chrome P 450 (Maines and Sinclair, 1976), and studies with heme oxygenase isolated from liver microsomes (Maines et al., manuscript in preparation) support the idea that heme oxygenase is a discrete enzyme protein whose prosthetic group is synonymous with its substrate.

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# Mechanism of Lysozyme Catalysis: Role of Ground-State Strain in Subsite D in Hen Egg-White and Human Lysozymes†

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ABSTRACT: The association constants for the binding of various saccharides to hen egg-white lysozyme and human lysozyme have been measured by fluorescence titration. Among these are the oligosaccharides GlcNAc-$\beta$(1 → 4)-MurNAc-$\beta$(1 → 4)-GlcNAc-$\beta$(1 → 4)-GlcNAc, GlcNAc-$\beta$(1 → 4)-MurNAc-$\beta$(1 → 4)-GlcNAc-$\beta$(1 → 4)-*N*-acetyl-D-xylosamine, and GlcNAc-$\beta$(1 → 4)-GlcNAc-$\beta$(1 → 4)-MurNAc, prepared here for the first time. The binding constants for saccharides which must have *N*-acetylmuramic acid, *N*-acetyl-D-glucosamine, or *N*-acetyl-D-xylosamine bound in subsite D indicate that there is no strain involved in the binding of *N*-acetyl-D-glucosamine in this site, and that the lactyl group of *N*-acetylmuramic acid (rather than the hydroxymethyl group) is responsible for the apparent strain previously reported for binding at this subsite. For hen egg-white lysozyme, the dependence of saccharide binding on pH or on a saturating concentration of Gd(III) suggests that the conformations of several of the complexes are different from one another and from that proposed for a productive complex. This is supported by fluorescence difference spectra of the various hen egg-white lysozyme–saccharide complexes.

Human lysozyme binds most saccharides studied more weakly than the hen egg-white enzyme, but binds GlcNAc-$\beta$(1 → 4)-MurNAc-$\beta$(1 → 4)-GlcNAc-$\beta$(1 → 4)-MurNAc more strongly. It is suggested that subsite C of the human enzyme is "looser" than the equivalent site in the hen egg enzyme, so that the rearrangement of a saccharide in this subsite in response to introduction of an *N*-acetylmuramic acid residue into subsite D destabilizes the saccharide complexes of human lysozyme less than it does the corresponding hen egg-white lysozyme complexes. This difference and the differences in the fluorescence difference spectra of hen egg-white lysozyme and human lysozyme are ascribed mainly to the replacement of Trp-62 in hen egg-white lysozyme by Tyr-63 in the human enzyme. The implications of our findings for the assumption of superposition and additivity of energies of binding in individual subsites, and for the estimation of the role of strain in lysozyme catalysis, are discussed.

The hypothesis that the active sites of enzymes are complementary in structure to the transition states of the reactions which they catalyze (Haldane, 1930; Fersht, 1974; Jencks, 1975; Pauling, 1948) is now widely accepted. A corollary to this hypothesis is that there must be strain in the interaction between an enzyme and the ground state of its substrate(s), although this strain need not necessarily be steric and need not lead to distortion of either the enzyme or the substrate in the complex formed between them (Fersht, 1974; Jencks, 1975). The role of strain in the lysozyme-catalyzed hydrolysis of oligosaccharides has been under active investigation ever since the proposal by Phillips and his co-workers of a model for a hen egg-white lysozyme (HEWL[1])–substrate complex (Blake et al., 1967; Phillips, 1966). According to this model, the enzyme

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[1] Abbreviations used are: HEWL, hen egg-white lysozyme; HL, human lysozyme; Mes, 4-morpholineethanesulfonic acid; TLC, thin-layer chromatography; GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid; XylNAc, 2-acetamido-2-deoxy-D-xylose. All oligosaccharides referred to are linked $\beta$(1 → 4).

can interact with up to six GlcNAc moieties, so long as the residue in the fourth subsite from the nonreducing end (subsite D) is distorted towards a partially planar conformation, in order to prevent prohibited steric interactions of its C(5) hydroxymethyl group with the protein (Blake et al., 1967; Ford et al., 1974). Since the transition state for cleavage of the glycosidic bond is expected to have oxo-carbonium ion like character (Dahlquist et al., 1969), planar about the C(1)-O(5) bond (Capon, 1969), and since D is the site of bond cleavage (Blake et al., 1967; Rupley and Gates, 1967), it was proposed that the distortion of the substrate towards the transition state geometry plays an important role in lysozyme catalysis (Blake et al., 1967; Imoto et al., 1972b; Vernon, 1967).

Experimental evidence for strain in the catalytic mechanism of lysozyme has in the main been concerned with binding interactions in subsite D. Studies with bacterial cell wall oligosaccharides have shown that the introduction of an *N*-acetylmuramic acid residue into subsite D destabilizes the HEWL-oligosaccharide complex (Chipman et al., 1967). On the other hand, analogues of *N*-acetyl-D-glucosamine, expected to fit subsite D *without* prohibited interactions, appeared to stabilize the enzyme-saccharide complex (Secemski et al., 1972; van Eikeren and Chipman, 1972). Largely on the basis of such data, it has been suggested that strain in subsite D is responsible for a reduction of the activation energy for bond cleavage in the enzymatic reaction by 5-10 kcal/mol (Chipman, 1971; Chipman and Sharon, 1969; Imoto et al., 1972b; Secemski et al., 1972; Thoma, 1974).

The binding of saccharides to human lysozyme, which shows considerable homology (Canfield et al., 1971; Jollès et al., 1974) and structural similarity (Banyard et al., 1974; Blake and Swan, 1971) to HEWL, has only been investigated to a limited extent (Kuramitsu et al., 1975; Mulvey et al., 1973; Teichberg et al., 1972). Further studies of the interaction between the human enzyme and oligosaccharides are of interest not only in order to provide additional information on the active site of this particular protein, but also to deepen our understanding of the relation between structure and function for other lysozymes with which it is homologous.

In this paper, we report studies of the binding of a number of new oligosaccharides to HEWL and HL under various conditions, as well as fluorescence difference spectra for several of the enzyme-saccharide complexes. These experiments were aimed at assessing quantitatively the role of strain in lysozyme catalysis. The results obtained with compounds which almost certainly bind in the region A-D in the enzyme cleft lead to the conclusion that the importance of strain in subsite D has been overestimated, and that this strain can account for only a small part of the observed catalytic effect of the enzyme.

## Materials and Methods

*Materials.* Hen egg-white lysozyme (twice crystallized, salt free) was from Worthington. Its concentration was determined spectrophotometrically using $E_{280}^{1\%} = 26.4$ (Imoto et al., 1972b). Human lysozyme, from the urine of patients with monocytic leukaemia, was a gift from Dr. E. F. Osserman of the College of Physicians and Surgeons of Columbia University (Osserman and Lawlor, 1966). Several experiments with enzyme obtained from Dr. R. E. Canfield (Canfield et al., 1971) gave indistinguishable results. The enzyme concentration was determined spectrophotometrically using $E_{280}^{1\%} = 24.6$ (Mulvey et al., 1973). $Gd_2O_3$ was from Alfa Inorganics and was converted to $GdCl_3$ by dissolution in warm aqueous HCl. The concentration of Gd(III) was determined by the method of Fritz et al. (1948).

*General Methods.* The following methods were used for the analytical and preparative separation of oligosaccharides: (1) descending paper chromatography with 1-butanol-acetic acid-water (4:1:5, v/v, upper phase); (2) high-voltage paper electrophoresis at pH 6.5 (1.2 M pyridine-acetic acid); (3) TLC on Analtech Silica Gel G plates with 1-propanol-ethanol-water (70:96:34, v/v); (4) TLC on silica gel with 1-propanol-water-concentrated $NH_3$ (70:30:1, v/v) (Powning and Irzykiewicz, 1967) with triple development. Paper chromatograms and electrophoretograms were visualized by the method of Sharon and Seifter (1964) and thin-layer chromatograms with 10% $H_2SO_4$ and charring. For the examination of product purity, the quantity of sample applied to a chromatogram was generally 10-20 times the limit of detection of the method used. Radioactive peaks were detected on a Packard Radiochromatogram Scanner. Saccharides were eluted from paper by the method of Eshdat and Mirelman (1972).

Amino sugars were determined on the amino acid analyzer after acid hydrolysis (2 N HCl, 2.5 h, 100 °C) as described by Mirelman and Sharon (1967). *N*-Acetyl-D-glucosamine, *N*-acetyl-D-xylosamine, and GlcNAc-MurNAc were run in parallel as standards. Norleucine served as internal standard for the determination of the concentrations of stock saccharide solutions by the above method.

*Preparation of Oligosaccharides.* Cell-wall oligosaccharides from *Micrococcus luteus* (Sharon, 1967; Chipman et al., 1968), chitin oligosaccharides (Rupley, 1964; Raftery et al., 1969) and GlcNAc-MurNAc-GlcNAc (Pollock and Sharon, 1970a) were prepared as reported. All lysozyme-catalyzed transglycosylations were carried out in a 0.1 M ammonium acetate buffer, pH 5.2.

$(GlcNAc)_3$-[5-$^3$H]XylNAc was prepared by lysozyme-catalyzed transglycosylation with [5-$^3$H]XylNAc as acceptor and a mixture of $(GlcNAc)_4$ and $(GlcNAc)_5$ as substrates (van Eikeren et al., 1973). A typical reaction mixture contained 70 mg of [5-$^3$H]XylNAc (520 cpm/nmol), 120 mg of a mixture of $(GlcNAc)_4$ and $(GlcNAc)_5$, and 5 mg of HEWL in 10 ml. After 24-h incubation at 40 °C, the reaction mixture was lyophilized and separated on a 1.5 × 70 cm charcoal-Celite column with a 4-l. gradient from water to 30% ethanol. Such columns also furnished GlcNAc-[5-$^3$H]XylNAc free of $(GlcNAc)_2$. The peaks containing $(GlcNAc)_3$-[5-$^3$H]XylNAc from four such reaction mixtures were pooled and rechromatographed, as shown in Figure 1. Approximately 4.5 mg of the product was obtained. It showed a single spot ($R_f$ 0.53) on TLC (system 4) coincident with the radioactivity. In the same system, the following $R_f$'s were obtained: $(GlcNAc)_4$, 0.51; $(GlcNAc)_5$, 0.41; $(GlcNAc)_6$, 0.31.

GlcNAc-MurNAc-GlcNAc-[5-$^3$H]XylNAc was also prepared by transglycosylation. $(GlcNAc\text{-}MurNAc)_2$ (46.5 mg) was preincubated with HEWL (3.4 mg) in 9 ml of buffer for 30 min at 36 °C (in order to ensure buildup of higher oligosaccharides (Chipman et al., 1968)) and then GlcNAc-[5-$^3$H]XylNAc (6.6 mg in 1.25 ml of buffer) was added. The incubation was continued for 48 h, with 1 mg of enzyme added after 24 h. After lyophilization, the reaction mixture was separated by preparative paper electrophoresis. The radioactive peak of mobility two-thirds that of GlcNAc-MurNAc was eluted and lyophilized. This material was successively purified by gel filtration on Sephadex G-15, electrophoresis, paper chromatography (system 1), and Sephadex G-15 gel filtration. About $3 \times 10^5$ cpm of the product (0.45 mg, assuming a specific activity of 520 cpm/nmol for [$^3$H]XylNAc) was obtained.

GlcNAc-MurNAc-GlcNAc-GlcNAc was prepared by incubation of $(GlcNAc\text{-}MurNAc)_2$ (50 mg), $(GlcNAc)_2$ (17 mg), and HEWL (1.5 mg) in 5 ml of buffer, for 18 h at 37 °C. After lyophilization, the reaction mixture was separated by preparative electrophoresis (system 2). A center strip was visualized and the band of the electrophoretogram approximately corresponding in mobility to the previously characterized GlcNAc-MurNAc-GlcNAc-XylNAc was cut out and eluted. This material was repurified by chromatography (system 1), to yield 3.5 mg of lyophilized product.

GlcNAc-GlcNAc-MurNAc was prepared by incubation of $(GlcNAc)_4$ (25 mg), GlcNAc-MurNAc (10 mg), and HEWL (1.1 mg) in 2 ml of buffer for 35 h at 37 °C. After lyophilization, the mixture was separated by preparative paper electrophoresis at pH 6.5 for an extended time (120 min). A center strip was visualized and the band of mobility similar to GlcNAc-MurNAc-GlcNAc was cut out and eluted. As this band was well separated from others, and showed one spot on chromatography (systems 1 and 3), no further purification was needed.

*Fluorescence measurements* were made on Aminco-Keirs or Hitachi-Perkin Elmer MPF-2A spectrofluorimeters. Association measurements were carried out with an excitation wavelength of 280 nm and emission at the wavelength of maximal spectral change for the complex (between 315–335 nm or near 365 nm for HEWL and 325 nm for HL) in buffers containing 10 mM Mes–0.1 M NaCl at pH 6.58 or in citrate–phosphate buffers (McIlvaine, 1921) at various pH's. The relative fluorescence, $F$, of the enzyme in the presence of saccharide was determined by comparison of two cuvettes containing identical enzyme concentrations (1–3 $\mu$M, $A_{280}$ = 0.04–0.12 in a 1-cm cell), one with and one without saccharide. The fluorescence was also corrected for absorbance of the saccharide, but this was never more than 10% of the change in fluorescence. In the case of $(GlcNAc\text{-}MurNAc)_3$, a good substrate of lysozyme, measurements were completed within 10–15 s of the addition of saccharide to the enzyme solution.

The concentrations of stock saccharide solutions were determined in one of the following ways: saccharides available in quantities of tens of milligrams (chitin oligosaccharides, $(GlcNAc\text{-}MurNAc)_2$) were dried over $P_2O_5$ and weighed. For $^3H$-labeled saccharides, liquid scintillation counting of aliquots was used. In other cases, amino sugar analysis was used.

The dissociation constants for the complexes were calculated by nonlinear least-squares fit (Wolberg, 1967) of the data to the equation:

$$F = 1 + \frac{(R-1)}{2E_0}\{(E_0 + L_0 + K) - [(E_0 + L_0 + K)^2 - 4E_0L_0]^{1/2}\} \quad (1)$$

where $E_0$ and $L_0$ are the total concentrations of enzyme and ligand, and the adjustable parameters $R$ and $K$ are the relative fluorescence of the enzyme saturated with ligand and the dissociation constant of the complex, respectively.[2] The fitting and plotting of the data were carried out on the CDC Cyber 73 computer of the Ben Gurion University.

Uncorrected fluorescence difference spectra were calculated by point-by-point subtraction of pairs of spectra of solutions with identical total enzyme concentration after at least two repeated alternating scans of the cuvettes. Aqueous tryptophan served as wavelength standard. The various difference spectra were normalized and are thus corrected for changes in instrument sensitivity. The fluorescence spectra of the enzyme saturated with certain compounds (e.g., $(GlcNAc\text{-}MurNAc)_2$) were corrected for the slight contribution of the saccharide above 360 nm. The concentration of saccharide was more than tenfold higher than $K_d$ for the complex in question in every case.

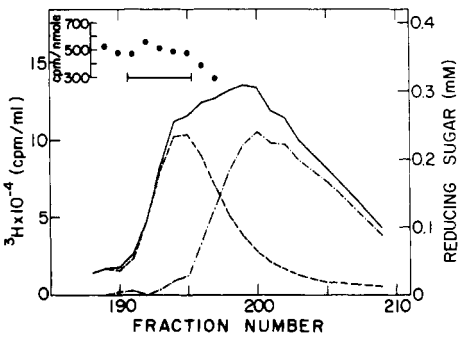


FIGURE 1: Purification of GlcNAc-GlcNAc-GlcNAc-[5-$^3$H]XylNAc by charcoal–Celite chromatography. Combined fractions from initial chromatography of four reaction mixtures (see Methods) were chromatographed on a 1.5 × 72 cm charcoal–Celite column, eluted with 4 l. of a 0–25% water–ethanol gradient. Fractions of 11 ml were collected and analyzed for reducing sugar (Park and Johnson, 1949) relative to GlcNAc (—), and for $^3H$ (- - -). Only relevant portion of profile is shown. The solid bar is fractions pooled (191–195) as GlcNAc-GlcNAc-GlcNAc-[5-$^3$H]XylNAc. The specific activity and color factor for this material were used to adjust the two ordinate scales so that the broken line (- - -) also represents its contribution to reducing sugar values for each fraction, which was subtracted from total reducing sugar to give calculated elution profile for $(GlcNAc)_4$ (- • - • -). Inset: calculated specific activities (●) of fractions around those pooled.

## Results and Discussion

For the studies reported here, we have prepared a number of new oligosaccharides containing *N*-acetylmuramic acid by lysozyme-catalyzed transglycosylation. In addition, $(GlcNAc)_3$-[5-$^3$H]XylNAc, whose preparation as a component of a mixture with $(GlcNAc)_4$ was previously reported (van Eikeren and Chipman, 1972; van Eikeren et al., 1973) has been prepared by a modified procedure leading to an essentially pure compound. Figure 1 demonstrates the resolution of $(GlcNAc)_3$-[5-$^3$H]XylNAc from $(GlcNAc)_4$ on a long charcoal–Celite column.

The purity of each of these compounds was demonstrated by electrophoretic and chromatographic analysis. The structures of the new compounds were deduced from the method of preparation (on the basis of the known behavior of lysozyme-catalyzed transglycosylations (Pollock and Sharon, 1970a,b), and from electrophoretic and chromatographic mobilities, as well as amino sugar analyses (Table I; see also Materials and Methods).

*Measurements of Saccharide Binding to Lysozymes.* Upon addition of the saccharides to either HEWL or HL, the fluorescence changes are as expected for the formation of 1:1 complexes of enzyme and saccharide. In the present study, we have used a nonlinear equation (eq 1) to calculate the dissociation constants from the data. This equation does not assume that the total ligand concentration is much greater than protein concentration ($L_0 > E_0$), as is commonly done, and thus allows the use of convenient enzyme concentrations even with ligands

[2] Equation 1 is simply the consequence of the four basic assumptions: (1) the equilibrium expression $K = [EL]/[E][L]$; (2) $E_0 = [E] + [EL]$, and (3) $L_0 = [L] + [EL]$, the conservation equations; and (4) $F = ([E] + R[EL])/E$, the assumption that fluorescence is additive (for solutions of low absorbance).

TABLE I: Characterization of Oligosaccharides.

| Saccharide | Relative Mobility: Paper Chromatography[b] | Relative Mobility: Paper Electrophoresis[c] | Amino Sugar Analysis[a]: MurNAc Calcd | MurNAc Found | XylNAc Calcd | XylNAc Found |
|---|---|---|---|---|---|---|
| GlcNAc-MurNAc | *1.00* | *1.00* | 1.00 | 1.00 | | |
| $(GlcNAc\text{-}MurNAc)_2$ | 0.50 | 1.23 (1.19[d]) | 1.00 | 1.16 | | |
| $(GlcNAc\text{-}MurNAc)_3$ | 0.27 | 1.34 (1.29[d]) | 1.00 | 1.05 | | |
| GlcNAc-MurNAc-GlcNAc | 0.50 | 0.80 (0.81[d]) | 0.50 | 0.46 | | |
| GlcNAc-MurNAc-GlcNAc-GlcNAc | 0.38 | 0.63 | 0.33 | 0.38 | | |
| GlcNAc-MurNAc-GlcNAc-XylNAc | 0.38 | 0.63 | 0.50 | 0.55 | 0.50 | 0.54 |
| GlcNAc-GlcNAc-MurNAc | 0.50 | 0.78 | 0.50 | 0.57 | | |
| GlcNAc-GlcNAc-GlcNAc-XylNAc | | | | | 0.33 | 0.36 |

[a] In terms of molar ratio relative to GlcNAc, by the method of Mirelman and Sharon (1967). [b] On Whatman 3MM paper, 1-butanol-acetic acid-water (4:1:5, v/v, upper phase). [c] On Whatman 3MM paper, 50 V/cm, 80 min, 1.2 M pyridinium acetate (pH 6.5). [d] From Pollock (1969).

TABLE II: Association of Saccharides with HEWL and HL.[a]

| Saccharide | HEWL: $K_d$ (M) | HEWL: $\Delta F_u$ (kcal/mol[b]) | HL: $K_d$ (M) | HL: $\Delta F_u$ kcal/mol[b] |
|---|---|---|---|---|
| GlcNAc | 32[c] | −4.5 | 0.6[d] | −2.7 |
| $(GlcNAc)_2$ | $3.1 \times 10^{-4}$ | −7.2 | $5.6 \times 10^{-4}$ | −6.8 |
| $(GlcNAc)_3$ | $8.6 \times 10^{-6}$ | −9.3 | $4.9 \times 10^{-5}$ | −8.3 |
| $(GlcNAc)_4$ | $5.6 \times 10^{-6}$ | −9.6 | $2.3 \times 10^{-5}$ | −8.7 |
| $(GlcNAc)_3$-XylNAc | $4.3 \times 10^{-6}$ | −9.7 | $3.3 \times 10^{-5}$ | −8.5 |
| GlcNAc-MurNAc-GlcNAc | $3.2 \times 10^{-6}$ | −9.9 | $1.3 \times 10^{-5}$ | −9.0 |
| $(GlcNAc\text{-}MurNAc)_2$ | $4.1 \times 10^{-4}$ | −7.0 | $1.7 \times 10^{-4}$ | −7.5 |
| $(GlcNAc\text{-}MurNAc)_3$ | $3.9 \times 10^{-5}$ | −8.4 | $5.1 \times 10^{-5}$ | −8.2 |
| GlcNAc-MurNAc-GlcNAc-XylNAc | $7.1 \times 10^{-7}$ | −10.8 | $3.6 \times 10^{-6}$ | −9.8 |
| GlcNAc-MurNAc-GlcNAc-GlcNAc | $1.1 \times 10^{-6}$ | $-10.5 \pm 0.2$ | $5.0 \times 10^{-6}$ | $-9.6 \pm 0.2$ |
| GlcNAc-GlcNAc-MurNAc | $7 \times 10^{-4}$ | $-6.7 \pm 0.6$ | | |

[a] At pH 6.58 (10 mM Mes–0.1 M NaCl), except where noted. [b] $\Delta F_u = RT \ln K_d - 2.4$ kcal/mol. All values are ±0.1 kcal/mol, except where noted. [c] At pH 5.72. This association is weakly dependent on pH (Imoto et al., 1972b). [d] At pH 7.6 (region of maximal spectral change). This association is very weakly dependent on pH, if at all (Kuramitsu et al., 1975).

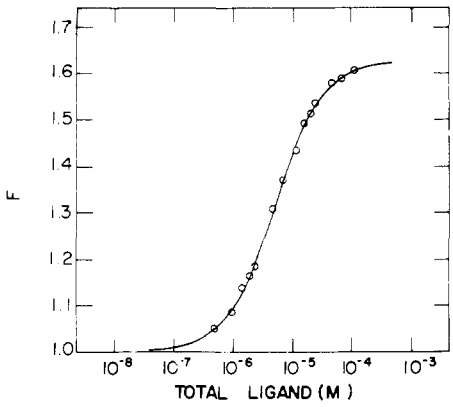


FIGURE 2: Fluorescence titration of HEWL with GlcNAc-GlcNAc-GlcNAc-XylNAc at pH 6.58. Open circles, experimental points, solid line, calculated best fit to data.

having high association constants. Typical data and the calculated best curve for fit to it are shown in Figure 2.

The dissociation constants obtained in this way are presented in Table II, together with the unitary free energies of binding, $\Delta F_u$. Typically, the uncertainty in the calculated $\Delta F_u$ from a single titration was less than 0.06 kcal/mol. The repetition of a titration (whether at the same or a different emission wavelength) gave results reproducible within the same uncertainty. There are two possible causes of error greater than about 0.1 kcal/mol in $\Delta F_u$ as measured in this work: (a) uncertainty in the concentration of the stock solution, particularly critical when $K$ is of the order of $E_0$ or smaller, and (b) inability to approach saturation because of a limited supply of a ligand of low affinity. The first of these difficulties led to the somewhat larger uncertainty reported for the binding of GlcNAc-MurNAc-GlcNAc-GlcNAc, while the second is responsible for the very large uncertainty reported for GlcNAc-GlcNAc-MurNAc, for which less than 30% saturation was achieved.

The data for HEWL (Table II) are in good agreement with values reported for those compounds previously studied (Imoto et al., 1972b), except for one crucial case. $\Delta F_u$ reported here for $(GlcNAc)_3$-XylNAc is less negative by nearly 1.9 kcal/mol than that reported by van Eikeren and Chipman (1972) for the same compound at pH 5.2. The value previously reported was obtained by fluorescence titration using an unresolved mixture of $(GlcNAc)_3$-XylNAc and $(GlcNAc)_4$. The dissociation constant, $K$, for $(GlcNAc)_3$-XylNAc was determined by a computer fit to the data, which assumed a given $K$ for $(GlcNAc)_4$ and ignored the effect of changing that parameter. The assumed value of $K$ was, in fact, too high, and when a lower value is used the data of van Eikeren and Chipman can be fit by the value of $K$ for $(GlcNAc)_3$-XylNAc given in Table II. The value given here is certainly far more reliable than the

TABLE III: Comparison of Unitary Free Energies of Binding of Various Saccharides to HEWL, Including Assumed Binding Regions.

| | Subsite A | B | C | D | $\Delta F_u$[a] | Contribution of Group in Italics[b] |
|---|---|---|---|---|---|---|
| 1. | | | GlcNAc | | −4.5 | |
| 2. | | GlcNAc- | GlcNAc | | −7.2 | |
| 3. | GlcNAc- | GlcNAc- | GlcNAc | | −9.3 | |
| 4. | {GlcNAc- | GlcNAc- | GlcNAc | } | −9.6 | |
| | | GlcNAc- | GlcNAc- | GlcNAc | | |
| 5. | GlcNAc- | GlcNAc- | GlcNAc- | *XylNAc* | −9.7 | −0.4 |
| 6. | | MurNAc- | GlcNAc | | −7.9[c] | |
| 7. | {GlcNAc- | MurNAc | | } | −4.1[c] | |
| | | | GlcNAc- | MurNAc | | |
| 8. | | GlcNAc- | GlcNAc- | *MurNAc* | −6.7 ± 0.6 | +0.5 (±0.6) |
| 9. | GlcNAc- | MurNAc- | GlcNAc | | −9.9 | |
| 10. | GlcNAc- | MurNAc- | GlcNAc- | *XylNAc* | −10.8 | −0.9 |
| 11. | GlcNAc- | MurNAc- | GlcNAc- | *GlcNAc* | −10.5 ± 0.2 | −0.6 |
| 12. | GlcNAc- | MurNAc- | GlcNAc- | *MurNAc* | −7.0 | +2.9 |
| 13. | GlcNAc- | GlcNAc- | GlcNAc- | (*GlcNAc lactone*) | −12.1[d] | −2.8 |
| 14. | GlcNAc- | MurNAc- | GlcNAc- | $\Delta^{2,3}$-*GlcNAc* | −9.6[e] | +0.3 |

[a] $\Delta F_u = RT \ln K_d - 2.4$ kcal/mol. All values are ±0.1 kcal/mol, except where otherwise noted. [b] Difference between $\Delta F_u$ for indicated compound and that for compound lacking residue in italics. [c] From Chipman et al. (1967) at pH 5.2. Error may be larger than 0.1 kcal/mol. [d] From Secemski et al. (1972). [e] From Schindler and Sharon (1976). $\Delta^{2,3}$-GlcNAc is $\Delta^{2,3}$-2-acetamido-2,3-dideoxy-D-glucose.

previous one, since it was obtained using highly purified $(GlcNAc)_3$-XylNAc, required no assumptions, and is reproducible.

Values in the literature for the binding of the chitin oligomers $(GlcNAc)_n$, $n$ = 1–4, to human lysozyme under similar conditions, measured either by spectrofluorimetry (Teichberg et al., 1972; Mulvey et al., 1973) or circular dichroism (Kuramitsu et al., 1975), do not differ from those given in Table II by more than 0.4 kcal/mol. The single exception is a low value for the affinity of $(GlcNAc)_2$ reported by Teichberg et al. (1972), which is presumably in error for reasons not clear to us.

*Apparent Contribution of Subsite D to Saccharide Binding to HEWL.* The original proposals by Phillips and his coworkers concerning the role of strain in lysozyme catalysis (Blake et al., 1967; Phillips, 1966) were implicitly based on the assumption of superposition; i.e., that in different enzyme–saccharide complexes, the conformation of the enzyme and the conformation and interactions of a saccharide residue in a given subsite are identical. Despite the fact that later crystallographic studies by the same group have indicated that residues in subsites B or C are not necessarily always in the positions observed for the HEWL–$(GlcNAc)_3$ complex (Beddell et al., 1970; Ford et al., 1974), the assumption of superimposition, and the corollary of additivity of subunit contributions to $\Delta F_u$ for binding, have remained common in the analysis of data concerning lysozyme in solution. The assumption of superposition is not appropriate for the analysis of the data presented here, however, and appears particularly unsuitable for saccharides occupying the region of subsite D in HEWL.

Table III summarizes the unitary free energy of binding of a number of saccharides to HEWL. The positions of binding proposed in the table are based on several considerations: (a) known binding modes in crystals (Imoto et al., 1972b; Ford et al., 1974), (b) the exclusion of MurNAc residues from subsites A, C, and E, and (c) the assumption that a residue hanging out of the cleft cannot affect $\Delta F_u$. For example, neither GlcNAc-GlcNAc-MurNAc nor $(GlcNAc\text{-}MurNAc)_2$ can reasonably be assumed to bind with their two reducing terminal groups in subsites A–B and the nonreducing terminal residue(s) out of the cleft; for were this so, GlcNAc-MurNAc should bind in A–B with a similar affinity. We feel that there are no reasonable alternative assignments for saccharides 9–12 in Table III, which are most significant for the following discussion. Some binding positions are, of course, uncertain. Ford et al. (1974) noted that crystals of a $(GlcNAc)_4$–HEWL complex show some occupancy of subsite D, but that this is considerably less than that of subsite C. Millett and Raftery (1972) concluded from $^{19}F$ NMR studies of the HEWL–tetra-*N*-trifluoroacetylchitotetraose complex that the reducing terminus of this analogue of $(GlcNAc)_4$ is bound in subsite C. Presumably, binding modes with the terminus in C or D are both possible for $(GlcNAc)_4$, with the former more favorable.

The assignment of binding positions to the saccharides makes possible the calculation of the apparent contribution of a group in subsite D to the unitary free energy of binding for an oligosaccharide (right-hand column of Table III). It is immediately obvious from these results that an *N*-acetyl-D-glucosamine residue in subsite D can slightly stabilize a HEWL–saccharide complex (decrease $\Delta F_u$). A XylNAc residue in subsite D stabilizes the complex only slightly more. A $\Delta^{2,3}$-2-acetamido-2,3-dideoxyglucoseen residue (compound 14, Schindler and Sharon, 1976) in subsite D has little or no effect on the stability of the complex. This implies that, at least for a tetrasaccharide with GlcNAc at it reducing terminus, the C(5) hydroxymethyl group (absent in XylNAc) leads to no serious steric interactions with the protein in subsite D. The strain of 2.9 kcal/mol for the introduction of a MurNAc residue into subsite D (Chipman et al., 1967) must therefore be largely due to the 3-*O*-(1-carboxyethyl) group of muramic acid.

*The Nature of Saccharide Binding in Subsite D.* If the residues at the reducing termini of tetrasaccharides 5, 10, 11, or 12 (Table III) were bound in subsite D in positions similar to that proposed by Phillips for the HEWL–$(GlcNAc)_6$ complex (Blake et al., 1967; Imoto et al., 1972b), or to that found (Ford et al., 1974) for the HEWL complex of the δ-

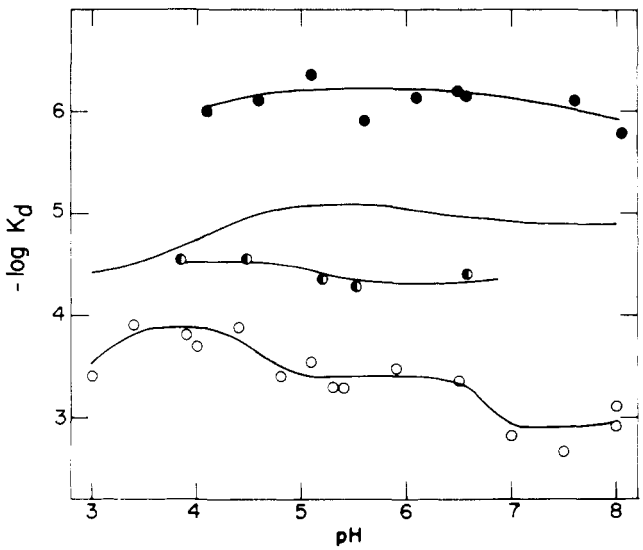


FIGURE 3: pH dependence of the binding of saccharides to HEWL. (○) $(GlcNAc\text{-}MurNAc)_2$; (◐) $(GlcNAc\text{-}MurNAc)_3$; (●) GlcNAc-MurNAc-GlcNAc-XylNAc; all at 22 °C in citrate–phosphate buffers (or Mes at pH 6.58). Solid line: calculated curve for $(GlcNAc)_3$ at 30 °C (Banerjee and Rupley, 1973).

TABLE IV: Effect of Gadolinium on Association of Saccharides with HEWL.[a]

| Saccharide | $-\Delta F_u$ (kcal/mol)[b] with 20 mM Gd(III) | $-\Delta F_u$ (kcal/mol)[b] No Gd(III) |
|---|---|---|
| $(GlcNAc)_3$ | 9.0 (9.2[c]) | 9.3 (9.3[c]) |
| $(GlcNAc)_4$ | 9.5 (9.4[c]) | 9.6 (9.7[c]) |
| $(GlcNAc)_3$-XylNAc | 9.5 | 9.7 |
| GlcNAc-MurNAc-GlcNAc | 9.5 | 9.9 |
| $(GlcNAc\text{-}MurNAc)_2$ | 6.7 | 7.0 |
| GlcNAc-MurNAc-GlcNAc-XylNAc | 10.5 | 10.8 |
| GlcNAc-MurNAc-GlcNAc-GlcNAc | 9.9 | 10.5 |

[a] pH 6.58 in 10 mM Mes buffer–0.1 N NaCl, with addition of 20 mM $GdCl_3$ where indicated, at 22–23 °C. [b] $\Delta F_u = RT \ln K_d - 2.4$ kcal/mol. [c] Data of Secemski and Lienhard (1974).

lactone derivative of $(GlcNAc)_4$ (Table III, compound 13), C(1) and O(1) of these residues would be between Asp-52 and Glu-35. Measurements of binding to HEWL were also carried out under conditions designed to detect possible interactions between the bound saccharide and these residues. Gadolinium(III) ion is known to bind between these two carboxyls in HEWL crystals and is thought to occupy the same site in solution (Dwek et al., 1971; Kurachi et al., 1975), with a dissociation constant of 0.5 mM at pH 6.5 (Secemski and Lienhard, 1974). The measured apparent unitary free energies of binding to HEWL in the presence of 20 mM Gd(III) are reported in Table IV and compared with those measured in the absence of Gd(III). The results of Secemski and Lienhard (1974) for two compounds are also given in the table. The presence of Gd(III) does not lead to a marked decrease in the affinity of HEWL for any of the saccharides tested.

The pH dependence of the dissociation constant for several saccharides is presented in Figure 3, together with the calculated curve for the binding of $(GlcNAc)_3$ to HEWL from the work of Banerjee and Rupley (1973). The dissociation constants for complexes of GlcNAc-MurNAc-GlcNAc-XylNAc, $(GlcNAc\text{-}MurNAc)_2$, and $(GlcNAc\text{-}MurNAc)_3$ do not show pH dependence significantly more marked than does that for $(GlcNAc)_3$ in the pH range 4.0–6.5, where Asp-52 and Glu-35 ionize (Imoto et al., 1972b). (The reason for the slight increase in the affinity of the enzyme for $(GlcNAc\text{-}MurNAc)_2$ and $(GlcNAc\text{-}MurNAc)_3$ upon going from pH 5 to 4 is not clear.)

These results suggest that the terminal groups of the tetrasaccharides in question are bound in subsite D in a very different position from that proposed for the HEWL–$(GlcNAc)_6$ model (Blake et al., 1967; Imoto et al., 1972b) or observed for the complex of HEWL with compound 13 (Ford et al., 1974). It is likely that these groups are not bound deeply in the cleft as in the above structures, but rather lie near the surface of the cleft, e.g., as observed for the glucose residue of GlcNAc-glucose (Beddell et al., 1970).

Examination of a three-dimensional model of HEWL–$(GlcNAc)_6$ shows that if a residue in subsite D is moved significantly out of the cleft relative to its position in the model, the position of the residue in subsite C must also change; such differences have been observed (Beddell et al., 1970). This is indicated for the saccharides studied here as well, by the data presented above. The calculated contribution of a given residue in subsite D to $\Delta F_u$ for a HEWL complex is variable (e.g., compare contributions of MurNAc for saccharides 8 and 12 in Table III), which implies that the complexes in question are not superimposable. The calculated contributions of residues are thus functions not only of contacts in subsite D, but also of the conformational changes elsewhere in the cleft caused by the introduction of the given residue.

*Comparison of Saccharide Binding to HL and HEWL.* The unitary free energies of binding of the various saccharides to HEWL and HL can be compared in Table II. As noted previously (Kuramitsu et al., 1975; Mulvey et al., 1973; Teichberg et al., 1972), the binding of chitin oligomers to HL is weaker than to HEWL. This is also the case for GlcNAc-MurNAc-GlcNAc and several of the other saccharides examined here. Differences in binding in subsites A–C of the two proteins are to be expected, as a number of amino acid residues in contact with $(GlcNAc)_3$ bound in this region in HEWL are substituted in HL (Banyard et al., 1974; Blake and Swan, 1971; Canfield et al., 1971; Jollès et al., 1974). Most important of these substitutions may be the replacement of Trp-62, which makes nonpolar contacts with residues in subsites A, B, and C and whose indole NH is hydrogen bonded to O(6) of the residue in subsite C in HEWL (Imoto et al., 1972b), by a tyrosine in the human enzyme (numbered Tyr-63 because of an insertion). This replacement is probably responsible for the very low affinity of HL for *N*-acetyl-D-glucosamine itself. We suggest that the localized peak in subsite C observed in the crystallographic map of the HL–*N*-acetyl-D-glucosamine complex (Banyard et al., 1974; Blake and Swan, 1971) may represent binding of the $\alpha$-anomer of the sugar, as it has been noted that in HEWL the $\alpha$-anomer, unlike the $\beta$-anomer, does not make a hydrogen bond with Trp-62 (Blake et al., 1967; Imoto et al., 1972b). In any case, the large difference between $\Delta F_u$ for the binding of *N*-acetyl-D-glucosamine in subsite C of the two enzymes appears to play a major role in the difference in their affinities for oligosaccharides (Table II).

In contrast to the weaker binding by human lysozyme of many oligosaccharides, this enzyme binds $(GlcNAc\text{-}MurNAc)_2$ more tightly than does HEWL. If one assumes that GlcNAc-MurNAc-GlcNAc is bound in subsites A–C and

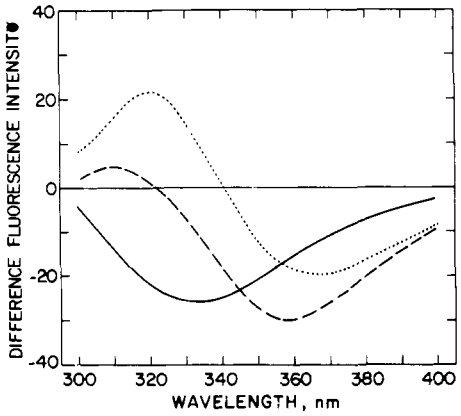


FIGURE 4: Difference fluorescence emission spectra (excitation 280 nm) of HEWL at pH 5.6: (···) between enzyme-$(GlcNAc)_3$ complex and free enzyme; (- - -) between enzyme-GlcNAc-MurNAc-GlcNAc and free enzyme; (—) between enzyme-$(GlcNAc)_3$ and enzyme-GlcNAc-MurNAc-GlcNAc. Enzyme concentration was $1 \times 10^{-6}$ M, and saccharide concentration 20 times $K_d$ for each complex.

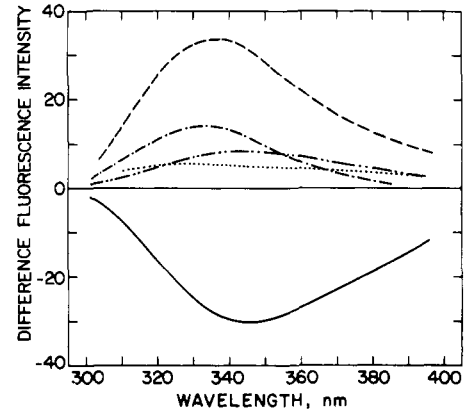


FIGURE 5: Difference fluorescence emission spectra between complexes of HEWL at pH 5.6. Complexes compared are of: $(GlcNAc)_3$-XylNAc — $(GlcNAc)_3$ (- • - • -); $(GlcNAc)_4$ $(GlcNAc)_3$ (- • • - • •); $(GlcNAc\text{-}MurNAc)_2$ (GlcNAc-MurNAc-GlcNAc) (—); GlcNAc-MurNAc-GlcNAc GlcNAc GlcNAc-MurNAc-GlcNAc (···); and GlcNAc-MurNAc-GlcNAc-XylNAc GlcNAc-MurNAc-GlcNAc (- - -). Enzyme concentration was $1 \times 10^{-6}$ M, and saccharide concentration 10-20 times $K_d$ for each complex.

$(GlcNAc\text{-}MurNAc)_2$ in A-D in HL as they are in HEWL,[3] the destabilization caused by adding a MurNAc residue in subsite D to the HL-GlcNAc-MurNAc-GlcNAc complex is only 1.5 kcal/mol. The difference in the destabilizing effect of MurNAc in subsite D for the two enzymes may be related as much to the nature of subsite C as it is to the nature of subsite D. "Looser" contacts in subsite C in HL as compared to HEWL would explain the lower apparent strain caused by introduction of MurNAc into subsite D in the former enzyme.

It would appear (Table II) that subsite D of HL can accommodate a GlcNAc residue, and that the destabilization caused by a MurNAc residue in this subsite is due to the 3-*O*-(1-carboxyethyl) group, as in the case of HEWL. Subsite D in HL may even be more favorable than subsite A for the binding of a GlcNAc residue; indeed, Banyard (1973) has suggested on the basis of low resolution crystallographic studies that $(GlcNAc)_3$ is bound in subsites B-D in HL.

*Fluorescence Difference Spectra.* Any enzyme-saccharide complex will attain the conformation of lowest energy by appropriate arrangements of both the saccharide and the protein. The fluorescence spectra of lysozyme complexes might be expected to reflect the conformational differences between them. Difference fluorescence emission spectra of HEWL-saccharide complexes at pH 5.6 in citrate-phosphate buffer are shown in Figures 4 and 5. On the basis of previous studies (Formoso and Forster, 1975; Imoto et al., 1972a; Halford, 1975; Lehrer and Fasman, 1966, 1967; Teichberg and Sharon, 1970), it is generally accepted that the major fluorophores of hen egg-white lysozyme are Trp-108 and Trp-62, with emission maxima at about 340 and 355 nm, respectively, and an additional residue (Trp-63?) with a short wavelength emission. Upon binding of $(GlcNAc)_3$ in subsites A-C at pH 5.6, the fluorescence emission maxima of Trp-108 and Trp-62 are shifted to the blue. Direct comparison of the HEWL complexes of GlcNAc-MurNAc-GlcNAc and $(GlcNAc)_3$ (Figure 4) shows that in the former a fluorophore (or fluorophores) of emission maximum about 330 nm is quenched compared to the latter. In the HEWL-$(GlcNAc)_3$ complex there is a hydrogen bond from O(3) of the residue in subsite B to O(5) of that in A (Imoto et al., 1972b). The substitution of O(3) with a 1-carboxyethyl group not only eliminates the possibility of forming this bond, but must also require some twisting of the residues in subsites A and B with respect to one another. The finding of a difference in the spectra of the two complexes is not surprising if the change in relative positions of these groups influences the conformation of the active site cleft in the vicinity of one of the tryptophans.

Each curve in Figure 5 is the directly measured difference between the emission of a tetrasaccharide complex of HEWL and the complex of the related trisaccharide which lacks the reducing terminal group of the tetrasaccharide. The further spectral differences observed upon adding a residue in subsite D to the HEWL-GlcNAc-MurNAc-GlcNAc complex are large. An added MurNAc residue leads to quenching (maximum ~345 nm), a GlcNAc residue causes little change, and a XylNAc residue leads to enhancement (maximum ~335 nm), compared to the parent complex. The wavelength of the maximum and the width of the difference spectrum for the addition of the MurNAc residue make it unlikely that the sole fluorophore affected is that in the proximity of subsite D, Trp-108. The detailed structure of a HEWL-$(GlcNAc\text{-}MurNAc)_2$ complex must, of course, await crystallographic examination, but it seems to us extremely likely that the conformation of the complex in the region of subsites A-C is very different from that of a GlcNAc-MurNAc-GlcNAc complex, or that of the other tetrasaccharide complexes studied.

The difference spectra (complex − enzyme) for the binding of a number of compounds to HL at pH 5.6 and 7.6 are given in Figure 6 and compared with difference spectra for HEWL. As Mulvey et al. (1973) have pointed out, the quantum efficiency of the fluorescence of human lysozyme is low compared to that of HEWL, presumably due to the absence of Trp-62, a major contributor to fluorescence in the latter protein. The absence of Trp-62 also affects the difference spectra, which are smaller on an absolute basis[4] for HL and lack the large

[3] This appears to be a very reasonable assumption for much the same reasons as it is for HEWL; the structure of the human enzyme (Banyard et al., 1974; Blake and Swan, 1971) also indicates that subsites A, C, and E would be prohibited to MurNAc residues.

[4] The difference spectra of Figure 6 are given relative to the peak height of the free enzyme in each case, so that the human lysozyme spectra are weaker, on an absolute scale, than they appear to be in the figure.

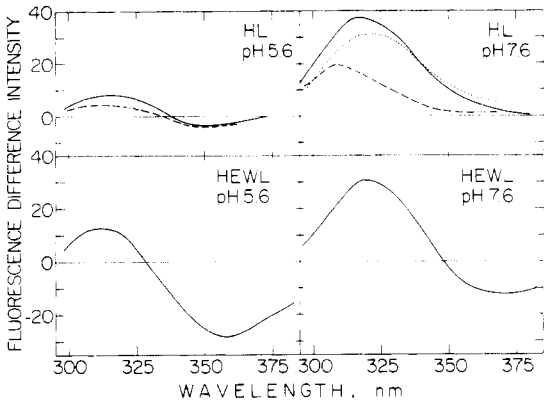


FIGURE 6: Difference fluorescence emission spectra (complex–enzyme) for complexes of HL and HEWL: (—) GlcNAc-MurNAc-GlcNAc-GlcNAc; (- - -) $(GlcNAc\text{-}MurNAc)_2$; (···) GlcNAc-MurNAc-GlcNAc-XylNAc. Enzyme concentration was $2.5 \times 10^{-6}$ M, and saccharide concentration 10–20 times $K_d$ for each complex.

negative limb ($\lambda_{max}$ 350–360) characteristic of difference spectra for saccharide binding to HEWL. The quenching of Trp-108 between pH 7.6 and 5.6 by the protonation of Glu-35 in the enzyme–saccharide complexes (Mulvey et al., 1973) decreases markedly the difference between the emission spectrum of HL and its saccharide complexes, with the difference spectrum for the $(GlcNAc\text{-}MurNAc)_2$ complex becoming extremely small. Caution must be taken in such interpretations, since the fluorescence properties of HL have not been studied in very great detail. Extrapolations from studies of HEWL are limited by the fact that the effects on fluorescence of aromatic replacements other than Trp-62 → Tyr-63 (e.g., Phe-34 → Trp-34 (Canfield et al., 1971)), and of subtle conformational changes, are unknown.

*The Catalytic Role of Enzyme-Transition State Complementarity.* The results discussed above have important implications for the mechanism of lysozyme catalysis. Estimates of the contribution to catalysis of steric strain at the C(5) hydroxymethyl group on the residue in subsite D, which are based on comparisons between the binding of $(GlcNAc\text{-}MurNAc)_2$ and any other saccharide (Chipman et al., 1967; Secemski et al., 1972; van Eikeren and Chipman, 1972), are not valid. A reassessment of the "transition state analogue" of highest affinity prepared to date (compound 13 in Table III, $K_d = 8 \times 10^{-8}$ M at pH 6.5 (Secemski et al., 1972)) in the light of our new data indicates that the terminal lactone of this compound is bound in subsite D only about 30-fold (2.2 kcal/mol) more tightly than a GlcNAc residue.[5] This is insignificant compared to the $10^7$–$10^9$-fold acceleration ascribed to lysozyme catalysis (Chipman, 1971).

Warshel and Levitt (1976) have concluded, on the basis of calculations of the potential energy of complexes of HEWL with GlcNAc oligomers, that there is no torsional distortion in the ground state of a lysozyme–substrate complex. While our results are in accord with this conclusion for tetrasaccharide complexes with reducing termini in subsite D, they do not necessarily rule out the possibility of "distortionless strain" (Fersht, 1974) in the lysozyme mechanism, e.g., that the transition state has additional binding interactions which are not realized in the enzyme–substrate complex. The positioning of residues in subsite D near the surface of the cleft, inferred above, suggests this sort of strain. More compelling evidence comes from the transglycosylation behavior of the enzyme (Chipman et al., 1968; Pollock and Sharon, 1970b; Rupley and Gates, 1967), which shows that the glycosyl-enzyme intermediate reacts with saccharides (of specific structure) far more readily than with water. Since microscopic reversibility requires that the transition state for the formation of a new glycosyl bond (transglycosylation) must be identical to that for bond cleavage, the same contacts in subsites E and F, which stabilize the former by 4–5 kcal/mol (Chipman, 1971), must stabilize the latter. Such contacts are absent from the ground-state complex of, say, $(GlcNAc\text{-}MurNAc)_3$, as witnessed by a difference of only −1.4 kcal/mol between $\Delta F_u$ for it and for $(GlcNAc\text{-}MurNAc)_2$ (Table II).

A number of other kinetic results support the proposal that the development of contacts between lysozyme and its substrates at the transition state are of catalytic importance. Capon and Dearie (1974) found that $k_{cat}/K_m$ for cleavage of the *p*-nitrophenyl group from $(GlcNAc)_4$ nitrophenyl glycoside is at least 500 times that for $(GlcNAc)_3$-XylNAc nitrophenyl glycoside. $k_{cat}/K_m$ is directly related to the energy difference between the enzyme–transition state complex and free enzyme plus free substrate, and is not affected by nonproductive binding. These results are of particular interest in light of our redetermination of the dissociation constant for the HEWL–$(GlcNAc)_3$-XylNAc complex, and may imply that, even though steric hindrance to the C(5) hydroxymethyl group in subsite D may not be important in *ground-state* binding, interactions of this group with the enzyme stabilize the *transition state* for bond cleavage.

Experiments in progress in our laboratories with substrates that do not undergo transglycosylation indicate that residues interacting with subsites E and F can increase $k_{cat}/K_m$ by at least two orders of magnitude. Despite the strain involved in the ground-state binding of an *N*-acetylmuramic acid residue in subsite D, $k_{cat}/K_m$ for the cleavage of $(GlcNAc\text{-}MurNAc)_3$ and $(GlcNAc)_6$ by HEWL are similar. It is also of interest, in view of the generally weaker binding of saccharides by HL, that HL is from two to ten times less active than HEWL in hydrolysis of a linear peptidoglycan from *M. luteus* (Mirelman et al., 1974), or $(GlcNAc\text{-}MurNAc)_3$ under various conditions (preliminary results from our laboratories).[6] Such observations must be accounted for in any mechanism proposed for lysozyme.

An assessment of the catalytic role of geometric complementarity between lysozyme and the transition state for cleavage of its substrates must take into consideration all the enzyme–saccharide interactions. Until recently, attention has been focused on the contacts between the enzyme and the C(5) hydroxymethyl group on the residue in subsite D. We have shown above that there is little or no ground-state strain due to this interaction. However, the interactions of the enzyme with various parts of the substrate presumably change on going from the ground state of the complex to the transition state. Any of these changes may be of catalytic significance and one must consider, in addition to the contacts of the hydroxymethyl group, those of the O(3) 1-carboxyethyl group of a MurNAc residue in subsite D, of leaving group moieties in subsites E and F, and even of residues in subsites A–C, which may undergo subtle changes during the course of the enzymatic reaction.

[5] The relevance of this analogue to steric strain is questionable in any case, as the lactone carbonyl oxygen forms a hydrogen bond to Asp-52 (Ford et al., 1974), which may account for part of its affinity.

[6] HL is more active than HEWL in the *M. luteus* clearing assay (Mulvey et al., 1973).

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