CHAPTER 5

Molecular modelling and molecular dynamics simulation studies
on *Agrocybe cylindracea* galectin and α(2,6)-linked sialyllactose
complexes

5.1 Introduction

Galectins are β-galactoside binding proteins which carry out intra- and
extra-cellular function through glycoconjugates mediated by a conserved
carbohydrate recognition domain (CRD) (Barondes et al. 1994a; Barondes et al.
1994b; Leffler, 2001; Cummings and Liu, 2009). *Agrocybe cylindracea* galectin
(ACG) is a fungal galectin isolated from an edible fungus which specifically
recognizes α(2,3)-linked sialyllactose (N23L) occurring at the cell surface and
plays an extensive role in many biological recognition processes (Yagi et al.,
1997; Leffler et al., 2004; Ban et al., 2005; Kuwabara et al., 2013). ACG galectin
is well known to have high affinity for the preference of α(2,3)-linked sialyllactose
[3'-sialyllactose or Neu5Acα(2-3)Galβ(1-4)Glc]. In addition to the 3'-sialyllactose,
ACG also recognizes extensively with various β-galactosides that include lactose,
N-Acetyl-lactosamines [Galβ(1-3)GlcNAc and Galβ(1-4)GlcNAc], Galβ(1-
3)GalNAc and NeuAca2-3Galβ(1-3)Glc (Yagi et al., 1997; Ban et al., 2005;
Imamura et al., 2011). The substitutions of one or two amino acid residues inside
the binding pocket of lectins alter the glycan-binding specificity (Sharon and Lis,
1994; Parasuraman et al., 2014). The carbohydrate binding specificity of ACG is
investigated by the substitutions of mutants at the binding pocket of ACG using
surface plasmon resonance (SPR) and frontal affinity chromatography (FAC)
(Salomonsson et al., 2010; Imamura et al., 2011). The β-galactose-binding
leguminous lectin of *Bauhinia purpurea* lectin (BPA) is used to design the artificial lectins (cyborg lectins) with distinct and desired carbohydrate binding properties by the random mutations at cDNA levels (Yamamoto et al., 2000). Hu and co-workers have showed the dramatic change of carbohydrate binding specificity after the substitution of single amino acid residue at the binding pocket of lectins (Hu et al., 2013). However, the binding specificity of ACG towards $\alpha$(2,6)-linked sialyllactose (N26L) and its complex dynamics in explicit water through theoretical methods are not yet investigated.

In the previous chapter, we have investigated the glycan-binding specificity of ACG towards 3'-sialyl lactose (N23L) (Parasuraman et al., 2015). In that investigation, the single point (N140Q) and double point (Y59R/N140Q) mutations at the binding pocket of ACG have been carried out using site-directed *in silico* mutations and the glycan-binding specificity of cyborg lectin towards 3'-sialyllactose is examined using 30 ns MD simulations. Plausible binding modes are proposed for ACGs–N23L complexes and the enhanced binding specificity has been achieved in the double point mutant of ACG2 towards N23L in which 26 (19D, 7W) direct and water-mediated hydrogen bonds stabilize the ACG2–N23L complex. In this present work, $\alpha$(2,6)-linked sialyllactose (N26L) is modelled into the binding pocket of ACG by rigid body dynamics to investigate their recognition mechanism. The single point and double point site-directed *in silico* mutations are performed at the binding pocket of ACG for tuning the carbohydrate binding specificity of ACG towards N26L. MD simulations of 30 ns durations are performed for wild-type (ACG), single point (ACG1) and double point (ACG2) mutated ACGs complexed with N26L to investigate the dynamical behavior of the mutants and the binding specificity of ACGs towards $\alpha$(2,6)-linked sialyllactose.
5.2 Computational details

5.2.1 Starting structure

Atomic coordinates of the three dimensional structure of ACG is taken from the Protein Data Bank (PDB ID: 1WW4) (Berman et al., 2000; Ban et al., 2005). As previously discussed in section 4.2.1, subunit A of 1WW4 is taken for site-directed in silico mutation study, since the four identical well separated subunits A, B, C and D are independent and the binding site residues are conserved (Figure 4.2). The schematic representation of α(2,6)-linked sialyllactose \([\text{Neu5Ac}\alpha(2-6)\text{Galβ(1-4)}\text{Glc}]\) with glycosidic torsional angles is shown in Figure 5.1.

![Figure 5.1](image)

**Figure 5.1.** Molecular structure of α(2,6)-linked sialyllactose. Where, \(\Phi_{N26G} = C1-C2-O2-C6; \Psi_{N26G} = C2-O2-C6-H61; \Phi_{G14GL} = H1-C1-O1-C4; \Psi_{G14GL} = C1-O1-C4-H4; \) R1: Acetamide group \([\text{NHCOCH}_3]\); R2: Glycerol side chain \([(\text{CHOH})_2\text{CH}_2\text{OH}]\); Neu5Ac: N-acetylleuraminic acid; Gal: Galactose; Glc: Glucose.
5.2.2 Rigid body dynamics

In order to get the initial model of carbohydrate inside the binding pocket of ACG without any stereo chemical clashes, rigid body dynamics fitting is used. Keeping the centroid of Neu5Ac from crystal structure, the structure of α(2,6)-linked sialyllactose is built inside the binding pocket of ACG. The Φ, θ and Ψ are varied from 0°–360°, 0°–180° and 0°–360° respectively. The orientations of α(2,6)-linked sialyllactose without any stereo chemical clashes are obtained and the orientation with more possible number of hydrogen bonds is chosen to be the initial starting structure. The entire program is FORTRAN based.

5.2.3 Amino acid mutations and mutant stability analysis

The amino acids at the binding pocket of ACG are mutated using in-house developed FORTRAN program. The binding site residue Asn140 is replaced by Gln140 (N140Q) in single point mutation and this modified protein is named as ACG1. In double point mutation, the binding site residues Tyr59 and Asn140 are replaced with Arg59 and Gln140 (Y59R/N140Q) respectively and named as ACG2. The native sequences and the mutant residues in single point and double point mutated ACGs (ACG, ACG1 and ACG2) are shown in Figure 5.2.

<table>
<thead>
<tr>
<th></th>
<th>51</th>
<th>68</th>
<th>133</th>
<th>150</th>
</tr>
</thead>
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<td>ACG</td>
<td>NLLSENGAYLLHIAFRLQ----------TTSSLSYNSTEGTSIFST</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACG1</td>
<td>NLLSENGAYLLHIAFRLQ----------TTSSLSYQSTEGTSIFST</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACG2</td>
<td>NLLSENGARLLHIAFRLQ----------TTSSLSYQSTEGTSIFST</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.2. The native sequences of ACG are blue in colour and the mutant residues are green and magenta in colour for ACG1 (N140Q) and ACG2 (Y59R/N140Q) respectively.
The stability changes of the ACGs upon mutation (ΔΔG) are predicted using the web server CUPSAT (Cologne University Protein Stability Analysis Tool) before carrying out the simulations. The positive and negative ΔΔG represents the stabilizing and destabilizing effects respectively. Predicted ΔΔG values for the mutants in the ACGs are 2.44 and 0.98 kcal/mol for Y59R and N140Q respectively (Topham et al., 1997; Gromiha et al., 1999; Parthiban et al., 2006).

5.2.4 Molecular Dynamics simulations

Molecular dynamics (MD) simulations of 30 ns durations are carried out for the α(2,6)-linked sialyllactose (N26L) bound complexes of wild-type, single point and double point mutated ACGs in explicit water. For MD simulations, the force field ff99 of AMBER12 is used for protein and GAFF (General Amber Force Field) for saccharide (Wang et al., 2000; Wang et al., 2004; Case et al., 2012). GAFF force field for carbohydrates produces reliable results when used alongside ff99 for proteins (Veluraja and Margulis, 2005; Yan et al., 2008; Priyadarzini et al., 2012). Necessary sodium ions are added to neutralize the ACGs–N26L complexes. To treat the long range electrostatic interactions, the Particle Mesh Ewald (PME) is used (Darden et al., 1993). The PME grid sizes of ACG, ACG1 and ACG2 are 54x58x58Å, 54x58x60Å and 56x59x58Å respectively. The complex system that includes ACG, 26SL and the sodium ions are solvated using TIP3P water (Jorgensen et al., 1983). The same number (5172) of water molecules are added for ACG, ACG1 and ACG2 complex systems separately. The solvated complex system is equilibrated for 2000 steps and then simulated for 30 ns duration using NAnoscale Molecular Dynamics (NAMD) (Phillips et al.,
Number of particles, pressure and temperature (NPT) of the systems are kept constant. Pressure is maintained at 1 atm during the production simulations and the temperature is maintained at 300K to mimic the biological environment throughout the simulations. The trajectories are recorded for every picosecond (ps) and 30,000 structures are collected for a typical 30 ns MD simulations. The trajectories are investigated using Visual Molecular Dynamics (VMD) (Humphrey et al., 1996). Atomic visualization of ACG–N26L interactions are rendered by using UCSF Chimera (University of California San Francisco–Chimera) and MolScript (Kraulis, 1991; Pettersen et al., 2004).

5.2.5 Pair-wise interaction energy and binding free energy analysis

The total pair-wise interaction energy (PIE) between interacting binding site residues of ACGs and the receptor 26SL has been calculated using NAMD (Phillips et al., 2005). For PIE calculations, the NAMD considers non-bonded interactions that include hydrogen bonds, hydrophobic contacts, electrostatic and van der Waals interactions between ACGs and N26L. The binding free energy calculations are performed by subtracting the free energies of unbound protein and carbohydrate from the free energy of the bound complex using Molecular Mechanics-Poisson Boltzmann Surface Area (MM-PBSA) of AmberTools13 and as follows:

\[ \Delta G_{\text{bind}} = \Delta G_{\text{complex}} - (\Delta G_{\text{ACG}} + \Delta G_{\text{26SL}}) \quad \ldots \quad (5.1) \]

Where, \( \Delta G_{\text{bind}} \) is the binding free energy of ACGs–N26L complexes. Water molecules and the sodium counter ions are stripped to hasten convergence by preventing solvent-solvent interactions before calculating the binding free energy of ACGs–N26L complexes (Srinivasan et al., 1998; Miller III et al., 2012). The
plausible binding modes are proposed for ACGs–N26L complexes using total pair-wise interaction energy and the binding free energy calculations.

5.3 Results and Discussion

5.3.1 Conformational flexibility of N26L at the binding pocket of ACGs

The bound conformational flexibility of α(2,6)-linked sialyllactose [Neu5Acα(2-6)Galβ(1-4)Glc or N26L] at the binding pocket of ACGs as a function of glycosidic torsional angles plotted from the trajectories of 30 ns MD simulations (30,000 structures) are shown in Figure 5.3. Figure 5.3 a1, b1 and c1 shows that (Φ_{N23G}, Ψ_{N23G}) of Neu5Acα(2-6)Gal (N26G) prefers (-150°, 70°) and (-70°, 70°) conformations and Figure 5.3 a2, b2 and c2 shows that (Φ_{G14GL}, Ψ_{G14GL}) of Galβ(1-4)Glc (G14GL) prefers (60°, 0°) conformation at the binding pocket of ACGs. The lactose (G14GL) exhibits multiple conformations in the ACGs binding pocket because of its higher flexibility. The plausible bound conformations observed are similar with the free sialyllactose in solution state. It is evidenced that in the α(2,6)-sialyllactose bound complex of ACGs, the favored glycosidic torsions fall into one of the local minima (Rao et al., 1998; Sharmila and Veluraja, 2004; Veluraja et al., 2010; Selvin et al., 2012).
Figure 5.3. Glycosidic torsional maps for Neu5Acα(2-6)Galβ(1-4)Glc (6'-sialyllactose) at the binding pockets of wild-type, single point and double point mutated ACGs (ACG, ACG1 and ACG2). (a1), (b1) and (c1) for Neu5Acα(2-6)Gal [N26G]; (a2), (b2) and (c2) for Galβ(1-4)Glc [G14GL] at the binding pocket of ACGs.
5.3.2 MD simulations on the wild-type ACG–N26L complex

MD simulations of 30 ns duration is performed for the wild-type ACG and α(2,6)-linked sialyllactose (N26L) complex in order to investigate the glycan-binding specificity to ACG galectin from the trajectories of MD simulations. The total pair-wise interaction energy between interacting binding site residues such as Ser44, Pro45, Asn46, His62, Arg66, Glu69, Asn75, Arg77, Trp83, Leu84, Glu86, Arg88 and N26L is calculated by NAMD and is shown in Figure 5.4.

![Interaction energy graph](image)

**Figure 5.4.** The total pair-wise interaction energy between α(2,6)-sialyllactose (N26L) and the interacting binding site residues of wild-type ACG over time in ps.

The binding free energy for wild-type ACG–N26L complex is also calculated using MM-PBSA of AmberTools13. An analysis of the trajectories reveals that a single binding mode (BM1) is observed for wild-type ACG–N26L complex based on the examinations of total pair-wise interaction energy between the interacting binding site residues of ACG and the receptor N26L using NAMD,
binding free energy of the complexes using MM-PBSA and the hydrogen bonding pattern. The hydrogen bond analysis is performed based on the distance between two electronegative atoms X and Y (2.6 to 3.2 Å) and the angle (H-X…Y ≤ 30°). In BM1 of ACG–N26L complex, 10 hydrogen bonds are observed in the structural stabilization of the complex of which 8 are direct hydrogen bonds (8D) and 2 are water-mediated hydrogen bonds (2W) as shown in Figure 5.5.

![Figure 5.5](image-url)

Figure 5.5. Direct and water-mediated hydrogen bonds observed in BM1 of ACG–N26L complex. W is water molecule involved in water-mediated hydrogen bonds. Structure of ACG is shown in bonds representation and α(2,6)-sialyllactose (N26L) is shown in ball and stick representation created using MolScript.
The Neu5Ac and glucose units of N26L contribute to six and four hydrogen bonds with the binding site residues of ACG respectively. The amino acid residues Tyr59 and Asn140 are not interacting with the receptor α(2,6)-linked sialyllactose but they are observed in the binding pocket of ACG. Also the penultimate galactose in BM1 of ACG–N26L complex is not contributed in making hydrogen bonds with the binding site residues of ACG because of its orientation at the binding pocket of ACG. The atomic level interactions between ACG and N26L along with the residential time of water involved in water-mediated hydrogen bonds are summarized in Table 5.1. The plausible binding modes, hydrogen bonds, interacting binding site residues, relative pair-wise interaction energy and the binding free energy observed in ACG–N26L complex are given in Table 5.2.

5.3.3 MD simulations on the single point mutant ACG1–N26L complex

Single point mutation is carried out at the binding pocket of ACG using site-directed in silico mutation to investigate the altered glycan-binding specificity. In single point mutation, the binding site residue Asn140 is replaced with Gln140 (N140Q). Because in wild-type ACG, the amino acid residue Asn140 does not interact with the substrate but it is present in the vicinity of the binding pocket. An MD simulation of 30 ns duration is carried out for the single point mutant ACG1–N26L complex. The pair-wise interaction energy is calculated between the interacting binding site residues such as Gly14, Ser16, Ser44, Pro45, Asn46, Leu53, Asn56, Gly57, Tyr59, Arg77, Pro79, Asn80, Ser138, Gln140 and N26L which is depicted in Figure 5.6.
Figure 5.6. Total pair-wise interaction energy (PIE) between the receptor $\alpha(2,6)$-linked sialyllactose (N26L) and the interacting binding site residues of single point mutant ACG1.

In ACG1–N26L complex, two binding modes BM1 and BM2 are proposed based on the NAMD pair-wise interaction energy from the trajectories of MD simulations. The binding mode with the global minimum energy is denoted as BM1 and the relatively higher energy is denoted as BM2. BM1 occurs from 21400 ps to 30000 ps and BM2 occurs from 5200 ps to 17400 ps. In BM1 of ACG1–N26L complex, 18 hydrogen bonds are observed of which 6 are direct hydrogen bonds and 12 are water-mediated hydrogen bonds (Figure 5.7a). In BM2 of ACG1–N26L complex, 13 hydrogen bonds (9D, 4W) are involved in the structural stabilization of the complex and the atomic level interactions observed are shown in Figure 5.7b. In BM1 and BM2 of single point mutant, the increased contribution to the binding specificity is given by Neu5Ac unit than the galactose and glucose units of N26L. BM1 is relatively $16.18 \pm 1.3$ kcal/mol energy lower

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than BM2 based on the pair-wise interaction energy, which can easily be accounted for the difference in the number of hydrogen bonds. The binding free energy calculated for two different binding modes observed in ACG1–N26L complex reveals that BM1 is 6.70 ± 4.7 kcal/mol energy lower than BM2 by using MM-PBSA calculations (Table 5.2). The binding specificity of single point mutant ACG1 shows enhanced binding when compared to the wild-type, since the N140Q mutant protein has shown increased number of hydrogen bonds. Also the mutated residue Gln140 (N140Q) contributes hydrogen bonds with the carbohydrate through water-mediation. These three water-mediated hydrogen bonding interactions between NE2 of Gln140 and carboxyl oxygen O1D, ring oxygen O4 and O10 in the acetamide group of Neu5Ac unit in the BM1 of ACG1–N26L complex (Table 5.1). The atomic level interactions observed in ACG1–N26L complex along with the residential time of water are summarized in Table 5.1. Hydrogen bonds observed in the plausible binding modes, interacting binding site residues, relative pair-wise interaction energy and the binding free energy of ACG1–N26L complex are listed in Table 5.2.
Figure 5.7. Direct and water-mediated hydrogen bonds observed in (a) BM1 and (b) BM2 of single point mutant of ACG1–N26L complex.
Table 5.1. Atomic level interactions observed between the receptor α(2,6)-linked sialyllactose and the interacting binding site residues of ACG, ACG1 and ACG2, and the residential time of water involved in water-mediated hydrogen bonds.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Binding modes</th>
<th>Receptor atom</th>
<th>Protein atoms in ACG</th>
<th>Protein atoms in ACG1</th>
<th>Protein atoms in ACG2</th>
<th>Residential time of water in ps</th>
</tr>
</thead>
<tbody>
<tr>
<td>N26L</td>
<td>Binding Mode1 (α(2,6)-sialyllactose) (BM1)</td>
<td>O1S</td>
<td>-</td>
<td>-</td>
<td>NH1-Arg77</td>
<td>W-NH1-Arg59</td>
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<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>W-NH1-Arg77</td>
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<td>W-NH2-Arg59</td>
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<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>W-NH2-Arg77</td>
<td>8.2</td>
<td>W-NE1-Tyr83</td>
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<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>W-N-Arg80</td>
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<td>NE1-Tyr83</td>
<td>W-OH-Tyr59</td>
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<td></td>
<td></td>
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<td>ND2-Asn75</td>
<td>W-OG-Ser138</td>
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<td>Binding Mode2 (α(2,6)-sialyllactose) (BM2)</td>
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<td>NH1-Arg77</td>
<td>W-NH2-Arg59</td>
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<tr>
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</table>

ACG, *Agrocybe cylindracea* galectin; N26L, α(2,6)-linked sialyllactose; W, water-mediated hydrogen bond; ps, picosecond.
Table 5.2. Plausible binding modes, direct and water-mediated hydrogen bonds, interacting binding site residues, relative interaction energy and the binding free energy for α(2,6)-sialyllactose bound complexes of ACG, ACG1 and ACG2.

<table>
<thead>
<tr>
<th>Wild-type and mutated ACGs</th>
<th>Plausible binding modes</th>
<th>Number of direct and water-mediated hydrogen bonds</th>
<th>Number of interacting residues</th>
<th>Interacting residues</th>
<th>Relative interaction Energy (kcal/mol)</th>
<th>Binding free energy(^{b}) with standard deviation for the complexes using MMPBSA</th>
<th>Relative binding free energy(^{b}) with standard deviation for the complexes using MMPBSA</th>
<th>Order of binding specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACG (wild-type)</td>
<td>BM1</td>
<td>10 (8D,2W)(^{a})</td>
<td>8</td>
<td>Asn46,Arg66,Glu69, Asn75,Trp83,Leu84, Glu86,Arg88</td>
<td>-</td>
<td>-20.76 ± 5.2</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>ACG1 single mutant (N140Q)</td>
<td>BM1</td>
<td>18 (6D,12W)</td>
<td>7</td>
<td>Gly14,Gly57,Tyr59, Arg77,Asn80, Ser138,Gln140</td>
<td>0.0 ± 1.7</td>
<td>-26.42 ± 3.2</td>
<td>0.0 ± 3.2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>BM2</td>
<td>13 (9D,4W)</td>
<td>4</td>
<td>Asn56,Tyr59,Arg77, Pro79</td>
<td>16.18 ± 1.3</td>
<td>-19.72 ± 4.7</td>
<td>6.70 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>ACG2 double mutant (N140Q/Y59R)</td>
<td>BM1</td>
<td>21 (14D,7W)</td>
<td>8</td>
<td>Asn46,Arg59,Arg77, Asn80,Ala81,Trp83,Ser138,Gln140</td>
<td>0.0 ± 0.7</td>
<td>-41.87 ± 4.3</td>
<td>0.0 ± 4.3</td>
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<tr>
<td></td>
<td>BM2</td>
<td>17 (13D,4W)</td>
<td>7</td>
<td>Asn51,Gly57,Arg59, Arg77,Ala81,Trp83,Ser138</td>
<td>14.05 ± 0.7</td>
<td>-32.63 ± 3.9</td>
<td>6.34 ± 3.9</td>
<td></td>
</tr>
</tbody>
</table>

ACG, *Agrocybe cylindracea* galectin; \(^{a}\) D, Direct hydrogen bond; W, Water-mediated hydrogen bond; \(^{b}\) Energy in kcal/mol.
5.3.4 MD simulations on the double point mutant ACG2–N26L complex

In double point mutant of ACG2–N26L complex, the amino acid residues Tyr59 and Asn140 at the binding site are replaced with Arg59 and Gln140 (Y59R/N140Q) respectively. MD simulation of 30 ns durations is carried out for the ACG2–N26L complex. The pair-wise interaction energy is calculated between the interacting binding site residues such as Ser44, Pro45, Asn46, Asn51, Gly57, Arg59, His62, Arg66, Asn75, Arg77, Asn80, Ala81, Trp83, Glu86, Arg88, Ser138, Gln140 and N26L and is depicted in Figure 5.8.

![Interaction energy plot](image)

**Figure 5.8.** Total pair-wise interaction energy (PIE) plot between the receptor α(2,6)-linked sialyllactose (N26L) and the interacting binding site residues of double point mutant ACG2.

Two binding modes (BM1 and BM2) are observed for ACG2–N26L complex of which BM1 occurs from 3400 ps to 12600 ps and BM2 occurs from 12600 ps to 30000 ps. In BM1 of ACG2–N26L complex, 21 hydrogen bonds are
observed of which 14 are direct hydrogen bonds and 7 are water-mediated hydrogen bonds. Among the 21 hydrogen bonds observed in BM1 of ACG2, 7 are contributed from the mutants Arg59 and Gln140 to the structural stability. The Neu5Ac unit of N26L has given the substantial contribution to the binding of ACG2 by 13 direct and water-mediated hydrogen bonds (Table 5.2). Hence the Neu5Ac unit of the receptor N26L shows high affinity towards the amino acids than the galactose and glucose units of N26L as reported earlier in the literature (Yagi et al., 1997; Imamura et al., 2011). A representative plot indicating these direct and water-mediated hydrogen bonds is given in Figure 5.9a.

![Image of hydrogen bonds](image)

**Figure 5.9.** Direct and water-mediated hydrogen bonds observed in (a) BM1 and (b) BM2 of double point mutant ACG2–N26L complex.
As far as mutant Y59R is concerned in the binding pocket of ACG2–N26L complex, it forms water-mediated hydrogen bonds and it is reflected in the hydrogen bond distance plot (Figure 5.10). Figure 5.10 shows the distance between carboxylate oxygens of Neu5Ac and NH1/NH2 of the mutant Arg59.

Figure 5.10 Hydrogen bond distances between the carboxyl oxygens of Neu5Ac and the nitrogens in the side chain of Arg59 of ACG2–N26L complex. These water-mediated hydrogen bonds persist throughout the simulation trajectories.

In BM2 of ACG2–N26L complex, 17 hydrogen bonds (13D, 4W) are contributed in the structural stability and are depicted in Figure 5.9b. The shift in the binding mode from BM1 to BM2 is noted at 12600 ps of the total pair-wise interaction energy plot (Figure 5.8). BM1 of ACG2–N26L complex has relatively 14.05 ± 0.7 kcal/mol lower energy than BM2 based on the analysis of pair-wise interaction energy plot. The binding free energy of the ACG2–N26L complex also shows that BM1 has relatively lower energy of 6.34±3.9 kcal/mol than BM2. The
stability of glycan-binding is investigated by the predominant factors that include direct and water-mediated hydrogen bonds, pair-wise interaction energy between the interacting binding site residues of ACGs and the receptor N26L using NAMD, binding free energy of ACGs–N26L complexes using MM-PBSA and the interaction pattern of α(2,6)-sialyllactose into the binding pocket of ACGs. Based on the observations, the double point mutant Y59R/N140Q of ACG2–N26L complex has given the substantial contribution to the increased stability of galectin–glycan interactions when compared with the single point mutant ACG1 and the wild-type ACG.

In all the binding modes observed in ACG, ACG1 and ACG2 complexes, the Neu5Ac and glucose units of N26L are highly interacting and contribute to the structural stability while the penultimate galactose unit loses its interactions with the binding site residues of ACGs. Though ACG belongs to the family of β-galactoside binding proteins (Barondes et al., 1994a; Barondes et al., 1994b), the present study shows a lesser binding specificity of ACG towards α(2,6)-linked sialyllactose (N26L) since the penultimate galactose forms only seven hydrogen bonds which are described as follows: the galactose unit in the BM1 of ACG and ACG1 does not interact with the binding site residues while in BM2 of ACG1, O4 of galactose forms hydrogen bond with the backbone oxygen of Pro79. In BM1 of ACG2, O4 and O5 of galactose form water-mediated hydrogen bond with NH1 of Arg77 and direct hydrogen bond with NH1 of Arg59 respectively. In BM2 of ACG2, O3 is hydrogen bonded with backbone oxygen of Gly57 and the O4 of galactose forms direct hydrogen bonds with NH1 of Arg59 and NH1 of Arg77 and water-mediated hydrogen bond with the backbone oxygen of Gly57. The substituted mutant Gln140 in BM1 of single point mutant forms only three
hydrogen bonds with ACG1. In BM1 of double point mutant, the substituted mutants Arg59 and Gln140 form eight hydrogen bonds with ACG2 and these bonds play crucial roles in the carbohydrate binding of ACG2–N26L complex. Atomic level interactions observed between N26L and the interacting binding site residues of ACG, ACG1 and ACG2 along with the residential time of water involved in water-mediated hydrogen bonds are summarized in Table 5.1. The plausible binding modes, number of direct and water-mediated hydrogen bonds, interacting binding site residues, relative pair-wise interaction energy and the binding free energy of the ACGs–N26L complexes are given in Table 5.2. Thus, it can be concluded that the affinity of double point mutant Y59R/N140Q of ACG2 complexed with N26L is found to be higher than the affinity of single point mutant and the wild-type as evidenced from Table 5.2.

The double point mutant (N140Q/Y59R) of ACG2–N26L complex has 21 hydrogen bonds of which 14 are direct hydrogen bonds and 7 are water-mediated hydrogen bonds. A total of 8 interacting binding site residues viz Asn46, Arg59, Arg77, Asn80, Ala81, Trp83, Ser138 and Gln140 are involved in forming the hydrogen bonds. Whereas in the previous chapter, the double point mutant N140Q/Y59R of ACG2–N23L complex has 26 hydrogen bonds of which 19 are direct hydrogen bonds and 7 are water-mediated hydrogen bonds. A total of 11 interacting binding site residues viz Pro45, Asn46, Arg59, His62, Arg66, Asn75, Arg77, Trp83, Glu86, Arg88 and Gln140 contribute to the structural stability of ACG2–N23L complex. The relatively lowest binding free energy of the ACG2–N26L complex is \(-41.87 \pm 4.3\) kcal/mol while the relatively lowest binding free energy of the ACG2–N23L complex is \(-47.52 \pm 5.2\) kcal/mol. Hence the theoretical binding specificity of Agrocybe cylindracea galectin towards \(\alpha(2,3)-\)
linked sialyllactose is high similar to the experimental specificity when compared with the binding specificity of *Agrocybe cylindracea* galectin towards α(2,6)-linked sialyllactose, based on the analysis of direct and water-mediated hydrogen bonds, pair-wise interaction energy between interacting binding site residues and the receptor sialylglycans, and the binding free energy of the complexes obtained from the trajectories of MD simulations.

5.4 Conclusions

Terminal sugar α(2,6)-linked sialyllactose (N26L) is modelled into the binding pocket of ACG and the enhanced carbohydrate binding has been investigated by site-directed *in silico* mutations. Molecular dynamics simulations of 30 ns durations have been performed for the α(2,6)-linked sialyllactose bound complexes of wild-type, single point and double point mutated ACGs (ACG, ACG1 and ACG2). The pair-wise interaction energy between the interacting binding site residues of ACGs and N26L using NAMD, and the binding free energy of the complexes using MM-PBSA have been computed and different binding modes are proposed for ACGs−N26L complexes. Single binding mode (BM1) is observed for wild-type ACG and two binding modes (BM1 and BM2) are observed for single point and double point mutated ACGs (ACG1 and ACG2) in complex with the N26L. Hydrogen bonds, either direct or water-mediated stabilize the proposed binding modes of ACGs−N26L complexes. The result shows that the binding specificity of double point mutant of ACG2−N26L complex is found to be higher due to the substantial contribution from the mutated residues Y59R/N140Q, when compared with the wild-type and single point mutated ACGs. The trajectory analysis of wild-type and mutated ACGs reveals that the
substituted mutants Y59R and N140Q play vital role in forming direct and water-mediated hydrogen bonds with the receptor N26L. An extensive analysis of direct and water-mediated hydrogen bonds, total pair-wise interaction energy between the interacting binding site residues and \( \alpha(2,6) \)-linked sialyllactose (N26L) and binding free energy of the complexes, reveal that the order of ACGs binding specificity towards \( \alpha(2,6) \)-linked sialyllactose to be ACG2 > ACG1 > ACG. Thus, it can be concluded that the double point mutant of ACG2 shows enhanced binding affinity towards \( \alpha(2,6) \)-linked sialyllactose. While comparing the binding specificity of \textit{Agrocybe cylindracea} galectin towards \( \alpha(2,3) \)-linked sialyllactose (N23L) which is carried out in the previous work (Chapter 4), the binding specificity of \textit{Agrocybe cylindracea} galectin towards \( \alpha(2,6) \)-linked sialyllactose (N26L) is low and the results are agreeing to the experimental evidence.