The Binding of Oligosaccharides Containing N-Acetylglucosamine and N-Acetylmuramic Acid to Lysozyme

THE SPECIFICITY OF BINDING SUBSITES*

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SUMMARY

The association constants with lysozyme of several saccharides containing N-acetyl-D-glucosamine and N-acetylmuramic acid, have been determined by fluorescence spectroscopy or, in one case, by equilibrium dialysis experiments. The association data may be understood on the assumption that the active site of lysozyme consists of a number of subsites binding individual 2-acetamido-2-deoxyglucopyranose rings. On the basis of these data, we have drawn detailed conclusions about the specificity of the individual subsites and their contributions to the total binding energy of oligosaccharide substrates and inhibitors. These conclusions are consistent with, and offer further support of, the mechanism of lysozyme action deduced by Phillips and his coworkers on the basis of x-ray crystallographic studies.

The nature of the interactions leading to binding of a substrate molecule to a specific site on an enzyme is one of the major aspects of the general problem of the mechanism of enzyme action. The variation of binding constants with systematic changes in structure within a series of related substrates and their analogues may provide considerable information about such interactions. Studies of this kind are particularly valuable in the case of hen's egg white lysozyme, for which the tertiary structure of the active site, and indeed, the position of binding within the site for a few small molecules, have been determined by the x-ray crystallographic studies of Blake et al. (1), Johnson and Phillips (2), Phillips (3), and Blake et al. (4).

METHODS

The enzyme used was hen's egg white lysozyme (recrystallized twice, salt free, Lot LYSF638) from Worthington. Paper chromatography was carried out by the descending

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technique with either Whatman No. 1 or No. 3 filter paper with the use of the following solvent systems: 1-butanol-acetic acid-water (25:6:25, upper phase), Solvent I; and 1-butanol-pyridine-water (2:2:1), Solvent II. Paper electrophoresis was carried out under a constant voltage of 50 volts per cm at pH 6.5 (1.2 M pyridine-acetic acid buffer).

The cell wall tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc and dihexa saccharide GlcNAc MurNAc were isolated from *M. lysodeikticus* cell walls as described by Sharon (5, 10) and further purified by preparative paper chromatography in Solvent I. GlcNAc-MurNAc-GlcNAc-MurNAc was prepared by digesting the cell wall tetrasaccharide with lysostaphin (11). It was isolated and purified by preparative paper electrophoresis. MurNAc-GlcNAc-MurNAc (12) was a gift of Dr. J. M. Ghuysen. The tetrasaccharide pentapeptide (GlcNAc-MurNAc-GlcNAc-MurNAc with the terminal muramic acid linked by an amide bond to L-Ala-n-Lys-n-AlaCOOH) was obtained from *M. Zysodeikticus* cell walls as previously described (13).

N-acetyl-glucosamine (Pfizer) was recrystallized from ethanol-acetone to melting point 202-203°. The N acetyl-glucosamine oligomers, GlcNAc-GlcNAc and (GlcNAc)s were isolated by the method of Rupley (14) from an acid hydrolysat e of chitin, and further purified by paper chromatography (Solvent I).

The dimethyl ester of GlcNAc-MurNAc-GlcNAc-MurNAc was prepared by methylation of the tetrasaccharide. The tetra saccharide (80 mg) was dissolved in dry methanol (50 ml) and dry ethereal diazomethane solution was added until a yellow color remained in the mixture. The mixture was evaporated to dryness under reduced pressure at room temperature, the gummy residue was redissolved in methanol, and the solution was filtered. A sample of the filtrate was chromatographed on paper with Solvent I and the paper was treated with the alkali reagent for N-acetylhexosamines (15). One strong fluorescent spot of a material migrating twice as fast as the starting compound, as well as other faint spots, were seen. No spot corresponding to the starting compound could be detected. The material corresponding to the major new spot was isolated by preparative paper chromatography. A yield of 55 mg of lyophilized white powder was obtained.

The product was found to be homogeneous on paper chromatography with both Solvent I and Solvent II when spots were revealed with alkali (15) or silver nitrate (16). It did not migrate on paper electrophoresis at pH 6.5. The identification of the methylation product as the dimethyl ester of GlcNAc-MurNAc-GlcNAc-MurNAc was further supported by its digestion by lysozyme to yield a product identical with authentic GlcNAc-MurNAc methyl ester. The latter compound was prepared from GlcNAc-MurNAc by methylation with diazomethane in a manner similar to that described above for the tetra saccharide.

The proton magnetic resonance spectra of the dimethyl ester of GlcNAc-MurNAc-GlcNAc-MurNAc and of the methyl ester of GlcNAc-MurNAc in D_{2}O are essentially identical in the high field region, and differ from the spectra of the parent free acids only in the addition of a sharp peak at 6.26H due to the methyl ester protons, superimposed over the complex group due to the methylene protons at C-6. The areas of this grouping, the acetamido methyl peak at 7.83r, and the lactyl methyl doublet (0.7 5 ppm) at 8.53r, are in the ratio 7:6:3, as is expected.

All saccharides used were tested for purity by paper chromatography (Solvent I) and paper electrophoresis. Spots were revealed with the alkali reagent for N-acetylhexosamines (15). Each material used showed only a single spot in these tests.

**Fluorescence Measurements**—Fluorescence measurements were made with the Aminco-Keir spectrophotofluorimeter. For determinations of association constants the illuminating wavelength was 285 mÅ. In experiments with the saccharides which contained muramic acid, the fluorescence emission intensity of lysozyme at 370 mÅ was measured, as it was found that the greatest change in emission intensity on binding occurs at this wavelength. In experiments with GlcNAc and its oligomers, the fluorescence emission intensity of lysozyme was measured at 325 mÅ.

Saccharide solutions in buffer were prepared by weighing out the saccharides (dried over P_{2}O_{5}) and the concentrations were checked by the Park-Johnson reducing sugar test (17) in the cases in which very small amounts were weighed out. In the case of acid saccharides, the pH of the solution was brought back to that of the buffer by addition of 1 M NaOH, so that the pH of the buffer and of the saccharide solution were within 0.05 unit of one another.

Solutions of lysozyme and saccharide were prepared in matched 1-cm square quartz fluorescence cells, by mixing 0.100 ml of enzyme solution (0.4 to 1.2 mg per ml), 0.005 ml to 0.30 ml of saccharide solution, and buffer, to bring the volume to 1 ml. The fluorescence intensities of such solutions relative to that of a reference solution containing only enzyme and buffer were immediately determined. The absorption at 980 mÅ and the fluorescence at the appropriate wavelengths of solutions of the saccharide alone in buffer were also measured, and were used to correct the observed fluorescence of the lysozyme-saccharide solutions. At the highest saccharide concentration used, such corrections were less than 10% of the total change in the lysozyme fluorescence.

The relative fluorescence intensity of lysozyme saturated with saccharide, F_{∞}, was extrapolated from the experimental data by plotting 1/(F_{0}-F) against 1/[S] where F is the measured fluorescence of a solution containing the enzyme with a given substrate concentration [S] and F_{0} is the fluorescence of a solution of enzyme alone. The highest concentration of saccharide used was always such that the enzyme was at least 85% saturated.

**Equilibrium Dialysis Experiments**—Tritium-labeled GlcNAc-MurNAc was prepared by the Wilzbach tritium recoil labeling technique (18) and purified by anion exchange resin chromatography, and paper chromatography. The preparation used had a specific activity (counted in Bray's scintillation solution (19) on a Tri-Carb scintillation counter) of 6.1 × 10^{6} cpm per mmole. It yielded a single symmetric peak of radioactivity on paper chromatography in Solvents I and II and on paper electrophoresis at pH 6.5.

On Dowex 1-X 8 (20) it chromatographed as a single peak with constant specific activity.

Technilab model 1E dialysis cells (Technilab Instruments, Los Angeles, California) with membranes of Visking cellulose dialysis casing were used for the equilibrium experiments. The cells were rotated on a Technilab rotating device, in a controlled temperature room at 25°. One milliliter of a 0.20 M acetic acid-ammonium acetate buffer solution, pH 5.30, was introduced into one side of the cell and 1 ml of lysozyme solution (30 mg per ml) in the same buffer was introduced into

2 D. M. Chipman, J. J. Pollock, and N. Sharon, manuscript in preparation.
the other side of the cell. From 15 to 100 μl of a 0.004 M GlcNAc-
MurNAc-3H solution was added to one or the other side of the
cell. The cells were then closed and allowed to equilibrate for
16 hours. Preliminary experiments indicated that the half-
time for equilibration of the saccharide concentrations under
these conditions is 90 min.

After equilibration, samples were taken with a microsyringe
fitted with a Chaney adapter. Protein concentrations were
determined by taking 50+1 aliquots from both the “protein
side” and the “buffer side” of each cell, diluting to 10 ml, and
measuring optical density at 280 μm. ε300 for lysozyme was
found to be 3.54 x 10^5. (Hamaguchi reported a value of 3.89
x 10^5 (21).) In every case, the protein concentration on the
“buffer side” was less than 1% that on the protein side. Four
50+1 aliquots were taken from each side of each cell for scintilla-
tion counting in Bray’s solution. In order to eliminate quench-
ing differences between samples, 50 μl of buffer were added to
the samples from the “protein side” and 50 μl of protein solution
were added to the samples from the “buffer” of a cell. Each
sample was then counted for 10 min, and the whole series was
counted through twice. To make sure that quenching had in
fact been equalized, a standard tritium sample was then added
to half the samples and the samples were recounted. The
added counts on “protein” and “buffer” sides were in fact the
same within experimental error (±0.5%).

RESULTS

Saccharides closely related to lysozyme substrates are capable
of inhibiting the lytic activity of the enzyme towards M. lysodeik-
ticus cells and cell walls and, in appropriate concentrations, lead
to a blue shift of about 10 μm in the fluorescence emission maxi-
mum of the enzyme (5, 6, 9). However, neither the lytic activity
nor the fluorescence spectrum of lysozyme are affected by con-

![Fig. 1. Fluorescence emission spectrum of lysozyme determined on Aminco-Keir spectrofluorimeter with exciting wave-
length 285 μm, at 25°C. A, 0.081 mg per ml of lysozyme in Mcllvaine buffer, pH 5.45. B, As A, with 7.4 x 10^-4 M; GlcNAc-
MurNAc-GlcNAc-MurNAc added. C, As A, with 2.0 x 10^-3 M; GlcNAc-GlcNAc added.](http://www.jbc.org/)

![Fig. 2. Sample plot of fluorescence data for lysozyme with varying concentration of GlcNAc-MurNAc-GlcNAc-MurNAc at
pH 5.45 and 25°C. F0, F, and F∞ are the relative fluorescence emis-
sion intensity at 370 μm of the enzyme alone, the enzyme in the
presence of a concentration [S] of saccharide, and the enzyme
saturated with saccharide, respectively. See the text for experi-
mental details.](http://www.jbc.org/)

centrations as high as 0.1 M of such saccharides as cellobiose or
glucosamine, which lack the 2-acetamido group. The changes
in fluorescence emission intensity vary with the nature of the
substrate. At pH 5.4, and at a concentration such that the
enzyme is almost saturated, (GlcNAc)r causes a 25% increase
in the maximum fluorescence intensity of lysozyme, GlcNAc-
MurNAc-GlcNAc-MurNAc and its dimethyl ester cause a 30%
decrease, and MurNAc-GlcNAc and GlcNAc-MurNAc-GlcNAc
cause little or no change. All the above compounds cause,
however, a similar shift in the wavelength of the fluorescence
emission maximum. Some representative fluorescence emis-
sion spectra are given in Fig. 1.

For each of the compounds discussed below except GlcNAc-
MurNAc, the fluorescence intensity of lysozyme at a given
wavelength varies with the concentration of saccharide in a man-
ner consistent with reversible formation of a one-to-one com-
plex of enzyme and substrate. Fig. 2 shows a typical plot for the
binding of the cell wall tetrasaccharide GlcNAc-MurNAc-
GlcNAc-MurNAc to lysozyme. Log ([F∞-F]/[F-F∞]) is plotted
against log [S], where F0, F, and F∞ are the fluorescence intensi-
"ys of solutions of enzyme alone, enzyme in the presence of a
centration [S] of saccharide, and enzyme saturated with
saccharide, respectively. The slope of the plot is unity, indicat-
ing formation of a one-to-one complex, and it can readily be
shown that pK∞ for the complex equals the value of log [S] at
log ([F∞-F]/[F-F∞]) = 0.

In the case of GlcNAc-MurNAc, no fluorescence studies were
possible since the absorption and fluorescence of the disaccharide
preparation itself are too high at the concentrations necessary
for significant binding to lysozyme. The constant for associ-
ation of GlcNAc-MurNAc with lysozyme was therefore determined
by the dialysis equilibrium technique, with the use of tritium-labeled
GlcNAc-MurNAc. Because of the low affinity of this saccharide
for the enzyme, the undesirability of using large enzyme concen-
trations (lysozyme is known to dimerize to an inactive species at
high concentrations (22)), and the limits of accuracy in the
sampling and counting process, the experiments were carried out
**Table I**

Equilibrium dialysis data for binding of Glc-NAc-MurNAc-3H to lysozyme

For experimental details, see the text.

<table>
<thead>
<tr>
<th>Total saccharide added</th>
<th>Difference between protein and buffer sides of cell at equilibrium&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Free disaccharide on buffer side</th>
<th>Enzyme concentration&lt;sup&gt;b&lt;/sup&gt;</th>
<th>K&lt;sub&gt;a&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>cpm</td>
<td>cpm/ml</td>
<td></td>
<td>X 10&lt;sup&gt;-5&lt;/sup&gt; µ</td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>1.5 X 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2,293 ± 890</td>
<td>66,407</td>
<td>1.82</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>2.4 X 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4,176 ± 1,024</td>
<td>112,531</td>
<td>1.79</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>3.4 X 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4,203 ± 944</td>
<td>156,492</td>
<td>1.70</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>5.9 X 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>8,493 ± 2,487</td>
<td>263,684</td>
<td>1.72</td>
</tr>
<tr>
<td>Experiment 5</td>
<td>9.8 X 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>18,608 ± 4,174</td>
<td>434,453</td>
<td>1.51</td>
</tr>
</tbody>
</table>

<sup>a</sup> The difference is due to bound disaccharide.

<sup>b</sup> The enzyme concentration is the measured total concentration (cpm for lysozyme, 3.54 X 10<sup>5</sup>) minus the enzyme-saccharide complex concentration, calculated as the difference in counts per min per ml between the two sides of a cell at equilibrium divided by the specific activity of the disaccharide (6.1 X 10<sup>4</sup> cpm per µmole).

<sup>c</sup> Calculated as the difference in counts per min per ml on the protein and buffer sides of the cell divided by the counts per min per ml of the free disaccharide on the buffer side times the enzyme concentration. Average K<sub>a</sub> = 20.3 ± 3.4.

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**Table II**

Association constants with hen's egg white lysozyme at pH 5.4 and 25°

All saccharides are β(1 → 4) linked. All constants, except for that of GlcNAc-MurNAc were determined by fluorescence emission measurements. The constant for GlcNAc-MurNAc was derived from equilibrium dialysis experiments.

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>K&lt;sub&gt;a&lt;/sub&gt;, M&lt;sup&gt;-1&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>GlcNAc</td>
<td>15-20</td>
</tr>
<tr>
<td>GlcNAc-GlcNAc</td>
<td>5.5 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>GlcNAc-GlcNAc-GlcNAc</td>
<td>1.1 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>GlcNAc-MurNAc</td>
<td>20</td>
</tr>
<tr>
<td>MurNAc-GlcNAc</td>
<td>1.1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>GlcNAc-MurNAc-GlcNAc</td>
<td>2.8 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>GlcNAc-MurNAc-GlcNAc-MurNAc</td>
<td>2.1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>GlcNAc-MurNAc-GlcNAc-MurNAc dimethyl ester</td>
<td>1.9 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>GlcNAc-MurNAc-GlcNAc-MurNAc pentapeptide</td>
<td>7 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
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</table>

at disaccharide concentrations much below saturation. The calculation of the association constant in this case is thus based on the assumption that 1 mole of enzyme binds 1 mole of substrate in the concentration range studied. If this is not the case, the constant reported may be considered an upper limit, which does not change any of the conclusions reached below. The averaged data for each of five experiments are presented in Table I. The difference between counts on “protein” and “buffer” sides of a cell is due to bound saccharide. The error in this difference is the limiting factor in the accuracy of these experiments; the error given is the standard error in this quantity in the eight pairs of counting data (four samples counted twice) for each cell.

The association constants for the saccharides studied are given in Table II. The values for the three N-acetyl-d-glucosamine oligomers which we have studied are in substantial agreement with those determined by other workers (6-8).

The pH dependence of the association constant, K<sub>a</sub>, of lysozyme with the cell wall tetrasaccharide, GlcNAc-MurNAc-GlcNAc-MurNAc, the binding constant was also measured at pH 3.70 and found to be 1.1 X 10<sup>5</sup>, which is about one-half the value at pH 5.4 for this compound.

**DISCUSSION**

The fluorescence of lysozyme changes upon the addition of substrate analogues in a manner consistent with formation of one-to-one enzyme-saccharide complexes, and the association constants determined by fluorescence measurements are in accord with those determined by other techniques (7, 8). For each of the saccharides studied, the fluorescence maximum of the enzyme-saccharide complex is shifted about 10 nm towards shorter wavelength than that of free lysozyme, although the peak intensity may be increased, decreased, or remain unchanged.

The fluorescence emission spectrum of lysozyme (upon illumination at 285 nm) is almost entirely due to the indole moieties of the six tryptophan groups in the enzyme (6, 24). Since the fluorescence maximum of an indole group generally shifts to shorter wavelengths as its environment becomes less polar (24), it has been suggested that the observed blue shift in the fluorescence spectrum of lysozyme on interaction with certain saccharides indicates that in the enzyme-saccharide complex 1 or more tryptophan residues are in a more hydrophobic environment than in the free enzyme (6, 9). The saccharides are not themselves particularly hydrophobic, and it seems therefore likely that a change in conformation of the enzyme on binding...
is responsible for the spectral shift. Indeed, Phillips (3) has observed that tryptophan 62, near the active site, is about 0.75 Å closer to the opposite face of the "cleft" in the enzyme-tri-N-acetyl-D-glucosamine complex than in the free enzyme. However, the same spectral shift is found to be accompanied by either enhancement or quenching of fluorescence and it is therefore unlikely that the shift in fluorescence maximum and intensity are due to the same process.

The enzyme-saccharide binding data may be understood if one assumes that the "active site" of lysozyme consists of a number of subsites, each of which binds 1 sugar unit, and that the interaction of a given subsite with a single sugar moiety is independent of the nature of the rest of the saccharide. In Table III a scheme of probable positions of binding for a number of saccharides is given, together with the respective free energies of association ($\Delta F^b = -RT\ln K$). A, B, and C are strong binding subsites, but subsite C is "prohibited" to the MurNAc moiety. Subsite D is "unfavorable", that is, contributes a positive term to the overall $\Delta F^b$ of binding, while X and Y are regions of little or no interaction, and may be completely outside of the active site.

This analysis of the properties of the active site of lysozyme is supported both by the data for complex formation in solution presented here and by x-ray crystallographic studies (1-4). The latter studies have shown that in lysozyme-saccharide complexes in the crystalline state (GlcNAc)$_3$ is situated in subsites A-B-C with its nonreducing terminus at one end of the "cleft" in the enzyme. The $\beta$ form of GlcNAc is observed to bind in subsite C. The binding of the cell wall disaccharide GlcNAc-MurNAc in the lysozyme crystal seems to be anomalous; it is largely bound so that it does not lie along the cleft, but rather across it.

Some workers (1-4) have also suggested, on the basis of the geometry of the cleft, that subsite C might be sterically prohibited to MurNAc, as the 3-hydroxy group of a GlcNAc moiety in this subsite is directed in towards groups on the enzyme, and there appears to be no room for a lactyl group in this position. Another important hypothesis of the same workers, which is not supported by direct evidence, is that, in order to fit into subsite D, the ring of a sugar residue would have to be distorted from the stable chair conformation into the less stable "half-chair."

Both of these hypotheses, as well as other features of the scheme presented above, are supported by the data for binding of saccharides to lysozyme. The observation that the association constants of GlcNAc oligomers do not increase with chain length beyond chitotriose (7, 8) indicates that there are three contiguous "favorable" subsites, with no favorable site immediately on either side of the three (i.e. neither Y nor D is favorable). Since GlcNAc-MurNAc-GlcNAc is bound as strongly as GlcNAc-GlcNAc-MurNAc, subsite B must be able to accommodate a MurNAc moiety. On the other hand, both GlcNAc-GlcNAc and MurNAc-GlcNAc are bound much more strongly (by factors of over 100) than GlcNAc-MurNAc, which should be able to fit in subsites A-D without restriction. This indicates that the position of strongest binding of a disaccharide is not A-B but rather B-C, and that subsite C is "prohibited" to MurNAc.

The contributions of the individual subsites of the enzyme to the free energy of association of a saccharide with lysozyme may be calculated by comparing $\Delta F^b$ for the various saccharides given in Table III. If one assumes that $\Delta F^b$ for any saccharide is equal to the sum of contributions from each subsite filled, plus a constant positive term (see below), consistent values for the contributions of each subsite are obtained. These are summarized in Table IV. For example, comparison of MurNAc-GlcNAc and GlcNAc-MurNAc-GlcNAc indicates that the GlcNAc moiety in subsite A contributes about $-1.9$ kcal per mole to $\Delta F^b$ for binding of the trisaccharide. Similar comparisons lead to calculated contributions of about $-3.5$ to $-3.9$ kcal per mole for GlcNAc and MurNAc, respectively, bound in subsite B.

Although the most likely position for the binding of GlcNAc-MurNAc to lysozyme which is consistent with the scheme of Table III is in subsites A-B, x-ray crystallographic evidence (3, 4) indicates that a more favorable binding arrangement for this compound may exist which involves an anomalous position not along the subsites considered here. If such an anomalous complex is responsible for the observed binding of GlcNAc-

### Table III

**Suggested arrangement of saccharides in subsites of lysozyme**

Saccharides are arranged in the subsites in which it is suggested (see the text) that they are bound. All saccharides are $\beta(1 \rightarrow 4)$ linked; the reducing end is to the right. $\Delta F^b = -RT\ln K$, where $K$ is the experimental association constant for the saccharide.

<table>
<thead>
<tr>
<th>Subsite</th>
<th>X</th>
<th>Y</th>
<th>A</th>
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* From the data of Dahlquist, Jao, and Raftery (7).

$^*$ A more favorable position may be one not related to this scheme. See the "Discussion."
MurNAc to lysozyme ($\Delta F^2_a = -1.7$ kcal per mole), $\Delta F^2_a$ for the formation of a complex of GlcNAc-MurNAc bound to subsites A-B of lysozyme must be even less negative than $-5.7$ kcal per mole. A comparison of this value with $\Delta F^2_a$ for GlcNAc-MurNAc-GlcNAc ($-7.4$ kcal per mole) suggests that the GlcNAc moiety in subsite C contributes at least $-5.7$ kcal per mole to the binding of the latter saccharide.

On the basis of the structure of the lysozyme (GlcNAc)$\alpha_8$ complex in the crystalline state, Phillips was able to deduce what the interactions are in each subsite which contribute to binding (3, 4). The relative magnitudes of the contributions of subsites A, B, and C to $\Delta F^2_a$ calculated above are consistent with these deductions.

It will be seen that $\Delta F^2_a$ for a saccharide is less negative by approximately 4 kcal per mole than the sum of the calculated individual contributions of the subsites filled. Such a difference is understandable. The "contribution of a subsite" calculated above is in effect the change in $\Delta F^2_a$ to be expected on allowing an additional monomer unit, attached to a saccharide already bound to lysozyme, to interact with the enzyme. It therefore does not take into account such factors as loss in translational entropy, the energy of the conformational change in lysozyme which accompanies saccharide binding (4), and so on, which enter into the overall $\Delta F^2_a$ for the association of a saccharide with the enzyme. Some workers use the "unitary free energy" (25, 26), a function corrected for the entropy of mixing, for the association of small molecules with proteins ($\Delta F^2_a - \Delta F^2_a$ kcal per mole in water at 25°). Rupley et al. (8) have reported data for saccharide binding to lysozyme in this form. If this difference of 2.4 kcal per mole between $\Delta F^2_a$ and $\Delta F^2_a$ is taken into account, their data are in reasonable agreement with ours. It is apparent, however, that the entropy of mixing is not the only factor of importance in the difference between $\Delta F^2_a$ for the binding of a saccharide to lysozyme and the sum of the contributions of the subsites involved.

The difference between the binding of GlcNAc-MurNAc-GlcNAc and GlcNAc-MurNAc-GlcNAc-MurNAc can be explained either if the latter is bound in A-B-C-D and subsite D is "unfavorable" for binding or if D is completely prohibited for a MurNAc moiety. It is unlikely that D is prohibited for MurNAc; if both C and D were prohibited to MurNAc, no "cell wall oligosaccharide" consisting of alternating GlcNAc-MurNAc units could be bound along the whole cleft of the enzyme, and no such saccharide could be more strongly bound than GlcNAc-MurNAc-MurNAc. Thus, the lower binding constant for GlcNAc-MurNAc-GlcNAc-MurNAc implies that subsite D is unfavorable and the calculation shows that it makes a net contribution of +2.9 kcal per mole to the free energy of association of this saccharide.

Equilibrium data cannot, of course, indicate the reason for the unfavorable interaction of a MurNAc moiety with subsite D. The possibility that this is due to a coulombic repulsion is eliminated by the similarity of the affinities of the cell wall tetrasaccharide and its dimethyl ester for lysozyme. The ring-distortion which Phillips (3) suggests is necessary for the binding of a sugar unit in subsite D may well be responsible for the unfavorable interaction. If there is some favorable interaction at subsite D as well (such as a hydrogen bond) the energy required for distortion may be even more than the +2.9 kcal per mole obtained above.

Although it has been suggested (4) that the lactyl residue of a muramic acid moiety bound in a "permitted" site would be pointing "out" of the enzyme cleft, several observations indicate that it does interact with the enzyme. It should be noted, for instance, that GlcNAc-MurNAc-GlcNAc and MurNAc-GlcNAc are somewhat more strongly bound than the GlcNAc oligomers of the same chain length.

The pH dependence of the association constant of GlcNAc-MurNAc-GlcNAc-MurNAc also indicates that the lactyl group interacts with the enzyme. The association constant for GlcNAc-MurNAc-GlcNAc-MurNAc, which has two ionizable carboxyl groups of $pK_a$ 3.7, increases threefold on going from pH 5 to 3.5. On the other hand, the binding constant of the diethyl ester of the tetrasaccharide, although close to that of the parent compound at pH 5, decreases significantly on going from pH 5 to 3.5. Lysozyme also shows maximum binding to (GlcNAc)$\alpha_3$ at pH 5.0 to 5.5 (6, 7). This suggests that the protonated carboxyl groups in GlcNAc-MurNAc-GlcNAc-MurNAc favor binding of the saccharide to lysozyme. One way in which a protonated carboxyl group might favor binding to a protein is by the participation of its proton in a hydrogen bond. An amide group can also act in this manner, and such an interaction may explain the increased binding constant of the tetrasaccharide pentapeptide as compared with the tetrasaccharide itself (see Table II). It should be noted that many of the muramic acid moieties in the bacterial cell wall saccharides are linked by amide bonds to short peptides. The question of possible interactions between lysozyme and nonsaccharide moieties of the cell wall substrates deserves further investigation.

The arrangement of the binding subsites in lysozyme described above explains one puzzling peculiarity of lysozyme specificity. Although "cell wall" oligosaccharides, which contain the repeating GlcNAc-MurNAc structure, are digested far more...
readily by lysozyme than are GlcNAc oligomers, the latter are much more strongly bound to the enzyme. However, the strong complexes of the GlcNAc oligomers and lysozyme probably do not involve the unfavorable subsite D. If, as Phillips suggests, the site of bond breaking is between subsites D and E, such complexes are nonproductive. Since subsite C is prohibited to MurNAc, however, an oligomer of the structure GlcNAc-MurNAc must either occupy both subsites C and D or neither of them. As the overall contribution of the two subsites to $\Delta F^*$ is negative (compare GlcNAc-MurNAc and GlcNAc-MurNAc-GlcNAc-MurNAc), such cell wall oligomers will in fact occupy these sites. If subsites beyond D have even slight favorable binding interactions with GlcNAc moieties (as the transglycosylation reactions of lysozyme lead one to believe), a hexasaccharide or longer cell wall oligosaccharide will tend to be bound across the bond-breaking position.

Phillips (3) has pointed out that the distorted half-chair form, which a pyranose ring in subsite D might have to adopt, is close to the geometry required in the transition state for the breaking of a glycoside bond. The energy required to put a saccharide in this subsite may then represent a saving in the activation energy for the bond-breaking step. Thus, for cell wall oligosaccharides, the special combination of subsite C, a strongly binding site sterically prohibited to MurNAc, and subsite D, which necessitates distortion of a sugar towards the half-chair conformation, leads specifically to a binding interaction which lowers the free energy of the enzyme substrate complex. N-Acetyl-glucosamine oligosaccharides, lacking MurNAc residues, may be preferentially bound in an inactive complex, and, as suggested recently by Rupley and Gates (28), cleavage of such saccharides may necessitate less probable modes of binding.

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REFERENCES
The Binding of Oligosaccharides Containing N-Acetylglucosamine and N-Acetylmuramic Acid to Lysozyme: THE SPECIFICITY OF BINDING SUBSITES

David M. Chipman, Vladimir Grisaro and Nathan Sharon


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