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Camel Single-Domain Antibody Inhibits Enzyme by Mimicking Carbohydrate Substrate

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ABSTRACT **Whereas antibodies have demonstrated the ability to mimic various compounds, classic heavy/light-chain antibodies may be limited in their applications. First, they tend not to bind enzyme active site clefts. Second, their size and complexity present problems in identifying key elements for binding and in using these elements to produce clinically valuable compounds. We have previously shown how cAb-Lys3, a single variable domain fragment derived from a lysozyme-specific camel antibody naturally lacking light chains, overcomes the first limitation to become the first antibody structure observed penetrating an enzyme active site. We now demonstrate how cAb-Lys3 mimics the oligosaccharide substrate functionally (inhibition constant for lysozyme, 50 nM) and structurally (lysozyme buried surface areas, hydrogen bond partners, and hydrophobic contacts are similar to those seen in sugar-complexed structures). Most striking is the mimicry by the antibody complementary determining region 3 (CDR3) loop, especially Ala104, which mimics the subsite C sugar 2-acetamido group; this group has previously been identified as a key feature in binding lysozyme. Comparative simplicity, high affinity and specificity, potential to reach and interact with active sites, and ability to mimic substrate suggest that camel heavy-chain antibodies present advantages over classic antibodies in the design, production, and application of clinically valuable compounds. Proteins 32: 515–522, 1998.** © **1998 Wiley-Liss, Inc.**

Key words: sugar; acetamido group; mimicry; inhibition; lysozyme; CDR loop; V_HH; heavy-chain immunoglobulin; solvent accessible surface area

INTRODUCTION

Molecular mimicry serves important functions in natural processes as well as in therapeutic and diagnostic applications. For example, an antagonist or an inhibitor must often mimic on a structural or functional level a hormone or a substrate to produce an effect on the corresponding receptor or enzyme. A remarkable example of mimicry is seen in the bean α-amylase inhibitor, α-AI, which mimics the carbohydrate substrate of its enzyme target.[1] Such naturally occurring examples, however, are generated under evolutionary pressures that help to optimize a functional molecular mimicry. Antibodies, which can be generated and optimized in vivo in a matter of days or weeks, have been shown to mimic proteins,[2,3] haptens,[4,5] and even oligosaccharides,[6–8] but a general lack of structural data has left many details of the molecular mimicry up to speculation. Structures are known, however, for both complexes in one well-studied[9–15] mimicking system in which the antibody E5.2 mimics lysozyme in binding to the antibody D1.3. This example also demonstrates how an anti-idiotypic antibody, Ab_2 (i.e., E5.2) can mimic the internal image of the original antigen (i.e., lysozyme) of a primary antibody, Ab_1 (i.e., D1.3).

Anti-carbohydrate antibodies and antibodies that mimic carbohydrates (such as anti-anti-carbohydrate antibodies) may provide tools to fight disease (e.g., viral infection,[16] tumors expressing carbohydrate antigens,[7,17] or pathogens expressing cell-surface carbohydrates[18–21]) either by direct action as immunotoxins[22,23] or as vaccine surrogates.[18,24] Unfortunately, carbohydrates tend to be only weakly immunogenic due in part to their inability to stimulate antigen-specific T-cell responses.[16,25] Moreover, evidence suggests[26] that anti-idiotypic antibodies raised against anti-carbohydrate antibodies may have trouble mimicking the original carbohydrate antigen. This is proposed to be a consequence of the fact that anti-carbohydrate antibodies tend to contain a cleft that binds the sugar but is too small to allow full penetration of an anti-idiotypic antibody.[26] Rather, the anti-idiotype makes contacts around the outside of the cleft but fails to form similar molecular interactions; therefore, it fails to elicit anti-carbohydrate antibodies (anti-anti-idiotypes).[26] Despite this observation, antibodies[6–8] and randomly generated phage-displayed peptides[27–29] have been shown to

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mimic carbohydrates on functional levels, but as yet without supporting structural data.

We hereby report functional and structural evidence for the mimicry of a carbohydrate substrate by cAb-Lys3,[30,31] a single-domain fragment termed V_HH to indicate that it is a V_H derived from a camelid heavy-chain antibody naturally lacking light chains.[32] The effective inhibition of the enzyme lysozyme is demonstrated to be due to specific interactions that mimic those of substrate binding. A detailed comparison of the complex structure[30] with three available crystal structures[33–35] of carbohydrate-complexed lysozyme has been performed. The results support the previous predictions[30,36] as to the potential advantages in clinical applications of camel single-domain antibodies over classic heavy/light-chain antibodies.

METHODS

The cAb-Lys3 fragment was prepared as described previously.[30,31] Inhibition studies were carried out by monitoring spectrophotometrically the change in turbidity associated with the lysis of *Micrococcus lysodeikticus* cells (Worthington Biochemical, Freehold, NJ) as described previously.[37] Hen egg-white lysozyme (Sigma, St. Louis, MO) was pre-incubated in phosphate-buffered saline with cAb-Lys3 before mixing with *M. lysodeikticus* cells.

Binding constants were measured as described previously by competitive enzyme-linked immunosorbent assay (ELISA)[38] and also by using an IAsys Biosensor (Affinity Sensors, Cambridge, UK),[39] in which lysozyme was immobilized covalently (surface density, 10 ng/mm^2) on carboxy-methyl dextran cuvettes with the EDC/NHS coupling chemistry.[40] Association kinetics were measured as described previously.[41]

Complexes with lysozyme were compared by superimposing (least-squares) all atoms of lysozyme. Changes in solvent accessibility (ΔSA) were calculated with the MSROLL program from the Molecular Surface package[42] using a probe radius of 1.4 Å.

RESULTS AND DISCUSSION

The cAb-Lys3 fragment, with specificity for lysozyme, effectively protects *M. lysodeikticus* cells against lysis by lysozyme. The initial lysis rates measured (Fig. 1) extrapolate to indicate a K_i value of 50 nM. A control using a different camel antibody V_HH fragment that binds to another epitope of lysozyme showed no inhibition of lysis.

The low K_i compares with the dissociation constant of the antibody fragment which we have measured by two independent methods. Competitive ELISA experiments[38] resulted in binding curves from which a K_D of 50 nM was calculated (Table I). The dissociation constant was also measured using an IAsys Biosensor.[39] The resulting K_D of 65 nM compares with the value measured by ELISA. In addition, the measured values of k_{on} (4.1×10^4 M^{-1}s^{-1}) and k_{off} (2.7×10^{-3} s^{-1}) compare with those measured previously for the lysozyme specific antibody D1.3: V_H domain alone (10×10^4 M^{-1}s^{-1} and 1.0×10^{-3} s^{-1}, respectively)[43] or scFv (90.0×10^4 M^{-1}s^{-1} and 30.0×10^{-3} s^{-1}, respectively).[44] Despite the reduced size of the single-domain antibody, binding is comparable to that of two-domain fragments of classic immunoglobulins. From the perspective of lysozyme, however, cAb-Lys3 binds significantly more tightly than do several oligosaccharide products (Table I).

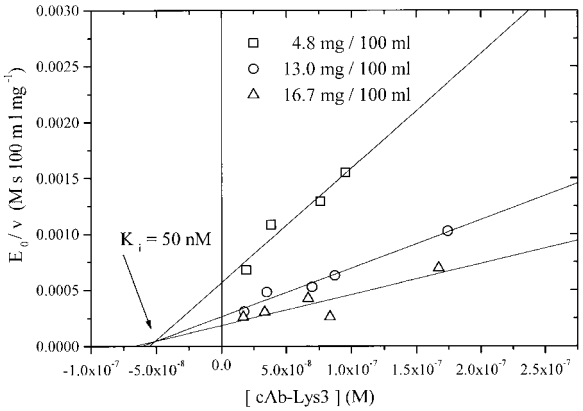

Fig. 1. Dixon plot showing the inhibition of lysozyme by cAb-Lys3 antibody fragment. Three concentrations of substrate (48, 130, and 167 µg/ml *M. lysodeikticus* cells) were mixed with samples of 20, 40, 80, 100, or 200 nM cAb-Lys3 (dilution factors account for apparent differences in plot) that were pre-incubated with 10 nM lysozyme. Initial rates of the reduction in turbidity (450 nm) were measured. E_0/v is the total enzyme concentration divided by the initial rate. The extrapolated K_i of 50 nM is indicated by the arrow.

TABLE I. Inhibition and Dissociation Constants for cAb-Lys3 and Lysozyme Compared With Dissociation Constants of Several Carbohydrate Product Inhibitors of Lysozyme Catalysis

K_i of cAb-Lys3	K_D of cAb-Lys3 (ELISA)	K_D of cAb-Lys3 (IAsys)	K_D of $(NAG)_3$	K_D of $(NAG)_4$	K_D of $(NAG\text{-}NAM)_2$
50 nM	50 nM	65 nM	7.7 µM[a]	7.1 µM[b]	170 µM[b]

[a]After Nakano et al.[59]
[b]After Imoto et al.[60]

It is immediately apparent from the cAb-Lys3-lysozyme crystal structure[30] that the antibody not only prevents access of substrate to the active site, but makes contact with many of the lysozyme residues that bind substrate and catalyze its hydrolysis. To compare lysozyme's interactions with cAb-Lys3 to those with substrate, we have superimposed lysozyme models from crystal structures of complexes with cAb-Lys3[30] and three independent carbohydrate ligands.[33–35] To best understand interactions in lysozyme's carbohydrate binding subsites B, C, and D, we considered the structure[33] containing the product consisting of *N*-acetyl muramic acid (NAM)

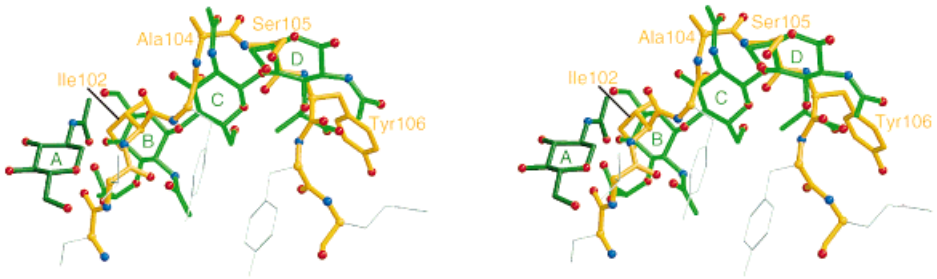

Fig. 2. Stereo figure comparing antibody and carbohydrate binding to lysozyme. Shown are carbohydrates NAM-NAG-NAM[33] (light green) and NAG in subsite A from a NAG_4 lysozyme complex[35] (dark green) superimposed with residues Ser100 to Glu107 of cAb-Lys3[30] (peptide backbone and selected side chains in orange, other side chains are light-faced in gray for clarity).

and *N*-acetyl glucosamine (NAG) in which NAM, NAG, and NAM occupy these subsites (respectively). Subsite A interactions were studied using structures containing $(NAG)_3$ and $(NAG)_4$ occupying subsites A to C[34] and A to D,[35] respectively. Antibody backbone atoms of the residues 102 to 107 (of the complementary determining region 3 [CDR3] loop) trace the substrate path passing within 1.0 Å of each superposed carbohydrate ring in the NAM-NAG-NAM structure (Fig. 2). This conformation of main chain shows that the antibody efficiently presents functional groups to lysozyme's binding site using primarily consecutive residues.

To further evaluate the structural mimicry of substrate by the antibody, we analyzed specific contacts in the sugar-lysozyme complexes and compared them with those of the antibody-lysozyme complex. As a quantitative measure of interaction with lysozyme, we have calculated the change in lysozyme solvent accessible surface area (ΔSA) of association with the carbohydrate substrate (total = 510 Å^2) or camel antibody (total = 780 Å^2). Figure 3A,B shows the molecular surface of lysozyme colored to indicate solvent excluded surface areas upon complexation with either carbohydrate or antibody. We expect a good mimic to affect the same residues of lysozyme that the substrate affects. We observe that 20 of 22 lysozyme residues that interact with substrate also interact with cAb-Lys3 (Fig. 3A–C). Only Arg45 and Gly102, with a total ΔSA of only 7.5 Å^2, are contacted by carbohydrate and not by cAb-Lys3. The antibody, however, also partially blocks solvent access to 6 residues (Gly71, Lys97, Asn106, Arg112, Asn113, and Lys116) that are not affected upon substrate binding. For comparison, we present in Figure 3D a similar analysis (using the same parameter settings) for the anti-idiotypic antibody E5.2 which mimics hen egg-white lysozyme binding to the D1.3 antibody.[12] Note that the occurrence of lysozyme in both examples is coincidental, and as the roles are unrelated, they should be considered independently. In the latter case, 21 of 22 D1.3 residues that interact with lysozyme also interact with the mimic E5.2, which in turn affects 5 additional D1.3 residues. In each mimicry example, some residues interact differently with the mimic and the mimicked compound, but for many, comparable surface areas become buried upon complexation. Note that in the E5.2 case, the mimic and the mimicked compound are both protein, whereas cAb-Lys3 mimics a non-homologous carbohydrate compound.

Previous studies have indicated that the lysozyme subsite C is the tightest binding of the subsites for pyranosyl groups.[33] The average crystallographic temperature factors for subsite C sugar atoms in the three available crystal structures are accordingly lower (28 Å^2) compared with A, B, and D subsite sugars (53, 42, and 49 Å^2, respectively). The average substrate ΔSA associated with subsite C sugar binding is correspondingly higher (207 Å^2) than that of A, B, and D subsite sugar binding (131, 118, and 178 Å^2, respectively). The acetamido substituent at the 2 position plays a key role by providing affinity and orientation for the sugar in the C subsite.[33] It is anchored sufficiently well to resist the van der Waals repulsion that distorts the D-ring aiding in catalysis. Several features of the lysozyme structure contribute to make the acetamido group binding highly favorable. The structural fold of lysozyme provides two important hydrogen bond partners located on either side of the acetamido binding site (Fig. 4A). The peptide nitrogen of residue Asn59 is made more acidic by the polarizing effect of the peptide hydrogen bond array of β-strand residues Asn44/Thr43, Asp52/Tyr53, and Ile58/Asn59. On the other side of the carbohydrate binding site, peptide hydrogen bonds from residues Asn103/Gly104, Met105/Asn106, and Ala107/Trp108 form a second hydrogen bond array ending in a similarly polarized carbonyl oxygen in residue Ala107. Thus, an excellent hydrogen bond donor and an excellent hydrogen bond acceptor are positioned approximately 8 Å apart in the active site of lysozyme. The subsite C NAG acetamido group amide inserts itself into this space, providing hydrogen bond partners for both (Fig. 4A). In addition, the

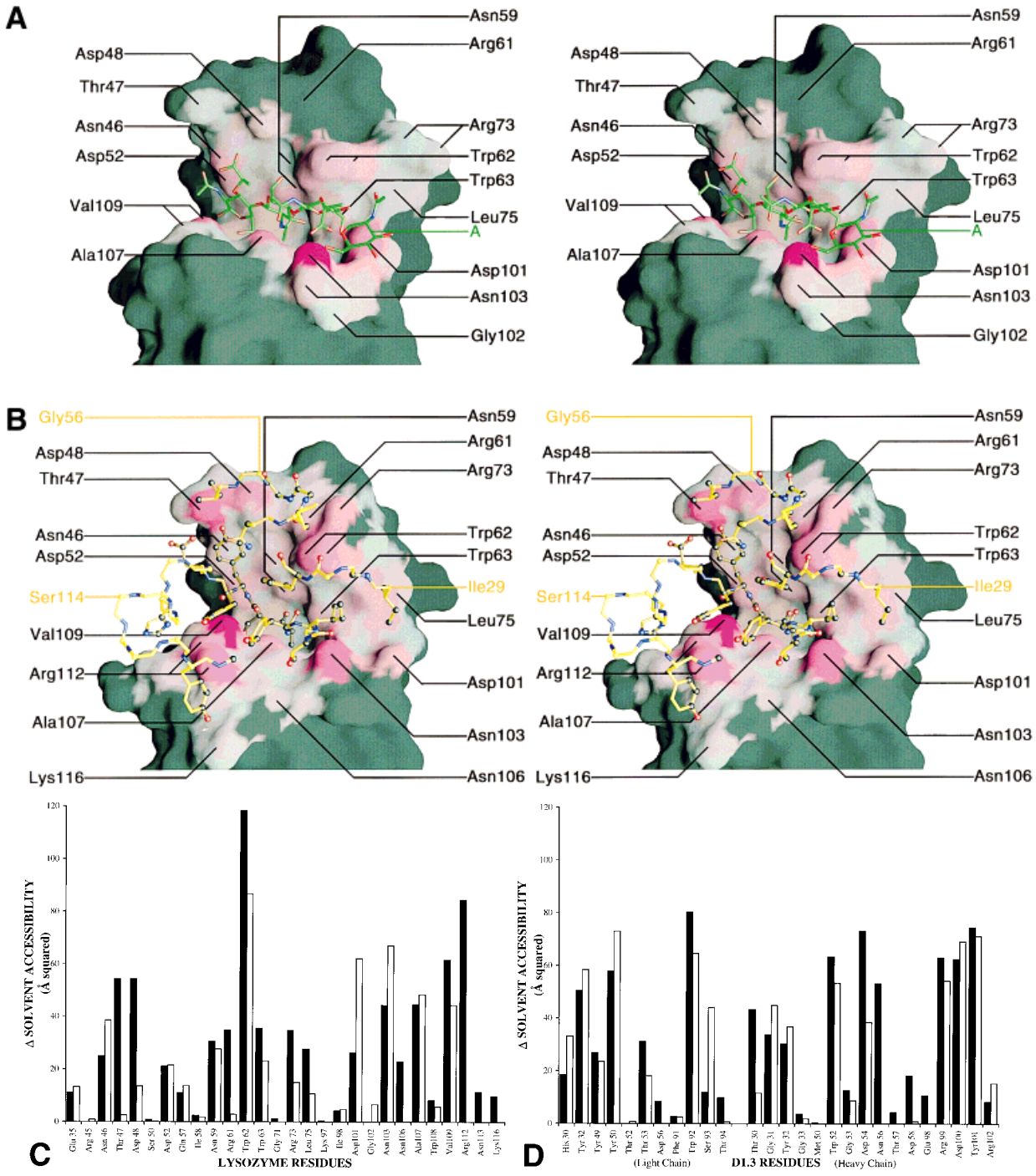

Fig. 3. Change in solvent accessibility (ΔSA) upon complexation. The solvent accessibility (probe radius 1.4 A) of lysozyme taken from crystal structures of complexes with cAb-Lys3[30] and each of three carbohydrates[33–35] were calculated[42] free and in complex. For each atom, the surface area accessible in complex was subtracted from that in the free molecule, resulting in ΔSA values. **A:** Stereo pair of the molecular surface of the lysozyme model from the NAM-NAG-NAM complex[33] calculated and displayed using GRASP.[57] The ΔSA values for each atom were mapped to the surface as colors to indicate no change (dark gray) or 0 to 50 Å^2 solvent excluded (light gray to magenta gradient). Left to right are shown stick models (carbon atoms in green) of sugars occupying subsites D (NAM), C (NAG), B (NAM), and A (NAG; labeled and shown in darker green to indicate that the model was taken from a different complex[35]). Selected residues of lysozyme are labeled. **B:** Similar to A except that the lysozyme model comes from the cAb-Lys3 complex[30] and is colored according to ΔSA upon complexation with cAb-Lys3. Three main chain segments (one from each CDR loop) are shown as stick models with side chains included if they contain atoms with a non-zero ΔSA of complexation (shown as red, blue, or black spheres for oxygen, nitrogen, or carbon, respectively). All other carbon atoms are shown in orange (spheres for reference in the case of Ca). One Ca from each loop is labeled for reference, and selected lysozyme residues are labeled. **C:** The ΔSA for each residue of lysozyme upon complexation with either cAb-Lys3 (solid bars) or carbohydrate (open bars). Values for carbohydrate are the NAM-NAG-NAM complex[33] values plus an average of the values obtained from subsite A NAGs of $(NAG)_3$[34] and $(NAG)_4$.[35] All residues not listed have zero ΔSA for both complexes. **D:** For comparison, the same calculations were made for residues of the anti-lysozyme D1.3 in its interactions with the anti-idiotypic E5.2[14] (solid bars) which mimics lysozyme[58] (open bars). All residues not listed have zero ΔSA for both complexes. Note that the occurrence of lysozyme in both examples of mimicry is coincidental; as their roles are different, they should not be compared directly.

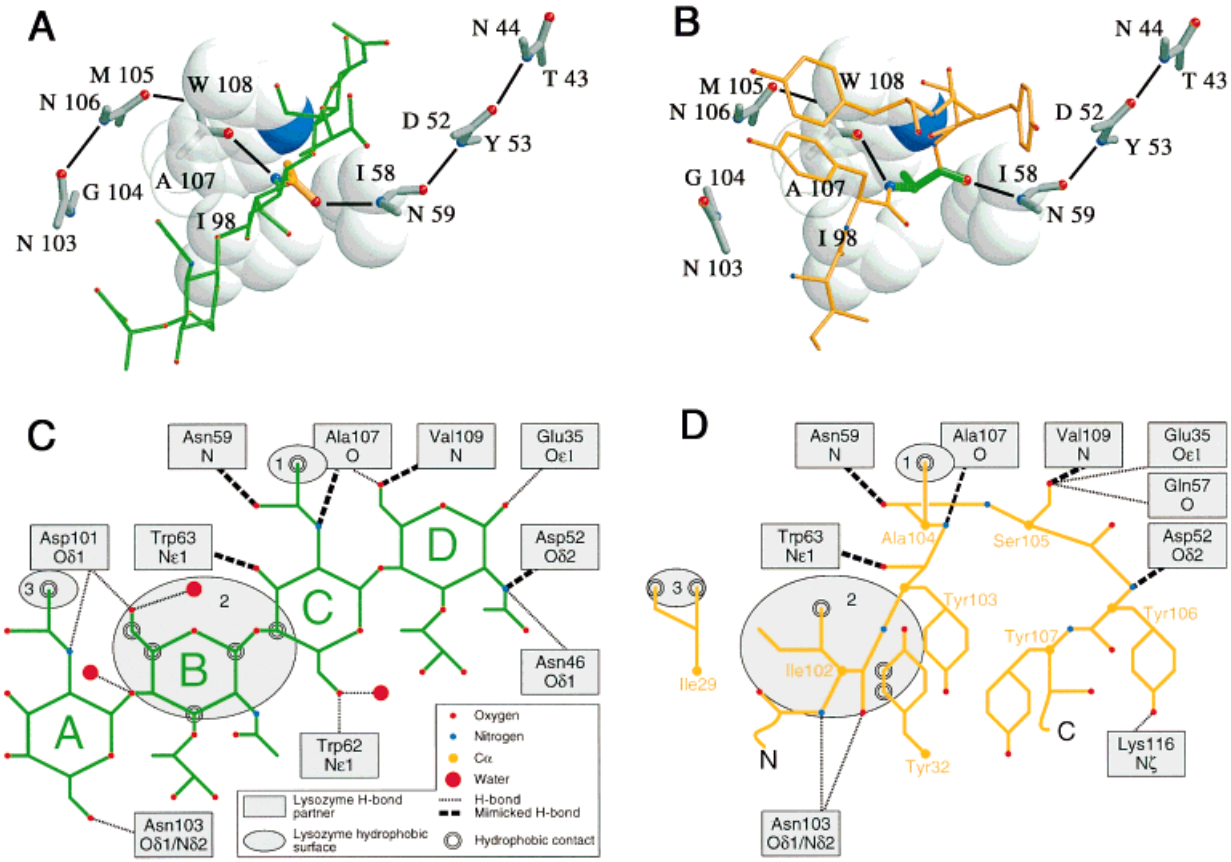

Fig. 4. **A, B:** Mimicry of the subsite C NAG 2-acetamido group (A) by Ala104 (B). The hydrophobic pocket of lysozyme contacting the methyl carbon in each case is composed of Ile58, Ile98, Ala107, and Trp108 (side chains shown in gray space filling models with Ala107 semi-transparent for clarity; Cα atoms are labeled except Ala107 where Cβ is labeled). Also shown and labeled are backbone atoms of lysozyme residues involved in hydrogen bond arrays forming similar hydrogen bonds with each ligand. **C, D:** Schematic of hydrogen bonds and hydrophobic interactions between lysozyme and carbohydrate (C) or antibody (D). Lysozyme atoms involved in hydrogen bonds are indicated as gray boxes. As indicated in the legend insert, hydrogen bonds are labeled as H-bond for non-mimicked hydrogen bonds (light-faced) or as Mimicked H-bond for hydrogen bonds mimicked by the antibody (bold-faced). Three hydrophobic patches of lysozyme are defined as gray ovals labeled as follows: 1, side chains of Ile58, Ile98, Ala107, and Trp108; 2, side chain of Trp62; and 3, side chains of Leu75 and Trp62 (edge). Carbohydrate or antibody carbon atoms shown on top of these surfaces and indicated by double circles make hydrophobic contacts with the given surface.

polarizability of the group itself results in a single array across the entire lysozyme molecule.

The cAb-Lys3 antibody mimics this interaction by presenting the backbone atoms of Ala104 into the equivalent position (Fig. 4B). The backbone amide nitrogen and oxygen of Ala104 serve the same function and take approximately the same position as the amide nitrogen and oxygen of the 2-acetamido group. While the presence of two carbons between the atoms does not allow the same polarization, it provides a longer spacer, thus reducing the hydrogen bond length to the Asn59 backbone nitrogen to 2.7 Å compared with 2.9 Å for similar bonds in the three sugar complexes. Rearrangement of a flexible loop of lysozyme disrupts the Asn103 to Asn106 backbone hydrogen bond in one hydrogen bond array, but all others remain intact (Fig. 4B).

The methyl carbon of the subsite C NAG acetamido group also appears very important in substrate binding,[33,34] as it fills a small hydrophobic pocket formed by Ile58, Ile98, Ala107, and Trp108. The side chain β-carbon of cAb-Lys3 Ala104 is perfectly positioned to occupy this site (Fig. 4A,B). Upon complexation with lysozyme, the ΔSA for this atom is 68 Å^2. This is 22 Å^2 more than for any other antibody atom. The similarly placed acetamido methyl carbon has an average of 64 Å^2 ΔSA, an average of 15 Å^2 more than for any other sugar atom. Thus, the 2-acetamido group of the subsite C sugar, arguably the most important functional group for substrate binding, has been efficiently mimicked by the antibody.

Although the 2-acetamido group mimicry is the most striking, other interactions between the carbohydrate substrate and lysozyme are mimicked by the camel antibody. In addition to the two previously mentioned, three other hydrogen bonds are mimicked by cAb-Lys3 (Fig. 4C,D). The backbone car-

bonyl oxygen of Tyr103 serves as an even better hydrogen bond acceptor for lysozyme's Trp63 ring nitrogen than the subsite C NAG hydroxyl O3. The hydroxyl group of antibody Ser105 mimics O6 of subsite D NAM in accepting a hydrogen bond from the backbone nitrogen of Val109. Also mimicking sugar interaction is the backbone nitrogen of cAb-Lys3 Tyr106, which like the 2-acetamido group NH of subsite D NAM, donates a hydrogen bond to the side chain carboxylate of catalytic residue Asp52. A total of 5 of the 12 hydrogen bonds between carbohydrate and lysozyme are mimicked by cAb-Lys3. Remarkably, we see that 4 of 5 mimicked hydrogen bonds in the cAb-Lys3 complex are formed by antibody backbone atoms. Moreover, 4 of 10 antigen atoms involved in hydrogen bonds are backbone atoms.

In addition to the hydrophobic pocket occupied by Ala104 Cβ mimicking the subsite C NAG acetamido methyl group, lysozyme Trp62 has previously been described as providing a hydrophobic surface for substrate contact.[33,45] Subsite B NAM atoms C1, C3, C5, and C6 as well as C4 of subsite C NAG make contact with this surface (number 2 in Fig. 4C) and are partly mimicked by Cγ2 of Ile102 and Cδ2 and Cϵ2 of Tyr32 of cAb-Lys3 (Fig. 4D). Accordingly, the ΔSA of Trp62 of lysozyme is the greatest in both carbohydrate and antibody complexes (Fig. 3C). We have also identified a smaller hydrophobic contact composed of lysozyme Leu75 and the indole ring edge of Trp62 (number 3 in Fig. 4C,D). This surface buries about 50 Å^2 of the acetamido methyl group of substrate in subsite A and, for the mimicking antibody residue Ile29, about 40 Å^2 of Cδ methyl group and about 35 Å^2 of the Cγ atoms. Interestingly, the amino acid at position 29 has a purely structural role in classic antibodies, buried in a hydrophobic core of the canonical H1 loop.[46] Therefore, it has not previously been observed contacting the antigen.[47] In this case, however, it contributes along with residues mentioned above in the effective mimicry of all three areas of substrate that interact hydrophobically with lysozyme.

With one significant exception, the lysozyme backbone from the cAb-Lys3 and three sugar complexes are superimposable. A flexible loop (residues 99 to 104) adopts slightly different conformations, but in all complexes lysozyme Asn103 makes hydrogen bonds with the bound molecule. That the hydrogen bonds are different even in the three sugar complexes suggests that this interaction may add to the affinity but probably does not play an important role in the specificity of substrate binding. Rather, the flexible loop of lysozyme can reorient to make different kinds of contacts. Similarly, one lysozyme side chain (Arg73) exhibits significantly different orientations in the antibody and carbohydrate complexes but still makes contacts with both ligands (Fig.3).

CONCLUSION

Immunoglobulins and their engineered fragments have great potential in therapeutic and diagnostic applications. Despite current molecular biology and protein engineering technology, classic heavy/light-chain antibodies still present some difficulties. Many of these are due in part to the size and complexity of the antigen-binding unit, which is composed of two polypeptide chains each presenting three complementary determining regions. The first consequence of this is the fact that the antigen-binding surface tends to form a cavity, groove, or planar surface (see MacCallum et al.[47] and the references therein), which is well suited to antigens presenting either a large flat surface or a small protrusion that can fit between the CDR loops of the V_H and V_L. With very few exceptions,[48,49] classic antibodies are not observed contacting clefts such as enzyme active sites. Indeed, estimates of free energy of binding based on solvent exclusion suggest that such antigenic cavities are energetically unfavorable epitopes for antibodies.[50] Thus, researchers wishing to target such sites are likely to have difficulty raising useful antibodies.

A second consequence of the size and complexity of the antigen-binding unit in classic antibodies results in difficulties in the design and engineering of useful fragments. Problems including low expression yields, aggregation, dissociation, and instability have challenged researchers to engineer fragments such as Fab, Fv, scFv, and dsFv.[23] Researchers have even attempted to generate isolated V_H fragments, sacrificing the V_L portion entirely to avoid some of these problems, but exposure to solvent of the hydrophobic "V_L-free" face (which normally interacts with the V_L domain) resulted in insolubility.[51]

Camelid heavy-chain antibodies[32] offer a new minimal antigen-binding unit[36] consisting of only a single variable domain selected and matured in vivo without light chains. It is more soluble than an isolated classic V_H due to differences in sequence[52] and in structure[30,53] of the "V_L-free" region. Moreover, as we presented previously in the crystal structure of cAb-Lys3,[30] smaller size and a longer CDR3 loop[52] allow greater access to clefts such as the active site of lysozyme, an epitope which is not contacted significantly by any of five antibodies for which there are lysozyme-complexed crystal structures.[54]

The results presented here describe the first detailed structural comparison of an antibody with the carbohydrate molecule that it mimics. The potential implications of such mimicry in the development of immunotoxins, vaccines, peptide mimics, or diagnostic reagents are made more far-reaching due to the enzyme inhibition and the advantages of camel V_HH fragments. In contrast to classic heavy/light-chain antibodies, camel antibodies may be raised against enzyme active sites that are not accessible to the

bulky V_H/V_L combining site.[55] In addition, the V_HH antigen-combining fragment does not present the same problems of synthesis, solubility, association, and stability associated with many classic antibody fragments. Also, the sequence homology between heavy-chain camel antibodies and human antibodies is even greater than that between mouse and human antibodies, thereby reducing their potential of immunogenicity.[56] Finally, even with three-dimensional structural data, it may be difficult to identify which contacts are most important in antigen-binding by a classic heavy/light-chain antibody. A peptide or other compound based on a linear sequence from a classic antibody CDR loop is expected to provide less of the total antigen-binding material than that from a camel single-domain antibody such as cAb-Lys3 in which the CDR3 loop accounts for the most important antigen-binding interactions.

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