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Structures of enzyme–substrate complexes of lysozyme

(GlcNAc/binding regions/conformational energy/active site)

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ABSTRACT **Conformational energy calculations were used to determine the binding structures of two oligosaccharides $(GlcNAc)_n$, in which $n = 5$ and 6, in the *rigid* active site of lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17). Starting with the lowest energy binding structures of $(GlcNAc)_4$ as determined in a previous publication, we added a fifth GlcNAc residue to this tetramer in three different conformations, corresponding to the left-handed and right-handed helical structures and an intermediate structure, and the energy of each complex was minimized. The most stable binding conformation of the fifth residue of the pentamer was closest to the left-handed helical one. During energy minimization, the fourth residue of the pentamer moved from its initial position near the surface of the active site farther into the active site cleft at binding site D. Binding structures of $(GlcNAc)_6$ were then examined by addition of a residue to the lowest energy structure of $(GlcNAc)_5$, and it was found that the sixth residue of the hexamer binds in a conformation again close to the left-handed helical one. Stable binding regions of the rigid active site for the fifth and sixth residues were found to be near arginyl 45 and asparaginyl 46, on the opposite side of the active site cleft from arginyl 114. When the calculated structure of the lysozyme–$(GlcNAc)_4$ complex (used here as the starting structure for addition of the fifth and sixth residues) is compared with recent experimental data, it is found that the calculated structure is a reasonable one. Of all binding regions available to the saccharide residues, the C site binds GlcNAc with the lowest energy, in agreement with experiments.**

In a previous publication (1), we presented an analysis of the manner in which oligosaccharides (substrates and inhibitors) containing up to four units of GlcNAc bound to the *rigid* active site of the enzyme lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17). In that analysis, we centered our attention on the first four contiguous binding regions of the active site (i.e., sites A through D). We now point out that the lowest-energy structure of the lysozyme-$(GlcNAc)_4$ (lysozyme-tetra-*N*-acetyl-D-glucosamine) complex determined earlier (1) is in agreement with experimental data published subsequently (2). We also show here that successive GlcNAc residues can be added to the tetramer structure (proposed in ref. 1) to give unique low-energy structures for the lysozyme–$(GlcNAc)_5$ and lysozyme–$(GlcNAc)_6$ complexes.

It must be emphasized that in this paper, as in the earlier one (1), the active site of the enzyme is assumed to be rigid throughout the calculations; only the atoms of the substrate are allowed to move. The purpose here is to determine whether the rigid native enzyme possesses the structural information that allows for the recognition of the substrate. Because we have not minimized the energy of the isolated enzyme, the results presented here are based on the reported x-ray coordinates and could be altered if the x-ray coordinates of lysozyme are refined further.

Abbreviation: $(GlcNAc)_2$, di-*N*-acetyl-D-glucosamine, etc.

METHODS

The methods used in the determination of the total conformational energy, E_{com}, of the enzyme–substrate complex have been described (1, 3). Briefly, we chose low-energy structures of the oligosaccharides having various inter-ring dihedral angles ϕ and ψ (internal variables) and certain rigid body variables [X, Y, Z, α, β, and γ that describe the orientation of the oligosaccharide substrate with respect to the active site of lysozyme (1)] (3) as starting conformations for subsequent minimization of the total energy E_{com} that includes the (intramolecular) conformational energy of the substrate (E_{sub}) and the intermolecular energy of interaction between the rigid enzyme and substrate (E_{int}). Forty-one residues of the protein, those surrounding the active site cleft, were included in the calculations (1). The geometry and energy parameters, the method of searching for low-energy regions of the active site, and the results of the analysis of oligomers $(GlcNAc)_n$, in which $n = 2$–4 were described in ref. 1.

RESULTS AND DISCUSSION

Tetrasaccharide. In the calculation of the most stable binding structure of $(GlcNAc)_4$ to lysozyme (1), in which possible binding structures in the regions of sites A–D (but not in the regions of sites E and F) were considered, it was found that the first three residues of the tetramer bound (to sites A, B, and C) in a manner very similar to that of the first three residues of the energy-minimized x-ray structure of the complex of lysozyme with tetra-*N*-acetylglucosamine-δ-lactone (1, 4). However, the fourth residue (at site D) was calculated to be farther outside of the active site cleft and more removed from the two acid residues aspartyl 52 and glutamyl 35 than was the fourth residue of the energy-minimized x-ray structure or that of the x-ray structure itself; compare figure 1*A* with figure 1 *B* and *C* in ref. 1. Recently, experimental evidence has become available that suggests that the fourth residue of $(GlcNAc)_4$ binds in a region substantially removed from both acid residues. In particular, it was shown that $(GlcNAc)_4$ exhibited a pH dependence of binding essentially identical to that of $(GlcNAc)_3$ (2). Thus, the same acid residues were involved in binding both $(GlcNAc)_4$ and $(GlcNAc)_3$. Further, it was shown that gadolinium ion, known to bind specifically between aspartyl 52 and glutamyl 35, did not perturb the affinity of $(GlcNAc)_4$ (or of a number of other tetramers) for lysozyme (2). Most strikingly, the tetramer $(GlcNAc)_3$-*N*-acetylxylosamine, the last residue of which lacked the C-6 methylene group of GlcNAc (5, 6) but bound with a higher affinity for the D site (2), was shown likewise to have bound in a disposition substantially removed from the region of the two acid residues (2). There appears to be, therefore, a close correlation between the mode of binding

Table 1. Binding dispositions and conformations for oligomers at the active site

Oligosaccharide*	Ref. residue†	Rigid body variables† X	Y	Z	α	β	γ	Inter-ring dihedral angles‡ φ	ψ	Binding sites§	(kcal/mol) E_{sub}†	E_{int}†	E_{com}†
1. $(GlcNAc)_4$	2	7.71	6.75	12.2	−7.2	10.4	116.6	−79.9	109.3	A–B	−36.9	−68.2	−105
Calculated,								−74.4	131.4	B–C			
ref.1								−69.4	106.2	C–D			
2. $(GlcNAc)_5$	3	7.63	6.42	6.03	50.5	−24.8	1.0	−77.3	112.0	A–B	−43.2	−85.0	−128
Calculated,								−81.3	130.8	B–C			
this work								−96.7	84.5	C–D			
								−89.9	107.4	D–E			
3. $(GlcNAc)_6$	3	7.69	6.45	6.05	50.4	−24.8	2.7	−77.7	113.8	A–B	−51.0	−102	−153
Calculated,	5	6.29	−0.40	−1.93	59.3	−25.9	16.9	−81.0	131.9	B–C			
this work								−97.8	83.1	C–D			
								−90.4	109.6	D–E			
								−42.3	117.8	E–F			
4. $(GlcNAc)_6$	3	7.01	6.02	4.80	39.0	−10.3	−0.2	−77.3	94.7	A–B	1.7×10^4	3.7×10^3	2.1×10^4
Model-built,	5	7.82	0.57	−4.32	59.6	−44.8	−47.0	−63.6	83.1	B–C			
ref. 5								−126.8	124.1	C–D			
								−154.0	83.0	D–E			
								−87.8	102.8	E–F			
5. $(GlcNAc)_6$	3	7.20	5.30	5.05	41.1	−12.2	−3.1	−77.4	90.5	A–B	−44.1	−74.2	−118
Energy-minimized								−62.4	97.6	B–C			
model-built								−120.1	122.7	C–D			
								−138.9	94.6	D–E			
								−75.0	118.2	E–F			

* In the lowest energy minimum.

† See table 1, footnote a, of ref. 1 for definition, There were several typographical errors in ref. 1 On page 4262, line 9 of column 1, isoleucyl 48 should be isoleucyl 58; in table 1, X should be 7.58 Å and γ should be 3.1° for oligosaccharide 3. E_{com}, total energy; E_{sub}, conformational energy of the substrate; E_{int}, intermolecular energy of interaction between the rigid enzyme and substrate.

‡ The convention used for dihedral angles is defined in ref. 2.

§ Residue location at active site. See table 1, footnote c, of ref. 1.

derived from calculations (1) and that implied by the experimental findings (2).

Pentasaccharide. To calculate the most stable conformations of $(GlcNAc)_5$ at the active site, we used the following approach. The fifth GlcNAc residue was added to $(GlcNAc)_4$ in the lowest energy conformation of the lysozyme–$(GlcNAc)_4$ complex. Three initial values for φ and ψ between residues 4 and 5 were taken as starting positions, namely, the left-handed helix (φ, ψ ≈ −75°, 110°), the right-handed helix (φ, ψ ≈ −140°, 70°), and an intermediate structure (φ, ψ ≈ −105°, 90°) as described (1). The side chains of the fifth residue were placed in their position of lowest (intramolecular) conformational energy for the particular values of φ and ψ (1, 3).

Upon energy minimization, one structure was found to have an energy significantly lower than that of the others. This structure [$(GlcNAc)_5$, conformer 2 in Table 1] was determined from starting conformations in both the left-handed helical and intermediate structure. Of great interest was the further finding that φ and ψ between residues 3 and 4 for the pentamer were altered from the left-handed helical region to the intermediate position. [Compare the values of φ and ψ between sites C and D in $(GlcNAc)_4$ and $(GlcNAc)_5$ in Table 1.] This change, as can be seen in Fig. 1, had the effect of pushing the fourth residue deeper into the active site and closer to aspartyl 52 and glutamyl 35 [which were implicated in the catalytic mechanism (5)], in a position similar to that of the fourth residue of the energy-minimized structure of $(GlcNAc)_4$–lactone (1, 4); compare Fig. 1 with figure 1*B* of ref. 1. This intermediate set of values for ϕ^3 ψ^3 (= −96.7°, 84.5°) was also a minimum-energy position for the fourth residue of $(GlcNAc)_4$ but represented a higher energy region (by 3 kcal/mol) for this molecule than did the left-handed helical region (in which ϕ^3, ψ^3 = −69.4°, 106.2°).

Upon our adding the fifth residue, the main interactions responsible for shifting the values of φ and ψ (between residues 3 and 4) away from their starting values in the left-handed helix were the unfavorable contacts (in the starting conformation) between several of the ring atoms, and C-6, of residue 5 of the substrate and the side-chain atoms of valyl 109 and, to a lesser extent, the side-chain atoms of asparaginyl 46. To relieve these interactions and to optimize favorable contacts between the enzyme and the substrate, we found that it was necessary for the inter-ring dihedral angles between saccharide residues at sites C and D to depart somewhat from the left-handed helix conformation.

By starting with the fifth residue of the saccharide initially in the *right*-handed helical conformation, we observed that minimization of the energy of the lysozyme–$(GlcNAc)_5$ complex led to a structure that was 26 kcal/mol higher in energy than the one given for $(GlcNAc)_5$ in Table 1. It may be possible that movement of the side chains of the enzyme would lower the energy of the complex, in which the fifth residue of the pentamer is added in the right-handed helical conformation, below that of the structure for $(GlcNAc)_5$ in Table 1. We are currently investigating this possibility. In a proposed structure of the lysozyme–hexamer complex based on model building (5), the fifth residue (in site E) from the nonreducing end had

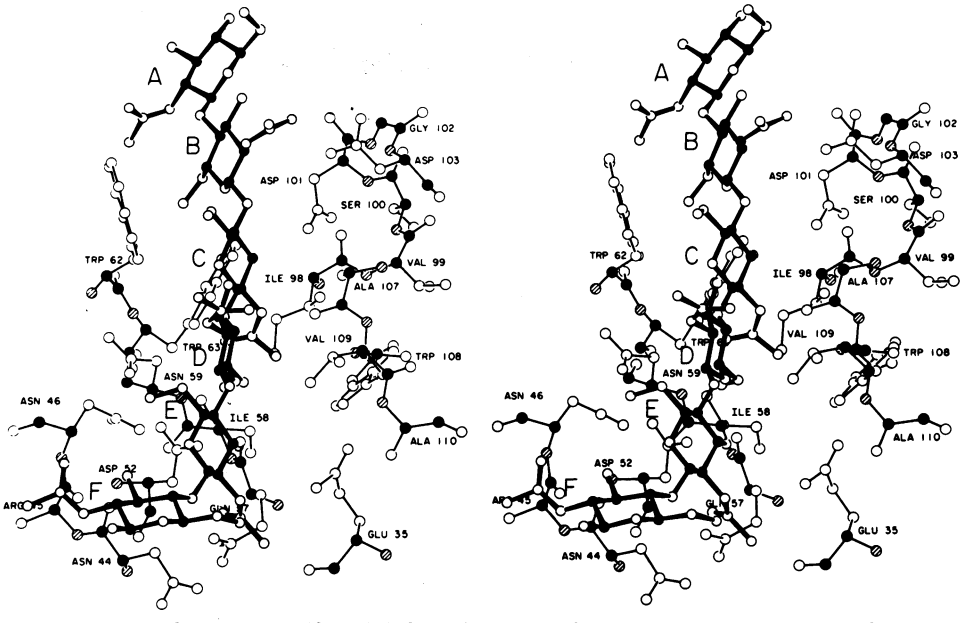

FIG. 1. Stereoview of the calculated structure of $(GlcNAc)_6$ bound to the rigid active site of lysozyme. All carbon atoms of the backbone of the enzyme and of the pyranosyl rings of the substrate are represented by dark circles. The backbone nitrogen atoms of the enzyme are indicated by circles filled with oblique lines. The first five residues of $(GlcNAc)_6$ are in approximately the same position as the lowest energy structure of the $(GlcNAc)_5$–lysozyme complex.

been placed in a conformation (ϕ, ψ = −154°, 83°) that corresponded to the right-handed helix.

Unfortunately, there are few experimental data with which to compare our calculated lowest-energy binding position for the fifth residue and the one proposed from model building (5). There are experimental data (6) that suggest that an *N*-acetylmuramic acid residue cannot be accommodated in the E site. It was concluded that a lactyl side chain attached to the O_8^5 (by the convention of ref. 3, or O_3 of residue 5 by the convention of ref. 6), changing the GlcNAc to a *N*-acetylmuramic acid residue, could not be accommodated in the E site because the lactyl O_8^5 pointed into the cleft in the model-built structure (5). Likewise, as can be seen in the calculated lowest-energy structure (Fig. 1), the oxygen to which the lactyl side chain would be attached (in site E of our calculated structure) would point into the active site cleft, in particular, toward the backbone atoms of glutaminyl 57 and isoleucyl 58. It is likely, therefore, that an *N*-acetylmuramic acid residue at site E would not be accommodated in our calculated conformation either, in agreement with experiments (6).

Hexasaccharide. A sixth residue was added to $(GlcNAc)_5$ in the lowest energy conformation of the lysozyme–$(GlcNAc)_5$ complex (Table 1), and stable binding structures were determined by the same procedure used for determining the structure of the enzyme–pentamer complex. Of the three sets of starting inter-ring dihedral angles ϕ and ψ (between residue 5 at site E and residue 6 at site F), only one, the left-handed helix, gave a low-energy minimum for the $(GlcNAc)_6$–lysozyme complex (structure 3 of Table 1). This structure is shown in Fig. 1.

It should be noted that the last residue, i.e., the saccharide on the reducing end, appeared to make favorable contacts with the protein on the "left side" of the active site as shown in Fig. 1 rather than on the "right side." In particular, the sixth residue made energetically favorable contacts with arginyl 45, asparaginyl 46, and threonyl 47 (not shown in Fig. 1). In the proposed structure based on model building (5), the last residue appeared to make favorable contacts chiefly with arginyl 114 (not shown in Fig. 1) on the "right side" of the active site cleft [to the right of alanyl 110 and glutamyl 35, or further along the X axis in the positive direction (1)]. The essential difference in orientation of the last two residues between the calculated and model-built structures can be seen by comparison of the rigid-body variables of residue 5 of conformers 3 and 4 in Table 1.

Because of the difference between the two conformers, the model-built structure for the lysozyme–$(GlcNAc)_6$ complex (5) was subjected to energy minimization by a procedure identical to the one described for the lysozyme–$(GlcNAc)_4$–lactone complex in ref. 1. The parameters for both the model-built structure and the energy-minimized model-built structure are listed in Table 1, conformers 4 and 5, respectively. It was seen that conformers 4 and 5 were quite similar, indicating that upon energy minimization the structure of the model-built complex did not undergo large changes. Furthermore, the results showed that the conformational energy for the energy-minimized model-built structure (no. 5) was higher than that for our calculated lysozyme–$(GlcNAc)_6$ structure (no. 3) by 35 kcal/mol.

The model-built structure *before* energy minimization has several bad contacts between the saccharide and the enzyme. Several somewhat unfavorable contacts occur between the *N*-acetyl group of the fourth residue from the nonreducing end of the hexasaccharide and the side-chain atoms of aspartyl 52 and asparaginyl 46, but the major bad contacts occur between the *N*-acetyl group of the fifth residue and the side-chain atoms, especially the nitrogen, of asparaginyl 44. The fifth residue of $(GlcNAc)_6$ makes additional bad contacts with valyl 109. Most of the unfavorable interactions between the fourth residue and aspartyl 52 involve contacts between backbone saccharide atoms and a generated hydrogen atom on the side-chain carboxyl group of aspartyl 52. Thus, it is possible that an energy minimization that allows movement of the side chains of the enzyme residues could lead to a structure of lower energy than the one found here with a rigid enzyme. We are currently investigating this possibility. It is clear, however, that the *rigid* active site of lysozyme has regions around arginyl 45, asparaginyl 46, and threonyl 47 (our sites E and F) that have a high affinity for the fourth and fifth residues of $(GlcNAc)_6$.

Recently, the structure of turkey egg-white lysozyme was published (7), and it was shown that the E and F sites of this enzyme were not blocked in the crystal (7), as was the case for the crystal of hen egg-white lysozyme (5, 6) used in this study. Hopefully, therefore, it will be possible to obtain crystal structures of saccharides bound to the E and F regions so that experimental structural information concerning the complex will be available.

It should be emphasized that the proposed structure shown in Fig. 1, based on conformational energy calculations, is a tentative one. A more definitive structure can be obtained only when all of the variables of the protein residues of the active site are allowed to vary as well as those of the substrate. These calculations are being performed at present. However, it is clear that for the rigid enzyme used here (*i*) the hexamer would most likely assume the conformation shown in Fig. 1—in which each residue takes up a conformation close to that of the left-handed helix or, in the case of the C–D site, a conformation between the left- and right-handed helix, but not the right-handed helical conformation itself, and (*ii*) a saccharide ring can readily bind to the D site without being distorted from the normal chair conformation to the half-chair form. Two recent experimental studies also show that oligosaccharides can bind to lysozyme without a distortion of the D residue, one study by Holler *et al.* (8) on the kinetics of displacement of the dye Biebrich scarlet from the active site of the enzyme by $(GlcNAc)_6$, and another study by Schindler *et al.* (2) on the binding of oligosaccharides to lysozyme molecules with a gadolinium ion already bound to the active site. Theoretical studies of Levitt (9) and Warshel and Levitt (10) similarly indicate that the GlcNAc residue in the chair form can be accommodated at the D site. Of course, these studies do not rule out the possibility that distortion can occur concomitantly with bond breaking during catalysis.

Affinities of Binding Subsites. Now that we have carried out an investigation of possible binding regions for oligosaccharides at the active site of the rigid enzyme, it is of interest to compare the relative affinities of each site available to the oligomers. Table 2 presents the relative affinities (E_{com}) that were obtained from a minimization of the conformational energy of the monomer (GlcNAc) in each binding region, along with free energies of binding determined from experimental data of Imoto *et al.* (5). The calculated data gave only approximate binding energies for oligomer residues because the monomers bound in slightly different orientations from those of the corresponding residues of oligomers, i.e., there were no constraints of an oligomer chain on the binding of a monomer. The affinity of a residue in an oligomer chain for a particular site will be influenced by the binding of the other residues in the chain.

The experimental data in Table 2 were determined under the assumption that binding of a saccharide residue at one site did not perturb the binding of another residue in the oligosaccharide chain to another site, i.e., under the assumption of independence of binding. Schindler *et al.* (2) have shown that this assumption may not be valid. Therefore, the data of Imoto *et al.* (5) should be considered only as rough estimates of free energies of binding.

From Table 2, it is seen that the calculated lowest-energy binding region was in the C site, a result in agreement with experiments (5). Crystallographic analyses of the binding of a variety of small ligands to lysozyme indicate that there is always a residue bound to the C site (5, 6), and that there exists a stable hydrogen bond between the *N*-acetyl group of the saccharide residue and the backbone of the protein, in addition to other favorable interactions. As can be seen in Fig. 1, two hydrogen bonds can exist at the C site in the calculated structure, one between the NH of the *N*-acetyl group and the C=O of alanyl 107, and another between the C=O of the *N*-acetyl group and the backbone NH of asparaginyl 59, as proposed from the crystal structure (5).

Table 2. Affinities of GlcNAc residues for each binding site of lysozyme

Site	Relative energy (kcal/mol)	
	Calculated*	Experimental†
A	21.0‡	2.3
B	6.6	1.9
C	0.0	0.0
D	3.1	7.5–10.6
E	9.6	0.6
F	8.6	2.9

* Calculated energy E_{com} expressed relative to that of the C site.

† The *free* energy of association relative to that of the C site as estimated by Imoto *et al.* (5) on the basis of binding data for various GlcNAc oligomers.

‡ Upon minimization of GlcNAc starting at site A, the saccharide moved to site B. The value given here was estimated from relative binding energies of two oligomers, i.e., the binding energy for the A site was computed from the difference in the binding energy of $(GlcNAc)_3$ in sites B–C–D and that of $(GlcNAc)_4$ in sites A–B–C–D. This value was then compared to that for the monomer in site C to obtain the value reported here.

It should be pointed out that in the region of the active site below the D site there are several binding locations of approximately equal affinity for $(GlcNAc)_2$. However, the E and F sites of $(GlcNAc)_6$ given in conformer 3 of Table 1 and shown in Fig. 1 were the only ones of high affinity for two saccharide residues attached to an oligomer chain already bound at sites A–D.

The only serious discrepancy between the calculated and experimental binding energies in Table 2 involved the A site. The calculations indicated that this site did not possess strong, favorable interactions like those found at the other sites, even though the region of the A site was searched carefully for possible binding locations (1). Three independent experimental observations, however, suggest that the A site does have a relatively high affinity (low binding energy) for saccharide residues: (*i*) $(GlcNAc)_4$ binds to lysozyme but it is not a substrate (5). If hydrolysis takes place between sites D and E, $(GlcNAc)_4$ presumably would be hydrolyzed if it were to bind to sites B–E or C–F, as suggested by the calculations, rather than A–D, as proposed by Imoto *et al.* (5). (*ii*) The hydrolysis products of the lysozyme + $(GlcNAc)_5$ reaction are mainly $(GlcNAc)_4$ and GlcNAc monomer (5, 11), suggesting that if hydrolysis takes place between sites D and E, $(GlcNAc)_5$ binds preferentially to sites A–E rather than B–F. (*iii*) $(GlcNAc)_6$ that must bind to sites A–F displaces the Biebrich scarlet dye, which presumably is bound to site F, whereas $(GlcNAc)_5$ does not displace the dye (8), suggesting that $(GlcNAc)_5$ prefers sites A–E over sites B–F, in contradiction to the calculated results shown in Table 2.

Thus, it appears that the calculations succeed in determining structures and relative energies of various low-energy enzyme–oligosaccharide complexes but fail to determine accurately the relative energy of binding to the A site. As mentioned in a previous paper (1), this failure may be due to uncertainties in the coordinates of the enzyme residues in this region. There are a number of unreasonable interatomic distances between nonbonded atoms around the A region in the x-ray structure, e.g., between the atoms in the carboxyl group of aspartyl 103 and the backbone atom of lysyl 97. It should be emphasized that

the results of these calculations are limited by the accuracy of the x-ray coordinates.

It is also important to note that there are apparent discrepancies among the experimental data. For example, if it is assumed that the binding energy of each site is independent of the other sites containing a saccharide residue, then the experimental binding energies given in Table 2 show that $(GlcNAc)_4$ should bind most stably to sites B–E, then (with slightly higher energy) to sites C–F, and finally (with still higher energy) to sites A–D. However, if binding occurs at sites B–E or C–F, the $(GlcNAc)_4$ molecule should be hydrolyzed, but this is not observed experimentally (5). Even if one uses the highest value for binding of the D residue of the tetramer in sites B–E and the lowest value for binding of the D residue of the tetramer in the sites A–D (see Table 2), a significant fraction (approximately 10%) of the tetramer would still bind to sites B–E at room temperature. Assuming that the rate of hydrolysis of a tetramer in sites B–E would be the same as a pentamer in sites A–E, one would expect a significant amount of hydrolysis of the tetramer, in contradiction to the experimental results (5). Moreover, significant splitting of $(GlcNAc)_5$ does occur between its third and fourth residues (5) indicating the occurrence of binding to sites B–F, as would also be expected from the experimental binding energies in Table 2. This suggests that $(GlcNAc)_5$ should displace, at least to some degree, the Biebrich scarlet dye bound at site F, but Holler *et al.* (8) do not observe this expected displacement. The fact that an appreciable amount of $(GlcNAc)_5$ binds to sites B–F also would suggest that $(GlcNAc)_4$ could bind to sites B–E or C–F and be a substrate of lysozyme. This, however, is not the case. Further experimental and theoretical work will be required to resolve these apparent discrepancies.

SUMMARY

We have determined favorable binding structures of $(GlcNAc)_n$, in which $n = 5$ and 6, with the *rigid* active site of hen egg-white lysozyme. The computed position of the saccharide residue of $(GlcNAc)_5$ in the D site, moved from its position in the computed lysozyme $(GlcNAc)_4$ structure (1) near the surface of the active site to a position deeper into the crevice of the active site, is very similar to that found for the fourth residue in the energy-minimized x-ray structure of $(GlcNAc)_4$–lactone (1). This suggests that a saccharide residue may exist in the active-site cleft at the D site while maintaining the chair conformation. Our calculations also show that the C site has the highest affinity for saccharide residues, in agreement with experiment. There is, however, a discrepancy between the calculated relative affinity of the A site for a GlcNAc residue and that determined experimentally. This discrepancy may be resolved when the positions of the atoms of the enzyme are allowed to vary during energy minimization.

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