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Crystallographic detection of a second ligand binding site in influenza virus hemagglutinin

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ABSTRACT **X-ray crystal structures have been determined for several complexes between influenza virus hemagglutinin and derivatives of its cell-surface receptor, sialic acid (Neu5Ac). Difference electron density maps establish the existence of a second binding site in addition to the primary site characterized previously. Three compounds bind to both sites: Neu5Ac(α2-3)Gal(β1-4)Glc [(α2-3)sialyllactose], α-2-*O*-(4′-benzylamidocarboxybutyl)-5-*N*-acetylneuraminic acid, and α-2-*O*-(4′-methylamidocarboxybutyl)-5-*N*-acetylneuraminic acid; and four other compounds bind only to the primary site: Neu5Ac(α2-6)Gal(β1-4)Glc [(α2-6)sialyllactose], α-2-*O*-methyl-5-*N*-acetylneuraminic acid, 4-*O*-acetyl-α-2-*O*-methyl-5-*N*-acetylneuraminic acid, and 9-amino-9-deoxy-α-2-*O*-methyl-5-*N*-acetylneuraminic acid. The maps also extend earlier results by showing the location of all three sugar residues of (α2-3)sialyllactose in the primary binding site. The affinity of (α2-3)sialyllactose for the second site was estimated by collecting x-ray diffraction data at various ligand concentrations and was found to be at least four times weaker than its affinity for the primary site. Although it is not yet known whether the second binding site participates in the infection process, it nevertheless offers a potential target for the design of antiviral drugs.**

Influenza infection begins when virus particles attach to cell-surface receptors terminating in sialic acid (Neu5Ac; for review, see ref. 1). α-Sialosides are recognized by the viral glycoprotein hemagglutinin, a membrane-bound trimer consisting of three HA1 and three HA2 polypeptide chains. The external portion of the hemagglutinin molecule was proteolytically released from the membrane and crystallized (2, 3), and the resulting structure at 3-Å resolution revealed a surface pocket containing several conserved amino acid residues, identifying this pocket as a possible sialoside binding site (4). The localization of the binding site was supported by the discovery of an amino acid mutation in this surface pocket that changes the binding specificity for α-sialosides. Viruses with hemagglutinin HA1 containing Leu-226 preferentially agglutinate erythrocytes derivatized with Neu5Ac(α2-6)Gal(β1-4)GlcNAc, whereas viruses with hemagglutinin containing Gln-226 preferentially agglutinate erythrocytes carrying Neu5Ac(α2-3)Gal(β1-3)GalNAc (5). More recently, crystals of hemagglutinin with either leucine or glutamine at position 226 have been incubated with mixtures of Neu5Ac(α2-3)Gal(β1-4)Glc [(α2-3)sialyllactose] and Neu5Ac(α2-6)Gal(β1-4)Glc [(α2-6)sialyllactose], and x-ray crystallographic analysis has shown that the Neu5Ac residue is indeed bound in the surface pocket, with very similar orientations in the two complexes (6). The atomic structures are consistent with ^{1}H NMR evidence showing that when an α-sialoside binds to hemagglutinin, the N-acetyl resonance exhibits an upfield chemical shift, presumably due to the proximity of the acetyl group to the side chain of Trp-153, which is one of the conserved amino acids in the surface pocket (7).

To define further the conformation of bound Neu5Ac residues, we have determined the crystal structures of hemagglutinin containing purified sialyllactose isomers or several synthetic α-sialosides.** Unexpectedly, we have obtained crystallographic evidence that some of these compounds bind not only to the surface pocket described above but also to a second site on the hemagglutinin molecule.

METHODS

Protein. A soluble ectodomain of hemagglutinin was released from purified X-31 (H3N2) influenza virus by bromelain digestion (2). The resulting bromelain-released hemagglutinin (BHA) contained residual neuraminidase activity that was eliminated by passing the protein through an immunoaffinity column containing anti-neuraminidase antibodies (7, 8). BHA crystals were grown by either microdialysis or vapor diffusion and were isomorphous to those studied previously (3, 4, 6, 9).

Ligands (Fig. 1). (α2-3)Sialyllactose was isolated by HPLC (8, 10) from a mixture of (α2-3)- and (α2-6)sialyllactose obtained from bovine colostrum (Sigma). The resulting compound was pure by ^{1}H NMR and was used for the high-resolution crystallographic study. Low-resolution crystallographic studies of sialyllactose complexed with BHA employed (α2-3)- and (α2-6)sialyllactose salts from BioCarb (Lund, Sweden). α-2-*O*-(4′-Benzylamidocarboxybutyl)-5-*N*-acetylneuraminic acid (Neu5Acα2Bac; Fig. 1, compound III), α-2-*O*-(4′-methylamidocarboxybutyl)-5-*N*-acetylneuraminic acid (Neu5Acα2Mac; Fig. 1, compound IV), α-2-*O*-methyl-5-*N*-acetylneuraminic acid (Neu5Acα2Me; Fig. 1, compound V), and 4-*O*-acetyl-Neu5Acα2Me were synthesized as described (7, 11). 9-Amino-9-deoxy-Neu5Acα2Me was synthesized by J. Hanson.

Preparation of Complexes. Protein–ligand complexes were prepared by soaking protein crystals in ligand solutions for 1–5 days prior to data collection. High-resolution data sets were collected using 30 mM (α2-3)sialyllactose, 25 mM

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Abbreviations: Neu5Ac, sialic acid; (α2-3)sialyllactose, Neu5Ac(α2-3)Gal(β1-4)Glc; (α2-6)sialyllactose, Neu5Ac(α2-6)Gal(β1-4)Glc; BHA, bromelain-released hemagglutinin; Neu5Acα2Bac, α-2-*O*-(4′-benzylamidocarboxybutyl)-5-*N*-acetylneuraminic acid; Neu5Acα2Mac, α-2-*O*-(4′-methylamidocarboxybutyl)-5-*N*-acetylneuraminic acid; Neu5Acα2Me, α-2-*O*-methyl-5-*N*-acetylneuraminic acid.

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**Atomic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (references 1HGF–1HGJ, R1HGFSF–R1HGSSF).

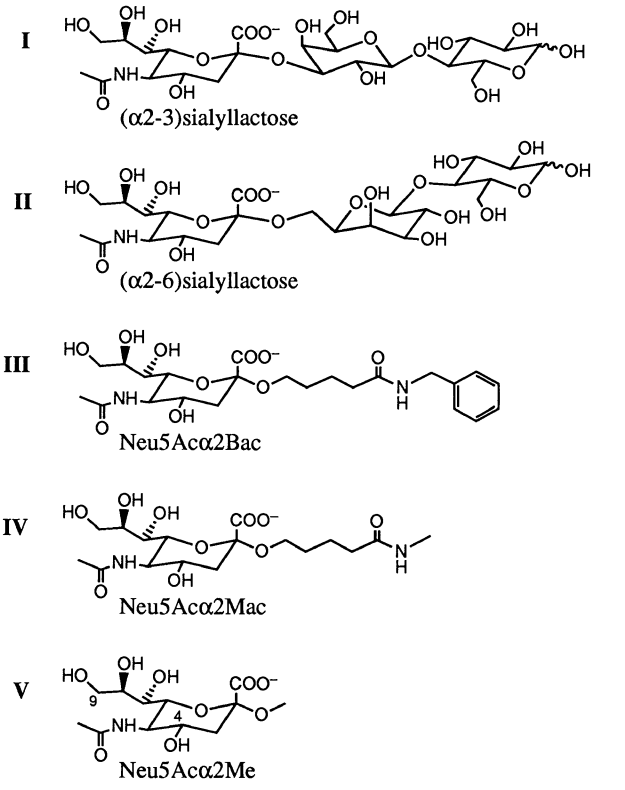

FIG. 1. Ligands used for crystallographic studies. 4-*O*-Acetyl-Neu5Acα2Me and 9-amino-9-deoxy-Neu5Acα2Me are derivatives of compound V.

Neu5Acα2Bac, 25 mM 4-*O*-acetyl-Neu5Acα2Me, and 200 mM 9-amino-9-deoxy-Neu5Acα2Me. Low-resolution data sets were collected using (α2-3)sialyllactose at 2, 8, and 32 mM; (α2-6)sialyllactose at 2, 8, and 32 mM; Neu5Acα2Mac at 50 mM; and Neu5Acα2Me at 2 and 100 mM.

Data Collection and Processing. High-resolution data (2.9-Å resolution) for the X-31–(α2-3)sialyllactose complex were collected on 1° oscillation photographs and processed as described (4, 6, 9, 12). Other high-resolution data sets (2.7-Å resolution) were collected on a multiwire area detector utilizing 0.04° or 0.1° oscillation frames and processed with the BUDDHA software package (8, 13, 14). Separate low-resolution data (5.8-Å resolution) were collected on 0.2° oscillation frames at an overall precession rate of 6°/hr, about 15 times faster than that used for high-resolution data. This rapid collection strategy yielded a complete low-resolution data set in <1 day and was, therefore, useful as a quick screen to assess whether a ligand was present in the protein binding site.

Model Building and Structure Refinement. Model building was performed using the FRODO molecular modeling program (15). Models were refined against high-resolution data with the program X-PLOR (16) as described (8, 17). Refinement converged to residual error (*R*) values of 22.9% (7.0–2.91 Å) for the X-31–(α2-3)sialyllactose complex and 22.6% (7.0–2.70 Å) for the X-31–Neu5Acα2Bac complex.†† rms deviation from bond-length ideality was 0.015 Å. Seventy-two water molecules were included in the model.

RESULTS

The Existence of a Second Binding Site. A difference electron density map at 3-Å resolution of the X-31–(α2-3)sialyllactose complex (Fig. 2) confirms the previously reported location (6) of three ligand binding sites on the hemagglutinin trimer, related to each other by threefold noncrystallographic symmetry. The map is good enough to locate the position of the lactose group (8), which was not seen previously. In contrast with our earlier results, the map exhibits a second set of three symmetry-related peaks in a different location (Fig. 2). The size and shape of these electron density peaks suggest that they represent (α2-3)sialyllactose molecules occupying a second ligand binding site. The electron density has clear protrusions corresponding to the glyceryl, N-acetamido, 4-hydroxyl, and 1-carboxylate groups of the α-sialoside (Fig. 3*a*); and together these data establish the position and orientation of the Neu5Ac moiety. The remaining electron density consists of two flattened globules corresponding to the pyranose rings of the galactose and glucose residues, plus an additional small protrusion that is interpreted here as corresponding to the 6″-hydroxyl group of glucose (Fig. 3*a*). These features, and the fact that the two glycosidic linkages in (α2-3)sialyllactose contain a total of only four dihedral rotational degrees of freedom, make it possible to build a model of the whole ligand molecule. The refined model is shown in Fig. 3*a*, and a schematic diagram appears in Fig. 4.

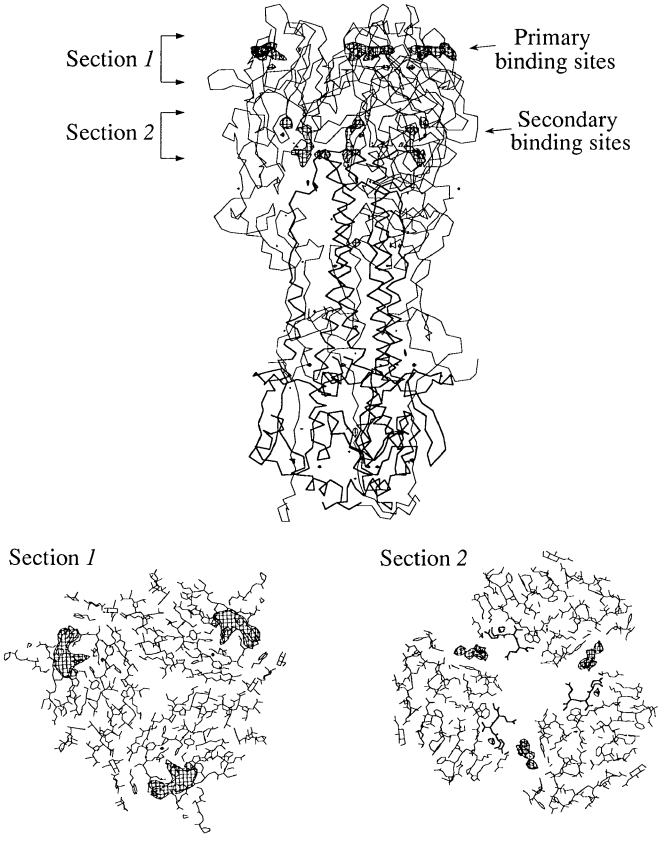

FIG. 2. Difference electron density map calculated with coefficients ($F_{obs}^{X\text{-}31+(\alpha 2\text{-}3)sialyllactose} - F_{obs}^{X\text{-}31}$) from 10- to 3.0-Å resolution, averaged about the threefold axis of noncrystallographic symmetry, and contoured at +4σ above the mean density. (*Upper*) The axis of noncrystallographic symmetry is vertical, and protein is represented by a trace connecting the α-carbons, with thin lines representing the three HA1 polypeptide chains and thick lines depicting the three HA2 polypeptide chains. (*Lower*) Two cross-sectional views projected on a plane perpendicular to the axis of noncrystallographic symmetry. Cross-section 1 shows electron density peaks corresponding to the reported ligand binding sites (6), and cross-section 2 shows the second set of peaks not previously observed. An unaveraged map (data not shown) was qualitatively similar, indicating that the primary and secondary sites were occupied in all three monomers of the BHA trimer.

††*R* values are given for the resolution range shown, except that data higher than 3.2-Å resolution were eliminated if $F < 2\sigma_F$ (where *F* is the structure factor and σ_F is its standard deviation).

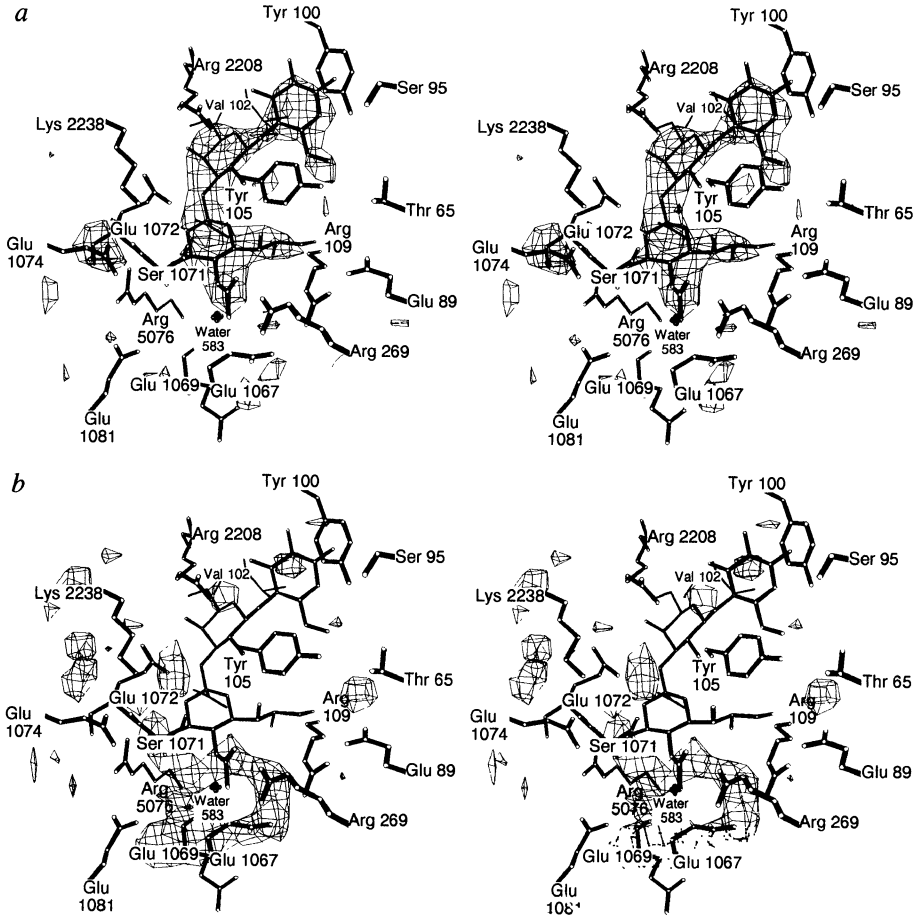

FIG. 3. (*a*) Stereoview of the second ligand binding site. A refined model of the X-31–(α2-3)sialyllactose complex is superimposed on the same difference map shown in Fig. 2, contoured here at $+3\sigma$ above the mean density. Refined isotropic B-factors for the pyranose ring atoms of Neu5Ac (44 Å^2), galactose (47 Å^2), and glucose (49 Å^2) suggest that the three residues are about equally ordered. (These values assume a ligand occupancy of 0.4.) The small difference peak near the left side of the image corresponds to a perturbation of residue 1071 relative to its position in uncomplexed hemagglutinin. (*b*) Same view with an $F_{obs} - F_{calc}$ map (20- to 2.91-Å resolution), averaged about the threefold axis of noncrystallographic symmetry and contoured at $+3\sigma$ above the mean density. The absence of any peaks overlapping the sialyllactose molecule indicates that the model agrees with the crystallographic data. The large ring-shaped $F_{obs} - F_{calc}$ peak seen here is located in a deep flat pocket within the protein and is surrounded by residues with low B-factors. With Neu5Ac occupying the second site, the pocket is nearly sequestered. It is unknown whether the density represents bound solvent, precipitant (citrate ion), or some other molecule.

Molecular Interactions in the Second Site. The second ligand binding site identified above is a concave pocket at the interface of the HA1 and HA2 polypeptide chains of a hemagglutinin monomer. The site is adjacent to one amino acid (residue 2208) from the HA1 chain of a second monomer, and the HA2 chain of a third monomer is also nearby (residue 5076 in Fig. 3; the numbering scheme is explained in Fig. 4). The carboxylate group of the sialoside does not form a salt bridge with the protein. However, other parts of the (α2-3)sialyllactose molecule make intermolecular polar contacts

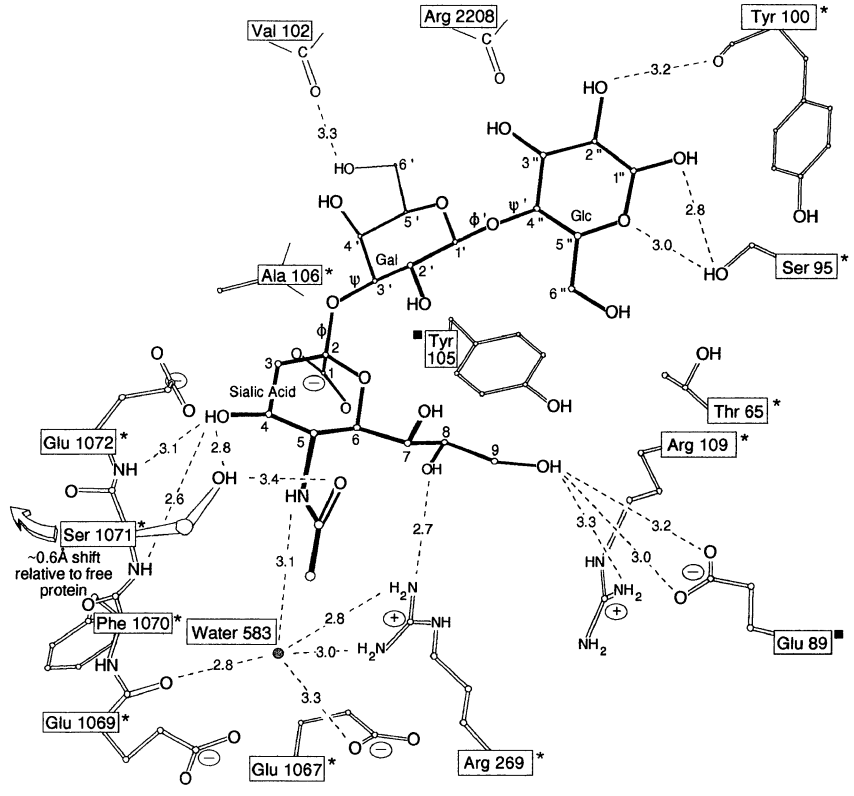

FIG. 4. Schematic diagram showing interactions between (α2-3)sialyllactose and protein in the second binding site of X-31 hemagglutinin. All protein side chains or main-chain segments within 4.3 Å of the ligand are shown. In addition, intermolecular contacts between polar atoms are represented by dashed lines if the contact distance is <3.4 Å. The coordinate error is ≈0.35 Å (17). The second binding site includes amino acid residues from different polypeptide chains of the hemagglutinin trimer. Residue numbers: 1–328, HA1 of monomer 1; 1001–1175, HA2 of monomer 1; 2001–2328, HA1 of monomer 2; 5001–5175, HA2 of monomer 3. Dihedral angles linking the sialyllactose sugars are as follows: ϕ (C1–C2–O2–C3′), −59°; ψ (C2–O2–C3′–H3′), 15°; ϕ' (H1′–C1′–O1′–C4″), −54°; ψ' (C1′–O1′–C4″–H4″), −8°. Amino acids conserved in human H3 hemagglutinin isolates are indicated by an asterisk (*), and amino acids that are identical in all influenza A isolates are indicated by squares (■). Even though the other residues undergo mutations, the particular atoms that interact with the ligand (main chain carbonyl oxygens of residues 102 and 2208) are conserved. (However, the conservation of amino acid residues does not imply that the second binding site is necessary for binding to the cell surface; when present on intact virus particles this part of the protein may simply be inaccessible to neutralizing antibodies and, therefore, free from selective pressure.)

(Fig. 4). These contacts involve the ring oxygen atom of the glucose residue, three hydroxyl groups on the glucose and galactose residues, and the 8- and 9-hydroxyl groups of the Neu5Ac residue. The 4-hydroxyl and 5-acetamido groups form a complex network of polar contacts with amino acid residues 1071 and 1072. These interactions are made possible by a shift in the position of residue 1071, which is perturbed about 0.6 Å relative to its position in the uncomplexed protein.

The refined model includes a bound water molecule, which makes hydrogen bonds to the acetamido nitrogen of Neu5Ac and to amino acid residues 269, 1067, and 1069 (Fig. 4). The presence of a water molecule is supported by crystallographic refinement against data from four protein–ligand complexes, two with ligands and two without ligands in the second site, in which the position and B-factor of the water molecule remained stable (8). However, the water structure must be interpreted with caution. The water molecule was initially included in the model because an electron density peak appeared at a level of $+4\sigma$ in a threefold averaged $F_{obs} - F_{calc}$ map. The peak was located near a very large unexplained ring-shaped peak (Fig. 3*b*) that appears in all structures studied to date, including those with no ligand present. It is therefore possible that the peak we interpret as a water molecule might not be distinct from this larger feature.

Measuring the Ligand Affinity of the Second Site. The relative affinity of the ligand for the second site was estimated by collecting separate low-resolution data sets at three concentrations of (α2-3)sialyllactose (2, 8, and 32 mM). The data were used to calculate difference electron density maps with coefficients in the resolution range of 10–5.8 Å. All three maps showed difference peaks corresponding to the entire ligand molecule (that is, to all three sugar residues) in the primary binding site. In the second binding site, the map obtained from the 32 mM complex contained density for the entire ligand, the map from the 8 mM complex showed density for the Neu5Ac and glucose residues, and the map from the 2 mM complex showed no difference density peak at all. These data suggest that under the crystallization conditions (1.4 M sodium citrate/0.1% sodium azide, pH 7.5), (α2-3)sialyllactose has an affinity for the second site that is at least four times weaker than that for the primary site.

Binding of Synthetic Sialosides to the Second Site. A difference electron density map at 3-Å resolution of X-31 hemagglutinin complexed with Neu5Acα2Bac (Fig. 1, compound III) shows that this compound binds to both ligand binding sites. Indeed, this was our first evidence for the existence of the second binding site (Fig. 5). A low-resolution electron density map indicates that Neu5Acα2Mac (Fig. 1, compound IV), a compound that lacks the phenyl ring of Neu5Acα2Bac, also binds to both sites (data not shown). The Neu5Ac residue of Neu5Acα2Bac binds to the second site in an orientation that is nearly identical to that of the Neu5Ac residue of (α2-3)sialyllactose (rms deviation for nonhydrogen atoms is 0.18 Å). It was not possible, however, to determine the conformation of the 2-*O*-(4′-benzylamidocarboxybutyl) side chain of Neu5Acα2Bac. The difference electron density corresponding to the ligand (Fig. 5), although suggesting the location of this side chain, was not large enough to accommodate it entirely, nor did the density have a shape characteristic of the side chain moiety. Furthermore, as the side chain contains eight dihedral rotational degrees of freedom, it is possible to build many different models that are stereochemically reasonable and consistent with the difference electron density.

Sialosides that Do Not Bind to the Second Site. Electron density maps identified four Neu5Ac derivatives that bind to the primary site but not to the second site of X-31 hemagglutinin at the concentrations examined: (α2-6)sialyllactose, Neu5Acα2Me, 4-*O*-acetyl-Neu5Acα2Me, and 9-amino-9-deoxy-Neu5Acα2Me. (Although low-resolution electron density maps showed that (α2-6)sialyllactose does not bind to the second site at concentrations ranging from 2 to 32 mM, the same maps contained density peaks that for the first time allowed us to locate all three sugar residues in the primary site.) It is puzzling to note that two compounds that bind to the second site, (α2-3)sialyllactose and Neu5Acα2Mac, have only the α-Neu5Ac residue as their common element, whereas a simpler α-sialoside, Neu5Acα2Me, does not bind to the second site. Further research will be needed to define the minimum set(s) of chemical groups necessary for second site binding.

Attempts to Affect the pH of Membrane Fusion. Hemagglutinin undergoes conformational changes at low pH that lead to the fusion of the viral and endosomal membranes (for review, see ref. 18). These changes can be prevented by the introduction of a covalent crosslink between adjacent HA1 monomers of the hemagglutinin trimer (L. Godley, J. Pfeifer, D. Steinhauer, G. Shaw, R. Kaufmann, E. Suchanek, C. Pabo, B. Ely, J.J.S., D.C.W., and S. Wharton, unpublished data). Since the second binding site is located in the interface between HA1 monomers, it is therefore possible that molecules binding here might either stabilize or destabilize the neutral pH conformation and thus lower or raise the pH of membrane fusion. This possibility is also suggested by the proximity of the second site to residue 1081 (Fig. 3), where the Glu → Gly mutation is known to increase the pH of fusion (20). However, a preliminary experiment with Neu5Acα2Bac failed to detect any effect of the ligand on the pH at which the conformational changes occur (S. Wharton, personal communication).

DISCUSSION

Previous crystallographic studies of BHA complexed with a mixture of sialyllactose isomers identified a Neu5Ac binding

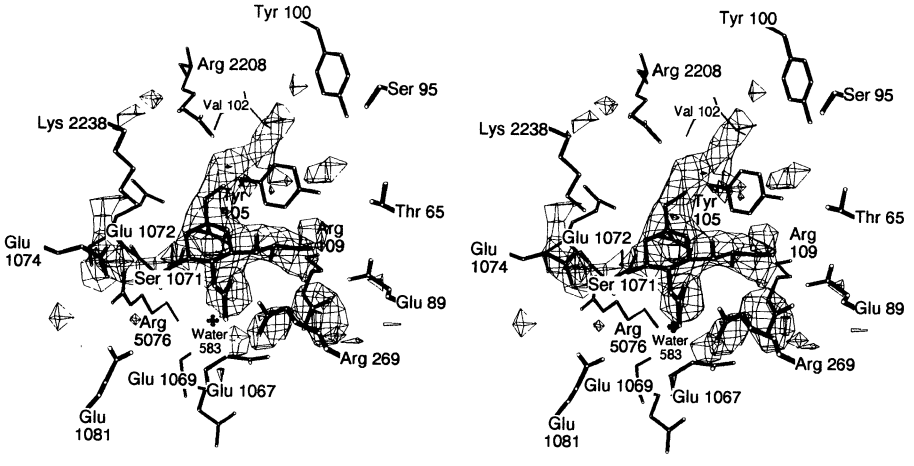

FIG. 5. Stereoview of the second ligand binding site, showing a difference electron density map calculated with coefficients ($F_{obs}^{X\text{-}31+Neu5Ac\alpha2Bac} - F_{obs}^{X\text{-}31}$) from 10- to 3.0-Å resolution, averaged about the threefold axis of noncrystallographic symmetry, and contoured at $+3\sigma$ above the mean density. Since the density does not clearly establish the conformation of the 2-*O*-(4′-benzylamidocarboxybutyl) side chain, a refined model of protein plus Neu5Acα2Me is shown instead. Isotropic B-factors for the pyranose ring atoms of Neu5Ac refine to 27 Å^2, assuming a ligand occupancy of 0.6.

site in a pocket on the protein surface (6). The peaks corresponding to Neu5Ac appeared only at a level of 5σ above the mean electron density in threefold averaged difference maps, possibly because the concentration of ligand in the crystals was low. For the experiments presented here, we wanted to be sure that the ligands were present as single isomers and at known concentrations. The sialosides were, therefore, purified by HPLC, and residual neuraminidase activity was removed from the BHA by immunoaffinity chromatography. Data from BHA complexed to purified (α2-3)sialyllactose yielded a difference map containing peaks at the significantly higher level of 14σ above the mean density (Fig. 2). The map revealed the conformation of all three of the sugar residues of (α2-3)sialyllactose in the known binding site (8) and also established the existence of a second binding site for (α2-3)sialyllactose.

Since (α2-3)sialyllactose binds to two sites, its interaction with hemagglutinin can be described by two microscopic dissociation constants. However, the ^{1}H NMR experiments as described (7) presumably measured only the microscopic constant of (α2-3)sialyllactose binding to the primary site. In that study, binding was quantified by observing the chemical shift in the N-acetyl resonance of Neu5Ac in the presence of protein, which likely derives from the acetyl group's proximity to an aromatic residue (Trp-153) in the primary site. The second site does not contain any aromatic residues near enough to the N-acetyl group to cause significant chemical shift changes (Fig. 4 and ref. 8).

Binding can also be detected by observing line broadening of ligand NMR resonances (the H_{3a} resonance in particular), but since line broadening can be caused by binding to either site, it is not possible to use these data to determine the two individual microscopic constants (21). However, for (α2-3)sialyllactose binding to X-31 hemagglutinin, the crystallographic evidence presented above suggests that the ligand binds to the primary site at least four times more strongly than to the second site. This makes it unlikely that binding to the second site would contribute significantly to line broadening. Consistent with this view, the line broadening of the H_{3a} resonance (N.K.S., unpublished data) and the chemical shift of the N-acetyl resonance (7) give dissociation constants that are the same within experimental error when analyzed with the assumption that there is only a single microscopic constant.

The binding of (α2-3)sialyllactose to two sites raises the possibility that a cell-surface receptor containing a terminal Neu5Ac residue might interact with the second site. (α2-3)Sialyllactose is the saccharide component of G_{M3}, a ganglioside that when incorporated into the cell membrane causes cells to be agglutinated by X-31 virus (22). In addition, (α2-3)sialyllactose shares the sequence Neu5Ac(α2-3)Galβ with N-linked saccharides commonly found in membrane glycoproteins (23). In the refined model (Fig. 3), the glucose residue of (α2-3)sialyllactose is partly buried, so that bulky glycosidic substituents on the glucose could not be accommodated. However, the fact that Neu5Acα2Bac and Neu5Acα2Mac also bind to the second site shows that the molecular contacts that we observe between glucose and the surrounding amino acids are not necessary for binding. It may therefore be possible to fit the Neu5Ac(α2-3)Galβ component of gangliosides or glycoproteins into the second site, provided that the third saccharide residue in those molecules adopts a conformation different from that seen in the (α2-3)sialyllactose complex studied here.

It should be stressed that our crystallographic results do not demonstrate that the second site is relevant to the infection process. However, whether or not it is necessary for infection, it may be possible to utilize the second site in the design of antiviral drugs. If a bivalent sialoside could be designed to bind to both sites simultaneously, it might bind tightly enough to hemagglutinin to be a useful inhibitor of hemagglutinin's interaction with cell membranes. Indeed, studies have shown that molecules containing multiple Neu5Ac residues, including the sialylglycoprotein α_2-macroglobulin (24), synthetic polymers carrying pendant α-sialosides (25, 26), and bivalent sialosides (19, 27), can be used at relatively low concentrations to inhibit viral agglutination of erythrocytes.

We have presented an atomic model for a second ligand binding site in influenza virus hemagglutinin. More research is needed to determine whether this site participates in the infection process and whether it can be utilized as a target in the design of drugs to inhibit either the membrane fusion event triggered by low pH or the attachment of viruses to cells.

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