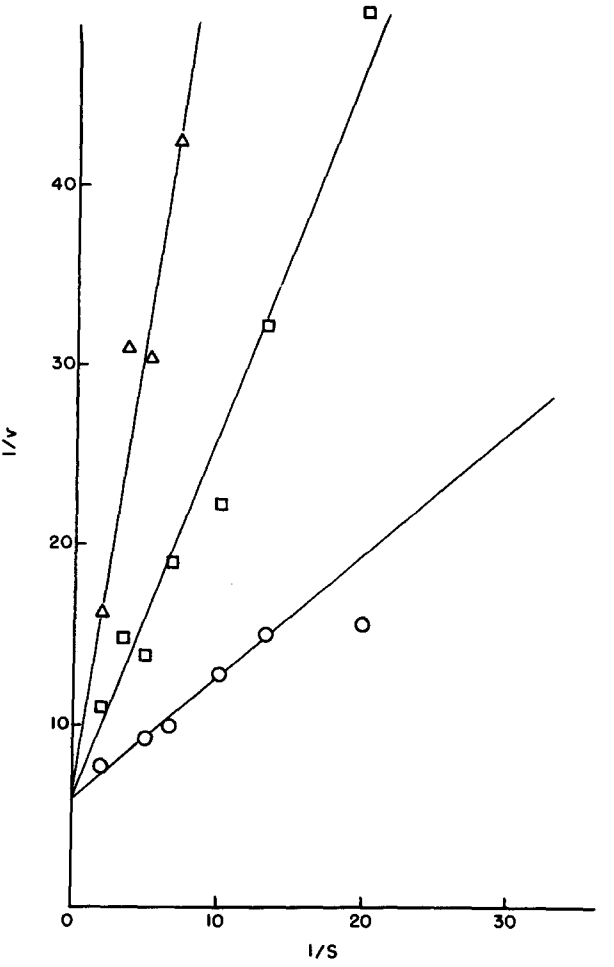


Fig. 2

Steady state reaction rates of lysozyme-catalyzed hydrolysis of cell wall material at pH 7.6 and 25°, showing inhibition effect of BS. Data are plotted according to the Lineweaver-Burk method. The reciprocal of initial reaction velocity (v), measured as rate of change of turbidity at 700 mμ, is plotted against the reciprocal of substrate concentration (S). O, no BS; □, 0.37 mM BS; Δ, 0.92 mM BS. All solutions contained 0.27 μM lysozyme. Substrate was dried cell walls of Micrococcus lysodeikticus (Mann); concentration in experimental solutions was in the range 11 μg/ml to 225 μg/ml. Units in the graph are arbitrary. $K_D$, the enzyme-dye dissociation constant, is calculated from the expression $R = 1 + (D/K_D)$, where R is equal to the ratio of the slopes of the lines obtained in the presence and the absence of dye.

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and Burk, 1934) of steady state rates observed for the lysozyme-catalyzed hydrolysis of cell walls of Micrococcus lysodeikticus in absence and presence of dye. The identical ordinate intercepts demonstrate that BS is a

competitive inhibitor. The apparent inhibition constant calculated from the slopes of these plots, approximately 0.2 mM, is in reasonable agreement with the value of 0.3 mM obtained from equilibrium measurements (Fig. 1). These experiments, together with the data shown in Fig. 3, indicate that BS binds to the catalytically active region of the cleft in the lysozyme molecule, and not to the A-C site. The experiments shown in Fig. 3 indicate that the binding of BS to the enzyme is not appreciably affected by the presence of tri-GlcNAc at concentrations known (Sharon, 1967; Rupley et al., 1967) to saturate the predominant (unproductive) binding site, A-C.

Measurement of the extent to which GlcNAc polymers displace BS from the enzyme is provided by difference spectrum measurements such as shown in Fig. 3 Evaluation of dissociation constants from these data was made on the basis of a model that is consistent with the available chemical and crystallographic evidence. According to this model, significant binding of the trimer to the reactive D-F site can only be observed after the A-C site is saturated.

Calculations of trimer binding were made according to the scheme:

$$\text{(I)}\quad E + S \overset{K_U}{\rightleftharpoons} EU + S \overset{K_S}{\rightleftharpoons} EUS \xrightarrow{k_{cat}} E + \text{products}; \qquad EU + D \overset{K_D}{\rightleftharpoons} EUD$$

It is assumed that the unproductive binding of trimer to the A-C site does not influence the binding of trimer or BS to the D-F site. EU represents the unproductive lysozyme-substrate complex, and $K_U$ the dissociation constant pertaining to this complex. $K_D$ and $K_S$ are the lysozyme-dye and the productive lysozyme-substrate dissociation constants, respectively. When $S_0 > K_U$, the only lysozyme complexes that need to be considered in the calculations are the ternary complexes EUD and EUS, which involve either the dye or the substrate bound to the productive site of lysozyme, D-F. When $S_0 \gg E_0 > D_0$, $K_S$ can be calculated from:

$$EUD = E_0\ (D_0 - EUD)\left[K_D\left(1 + \frac{S_0}{K_S} + \frac{D_0 - EUD}{K_D}\right)\right]^{-1}$$

This was found to be $2 \times 10^{-2}$ M (see legend of Fig. 3). This value is to be compared with a previously measured dissociation constant of $\sim 5\ \mu M$, pertaining

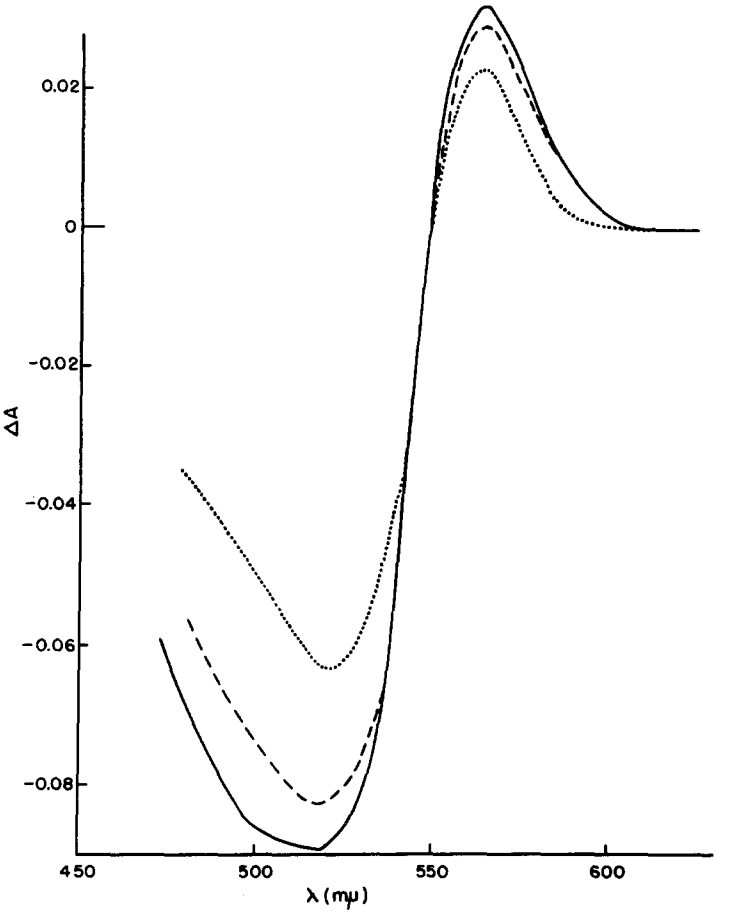


Fig. 3

Difference spectra between BS and (1) ____, BS-lysozyme; (2) ----, BS-lysozyme-tri-GlcNAc; (3) ...., BS-lysozyme-hexa-GlcNAc. Concentrations were 0.05 mM BS, 0.18 mM lysozyme, and 5 mM polymer. The sugars were prepared according to a previously published method (Rupley, 1964). For other experimental details see Fig. 1 legend.

The fraction of dye that remains bound to the enzyme in the presence of excess polysaccharide is measured by the ratio of the magnitudes of difference spectra in the presence and in the absence of polysaccharide: $A_{565} - A_{500}$ (presence of polymer) divided by $A_{565} - A_{500}$ (absence of polymer). The concentration of enzyme-dye complex in absence of polysaccharide and under conditions $E_0 > D_0$ can be calculated from the relation $ED = E_0D_0/(E_0+K_D)$, with the use of known initial concentrations of dye and enzyme, and the known value of $K_D$, the enzyme-dye dissociation constant. The total concentration of dye-containing complexes in the presence of substrate can thus be determined and used in the calculation of $K_S$ as described in the text.

A possible interference due to complex formation between dye and polysaccharides was ruled out by difference spectrum measurements which were the same for enzyme-dye-polymer against dye and for enzyme-dye-polymer against dye-polymer.

The table shows a summary of the data obtained in a series of experiments such as illustrated in the figure.

| Polymer | Concentration (mM) | $\frac{[A_{565} - A_{500}] \text{ (substrate present)}}{[A_{565} - A_{500}] \text{ (substrate absent)}}$ | $K_S$ (M) |
|---|---|---|---|
| Hexa-GlcNAc | 5 | 0.078 | $5 \times 10^{-6}$ |
| Tri-GlcNAc | 5 | 0.09 | $2 \times 10^{-2}$ |

Experiments performed at different polymer concentrations (in the range 1 to 10 mM for the trimer and 1 to 5 mM for the hexamer) yielded the same average values for $K_S$.

to unproductive binding at sites A-C (Rupley et al., 1967).

Independent evidence for the mechanism shown in (I), and independent measurement of the binding constants are provided by the experiments shown in Fig. 4 inset. The addition of trimer to solutions of lysozyme is accompanied by hydrogen ion uptake from solution at low trimer concentrations; at higher trimer concentrations, a hydrogen ion release is superimposed. A plot of $[\Delta H^+]/E_0$ against $[\Delta H^+]/E_0S_0$ (Fig. 4) gives the lysozyme-substrate dissociation constant, provided that $[\Delta H^+]$ is a result of substrate binding (McConn, Ku, Odell, Czerlinski and Hess, 1968). The uptake of hydrogen ion at low substrate concentration gives a dissociation constant of $1 \times 10^{-5}$ M, in excellent agreement with the dissociation constant of the trimer bound to the A-C site of lysozyme. The hydrogen ion release at high substrate concentration yields a value of $1 \times 10^{-2}$ M, in excellent agreement with the data obtained with BS.

In the case of the hexamer, calculations were made according to the scheme

$$\text{(II)} \qquad EU \overset{K_U}{\rightleftharpoons} E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + \text{products};$$

$$E + D \overset{K_D}{\rightleftharpoons} ED; \qquad E + S + D \overset{K_D K_U}{\rightleftharpoons} EUD$$

In these equations, EU represents unproductive complex between lysozyme and hexamer, with binding only at the A-C sites; ES is productive complex, with binding to A-F sites; ED is enzyme-dye complex; and EUD is a ternary complex in which the saccharide is bound to the unproductive site. (A ternary complex

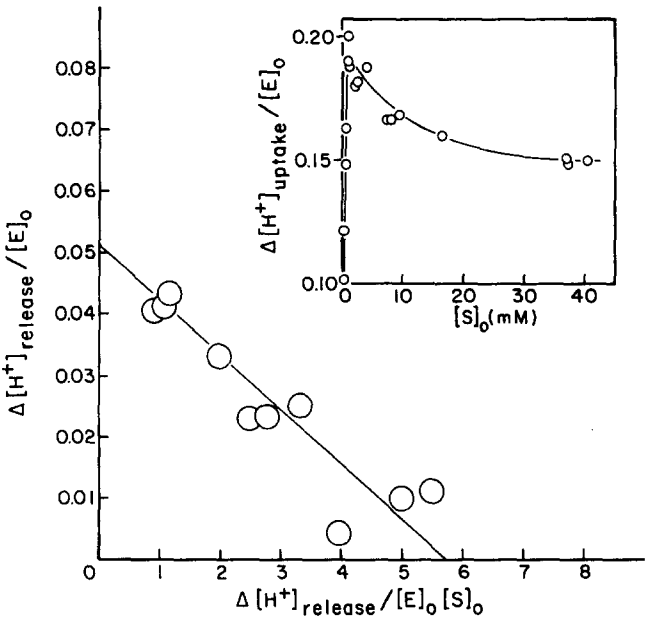


Fig. 4

Change in hydrogen ion concentration upon addition of GlcNAc trimer to lysozyme solutions at pH 6.5 and 25°. The method used in obtaining these data has been described in detail (McConn et al., 1968). Initial concentration ranges are 1 mM to 40 mM substrate and 0.2 mM to 0.5 mM enzyme. Inset, proton uptake as a function of substrate concentration. The maximum uptake occurred at a substrate concentration of ~1 mM; the presence of additional substrate resulted in less proton uptake. This decrease in proton uptake, expressed as a release of proton with reference to the maximum uptake value at $S_0 \cong 1$ mM, is plotted in the form of an Eadie plot in the main part of the figure. The slope of the line gives a $K_S$ value of $1 \times 10^{-2}$ M, in excellent agreement with the $K_S$ value for trimer obtained by use of BS.

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ESD cannot be formed, because according to the evidence presented in this paper, BS occupies the catalytically active part of the cleft.) It is assumed that the presence of dye bound to the catalytically active site does not affect the binding of hexamer to sites A-C, and vice versa; therefore, $K_D$ refers to the binding of dye in both the ED and EUD complexes. The value assumed for $K_U$ was obtained from previous measurements of Rupley et al. (1967), who considered $K_U$ to be much smaller than $K_S$ and approximately equal to 5 μM. $K_S$, pertaining to the productive binding of hexamer, was calculated from an expression based on the mechanism give above, for initial conditions $S_0 \gg E_0 > D_0$:

$$ED + EUD = E_0 \left[D_0-(ED+EUD)\right]\left(1+\frac{S_0}{K_U}\right)\left\{K_D\left(1+\frac{S_0}{K_U}\right)+\frac{S_0}{K_S} + \left[D_0-(ED+EUD)\right]\left(1+\frac{S_0}{K_U}\right)\right\}^{-1}$$

The quantity (ED+EUD), which represents the total concentration of dye-containing complexes in the enzyme-dye-hexamer system, was obtained as described in the legend of Fig. 3. The average value of $K_S$ calculated for hexa-GlcNAc is $\sim$5 μM (see Fig. 3 legend), which is very close to the previously estimated value of $K_U$, the dissociation constant for the unproductive complex (Rupley et al., 1967). These results indicate that hexa-GlcNAc binds productively about as strongly as it and smaller polysaccharides bind unproductively to sites A-C.

These experiments with BS·provide a modified view of the mechanism of the lysozyme-catalyzed reaction. A previous interpretation (Rupley and Gates, 1967) is that only a small amount of substrate is productively bound to the enzyme at any given time, but that this amount is hydrolyzed very rapidly. We interpret the data presented here as evidence that the hexamer of GlcNAc binds to give a relatively high proportion of productive complex. The ability to differentiate between productive and unproductive enzyme-substrate complexes, made possible by use of this particular dye, opens to investigation the individual steps in lysozyme-catalyzed reactions, a kind of study that has invariably been found necessary for an understanding of even simple reaction mechanisms.

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