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Experimental Identification of a Theoretically Predicted "Left-Sided" Binding Mode for $(GlcNAc)_6$ in the Active Site of Lysozyme†

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ABSTRACT: Using conformational energy calculations, we previously predicted that there are two distinct binding modes for hexasaccharide substrates of hen egg white lysozyme (HEWL), a "left-sided" binding mode and a "right-sided" one. The former involves such residues as Arg-45, Asn-46, and Thr-47, while the latter involves such residues as Asn-113 and Arg-114. The left-sided binding mode was predicted to predominate for $(GlcNAc)_6$. We now present two lines of experimental evidence that indicate that left-sided binding occurs for this substrate. First, we show that ring-necked pheasant lysozyme (RNPL), in which Lys and His replace Asn and Arg at positions 113 and 114, respectively, has the same affinity for $(GlcNAc)_6$ as does HEWL, indicating that the "right" side is *not* involved in equilibrium binding to the substrate. Second, we show that a monoclonal antibody, HyHEL-5, which binds specifically to an epitope including residues Arg-45, Asn-46, Thr-47, Asp-48, and Arg-68 on the far "left" side of HEWL, is competitively displaced by $(GlcNAc)_5$ and $(GlcNAc)_6$ but not by GlcNAc, $(GlcNAc)_2$, or $(GlcNAc)_4$. Only the former two substrates can bind in site F in the lower active site. Since these two substrates are the only ones that competitively displace HyHEL-5, our results suggest that the terminal saccharide residues of these substrates bind to the left side of the active site cleft, as predicted from theory.

On the basis of theoretical considerations (Pincus & Scheraga, 1979, 1981a,b; Scheraga et al., 1982), we found that hexasaccharide substrates, including the homopolymer $(GlcNAc)_6$[1] and copolymers of GlcNAc and MurNAc, exhibit two distinct binding modes to the active site of hen egg white lysozyme, a "left-" and "right-sided" mode. For the homopolymer $(GlcNAc)_6$, the preferred binding mode is one involving the left side of the active site in which the terminal (reducing) sugar binds to a β-sheet region formed in part by Arg-45, Asn-46, and Thr-47. In this binding mode, the sugar residue in the D site adopts the full-chair conformation (i.e., no distortion of the D-site saccharide residue is necessary) and occupies a position somewhat removed from the deep-cleft region between the two acid catalytic residues, Glu-35 and Asp-52. The first three residues from the nonreducing end in sites A–C occupy positions quite similar to those determined by X-ray diffraction analysis (Imoto et al., 1972; Pincus et

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[1] Abbreviations: GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; $(GlcNAc)_n$, n β-1,4-linked *N*-acetylglucosamine units; HEWL, hen egg white lysozyme; RNPL, ring-necked pheasant lysozyme; HyHEL-5, hybridoma anti-hen egg white lysozyme, clone number 5; mAb, monoclonal antibody; BSA, bovine serum albumin, Pentex fraction V; ELISA, enzyme-linked immunosorbant assay.

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al., 1976). Our calculated D-site binding disposition has been confirmed both by solution studies (Schindler et al., 1977) and by X-ray crystallographic studies (Kelly et al., 1979). Extension of the polysaccharide chain bound to sites A–D in our calculated mode results uniquely in the left-sided binding mode described above. Another, energetically less favorable, right-sided binding mode also exists in which the D-site sugar residue is buried more deeply in the cleft and in which the terminal reducing residue in site F makes contacts with such residues as Asn-113 and Arg-114. This calculated right-sided binding structure is similar to the one proposed on the basis of X-ray crystallographic model-building studies (Imoto et al., 1972). Our calculations predict that the left-sided binding mode should predominate (Pincus & Scheraga, 1979, 1981b; Scheraga et al., 1982). Color stereo views, as well as line drawings showing the residues of the enzyme that contact the substrate, in both binding modes have been presented elsewhere (Pincus & Scheraga, 1979).

In extensive studies on the kinetics and thermodynamics of the interactions of different homopolymers of GlcNAc with lysozyme (Banerjee et al., 1975), it has been found that two types of equilibrium complexes form between $(GlcNAc)_6$ and lysozyme. The first is one (called the α-complex) in which only three residues of the substrate bind to the high affinity sites A–C, and the other is one in which all six residues make contact with the enzyme. In a second step, the "productive" binding mode (also referred to as the γ-complex), the interactions between enzyme and substrate become enhanced while catalysis occurs (Banerjee et al., 1975). It is in this step that D-ring distortion is thought to occur. Our calculations pertain to the first or recognition step in which the enzyme forms an initial stable complex with the substrate.

Until now, there have been no experimental studies to determine which residues of the enzyme are involved in substrate (hexasaccharide) binding in the "lower" active site, i.e., sites E and F, although binding of GlcNAc-MurNAc to the lower active site of turkey egg white lysozyme has been observed (Sarma & Bott, 1977). In the present study, we present two independent lines of experimental evidence that show directly (1) that the right side of the active site cleft (involving such residues as Asn-113 and Arg-114) is *not* involved in the binding of $(GlcNAc)_6$ to lysozyme in the initial binding step and (2) that the left side of the active site (involving such residues as Arg-45, Asn-46, and Thr-47) *is* involved in the initial binding step, as predicted previously from conformational energy calculations (Pincus & Scheraga, 1979, 1981a,b; Scheraga et al., 1982). In the first type of experiment, we show that pheasant lysozyme, which has radical substitutions at positions 113 and 114 [Lys and His replace Asn and Arg, respectively, at these positions (Jollès et al., 1979)], nevertheless exhibits exactly the same affinity for $(GlcNAc)_6$ as does hen egg white lysozyme. In the second type of experiment, we show that a monoclonal anti-lysozyme antibody HyHEL-5 (Smith-Gill et al., 1982) directed specifically against the region of the molecule containing residues Arg-45, Asn-46, and Thr-47, i.e., the left side of the active site, is competitively displaced by the substrate $(GlcNAc)_6$.

Materials and Methods

Lysozymes. Hen egg white lysozyme (3 times recrystallized) was obtained from Worthington. Ring-necked pheasant eggs were obtained from LL Pheasantry, Hegins, PA, and the lysozyme was isolated and purified as described previously (Smith-Gill et al., 1982).

Saccharides. GlcNAc and its oligomers were prepared by acid hydrolysis of chitin (Sigma) as described previously (Raftery et al., 1969; Banerjee et al., 1973).

Steady-State Kinetics. The rate of hydrolysis of $(GlcNAc)_6$ by hen egg white or pheasant lysozyme was determined by product analysis, as described previously (Banerjee et al., 1973) with the following modifications. Lysozyme was reacted with $(GlcNAc)_6$ at 40 ± 0.1 °C in a 0.01 M ammonium acetate buffer solution of pH 5.2, for a time sufficient to give 16–50% hydrolysis of the substrate. The volume of the reaction mixture was chosen to give a total saccharide content suitable for product analysis. Saccharide concentration was varied between 8×10^{-6} and 8×10^{-5} M and the enzyme concentration between 10^{-8} and 10^{-7} M. Enzyme concentration was determined by measurement of the absorbance of the stock solution used to prepare the reaction mixture, with a 280-nm molar extinction coefficient of 38 000 M^{-1} cm^{-1}. At the end of the reaction, the solution was lyophilized and, if necessary, redissolved and lyophilized again to remove volatile salt. The dry sample was dissolved in 0.2 mL of water. A 0.05-mL aliquot was analyzed by high-performance liquid chromatography, with a Waters apparatus equipped with a μBondapak NH_2 carbohydrate column and an AX/CORASIL precolumn, run at a 2 mL/min flow rate with a 75:25 acetonitrile–water eluent. The concentrations of the GlcNAc oligomers were determined by comparison of the areas of eluted peaks between unknown and standard mixtures. The rate of reaction was calculated from the extent of conversion of $(GlcNAc)_6$ to the hydrolysis products, predominantly the dimer and tetramer. Estimates of the kinetic parameters K_M and k_{cat} were obtained by nonlinear least-squares analysis of the reaction velocity as a function of enzyme and substrate concentration (Banerjee et al., 1973).

Monoclonal Antibody. In a previous paper, a method for developing monoclonal antibodies (mAb) direct against hen egg white lysozyme was described (Smith-Gill et al., 1982). One of these antibodies, designated as HyHEL-5, was shown to interact strongly with a determinant on hen egg white lysozyme involving the following residues: Arg-45, Asn-46, Thr-47, Asp-48, and Arg-68 (Smith-Gill et al., 1982). It was possible to identify this determinant from a study of the ability of this antibody to cross-react with eight other avian lysozymes of known sequence. In the case of the HyHEL-5 antibody, any lysozyme species that had changes in one or more of these residues exhibited a markedly decreased affinity for the antibody. Conversely, lysozymes that had no changes in these residues but had major substitutions elsewhere (such as pheasant lysozyme at positions 113 and 114) exhibited identical affinities to that of hen egg white lysozyme (Smith-Gill et al., 1982).

Competitive Binding Experiments. The purpose of these experiments was to determine whether there was competition between the binding of the monoclonal HyHEL-5 antibody and various substrates and inhibitors of lysozyme, i.e., whether substrates and inhibitors could displace the antibody from HEWL. Substrate inhibition of antibody binding to HEWL was examined in a low-temperature ELISA plate-binding assay. It is known from previous work (Douzou et al, 1974; Fink et al., 1980) that, at temperatures below −20 °C in a variety of antifreeze buffers, the rate of lysozyme-catalyzed hydrolysis approaches zero (i.e., $k_{cat} = 0$) for cell wall substrates and for $(GlcNAc)_6$. However, the binding of substrates to the enzyme is actually enhanced (Douzou et al., 1974; Fink et al., 1980). Furthermore, the fact that Arrhenius plots of the logarithm of the rate of reaction vs. temperature over all temperatures between 25 and −20 °C are linear suggests that no major structural changes occur in the enzyme–substrate

complex (Douzou et al., 1974; Fink et al., 1980). In the competitive binding experiments, 96-well V-bottom microtiter plates were coated with 50 μL of 1 μg/mL HEWL for 1 h followed by 1% BSA for 1 h at room temperature and then frozen overnight at −22 °C. Tissue culture supernatants containing HyHEL-5 were diluted 1/1200, giving a final estimated concentration of antibody ranging between approximately 1×10^{-12} and 6×10^{-12} M (Smith-Gill et al., 1982). Saccharide solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.6, and diluted 1:1 with 50% methanol just prior to use. Antibody and saccharide solutions were kept on ice. The prechilled plates were placed on a block of dry ice, and 25 μL each of both saccharide and antibody was added to each well. The final concentration of methanol was 12.5%. The plates were covered, incubated overnight at −22 °C, and developed the following day to determine the amount of bound antibody as described previously (Smith-Gill et al., 1982).

For every saccharide incubation experiment, an appropriate control experiment was performed in which 0.1% bovine serum albumin replaced the saccharide in the incubation mixture, as described previously (Smith-Gill et al., 1982). To determine whether the antifreeze buffer or other assay conditions caused inactivation of HyHEL-5, the following 12-h incubations with lysozyme-coated microtiter plates were carried out under conditions identical with those described for the saccharide incubation experiments: antibody in buffer, antibody plus HEWL in buffer, and antibody plus bobwhite quail lysozyme in buffer. In the second experiment, competitive binding from HEWL in solution was able to inhibit antibody binding to the plate completely. In the last experiment, bobwhite quail lysozyme failed to inhibit antibody binding to the plate competitively (Smith-Gill et al., 1982). HyHEL-5 is known not to bind to bobwhite quail lysozyme because this enzyme has a substitution at Arg-68 on the left side of the active site (Smith-Gill et al., 1982). The fact that HEWL inhibits HyHEL-5 from binding to the plate, while bobwhite quail lysozyme does not inhibit binding, indicates the functional specificity of HyHEL-5 for HEWL is preserved.

Results and Discussion

Steady-State Kinetics. Among the many known homologous avian lysozyme species, there is one, ring-necked pheasant, that has a sequence around the active site that is identical with that of HEWL except at positions 113 and 114 where Lys-113 and His-114 replace Asn-113 and Arg-114 of HEWL (Jollès et al., 1979). If these residues are critical in binding to the substrate in the equilibrium complex, such major changes in residues, which change not only side-chain length and structure but also net charge, should influence the affinity of the enzyme for the substrate.

We have thus obtained the parameters K_M and k_{cat} for both of these enzymes in their reactions with $(GlcNAc)_6$. In prior studies (Banerjee et al., 1973), K_M has been shown to be a true dissociation constant. Table I compares the steady-state kinetic parameters for HEWL and RNPL. There is no significant difference in K_M between the two enzymes. We conclude that right-sided binding is not involved in the formation of the equilibrium complexes.

It is interesting that, while the K_M's of these two enzymes do not differ significantly, there is a small difference in k_{cat}, wherein the value for HEWL is about 3 times greater than that for RNPL. This difference may reflect differences in the abilities of the right-sided residues to interact with the substrate in the *transition state*. This conclusion is also compatible with our calculated results (Pincus & Scheraga, 1979) in which increased stability of the right-sided complex occurs if the ring of the saccharide unit in the D site is distorted, as it would be in the transition state.

Table I: Results of Steady-State Kinetic Studies on the Binding of $(GlcNAc)_6$ to Hen Egg White and Ring-Necked Pheasant Lysozymes[a]

enzyme	$K_M \times 10^6$ (M)	k_{cat} (s^{-1})
hen egg white	15.6 ± 9.5	0.160 ± 0.032
(Asn-113, Arg-114)	9.7 ± 1.9[b]	0.142 ± 0.005[b]
ring-necked pheasant	12.6 ± 3.1	0.059 ± 0.004
(Lys-113, His-114)		

[a] Reactions were carried out at 40 °C in 0.01 M ammonium acetate buffer solution at pH 5.2. Uncertainties are standard deviations. [b] Values from Banerjee et al. (1973), for reaction at 40 °C, pH 5, and ionic strength 0.1.

Competitive Displacement of Monoclonal Antibody HyHEL-5 Directed against the Left Side of the "Lower Cleft". The above comparative approach used for the right side could not be used on the left side, both because only conservative substitutions occur at positions 45–47 and because the substrate interacts exclusively with the *backbone* atoms of these residues, which are in the extended conformation (Pincus & Scheraga, 1979, 1981a,b; Scheraga et al., 1982). Thus, as long as the extended conformation of the residues of the protein in this region is preserved, binding to the substrate on the left would be expected to remain unchanged, in spite of substitutions in this sequence.

However, it is known from comparative studies on the relative affinity of a monoclonal antibody, HyHEL-5, toward different avian lysozymes (Smith-Gill et al., 1982) that even conservative substitutions of the residues on the left side of the cleft and also at Arg-68 drastically affect the affinity of HyHEL-5 for the enzyme, indicating that it binds to this left-sided region of the enzyme. This antibody exhibits the same affinity for HEWL and RNPL, which have the same residues on the left side (Smith-Gill et al., 1982), indicating that it does not make contact with the right side of the cleft, where the residues differ. It can therefore be used as a probe to determine whether $(GlcNAc)_6$ binds, in the equilibrium complex, on the left side of the lower active site cleft.

Previous studies (Smith-Gill et al., 1982) have already shown that the dye Biebrich Scarlet [known to bind exclusively to the F site of HEWL (Holler et al., 1975)] is displaced by HyHEL-5 from HEWL, indicating that the dye itself lies at least partially in the region of residues 45–47. In Figure 1, the results of competition experiments are shown in which different oligosaccharides of GlcNAc are allowed to compete with HyHEL-5 in binding to HEWL. These experiments were conducted at −20 °C, at which temperature no catalysis occurs but binding is actually stronger (Douzou et al., 1974; Fink et al., 1980).

As may be seen in Figure 1, only $(GlcNAc)_6$ and $(GlcNAc)_5$ cause significant inhibition (>50%) of antibody binding. The substrate $(GlcNAc)_6$, in fact, at the highest concentrations studied ($\sim 1 \times 10^{-3}$ M) displaces almost 75% of the lysozyme-bound antibody. In contrast, neither GlcNAc nor $(GlcNAc)_2$, which bind to site C and to sites B and C, respectively (Imoto et al., 1972) inhibits antibody binding to an appreciable extent, even at the highest concentrations. The inhibitor $(GlcNAc)_4$ appears to cause some inhibition of antibody binding although the errors in the experiments with this saccharide were large. As noted in the legend of Figure 1, the range in percent antibody bound at the highest concentration of $(GlcNAc)_4$ was 40–95%. However, in Figure 1, the level of inhibition caused by $(GlcNAc)_4$ appears to be relatively constant and never reaches 50% even at the highest concen-

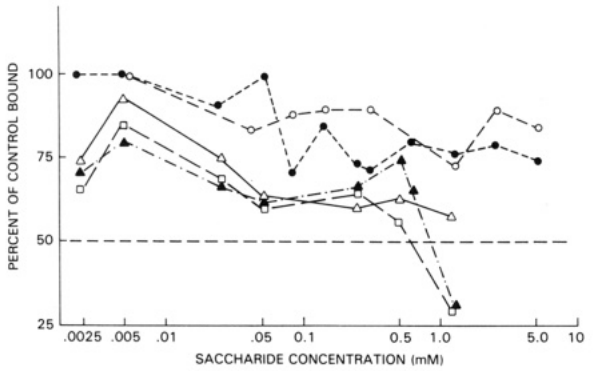

FIGURE 1: Semilogarithmic plot of the binding of HyHEL-5 to HEWL at −20 °C in plate-binding ELISA in the presence of (○) GlcNAc, (●) $(GlcNAc)_2$, (△) $(GlcNAc)_4$, (▲) $(GlcNAc)_5$, and (□) $(GlcNAc)_6$. Antibody was in tissue culture supernatant diluted 1/1200, giving a final estimated concentration of approximately 1×10^{-12} M. All points represent the means of at least six independent determinations. The 100% binding value was determined for each experimental plate with 0.1% BSA in 0.1 M potassium phosphate buffer, pH 7.6, in place of a saccharide solution. The ranges of values for percent of antibody bound, observed for each saccharide at the highest concentration of 1.25 mM, were (GlcNAc) 70–90%, $[(GlcNAc)_2]$ 70–86%, $[(GlcNAc)_4]$ 40–95%, $[(GlcNAc)_5]$ 21–37%, and $[(GlcNAc)_6]$ 17–38%.

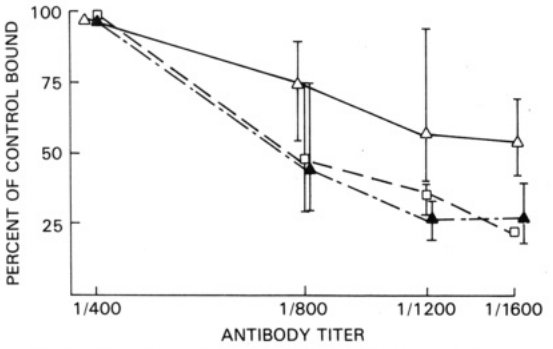

FIGURE 2: Semilogarithmic plot of different dilutions of tissue culture supernatant containing HyHEL-5 binding to HEWL at −20 °C in plate-binding ELISA, in the presence of 1.25 mM (△) $(GlcNAc)_4$, (▲) $(GlcNAc)_5$, and (□) $(GlcNAc)_6$. Vertical bars indicate range of values from independently run replicates.

trations ($\sim 1 \times 10^{-3}$ M) in approximately 100-fold excess of its K_d (Banerjee et al., 1973) (the limit of solubility for these saccharides). At this concentration, it is likely that 2 mol of $(GlcNAc)_4$ is bound per mol of lysozyme: one molecule binding in the high-affinity sites A–D or A–C (Imoto et al., 1972; Pincus & Scheraga, 1979; Pincus et al., 1976) and one in the lower affinity sites E–F (Imoto et al., 1972). In the latter binding mode, $(GlcNAc)_4$ would be expected to cause some displacement of the antibody.

It may be observed in Figure 1 that, even at low concentrations of $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$, some inhibition of antibody binding is seen even though these concentrations (e.g., 0.0025 mM) are below the K_d's or K_M's for these saccharides. In fact, at a concentration of 1×10^{-7} M, $(GlcNAc)_5$ and $(GlcNAc)_6$ produced 29 and 37% inhibition, respectively (data not shown). It is unlikely that this inhibition at low concentration results from direct displacement of the antibody. It is more likely that it is caused by binding of the oligosaccharides to sites (such as A–C) *not* occupied by the antibody. For $(GlcNAc)_6$, this binding would be the α-mode (Banerjee et al., 1973) and may indirectly lower antibody affinity. This explanation is supported by the observation that GlcNAc and $(GlcNAc)_2$, which bind exclusively to site C and to sites B and C, respectively (Imoto et al., 1972), cause some displacement of the antibody (Figure 1). The level of inhibition plateaus at 15–20% and remains constant *even* at concentrations in 50–100-fold excess of their K_d's (Imoto et al., 1972). If these two saccharides bind only to site C and to sites B and C and saturate these sites at high concentration, i.e., 5×10^{-3} M, the inhibition of antibody binding by monomer and dimer seen in Figure 1 must be caused by an indirect effect. A direct competition for, say, site C would results in increased displacement of the antibody (P. V. Hornbeck and A. C. Wilson, unpublished results).

Thus, the inhibition curves for $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$ in Figure 1 very likely have two components: an indirect or noncompetitive component due to binding to sites A–C (or D) causing *lowered* antibody affinity at the lower saccharide concentrations and a direct or competitive component due to binding in site F causing progressive *displacement* of the antibody at higher concentrations as occurs for $(GlcNAc)_5$ and $(GlcNAc)_6$ at concentrations around 1×10^{-3} M (Figure 1). Because the inhibition curve for $(GlcNAc)_4$ seems to reach a constant level of inhibition at high saccharide concentrations, it appears that the inhibition of antibody binding caused by $(GlcNAc)_4$ is mainly of the indirect type, although binding of this saccharide to sites E and F at these high concentrations may also cause some displacement of the antibody.

The quantitative differences between $(GlcNAc)_4$ and the two (penta- and hexasaccharide) substrates in inhibition of

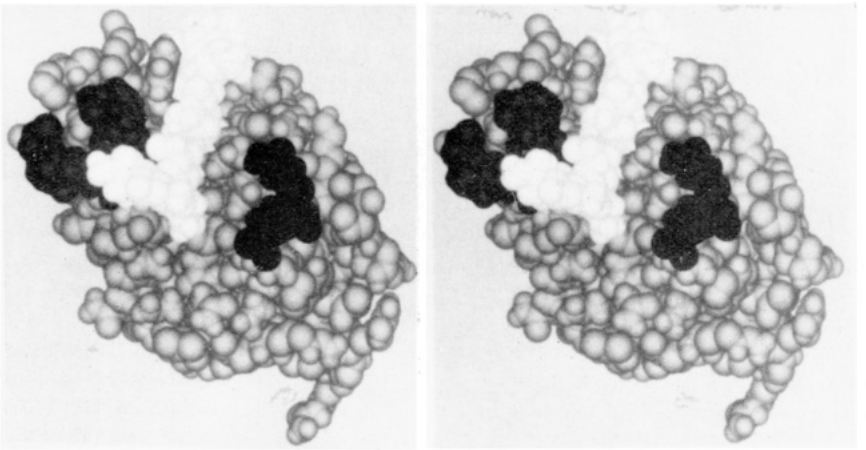

FIGURE 3: Space-filling stereo view of the computed equilibrium complex between $(GlcNAc)_6$ (white) and HEWL (Pincus & Scheraga, 1979). Most of the residues of the enzyme are gray except for the ones on the left side (Arg-45, Asn-46, Thr-47, Asp-48, and Arg-68) and those on the right side (Asn-113 and Arg-114), which are shaded more darkly. The dark-shaded residues on the left are those that interact with the antibody HyHEL-5.

antibody binding are illustrated further in Figure 2. In this set of experiments (plate-binding ELISA at −20 °C), each saccharide was held at a fixed saturating high concentration (1.25 mM) and tested for its ability to displace antibody, present in serial titers, from HEWL. As may be seen from this figure, at the three higher dilutions of antibody (1/800, 1/1200, and 1/1600), both substrates inhibited over 50% of antibody binding while the inhibition from $(GlcNAc)_4$ was less than 50%. (The titer of 1/1600 is the most dilute titer at which antibody can still be detected by ELISA.) At the three highest dilutions of antibody, there was no overlap in the values observed for $(GlcNAc)_4$ with those for either $(GlcNAc)_5$ or $(GlcNAc)_6$.

Thus, it is clear that only the longer saccharides that bind in the lower active site of HEWL, i.e., $(GlcNAc)_5$ and $GlcNAc)_6$, cause signficant competitive displacement of the antibody HyHEL-5. Because this monoclonal antibody binds only to the left side of the cleft, the equilibrium complex for binding of $(GlcNAc)_6$ must involve the residues on this side, vis., Arg-45, Asn-46, and Thr-47.

Steric Considerations. Our conclusions about the competitive binding of saccharides and HyHEL-5 to HEWL are based on the assumption that, when the antibody binds to HEWL in the left lower cleft region, it does not extend far across the cleft, thereby nonspecifically blocking access to *both* left- and right-sided binding regions. Several lines of evidence suggest that this type of steric blockage does not occur. First, as we have already noted, HyHEL-5 binds with the same affinity to both HEWL and RNPL. RNPL has drastic substitutions on the right side of the cleft that change side-chain length and structure and charge (one extra charge is introduced with the Lys-for-Asn substitution at position 113). If HyHEL-5 extended significantly far across the cleft, these changes would be expected to affect its affinity. Second, previous studies (Smith-Gill et al., 1982) have shown that substitution of a Lys for an Arg residue at position 68 in bobwhite quail lysozyme drastically lowers the affinity of HyHEL-5 for this enzyme. Arg-68 in HEWL occupies a position far removed from the active site cleft on the left side and far in back of it (Smith-Gill et al., 1982). It is unlikely that an antibody that binds to Arg-68 could, at the same time, stretch across the active site cleft.

Finally, the conclusion that HyHEL-5 does not sterically block the right side is supported directly by recent experiments (Smith-Gill et al., 1984) demonstrating that HEWL can simultaneously bind HyHEL-5 and another monoclonal antibody (HyHEL-9); the latter exclusively recognizes the right side of the cleft, i.e., Asn-113 and Arg-114, and fails to bind to RNPL. Figure 3 is a space-filling stereo view of the lowest energy structure for $(GlcNAc)_6$ (shown in white) bound to HEWL (Pincus & Scheraga, 1979). The dark-shaded residues on the left side of the enzyme (Arg-45, Asn-46, Thr-47, Asp-48, and Arg-68) are included in the epitope recognized by the antibody HyHEL-5 and interact with the reducing residue of the hexasaccharide substrate. The dark-shaded residues on the right side of the enzyme (Asn-113 and Arg-114) do not influence binding of HyHEL-5 or of the substrate in the equilibrium complex. The separation of the two sets of dark-shaded residues is consistent with the experimental evidence cited above.

Conclusions

We have shown that the HEWL substrate $(GlcNAc)_6$ probably does not interact with residues Asn-113 and Arg-114 in equilibrium complexes with lysozyme. Further, by employing an antibody (HyHEL-5), known to bind to the far left side of the cleft of the enzyme as a probe in competition experiments with substrates, we have at least tentatively identified the equilibrium binding position to be in the region of the left-sided residues Arg-45, Asn-46, and Thr-47, as predicted from theoretical conformational energy calculations.

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Registry No. $(GlcNAc)_6$, 38854-46-5; $(GlcNAc)_5$, 16334-31-9; lysozyme, 9001-63-2.

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