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Receptor Binding by Influenza Virus: Using Computational Techniques To Extend Structural Data

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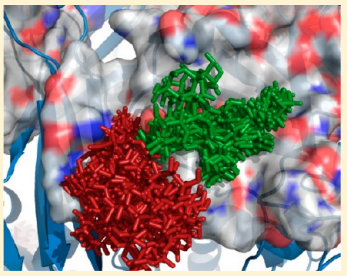
ABSTRACT: Influenza attaches to host cells via hemagglutinin binding of cell-surface glycans. These relatively low-affinity interactions involving flexible ligands are critical in determining tissue and host specificity, but their dynamic nature complicates structural characterization of hemagglutinin−receptor complexes. Molecular simulation can assist in analyzing glycan and protein flexibility in crystallized complexes, assessing how binding might change under mutation or altered glycosylation patterns, and evaluating how soluble ligands may relate to physiological presentation on the plasma membrane. Molecular dynamics simulation also has the potential to help integrate structural and dynamic data sources. Here we review recent progress from analysis of molecular dynamics simulation and outline challenges for the future.



Influenza virus enters host cells via binding of hemagglutinin to sialic acid-terminated glycans on the cell surface. This glycan-recognition event controls internalization and eventual membrane fusion to release the viral genome into the cytoplasm. While individual hemagglutinin−glycan interactions are of relatively weak millimolar affinity,[1,2] the highly multivalent interaction between many hemagglutinin copies on the viral surface and many branched ligands on the cell surface means that avidity plays an important role in determining viral attachment. Glycan recognition is thought to be an important factor in determining host range: avian influenza virus preferentially binds to avian respiratory tract glycans, while human influenza virus preferentially binds to human upper respiratory tract glycans.[3−5] Changes to glycan specificity are therefore thought to be one of the key permissive factors for interspecies transmission of influenza.

In addition, the hemagglutinin−neuraminidase affinity balance is important both to viral infectivity and to drug resistance.[6,7] Clinical isolates of influenza and lab strains that have lower glycan binding affinity and moderately reduced efficiency of infection but display neuraminidase independence have been identified:[8,9] they do not require neuraminidase function and are thus a priori resistant to the neuraminidase inhibitors that are the current mainstay of pharmacologic therapy for influenza. Understanding the determinants of glycan binding by influenza hemagglutinin is thus necessary to predict the impacts of mutations on viral biology and clinical outcomes.

Binding of hemagglutinin to host glycans is often considered a multivalent but two-component reaction between hemagglutinin and cellular receptors. However, biochemical and infectivity data increasingly show that the outcome of viral attachment is dependent on at least three components: the hemagglutinin protein, host glycans, and N-linked glycans that are covalently attached to the viral hemagglutinin. Hemagglutinin is extensively glycosylated (viral glycans contribute ∼20% of hemagglutinin mass[10]), and these glycans serve an important purpose in immune evasion. Because the viral glycans are host-derived, the extent and composition of the glycans also depend on the cell type in which the virus was grown (Figure 1).[11,12] Recent data show that this cell-type-dependent glycosylation can affect both viral affinity for cell-surface glycans and receptor binding specificity.[13,14]

Challenges in Studying Glycan Binding by Influenza: Studies of Soluble Ligands. Solution-phase nuclear magnetic resonance (NMR) and crystallographic experiments have yielded the highest-resolution structural and dynamic information about glycan binding by influenza hemagglutinin, but their physiologic applicability is limited by two factors. (1) Experiments have largely been performed using short soluble oligosaccharides that may not fully capture the way in which longer glycans, densely patterned on the cell membrane, interact with the virus. (2) Because the glycan residues are highly flexible, they are typically poorly ordered in crystal structures, and indeed, N-linked glycans on proteins are often truncated to facilitate crystallization. The crystallographic data thus provide incomplete structural information about the residues of interest and represent a simplified molecular system without some of the distal glycan interactions that have been shown to affect ligand binding affinity and specificity.

Despite these challenges, crystallographic data have yielded important structural information regarding how hemagglutinin binds its receptors. As shown in Figure 2, the terminal sialic acid binds in a well-formed pocket, while Gal-2 and Glc-3 lie more loosely across the protein surface. Only a few studies have reported electron density for hemagglutinin bound to both human-type α2,3 sialyllactose and avian-type α2,6 sialyllactose ligands or similar pentasaccharides, but the canonical

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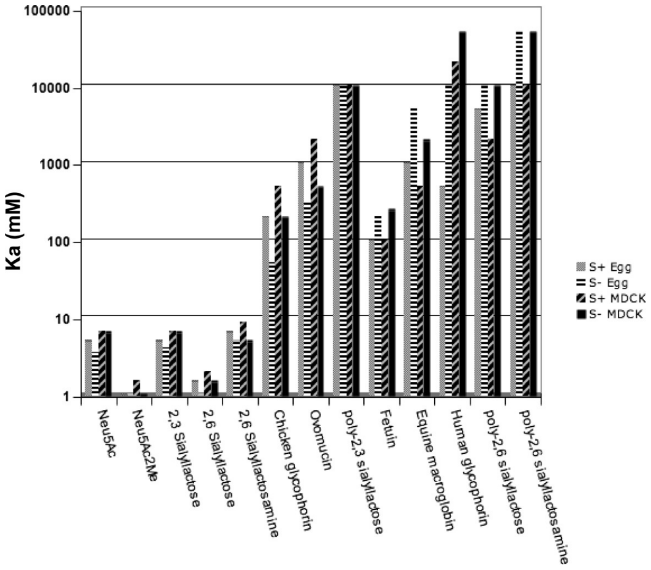

Figure 1. Influence of viral glycosylation on ligand binding. Binding of influenza virus grown in eggs or MDCK cells was assayed against a variety of carbohydrate ligands. Virus was additionally tested with and without deletion of the N131 glycosylation site (S+/S−). Data replotted from ref 11.

interpretation of these structures is that the α2,3 ligands extend more linearly in the binding pocket while the α2,6 ligands curl up to contact the 190-helix along one side of the binding pocket. While the electron density shows considerable flexibility for these contacts and the structural details should not be regarded as fixed, the data do suggest that distinct sets of hemagglutinin may be involved in binding α2,3 and α2,6 ligands beyond the terminal sialic acid.

Solid-Phase Assays of Glycan Binding. Solid-phase assays have yielded substantial insight into receptor binding by influenza hemagglutinin. One key innovation in the study of glycan binding by influenza hemagglutinin has been the development of glycan arrays, where spatial patterning on a solid support allows screening of many glycan molecules in a single experiment.[15−19] This technique has been used to study both purified hemagglutinin and whole virus. Use of glycan arrays has allowed broad screening of a large number of glycan structures, allowing large-scale analysis and profiling of binding. Such analyses have yielded hypotheses such as the "cone versus umbrella" model,[20] in which avian-tropic hemagglutinins tend to bind glycans with a narrower "cone-like" topology, while human-tropic hemagglutinins tend to bind glycans that can adopt a broader "umbrella-like" topology.

Surface plasmon resonance (SPR) experiments can provide extremely good measures of ligand binding and dissociation. Several SPR studies have utilized glycans coupled to a solid support to probe association and dissociation kinetics of influenza hemagglutinin with different ligands.[1,21] Despite the utility of this technique, the solid support may substantially affect the presentation mode and recognition of ligand, as assay conditions and spacer choice can greatly alter the results.[22]

Glycan arrays and SPR are prone to some of the same pitfalls: surface artifacts and an unknown physical presentation mode of the glycans on the array (and how that relates to physiologic presentation). There have been important discrepancies observed in a few cases between array binding and animal infectivity data.[23] Resolving these issues and better aligning the array results with both biochemical−structural assays and infectivity studies remain a major focus of array technology development. Among the many promising approaches is the use of supported lipid bilayers rather than a pure solid substrate to anchor the glycans, creating a context designed to mimic native membrane presentation of cell-surface glycans.[24,25]

Molecular Simulation as a Means of Extended Data Analysis. Molecular simulation of receptor binding by influenza hemagglutinin has roughly paralleled experimental approaches in its emphasis: (1) understanding structural subtleties and dynamics in the model systems used for crystallization, (2) extending these systems to understand solution-phase binding of more extensive ligands by hemagglutinin, and (3) analyzing how glycans may be presented and recognized in a membrane environment. Molecular simulation should best be viewed as an extended analysis of structural and chemical data: given a set of rules from basic physics and chemical parametrization,[26,27] simulations calculate the physical implications of a given set of structural data and hypotheses. Because glycans display extensive flexibility in their binding to hemagglutinin, we primarily consider simulation approaches that capture flexibility and dynamics: molecular dynamics simulation and related techniques. Particularly because of the

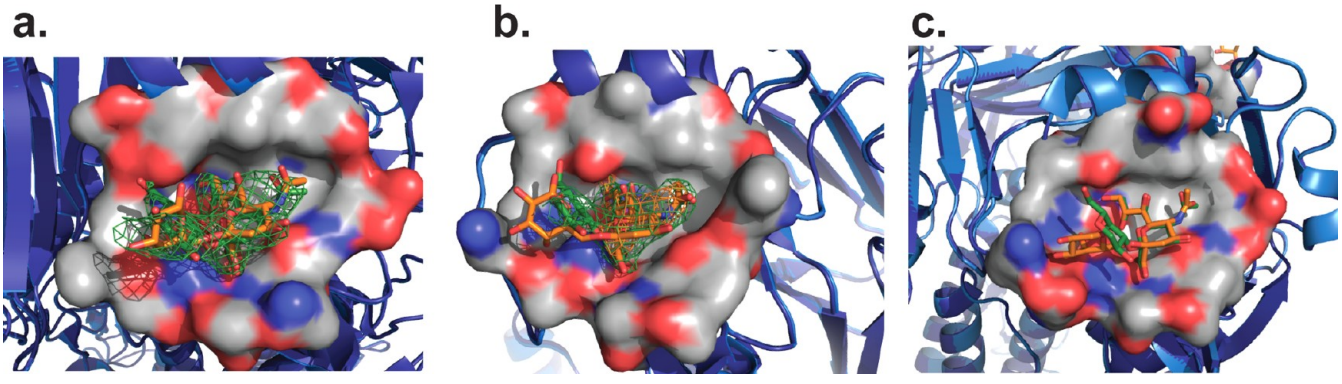

Figure 2. Crystallographic data for modes of binding of α2,3 and α2,6 ligands to influenza hemagglutinin. Protein structures are rendered in ribbon form with the ligand-binding pocket (defined as all atoms within 7 Å of the ligand) in surface form. Ordered residues from LSTa and LSTc ligands are colored green and red, respectively, with $2F_o - F_c$ maps contoured at 1.5σ where electron density is available: (a) an avian H3 hemagglutinin related to the 1968 H3N2 human strain,[42] (b) an avian H1 hemagglutinin,[45] and (c) a 1934 human H1 hemagglutinin.[44]

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extensive glycan flexibility, it is likely more appropriate to think of hemagglutinin and its complexes with ligand in terms of thermodynamic ensembles than static structures. Each molecular dynamics trajectory approximates the random sampling from this ensemble that a single-molecule trajectory would take; the challenge is then to systematically extract information about the underlying ensemble from a number of such random samples. Techniques for this process have been developed and studied for the folding of small proteins;[28−30] recent applications have begun to examine more complex systems such as ligand binding and conformational change.

Glycan Molecular Structure: A Statistical Mechanics Perspective. From the perspective of statistical mechanics, any molecule or molecular complex at a given time occupies a point in phase space $\mathcal{R}^d$, where d is the number of spatial and velocity degrees of freedom. Given a fixed number of such molecules at a constant temperature, the probability of any structure (or more formally any point in phase space) x is $P(x) = Z \times \exp[-H(x)/(k_B T)]$, where H is the Hamiltonian, k_B is Boltzmann's constant, T is the temperature, and Z is the normalization coefficient. If we let the number of molecules vary, as in a chemical reaction, the chemical potential will also enter into this equation and a grand-canonical ensemble formulation will be used instead of a canonical ensemble. This simple relationship has several important implications for glycan−protein interactions, as detailed below.

Flexibility and Experimental Structure Determination. Most protein crystallography and NMR methods determine a structure by taking a single candidate model to fit the data (or in NMR giving a series of models each singly fit to the data). Where H has a sharp minimum, this is a good approximation. However, the notion of structural flexibility implies a Hamiltonian with lower energetic penalties along some degrees of freedom. In the case of such flexibility, as well as other multiminimum situations, solving for an ensemble of structures that together fit the data may be most appropriate. The opportunity for such an approach is readily apparent in poorly defined regions of glycan density present in most influenza−ligand cocrystal structures. There have been some important recent efforts to incorporate ensemble or multiconformer methods for both crystallographic refinement and NMR structure solution;[31−33] however, this is still not the dominant paradigm, and indeed, the data may not support a many-parameter model for poorly defined glycan density. One must further note that crystallization conditions are explicitly engineered to reduce multiple conformations, and crystals are typically "observed" in a synchrotron at low temperatures.

Ergodicity, Sampling, and Computational Methods. One strength of molecular dynamics simulation is the possibility of sampling this conformational ensemble. Via the ergodic hypothesis, a single molecular trajectory will, over sufficient time, yield the probability-weighted thermodynamic ensemble for that molecule. There are of course more efficient ways to sample this ensemble, and molecular dynamics yields dynamical as well as thermodynamic information. However, this sampling property is a fundamental strength of dynamics simulation used as an adjunct to conventional structure solution. It also constitutes a fundamental weakness: given a set of starting conformations S for simulation, other conformations that are rare or slow to access from S will be unlikely to be sampled. Formally, one can think of this as conformations T where the expectation value of the transit time $E(s \in S \rightarrow t \in T)$ is slow. So if there are conformations separated by a time scale of minutes and our aggregate sampling power via molecular simulation is milliseconds, the system may be fundamentally undersampled. This is likely the case for hemagglutinin−glycan interactions: k_{on} and k_{off} have not been determined experimentally for monovalent interactions, but analysis of binding of surface-immobilized substrate to X-31 hemagglutinin rosettes yielded a multivalent k_{on} of 2×10^3 M^{-1} s^{-1} and a k_{off} of 2×10^{-4} s^{-1}.[1,1] If monovalent interactions are even 2 orders of magnitude faster, this is still a challenging regime for molecular dynamics. A number of strategies have been developed to alleviate this issue, including more efficient sampling techniques and reweighting of the Hamiltonian,[34−38] but ultimately there is no "free lunch". Complex systems with slow dynamics are difficult to sample.

Assessing Simulation-Assisted Models and Sources of Error. The strength of simulation-assisted models in probing dynamics and multiple conformations also presents a major challenge: if one performs simulations to assess behavior not included in structural models, how one does one assess the simulation models? There are several potential sources of error in glycan binding models developed using simulation. We would categorize them as follows, although this is certainly not an exhaustive list: (1) input chemical parameters and model fidelity, (2) how well the simulated molecular system approximates the physical system of interest, (3) sampling, (4) model construction and interpretation, and (5) how well the physical system of interest approximates the physiological system of interest.

One approach has been to validate on smaller test systems such as oligosaccharides in solution for which there are dynamical data available. This is frequently performed as part of chemical parameter development for molecular dynamics force fields.[26,27,39] The chemical parameters for major contemporary carbohydrate force fields are derived by fitting to high-level quantum mechanical calculations, typically for small carbohydrate molecules in the gas phase, or spectroscopic data such as vibrational frequencies.[26,27,39] A first step in validation is evaluating the goodness of fit involved in this parametrization. The parameters thus derived can be readily evaluated on small molecules for which structural and dynamic data are available and can be extensively sampled via simulation. Because carbohydrates display such conformational flexibility, solution NMR is particularly well-suited for evaluating the dynamics of oligosaccharides. Most contemporary carbohydrate force fields have been evaluated this way.[26,39] While this kind of testing gives a good metric for force field improvement, success at predicting small molecule dynamics and conformational ensembles is not necessarily sufficient to ensure accurate modeling of large protein−glycan complexes, and indeed, the relative sensitivity of the end observables to fine improvements in force fields versus, for example, the extent of sampling has not been well established. For large systems, a detailed sensitivity analysis on a large number of chemical parameters is simply not computationally feasible.

Another approach to validation is to utilize indirect readouts on structural heterogeneity in the high-resolution structural data. Correlations of root-mean-square fluctuation in simulation trajectories with crystallographic B factors can be helpful,[40] although elevated B factors can result from a number of causes and should not be interpreted as a quantitative indicator of spatial heterogeneity or mobility.[41] Where feasible, prediction of spectroscopic observables from the dynamical models and

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direct comparison of simulated and observed spectra can be a particularly powerful technique.

Finally and perhaps most importantly, one can assess the utility of the models: Can simulation-assisted dynamical models rationalize other experimental data? Can they generate novel testable hypotheses? Are they successful in blindly predicting new experimental findings? While none of these properties directly validate the models themselves, they are perhaps the most important properties we desire from a model, the ability to explain and predict experimental findings.

Glycan Dynamics in Crystallized Hemagglutinin−Oligosaccharide Complexes. Molecular dynamics simulation has been an effective adjunct to crystallographic study of hemagglutinin−oligosaccharide complexes, permitting analysis of the structure and dynamics of ligand molecules that are poorly ordered in the crystal structure. Physiological glycans are quite long, but for ease of crystallization and simplicity of solution study, investigators have typically used either the trisaccharides α2,3 sialyllactose (3SLN) and α2,6 sialyllactose (6SLN) or the pentasaccharides LSTa (Neu5Acα2−3Galβ1−3GlcNAcβ1−3Galβ1−4Glc) and LSTc (Neu5Acα2−6Galβ1−3GlcNAcβ1−3Galβ1−4Glc) to mimic avian-type and human-type cell surface glycans, respectively.[42−47] Although ligand is clearly present in the binding pocket of HA−oligosaccharide cocrystal structures, the ligand density is much less well resolved than that of the protein, suggesting substantial flexibility. This is well supported by molecular dynamics simulations of the hemagglutinin−oligosaccharide complex,[40,48] which predict substantial movement of the distal glycan residues, allowing contact with different portions of the binding pocket. Simulations by Xu et al. have examined the binding of pentasaccharide to hemagglutinin molecules from different viral subtypes in the presence of either LSTa or LSTc ligands.[48] In those simulations, both LSTa and LSTc exhibited substantial conformational diversity but, most interestingly, displayed different conformational profiles when bound to different hemagglutinin molecules.

Predicting how protein mutations affect ligand binding affinity and specificity has long been a goal of molecular simulation efforts in this field, and this difference in dynamic binding profiles reported in the Xu study suggests that conformational dynamics may have an important role to play in such efforts. The crystallographic data directly show ligand flexibility, but the simulation data furthermore suggest that conformational states may be differently populated in different HA−ligand complexes. In such a case, molecular dynamics simulation as opposed to purely static or multiconformer approaches becomes an important component to prediction of binding affinity, as generation of conformational substates and estimation of their relative population are critical to estimating the binding free energy of a conformational ensemble.

Because monomeric hemagglutinin−glycan dissociation coefficients are on the order of 1 mM, with slow association and dissociation rates,[1] accurately sampling bound and unbound states and predicting free energies of binding are challenging tasks. Two recent studies have made progress in this direction, examining how point mutations to hemagglutinin affect ligand binding affinity and specificity. Work by Newhouse et al. examined binding of LSTa and LSTc to human and avian hemagglutinins, linking molecular dynamics simulation to MM-GBSA analysis and predicting which protein residues in the ligand-binding domain of each hemagglutinin contribute most strongly to binding.[49] Comparing these contributions across human- and avian-type hemagglutinins and receptors yielded a set of predictions regarding mutation sites that may control glycan binding specificity.

One advantage of simulation relative to crystallographic studies is that protein mutagenesis is fairly straightforward to simulate, particularly if no large-scale structural changes result. Simulations can therefore be used as an effective intermediate screening method to predict the effect of mutations on ligand binding before experimental mutagenesis and structural or biophysical studies. Such an approach has been employed recently to predict ligand binding mutations in H5 avian influenza hemagglutinin, a molecule of particular interest because H5N1 influenza has sporadically infected humans but has not displayed efficient human-to-human transmission.[50,51] An H5 hemagglutinin−3SLN complex was first simulated on the basis of crystallographic data, and residues were evaluated on the basis of their influence on ligand conformation, measured as excess positional mutual information to the ligand relative to the rest of the protein. This was combined with sequence analysis to yield a refined set of mutation sites. A number of point mutations were tested at each site, with multimicrosecond ensemble molecular dynamics simulation performed for each mutation. The resulting data set was then used to score mutants by how much the ligand dissociation rate was accelerated; the top-scoring mutant also had weakened binding in red-blood-cell assays. A related approach was taken by Das and co-workers, where free energy perturbation was performed over the course of <10 ns runs for each of several starting configurations for a series of H5 hemagglutinin mutants.[52]

A fundamental challenge in studying ligand binding mutations of hemagglutinin is that for a 328-amino acid HA1 chain the protein mutation space is 20^{328}, and even excluding allosteric mutations to consider only the receptor binding domain still yields a space too large to explore fully via experimental mutagenesis. A computational method to effectively narrow this space of mutations for experimental testing would be of great help in allocating experimental resources and providing an integrated framework to explain the results. The approaches described above for predicting ligand binding mutations in hemagglutinin show great promise, although neither has yet yielded quantitative, experimentally comparable ligand binding affinities or association or dissociation rates. While of course quite a high bar, this remains an important goal in the field and the best standard by which to judge to success of the computational methodology.

Simulations of Three-Component Recognition: Protein, Ligand, and Viral Glycan. In addition to protein−ligand interactions, molecular simulation provides an effective means of probing interactions of ligands with N-linked glycans on the viral hemagglutinin. Biochemical studies have demonstrated that these interactions can have an important effect,[11,13,14] but they are extremely difficult to assay directly. Crystallographic data show ligand density in the proximity of viral glycan density but are not sufficient to establish glycan−glycan contact. To further explore this, molecular dynamics simulations were performed with the 3SLN ligand used for crystallographic studies in complex with hemagglutinin containing just the crystallographically modeled N-glycans.[40] Even in this minimal system, significant contact was predicted between the viral glycans and the ligand that approximates the host glycan (Figure 3). When the simulations were repeated with a glycan corresponding to that observed via mass

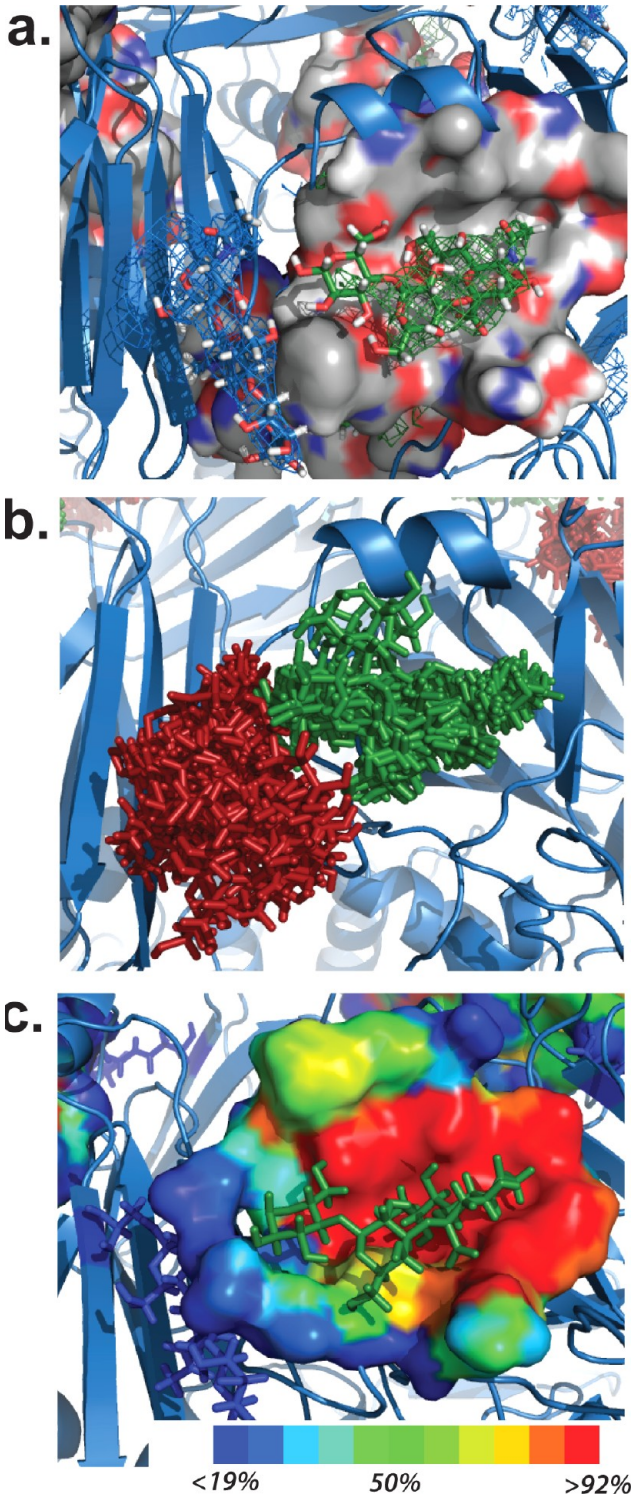

Figure 3. Structural flexibiliy of trisaccharide ligand and core viral glycans by crystallography and molecular dynamics simulation. (a) Crystallographic model and $2F_o - F_c$ density contoured at 1.5σ for α2,3 sialyllactose bound to human H3 hemagglutinin.[2] Viral glycans refined in the crystallographic model are colored blue, and the 3SLN ligand is colored green. (b) Same molecules as in panel a, with 20 structural snapshots of the ligand and glycan taken from a 100 ns molecular dynamics simulation of the full hemagglutinin–ligand complex as refined in the crystal structure.[40] Viral N-linked glycan is colored red and the ligand green. (c) Map of the binding pocket colored by the fraction of simulation time that each atom is in contact with the ligand. The ligand and viral glycan both show substantial flexibility, contacting each other and a relatively wide range of the binding pocket over the course of a relatively short 100 ns simulation.

spectrometry of virus grown in mammalian cell culture,[53] this glycan–glycan contact was even more extensive. Prior to these observations, it had been assumed that viral glycosylation effects on glycan binding occurred far from the binding pocket on the protein; these simulations suggested they could occur much closer to the pocket. Subsequent to these predictions, additional biochemical studies showed glycosylation-dependent effects on ligand binding even for relatively short oligosaccharide ligands,[13,14] providing further support for this idea. In addition to affecting ligand binding affinity and specificity, three-way protein–N-glycan–ligand interactions mean that glycosylation state should be considered when screening for receptor binding mutants of influenza and in designing small-molecule inhibitors.

Simulations of Presentation of Glycan on the Membrane. In a physiologic context, influenza binds to ligands presented on the plasma membrane, but most structural and biochemical data measure binding of hemagglutinin to soluble ligand or ligand coupled to a solid support (Figure 4). Just as biochemical assay development is moving to treat the membrane environment explicitly, recent simulation studies have examined the membrane effects on ligand presentation for recognition by hemagglutinin.[54] Simulations of the glycosphingolipid GM1 in a DMPC bilayer showed that the membrane-proximal sugar residues were largely shielded from solvent (and potentially from protein recognition) by lipid headgroups. The GM1 dynamics were validated by comparison to paramagnetic relaxation rate enhancement data from NMR. The GM1–DMPC system, though a much-simplified model of glycan presentation on a dense plasma membrane, provides an elegant proof of concept that simulation can be used to predict physical modes of ligand presentation on the membrane. As with all of these studies, developing a predictive simulated-assisted model of the physical model system under study is an important first step; the next challenge is to utilize the insight thus gained to understand the physiological system of interest in terms of specific, experimentally testable hypotheses.

Outlook: High-Resolution Models for Physiological Virus–Receptor Interaction. Decades of study have yielded a wealth of structural and biochemical data about ligand recognition by influenza virus, yet we lack a good predictive model for the basis of receptor specificity. Faced with a new viral mutation or a perturbation to the cell-surface glycans, we would like to understand how ligand recognition and viral infection might differ. Molecular simulation has shown good progress in extending crystallographic data to analyze protein–ligand dynamics in soluble complexes; two main challenges that lie ahead are increasing the quantitative fidelity with which mutational effects can be predicted and increasing the integrative reach of simulation. Progress in this latter realm will entail bringing together the virus–ligand interactions with ligand–membrane interactions and, perhaps most challenging, moving beyond single receptor–ligand pairs to consider the avidity and crowding effects that are believed to be key to determining binding outcomes. Influenza turns relatively modest affinities and affinity differences into efficient infection and robust host specificity; one challenge and opportunity for

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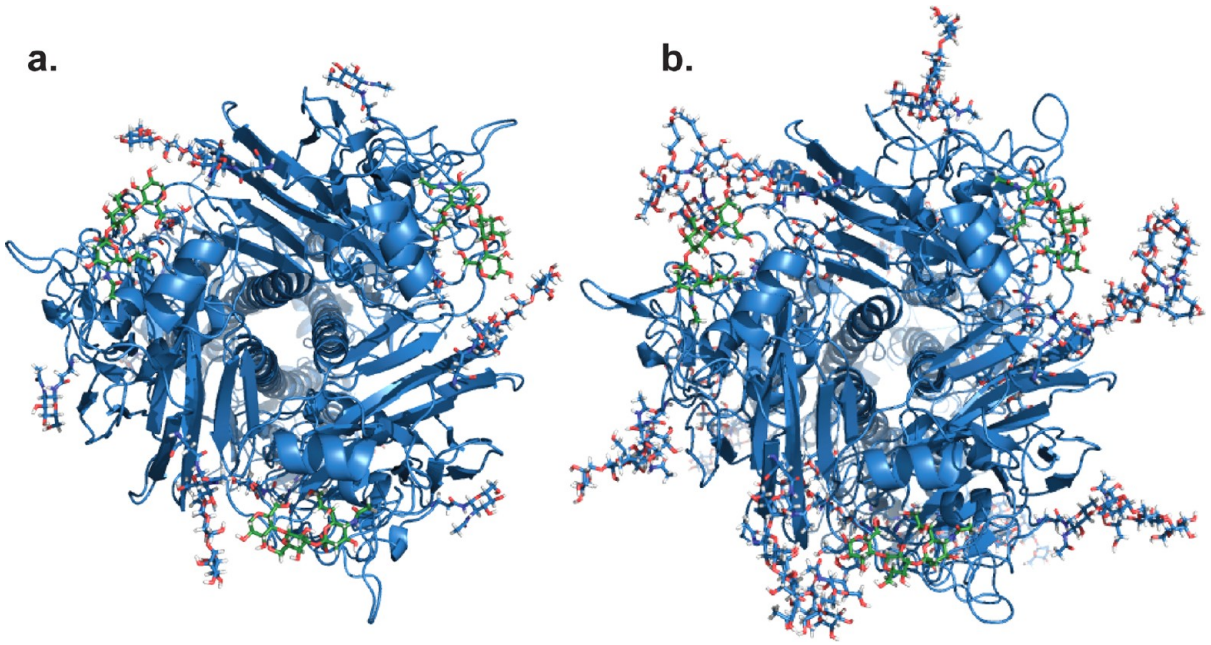

Figure 4. Human H3 influenza hemagglutinin with truncated crystallographic glycans and longer glycans corresponding to mammalian host. (a) Human H3 molecule used for Figure 3 rendered with only the crystallographically ordered glycans. (b) Same molecule rendered with glycans modeled corresponding to the shortest glycans detected via mass spectrometry when influenza was grown in a mammalian cell culture.[53] In both renderings, the 3SLN ligand is shown as green sticks, the protein as blue ribbons, and the viral N-linked glycan as blue sticks.

molecular simulation is to explain the physical basis for these effects.

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Notes

The authors declare no competing financial interest.

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