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Specificity of Furanoside–Protein Recognition through Antibody Engineering and Molecular Modeling

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Abstract

Recognition of furanosides (five-membered ring sugars) by proteins plays important roles in host–pathogen interactions. In comparison to their six-membered ring counterparts (pyranosides), detailed studies of the molecular motifs involved in the recognition of furanosides by proteins are scarce. Here the first in-depth molecular characterization of a furanoside–protein interaction system, between an antibody (CS-35) and cell wall polysaccharides of mycobacteria, including the organism responsible for tuberculosis is reported. The approach was centered on the generation of the single chain variable fragment of CS-35 and a rational library of its mutants. Investigating the interaction from various aspects revealed the structural motifs that govern the interaction, as well as the relative contribution of molecular forces involved in the recognition. The specificity of the recognition was shown to originate mainly from multiple CH– π interactions and, to a lesser degree, hydrogen bonds formed in critical distances and geometries.

Keywords

antibodies; furanosides; hydrogen bonds; hydrophobic interactions; molecular recognition

Introduction

Carbohydrate–protein interactions are involved in many important physiological events such as cell adhesion, tumor metastasis, and immunological responses.^[1, 2] Studies on the recognition of carbohydrates by proteins has led to an understanding of the molecular forces that stabilize these complexes.^[3–6] To date, the overwhelming majority of these studies have focused on the recognition of pyranosides by proteins. In contrast, similar detailed studies involving molecular interaction of proteins with furanose-containing carbohydrates are more limited.^[7–9] Furanose moieties are key components of glycoconjugates in many pathogenic bacteria, fungi and parasites.^[10–17] Often found on the cell surface of these organisms,

furanoside-containing glycans participate in a range of host–pathogen interactions that are essential for disease progression. For example, galactofuranose moieties from pathogens are recognized by intelectin^[2] and DC-SIGN,^[18] which result in modulation of the innate immune response. Moreover, furanoside glycans are often highly immunogenic; individuals infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis, have high antibody titers to cell wall glycans containing D-arabinofuranose residues.^[19]

Although some aspects of furanoside–protein recognition can be assumed to be similar to pyranoside–protein interaction (e.g., hydrogen bonding), others cannot. Furanoside rings are significantly more flexible than pyranoside rings, leading to questions about their plasticity upon protein binding.^[20] In addition, the hydrophobic and CH– π interactions that stabilize many pyranoside–protein binding events^[21] might be less important, as many biologically relevant furanosides do not possess hydrophobic faces, at least in their lowest energy conformations.^[22–24] By using a combination of electrospray mass spectrometry (ES-MS) and NMR spectroscopy, we have previously studied^[7] the binding of a panel of synthetic arabinofuranosyl-containing oligosaccharides to a monoclonal antibody (CS-35) that recognizes a terminal arabinofuranosyl-containing hexasaccharide motif (Ara6, **1**, Figure 1 a) in mycobacterial lipoarabinomannan (LAM). This study led to defining the epitope of Ara6 that interacts with CS-35.^[7, 25] However, the molecular details of the binding from the protein perspective, the dynamics of the interaction, and the molecular mechanism by which a specific epitope of Ara6 was preferred for interaction remained among the unanswered questions. This was subsequently corroborated by an X-ray crystallographic investigation of the CS-35 F_{ab} bound to a synthetic ligand (**2**, Figure 1 a).^[8] The crystal structure also raised a new number of questions and ambiguities about the nature of the recognition, such as the unusual structural and conformational features of the Ara6 and CS-35 in the combining site, and in particular, the importance of various amino acid residues and molecular forces in the interaction. To answer these questions and to provide a detailed picture of a furanoside–antibody interaction at the molecular level, we carried out an in-depth evaluation of this binding.

We describe here the preparation of a single chain variable fragment (scFv) of CS-35, the first scFv reported to recognize a mycobacterial glycan. By using available crystallographic data, we designed a series of scFv mutants as probes of the protein–ligand binding (Figure 1b). Computational analysis, which provides unique insight into the origin of the interaction energies at the per-residue level in both the antibody and the ligand, was applied to lay the groundwork for further rational design. Various aspects of the binding of wild-type scFv and the mutants to hexasaccharide **3** or **4** (Figure 1 a) were characterized using a combination of surface plasmon resonance (SPR) spectroscopy, saturation transfer difference (STD) NMR spectroscopy, circular dichroism (CD) spectroscopy, and computational energy calculations.

Results

Generation of the CS-35 scFv

The scFv was produced with the yield of 2 mgL^{−1}, after expression in inclusion bodies and refolding of the denatured proteins. Different buffer and pH conditions were tested in various steps of expression, refolding, and purification of monomer fragments. A key step in

this process is the proper folding of the protein to result in a functional scFv. CS-35 scFv was shown to be produced in high purity, even before purification by the Ni-NTA column, properly folded, and functional. Similar conditions resulted in the successful production of scFv mutants. The scFv and its mutants were stable in 50 mM ammonium acetate buffer pH 6.8 in 4°C for 2–3 weeks.

Computational analysis

CS-35 scFv—The average ligand displacement RMSD value (1.8 Å) was relatively small, and remained stable (± 0.5 Å) over the simulation period 5–50 ns, indicating that the MD trajectory had equilibrated and was suitable for further analysis (Figure S1 in the Supporting Information). The total interaction energy was decomposed into per-residue contributions from each amino acid in the protein and from each monosaccharide residue (Table 1, Table S1 in the Supporting Information). As expected, all the protein residues involved in forming key interactions with the glycan in the crystal structure were found to make favorable energetic contributions to binding, ranging from approximately 1–5 kcalmol⁻¹. Based on the per-residue interaction energies, the key residues were ranked in order of binding energy contribution, and the following residues were selected for site-directed mutagenesis in order to quantify their relative contributions to binding: Trp33^H, His35^H, Asn58^H, Ser50^H, Phe95^H, Asn97^H, Tyr98^H, Tyr98^H, Asn34^L, Tyr50^L (the superscripts L and H denote the light and heavy chains, respectively). In addition to providing insight into the glycan recognition motif, binding data for these mutants aid in validating the computational energy predictions. The majority of the theoretical binding data were consistent with expectations based on stabilizing interactions observed in the crystal structure, with the exception of two residues. Notably, Asn97^H, which is involved in forming a hydrogen bond with glycan residue D, was predicted not to make a net favorable electrostatic interaction with the glycan. This arose due to a cancellation of the direct electrostatic energy (−2.6 kcal mol⁻¹) by the desolvation free energy (4.2 kcalmol⁻¹). As the desolvation energy calculations are highly sensitive to the particular choice of parameters in the generalized Born (GB) model, the effect of replacing the residue with aspartate was also examined. A second asparagine residue (Asn34^L) was also selected for conversion to aspartate, based on the loss of the hydrogen bond between residue Asn34^L and residue A during the MD simulation.

Antigen—The ability to partition the binding energy between the monosaccharide residues in the antigen is a unique strength of the computational analysis, and indicated that approximately 93% of the affinity is provided by only three residues (A, 55%; B, 19%; E, 19%). This analysis suggests that residue A is the immunodominant component of the antigen. Although ring F makes no significant net contribution to binding, due to its distance from the antibody surface, it is the only antigen constituent that makes unfavorable electrostatic interactions (7.4 kcalmol⁻¹) with the antibody. A further analysis of this feature indicated that the net positive electrostatic energy involving ring F arises principally from unfavorable interactions with positively charged protein residues (Table S2 in the Supporting Information). This property is consistent with an interaction between the charged protein residues and the dipole (3.7 debye ± 1.8) associated with ring F that, during the simulation, frequently adopts orientations in which the positive terminus is directed toward

the protein surface, thus leading to repulsive charge–dipole interactions with Lys and Arg residues.

Mutants—The difference between binding energies (ΔG) of the wild type and mutants were calculated and compared to the experimental binding energy values. Generally the MM-GBSA calculations (from all residues, with $igb=1$) reproduce the experimental binding energies for mutants, and correctly predict that certain mutations will decrease affinity below the experimental detection limit (approximately 4 kcal mol^{-1}). Consideration of the local contribution from only the mutated residue was insufficient to describe the effects of the mutation, which required at a minimum the inclusion of the neighboring residues within $3 \text{ \AA}$ (Figure 2). However, there are notable exceptions where the MM-GBSA analyses failed to correctly estimate the effect of mutations, specifically in mutants N58A, S50A, S31A and N97D. Notably, in the case of S31A, in which there is no interaction between the residue and the ligand in either the wild type or mutant, the observed unfavorable effect of the mutation in the experiments may be due to indirect effects, such as a change in the conformation of the backbone. It should be noted that during the MD simulations the C α atoms were weakly restrained to the crystallographic positions, which would prevent the observation of any effects arising from changes in the fold conformation.

Circular dichroism spectroscopy

We used CD spectroscopy to study the structural features of the wild-type CS-35 scFv in the presence and absence of ligand **4**. The far UV spectra of the scFv, the F_{ab}, and the monoclonal antibody (mAb; Figure S2 in the Supporting Information) showed a positive band at 195 nm and a negative band at 218 nm, typical for the secondary structures of antibodies, suggesting that the scFv was properly folded. The far UV spectra of the scFv in complex with ligand **4** and that of the scFv alone were almost identical, implying that binding to **4** did not alter the secondary structure of the protein.

CD spectra in the near UV region (260–320 nm) arise from aromatic amino acid residues.^[26] Binding of **4** to the antibody, however, led to an increase in ellipticity in the near UV (Figure 3 a), suggesting that aromatic residues are involved in the binding, and that the tertiary structure of the protein is altered upon binding. The X-ray structure of the CS-35 F_{ab} in complex with **2** had revealed that Trp, Tyr and Phe residues are in close proximity to the ligand (Figure 1 b), supporting this observation. For further insights into the nature of this interaction, the scFv mutants were investigated.

Surface plasmon resonance spectroscopic studies of wild-type ScFv and **3**

We analyzed the binding profiles of the CS-35 scFv, its mutants and the CS-35 F_{ab} to **3** by SPR spectroscopy using the BIAevaluation software.^[51] The wild-type scFv showed 1:1 binding with **3** (Figure 3b), and the dissociation constant (K_D) was calculated to be $5.0 \times 10^{-7} \text{ M}$. This K_D value was consistent with the value calculated from equilibrium binding analysis ($K_D = 5.6(\pm 0.05) \times 10^{-7} \text{ M}$, Equation (1), Figure S3 in the Supporting Information). Both values are also in close agreement to that of the F_{ab}, which was measured as a reference by SPR spectroscopy ($K_D = 4.9 \times 10^{-7} \text{ M}$, Figure S4 in the Supporting Information). These affinities are also consistent with the value that was previously reported for the

interaction of the CS-35 F_{ab} with ligand **2** measured by ESI-MS and isothermal titration calorimetry, $K_D \approx 6 \times 10^{-6} \text{ M}$.^[7] The approximately one order of magnitude improvement in the affinity for ligand **3** compared to **2** is likely due to hydrophobic interactions that the octyl chain of **3** can make with the nonpolar amino acid side-chain residues in a hydrophobic pocket of CS-35 (Figure 4a). The scFv dimer showed more complicated binding modes, with fast on-rate ($2.69 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) and slow off-rate ($2.43 \times 10^{-6} \text{ s}^{-1}$), due to the avidity effects^[27, 28] (Figure S5 in the Supporting Information).

Surface plasmon resonance spectroscopic studies of mutant ScFv proteins and **3**

After characterizing the binding of the wild-type scFv, its mutants were screened for their ability to bind to ligand **3** by SPR spectroscopy. Of the twelve generated mutants, six did not recognize the antigen (Table 2). These included three aromatic mutants (Phe95Ala, Tyr98Ala, and Trp33Ala), two hydrogen-bond forming residues (Asn34Ala and Asn97Asp), and Ser31Ala, which is not in direct contact with the antigen. These six residues are therefore essential for recognition. In contrast, six other residues retained some affinity for **3** after mutation, and were further investigated to gain more information about the nature of the binding. Three of these mutants (Tyr50^LAla, Asn34^LAsp, and Tyr98^HPhe) showed comparable affinities to **3** in comparison to the wild type, and were not significantly affected by mutation (Table 2, Figure S6 in the Supporting Information). Among these mutants, there is Tyr98Phe; while mutation of this Tyr residue to Ala destroys binding, mutation to Phe retains comparable affinity. Therefore, the aromatic motif is essential for the interaction. However, three other mutants (His35^HAla, Asn58^HAla, and Ser50^HAla) exhibited substantially decreased affinities in comparison to the wild-type scFv, being in the range of millimolar. These three residues were shown to be involved in hydrogen bonding with the ligand in the crystal structure (Figure 4 b). More details about these three mutants were further obtained by STD NMR spectroscopy.

STD NMR spectroscopy of wild-type and mutant proteins with **3**

We used STD NMR spectroscopy to examine the epitope of **4** in binding to CS-35, and the effect of mutation on the epitope recognition by CS-35 (Figure 5, and Figure S9 in the Supporting Information). Such epitope mapping experiments can be useful in the absence of the crystal structures of the mutants. The STD NMR spectra of the F_{ab} and wild-type scFv with hexa-saccharide **4** were obtained (Figure S7 in the Supporting Information). An STD NMR spectrum was obtained for **4** only as a control. The STD NMR spectra of the four mutants (Tyr50^LAla, His35^HAla, Asn58^HAla, and Ser50^HAla) that recognized the ligand comparably to the wild-type scFv in the SPR spectroscopic experiments were obtained in combination with **4** (Figure S7). The focus was on proteins that were expressed in yields suitable to produce enough protein for these NMR experiments.

The results revealed the involvement of the hydrophobic octyl group of **4** in binding to the protein. The rest of the proton chemical shifts were consistent with the previously studied ligand **2** with CS-35 F_{ab}.^[7] The results also confirmed the involvement of rings ABCE in the binding as the dominant epitope, while the DF branch showed significantly less signal intensities than CE branch.

The spectra were investigated for the effect of mutations on the signal intensities of various protons (Figure S7 in the Supporting Information). Mutant Tyr50Ala showed noticeable decrease in the intensities of the protons of ring A, as would be expected. However, the STD effects for the octyl chain were not affected significantly. This is presumably because of the space it creates in the binding site for accommodating the long alkyl chain when the Tyr is replaced by Ala.

Three other mutants (His35Ala, Ser50Ala, and Asn58Ala) involve amino acids that, based on the crystal structure of the complex, interact with ring E by forming hydrogen bonds at the bottom of the binding pocket. Among these, the signal intensities were decreased the most for all protons in the His35Ala STD NMR results. This residue makes the shortest hydrogen bond with ring E (Figure 4b), and its mutation to Ala led to significant decrease in SPR and STD NMR results.

Discussion

Ara6–antibody recognition

By generating a stable and functional scFv for the CS-35 antibody, which retained its ability to bind to the Ara6 ligand, we were able to manipulate the binding site by producing a rational library of CS-35 scFv mutants. The generation of this fragment and its mutants enabled us to measure the kinetics and affinities of the binding. The one order of magnitude improvement in the affinity for Ara6 **3** compared to Ara6 **2** presumably results from the improved hydrophobic interactions between the octyl chain of the ligand and the binding site, which is rich in nonpolar amino acid side-chain residues in that region of the protein (Figure 4 a). A slow off-rate for the scFv–furanoside binding ($k_{\text{off}}=3.56\times 10^{-3} \text{ s}^{-1}$) was observed. The relatively high affinity of this interaction is therefore caused by slow dissociation. This is in agreement with the previous suggestion that the low affinities of carbohydrate–protein interactions are caused by fast dissociation of the protein from carbohydrate.^[29]

Specificity motifs and molecular forces involved in the recognition of arabinofuranoside and protein

Access to these mutants allowed us to investigate the details of the binding to the Ara6 antigen. Mutants were selected to probe the contribution of hydrogen bonds, CH– π interactions, and van der Waals interactions to the binding. SPR spectroscopy was used to screen CS-35 scFv mutants for their ability to bind to an Ara6-containing ligand immobilized on a CM5 chip. The screening results revealed the tolerance of the binding to the change of some hydrogen bonds, and the eradication of the recognition upon manipulating some aromatic residues. A study of the CS-35 scFv with Ara6 **4** in a near UV CD spectroscopic experiment also revealed the involvement of aromatic residues in the binding (Figure 3 a).

The residues that were essential to ligand binding, as determined by the abolition of binding upon mutation to alanine, were Ser31, three aromatic amino acids (Trp33, Tyr98, and Phe95), and two hydrogen-bond forming residues (Asn34 and Asn97). Ser31 is not in direct contact with Ara6; however, mutating this residue prevents the interaction. We propose that

this residue is involved in properly orienting other adjacent residues that are key to binding to Ara6 from ring A, and therefore indirectly affecting the interaction. Ring A was shown to be one of the three critical residues of the epitope (the other two being rings B and E) in the energy calculations, and was also shown to form an essential hydrogen bond with the protein. This residue extends the octyl chain into a hydrophobic pocket in the binding site, leading to higher affinity in comparison to the methyl furanoside.

The three aromatic residues were shown to make the highest per-residue energy contribution to the binding mostly by van der Waals interaction with the ligand (Table 1). Inspection of the binding site showed that these residues are at suitable distances and configurations to form CH- π bonds with Ara6 C-H bonds. While Tyr98 also involves its OH group in a water-mediated hydrogen bond with the C2 hydroxyl group of ring B, the mutation results showed that removal of this hydrogen bond in Tyr98Phe mutant does not significantly affect the binding. Therefore, the CH- π bond between the aromatic ring of Tyr98 and H2 of ring B at 2.63 Å distance is the main molecular interaction for the recognition of this essential residue. Inspection of the CS-35F_{ab}-2 crystal structure revealed that the aromatic rings of Trp33 and Phe95 are also in close enough proximities to CH groups of ring E (C1-H1 and C3-H3) to form CH- π interactions with T-shaped geometries.^[30, 31] The distances of these interactions of ring E with Phe95 and Trp33 are 2.85 and 2.63 Å, respectively (Figure 4c).

Hydrophobic interactions have been frequently reported for the protein recognition of pyranosides, which have discernible hydrophobic faces.^[21] Arabinofuranoside rings, particularly α -arabinofuranoside rings, often lack defined hydrophobic faces in their lowest energy conformations.^[22, 23] However, the CD data and mutation results suggest that there are considerable interactions between the furanoside rings of **4** and aromatic amino acid residues of CS-35. The data obtained with the Trp33Ala, Tyr98Ala, and Phe95Ala mutants demonstrate that CH- π interactions are also key contributors in building the affinity and specificity of Ara6-CS-35 binding. One of these interactions is with ring B of Ara6, and the other two, involve ring E of Ara6. Ring B of Ara6 which forms the essential CH- π interaction with Tyr98, adopts an unusual conformation, different from those observed in the solution. This residue is at the center of the Ara6, hinging the structure to orient its two branches properly into the binding pocket, and also makes a critical CH- π interaction with Tyr98. Such simultaneous functions from ring B appear to be facilitated by conformational flexibility of the furanose ring, which assists this α -furanoside to undergo an induced fit to a higher-energy conformation, allowing a critical interaction with the protein and placing other ligand structural domains in optimal orientations to interact with the protein. We hypothesize that ring plasticity may be a general feature of furanoside-protein binding events, which thus differentiates them from most pyranoside-protein interactions, aside from conformational changes that occur upon enzymatic catalysis. Ring E is also sandwiched between two other essential aromatic residues (Phe95 and Trp33), contributing significantly to the binding by the two critical CH- π interactions.

Two other essential amino acid residues of the protein are involved in hydrogen bonds with Ara6, one of which is Asn97, that forms a critical water-mediated hydrogen bond to the ring oxygen of residue C via its side chain nitrogen atom and a hydrogen bond with ring D. Mutation of this residue to Asn destroys the binding, that can be due to the change in the

conformation at this region, which is the flexible CDR H3 loop of the antibody. Asn34 is another essential residue forming a hydrogen bond with ring A, mutation of which to Ala, leads to the abolition of binding. However, when it is replaced with Asp, the mutant retains some affinity for the ligand.

A particularly interesting structural feature of the protein is a segment in the CDR H3 loop of the antibody, Phe95-Gly96-Asn97-Tyr98. Indeed, three of the essential residues (Phe95, Asn97, and Tyr98) occur in this sequence, a key segment to the specificity of the recognition. The Phe95-Gly96-Asn97-Tyr98 sequence contains a *cis* peptide bond between Phe95 and Gly96 in the crystal structure,^[8] representing a rare case of a *cis* peptide bond involving a non-proline amino acid. Such *cis* bonds are believed to be functionally important and engaged in regulating biochemical properties.^[32]

In the current system, the *cis* peptide bond assists the loop in positioning Phe95 and next important residues properly to form critical contacts with the ligand.

However, the CS-35-Ara6 interaction appears, to some extent, to be tolerant of the removal of some hydrogen-binding interactions. For example, when Ala replaces His35 (hydrogen bond to 2-OH, ring E), Ser50 (hydrogen bond to 3-OH, ring C), and Asn58 (hydrogen bond to 3-OH, ring C), the affinities decrease to the millimolar range, but binding is not abolished. The hydrogen-bond network from these residues to ring E cooperatively strengthen the binding, by interacting with ring E, which satisfies the proper distance and conformation for the interaction. Based on these results, the preference of the protein for the ABCE epitope of Ara6 over the ABDF epitope can be explained. The only difference between the CE branch and DF branch of the Ara6 is a methylene group. The CE branch is connected to ring B through a methylene group (C5 of ring B), while the connection of the DF arm is to O3. The C5 of the ring B connecting to ring E extends the ring E deeply into the binding site to be buried in this region of hydrogen-bond network. This spacer satisfies the distances that are required for hydrogen-bond formation. In particular His35, which significantly affected the interaction upon mutation, as shown in the STD NMR spectra, and is the most important residue in this hydrogen-bond network, makes a hydrogen bond in very close proximity with ring E. In the absence of methylene group, the DF branch is not able to extend deeply enough to the binding pocket to satisfy the critical distances for efficient hydrogen bonds. Moreover, the methylene group can provide more flexibility to the linkage for ring E to simultaneously make two essential CH- π interactions and three important hydrogen bonds with the protein (Figure 4 d). This subtle difference between two branches of Ara6 (CE and DE branch) makes ABCE the dominant epitope from the STD NMR results.

Finally, in the Tyr50Ala mutant, the overall affinity was not drastically decreased compared to the wild-type scFv (7.7×10^{-6} and 5.6×10^{-7} M, respectively). This residue is presumably guiding the octyl chain to a hydrophobic pocket, occupied by several aromatic and nonpolar residues, resulting in higher affinity compared to the corresponding methyl furanoside **2**. It should be noted that the Ara6 motif is present at the nonreducing terminus of LAM. This channel occupied by the octyl group, serves the region of the protein at which additional arabinofuranose residues in the polysaccharide would be bound.

Comparison of this furanoside–antibody interaction system with the previously reported carbohydrate–antibody systems is instructive. The most well-studied example of such interaction is the binding of the Se155-4 antibody to a trisaccharide fragment of the *Salmonella* serogroup B O-chain.^[29, 33–37] Since its crystal structure was reported as the first carbohydrate–antibody complex,^[38] several pyranoside–antibody systems have highlighted the essence of aromatic residues for the recognition.^[39–43] Comparison of Ara6–CS-35 interaction with the binding of **5** with the Se155-4 antibody revealed that the positioning of key tryptophan and histidine in the two systems is very similar (Figure 4 e and f). These two residues shape the specificity for arabinose and mannose rings (Figure 6) in the Se155-4 system,^[45] and are shown to be important for the recognition of arabinofuranose ring E in the CS-35 system.

The role of aromatic–carbohydrate interactions in carbohydrate–protein interactions has been discussed for pyranosides.^[21] While aromatic rings stack against the more hydrophobic face of pyranosides, such surfaces are not present in many furanose rings, in particular α -arabinofuranose residues, in their low-energy conformations. The binding of Ara6 to CS-35 scFv however, revealed key CH– π interactions involving both α -arabinofuranose and β -arabinofuranose residues. The β -arabinofuranose structure of ring E places H1 in close proximity (2.63 Å) to Trp33 at the anomeric position. The unusual conformation of ring B is also a consequence of being involved in a vital CH– π bond with Tyr98, and optimally placing rings A and CE branch simultaneously in the binding site.

The importance of hydrogen bonds and water-mediated hydrogen bonds were also in agreement with pyranoside–antibody interactions. The role of ring E in the binding site to make three essential hydrogen bonds and two critical CH– π interactions is interesting. These findings demonstrate how the arabinofuranoside antigen positions each part of the epitope in the right distance and conformation to interact with CS-35 antibody with a combination of different molecular forces.

Conclusion

Through the systematic evaluation of the recognition of Ara6 by the CS-35 antibody, we provide the first report of the detailed molecular motifs involved in a furanoside–protein interaction. This investigation consisted the generation of a scFv fragment of the CS-35 antibody and a panel of rationally designed mutants to probe the importance of various molecular forces and amino acids in the binding. Subsequent investigation of the interaction of these proteins with the Ara6 antigen by SPR spectroscopy and STD NMR spectroscopy indicated that the specificity of the recognition is achieved primarily by the interactions between multiple aromatic residues of the antibody and the CH groups of the ligand. Although some hydrogen-bonding interactions strengthen the binding, it appears that in general the interaction is tolerant to some extent to the manipulation of the hydrogen bonds, and that such interactions are of secondary importance to binding affinity and specificity.

Experimental Section

Production and purification of CS-35 scFv

The scFv of the CS-35 antibody was constructed based on the corresponding F_{ab} sequence (PDB ID: 3HNS)^[8] as the template, with a V_H -linker- V_L orientation by splice overlap PCR. The linker separating variable domains was the flexible peptide: (Gly₄Ser)₃. Sequencing of the product revealed a mutation, S31A, in the light chain. The mutant gene was stored for expression and was also used as a template to generate the original sequence of the wild-type scFv by single-site mutation. The correct sequence of the wild-type scFv product was confirmed by sequencing. The pET-28a plasmid (200 ng) harboring the scFv gene between BamHI and HindIII restriction sites was transformed into *Escherichia coli* BL21 (DE3) host cells by heat shock. Cells were cultured in LB medium (50 mL), then shaken at 37°C for 8 h. This culture (10 mL) was transferred to a 400 mL of LB medium and was shaken in 37 °C for almost 2 h. Expression was induced by adding 1 M isopropyl β -D-1-thiogalacto-pyranoside and the culture was then shaken at 20°C, overnight. Cells were then centrifuged at 8000 rpm for 30 min, the pellet was dispersed in buffer (50 mM Tris-HCl, pH 8, 1% Triton X-100, 150 mM NaCl) and followed by cell disruption by French press. Inclusion bodies^[44] were centrifuged and washed six times successively in buffer (50 mM Tris-HCl, pH 8, 200 mM NaCl). Inclusion bodies were then shaken at room temperature in buffer (4 M guanidine, 100 mM Tris-HCl, pH 8) for 2 h. The solution was further injected into buffer (0.5 M arginine, 100 mM Tris-HCl pH 8), and was incubated at 4°C for 48 h. Denatured proteins were then dialyzed against refolding buffer (50 mM Tris-HCl, pH 8, 300 mM NaCl) for 10 h, and the folded scFv was purified by affinity chromatography using Ni-NTA Superflow Qiagen resin. The size (29 kDa) and purity of the scFv was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and electrospray ionization mass spectrometry (ESI-MS; Figure S8 in the Supporting Information). Mass spectrometry also confirmed the existence of two conserved intradomain disulfide bonds in the variable domains. The scFv fragments were analyzed for monomer, dimer, and higher oligomer content by size-exclusion fast protein liquid chromatography (FPLC) using a Superdex 75 10/30 (GE Healthcare Life Sciences) column. Protein samples were eluted in ammonium acetate buffer (50 mM, pH 6.8). The mixture was shown to contain monomer and dimer scFv fragments (Figure S9 in the Supporting Information). The monomer fragment was collected to study the binding kinetics of a single antigen binding site. The dimer fragment was also collected for comparison. All samples were stored at 4°C for further studies.

Molecular dynamics calculations

Coordinates for the variable domain of the antibody (residues 1–107^L and 1–113^H) were extracted from the crystal structure (PDB ID: 3HNS). Waters of crystallization were removed prior to adding hydrogen atoms using the tLeap module of AMBERTOOLS version 12.^[45] The alanine mutants (Trp33^HAla, His35^HAla, Asn58^HAla, Ser50^HAla, Phe95^HAla, Tyr98^HAla, Asn34^LAla, Tyr50^LAla and Ser31^LAla) were generated by removing the side-chain groups of each of these residues using tLeap in AMBER.^[45] All the MD simulations were performed with the GPU implementation of the pmed code, pmed.cud_SPDP,^[47] from AMBER12.^[45] The antibody and carbohydrate residues were parameterized using the ff99SSB protein force field and the GLYCAM06h force field,^[48]

respectively. The system was solvated in a cubic water box (10 Å per side with TIP3P water). An energy minimization protocol consisting of 10 000 cycles of steepest descent followed by 10000 cycles of conjugate gradient was applied to the entire system. During the energy minimization, the protein residues were restrained with a force constant of 100 kcalmol⁻¹ Å². This was followed by heating in NVT ensemble from 5 to 300 K over the course of 50 ps. Production MD simulations were performed for 50 ns at constant pressure (NPT ensemble) with the temperature held constant at 300 K using a Langevin thermostat. To ensure that the scFv maintained a proper fold, the backbone atoms of the protein were restrained with a force constant of 5 kcalmol⁻¹ Å², and the protein side chains and ligand atoms were allowed to be flexible. All covalent bonds involving hydrogen atoms were constrained using the SHAKE^[51] algorithm, allowing a simulation time step of 2 fs. A nonbonded cut-off of 8 Å was used and long-range electrostatics were employed using the particle mesh Ewald (PME) method.^[49] The system was equilibrated for 1 ns prior to the production simulations, and snapshots were collected at 1 ps intervals for subsequent analysis. Prior to the binding energy calculations, to assess the stability of the system ligand displacement RMSD values and hydrogen bonds between the protein and the ligand were calculated using the ptraj module^[50] in AMBERTOOLS 12. The RMSD values were calculated relative to the first frame of the simulation and hydrogen-bond analysis was carried out with cut-off values of 3.5 Å and 120°. Binding free energies were calculated with the MMGB module in AMBER.^[49, 52] All the water molecules were removed prior to the MM-GBSA calculation, and desolvation free energies approximated using the generalized born implicit solvation models (igb=1, 2, 5, 7 and 8).

Generation of mutants

A library of mutants was designed rationally based on the crystal structure of CS-35 F_{ab} in combination with **2** (Figure 1B)^[8] and molecular dynamics calculations for possible contribution to the binding. The mutants chosen were Trp33^HAla, His35^HAla, Asn58^HAla, Ser50^HAla, Phe95^HAla, Asn97^HAsp, Tyr98^HAla, Tyr98^HPhe, Asn34^LAla, Asn34^LAsp, Tyr50^LAla, as well as Ser31^LAla, which was the product of the overlap PCR. The scFv was used as the template, and point mutations were generated by QuikChange site-directed mutagenesis (Agilent Technologies). Mutant scFv proteins were expressed and purified under the same conditions as those used for the wild-type scFv. SDS PAGE confirmed the size (29 kDa) and the purity of each monomer. The monomer fraction of each mutant was collected using FPLC as described above for the wild-type protein. Pure monomer fractions of mutants were used for further studies.

Circular dichroism spectroscopy

CD spectra were obtained in phosphate buffer (50 mM, pH 7.2) for wild-type scFv, using an Olis DSM 17 s spectrometer at room temperature. The far UV spectra were obtained for 27 μM of the CS-35 scFv with and without **4** in a 1 mm cell. An aliquot containing 200 ng of **4** in the same buffer was added to the wild-type scFv sample, and the far UV spectrum of the complex was recorded. The far UV spectra of the CS-35 mAb and F_{ab} fragment were also collected at 10 and 15 μM concentrations of the protein samples, respectively, under the same conditions, for comparison (Figure S2 in the Supporting Information). The near UV

spectrum was obtained for 27 μM of the CS-35 scFv in phosphate buffer (50 mM, pH 7.2) with and without **4** in a 3 mm cell.

Surface plasmon resonance spectroscopy

Binding affinities and kinetics were investigated by SPR spectroscopy using a BIAcore 3000 (GE Healthcare Life Sciences) instrument at 25 °C. Ligand **4** (5 μM) was covalently immobilized on the surface of CM5 chip up to the response unit 120, by amine coupling. A solution of 0.5M *N*-hydroxysuccinimide (NHS) and 10 mM etha-nolamine (EDC) was first injected on the chip surface to activate the carboxylate groups. Ligand **4** was then passed over the flow channel in buffer (10 mM HEPES, 150 mM NaCl, pH 7.2) to generate the surface bound ligand **3**. A reference flow channel, was also injected with ethanolamine (1 M, pH 8.5) after the activation of the surface with NHS (0.5 M) and EDC (10 mM). Freshly prepared scFv samples and mutants across a range of concentrations (0.26, 0.53, 1.6, 2.6, 4.8 μM) were injected to the flow channels in buffer (10 mM HEPES, pH 7.2, 150 mM NaCl) with a 20 μLmin^{-1} flow rate. The resonance units were recorded after the subtraction of the two flow channels. The F_{ab} was also evaluated and analyzed as a reference under the same conditions (Figure S4 in the Supporting Information).

Both association and dissociation phases showed good fitting to a 1:1 binding model in the BIAevaluation^[53] software. The equilibrium binding of the wild-type scFv, scFv mutants and F_{ab} were also determined by equilibrium binding analysis using the equation:

$$\frac{R_{\text{eq}}}{C} = K_A - K_A R_{\text{eq}} R_{\text{max}} \quad (1)$$

where R_{eq} is the equilibrium response units, R_{max} is the resonance signal at saturation, c is the concentration of the protein, and K_A is the association constant. A plot of R_{eq}/c versus c has a slope of $-K_A$ (Figure S3 in the Supporting Information). Therefore, to obtain K_A and R_{max} from the data, one can perform a first-degree (i.e., linear) polynomial regression to these variables (R_{eq}/c vs. R_{eq}). We performed this using the “polyfit” command in MATLAB. The K_A is the reciprocal of the K_D .

Saturation transfer difference NMR spectroscopy

Lyophilized scFv mutant samples were dissolved in deuterated ammonium acetate ($\text{CD}_3\text{CO}_2\text{ND}_4$; 50 mM, pH 6.8), with concentrations in the range of 27–30 μM . Wild-type scFv and F_{ab} samples were buffer exchanged into the deuterated phosphate buffer (50 mM, pH 7.2) with the concentration of 27–30 μM . An aliquot of ligand **4** was added to each sample with the final concentration of 0.5 mM. STD NMR experiments for scFv mutants were recorded on a Varian Inova 600 MHz spectrometer. The STD experiments for wild-type scFv and F_{ab} were performed on a Varian Inova 700 MHz instrument. Measurements were carried out in 3 mm NMR tubes with water suppression for mutants, using 0.1% external acetone set to 2.225 ppm, and without water suppression for wild-type scFv and F_{ab} . 1D STD experiments^[54] were performed at 298 K with saturation at 0.5 ppm, with 2 or 3.8 s relaxation delay, and 1–2 s acquisition time. The off-resonance frequency for saturations was set to 29.89 ppm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

a) Structure of the terminal hexasaccharide motif (Ara6, **1**) in mycobacterial LAM that is recognized by CS-35, and three synthetic derivatives, **2–4**. b) Binding pocket of CS-35 F_{ab} in complex with **2** (PDB ID: 3HNS).^[8] Compound **2** is shown in yellow. Oxygen and nitrogen are in red and blue, respectively. The highlighted residues were picked for mutation studies (see main text).

Figure 2. Comparison of experimental binding free energies (black) and calculated values: net contribution from all residues MM-GBSA (white), contribution from mutated position only (dark gray), and contribution from all residues within 3 Å of the mutation site (light gray). Mutants identified experimentally as weak binders or nonbinders are indicated by boxes I and II, respectively.

(a)

(b)

Figure 3.

a) The near UV CD spectrum of the wild-type CS-35 scFv with (dashed) and without **4**. b) SPR spectroscopic binding of the wild-type CS-35 scFv with **3** fit to a 1:1 binding model. Concentrations of the scFv injections were 0.26, 0.53, 1.6, 2.6 and 4.8 μM . The theoretical R_{max} is 102 RU.

Figure 4.

a) The hydrophobic pocket that can accommodate octyl chain extending from ring A (PDB: 3HNS). b) Hydrogen-bond network involving His35, Ser50, and Asn58 around ring E as observed in the crystal structure of CS-35 F_{ab} with hexasaccharide **2**. Compound **2** is shown in yellow. c) CH– π interactions of Tyr98, Phe95, and Trp33 with **2** from the crystal structure. The distances are shown in Ångström. d) β -Arabinofuranose ring E making five simultaneous interactions with CS-35. e) Trp and His residues in the binding site of CS-35; compound **2** is shown in orange. f) Trp and His residues in the binding site of Se155-4 (PDB: 1MFA). Trisaccharide antigen is shown in red.

Figure 5. Protons with strong (circled) medium (dark gray) and weak (gray) STD NMR signals for: a) CS-35 wild-type scFv, b) His35Ala mutant, c) Ser50Ala mutant, d) Asn58Ala mutant, e) Tyr50Ala mutant. (Figure S7 in the Supporting Information depicts the impact of mutations on the intensity of various protons in STD NMR experiments.)

Figure 6.
Trisaccharide antigen recognition by Se155-4 (PDB ID: 1MFA).

Table 1

Average per-residue energy^[a] contributions for key^[b] residues in the protein and the ligand.

Residue	CDR ^[c]	H bond ^[d]	vdW ^[e]	electrostatic	Polar desolvation	Nonpolar desolvation	Total
Protein							
Trp33	H1	Y	-4.2	-2.4	1.6	-0.5	-5.5
Tyr98	H3	Y	-4.6	-3.8	4.1	-0.8	-5.0
Asp91	L3	Y	0.6	-16.7	13.5	-0.1	-2.5
His35	H1	Y	-0.1	-3.4	1.2	0.0	-2.4
Asn97	H3	Y	-2.8	-2.6	4.2	-0.3	-1.5
Ser50	H2	Y	-0.1	-2.1	0.8	0.0	-1.5
Tyr96	L3	Y	-1.0	-0.8	1.0	-0.1	-0.9
Asn58	H2	Y	-0.6	-0.7	0.7	-0.1	-0.6
Phe95	H3	N	-3.7	-0.2	0.5	-0.4	-3.8
Val99	H3	N	-1.9	-0.3	0.7	-0.1	-1.5
Tyr50	L2	N	-1.9	0.4	0.3	-0.2	-1.5
Pro100	H3	N	-1.2	0.4	-0.3	0.0	-1.2
Pro94	L3	N	-1.0	-0.2	0.3	-0.1	-1.1
Tyr49	L2	N	-0.9	-0.2	0.2	0.0	-0.9
Tyr32	L1	N	-1.1	0.2	0.4	-0.1	-0.6
Tyr52	H2	N	-0.5	-0.3	0.4	-0.1	-0.5
Gly96	H3	N	-0.8	-0.6	0.9	0.0	-0.5
subtotal			-25.8	-33.3	30.5	-2.9	-31.5
Ligand							
A	-	Y	-10.7	-29.7	27.5	-1.9	-14.9
B	-	N	-5.4	-11.0	11.9	-0.5	-5.1
E	-	Y	-7.4	-1.8	5.5	-1.4	-5.1
D	-	N	-2.8	-1.2	3.3	-0.6	-1.3
C	-	Y	-3.6	-0.6	3.4	-0.3	-1.0
F	-	N	-0.6	7.4	-6.5	-0.1	0.1
subtotal			-30.6	-36.9	45.1	-4.8	-27.2

^[a] In kcalmol⁻¹.

- [b] Residues that contribute greater than 0.5 kcalmol^{-1} to the total binding energy.
- [c] Complementarity determining regions.
- [d] Intermolecular hydrogen bonds observed, based on a distance cut-off of $3.5 \text{ \AA}$.
- [e] vdW : van der Waals.

Table 2

Binding evaluation of the scFv mutants to **3** by SPR spectroscopy.^[a]

CDR	Mutant	K_D [M] equilibrium ^[b]	K_D [M] k_{off}/k_{on} [a]	k_{on} [M ⁻¹ s ⁻¹]	k_{off} [s ⁻¹]
1	L1 Ser31Ala	NB ^[c]	NB	–	–
2	H1 Trp33Ala	NB	NB	–	–
3	L1 Asn34Ala	NB	NB	–	–
4	H3 Phe95Ala	NB	NB	–	–
5	H3 Asn97Asp	NB	NB	–	–
6	H3 Tyr98Ala	NB	NB	–	–
7	H1 His35Ala	<10 ³	–	–	–
8	H1 Asn58Ala	<10 ³	–	–	–
9	H2 Ser50Ala	<10 ³	–	–	–
10	H3 Tyr98Phe	$1.8 \pm 0.21 \times 10^{-6}$	1.85×10^{-6}	2.66×10^3	4.91×10^{-3}
11	L1 Asn34Asp	$4.02 \pm 4.2 \times 10^{-6}$	1.85×10^{-6}	40	1.21×10^{-4}
12	L2 Tyr50Ala	$7.7 \pm 0.85 \times 10^{-6}$	7.40×10^{-6}	56.7	4.35×10^{-4}
–	wild-type scFv	$5.6 \pm 0.05 \times 10^{-7}$	5.0×10^{-7}	3.86×10^3	3.56×10^{-3}

^[a] K_D was calculated from k_{off}/k_{on} values obtained from BIAcore.

^[b] K_D was calculated from equilibrium binding analysis, Equation (1), Figure S3 in the Supporting Information.

^[c] Non-binding.