Supplemental data for:

Antibody fucosylation lowers FcγRIIIa/CD16a affinity by limiting the conformations sampled by the N162-glycan

Daniel J. Falconer, Ganesh P. Subedi, Aaron M. Marcella and Adam W. Barb

Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology
2437 Pammel Drive
Molecular Biology Building, rm 4210
Iowa State University, Ames, IA 50011

Supplemental Table 1. Summary of crystallographic statistics.

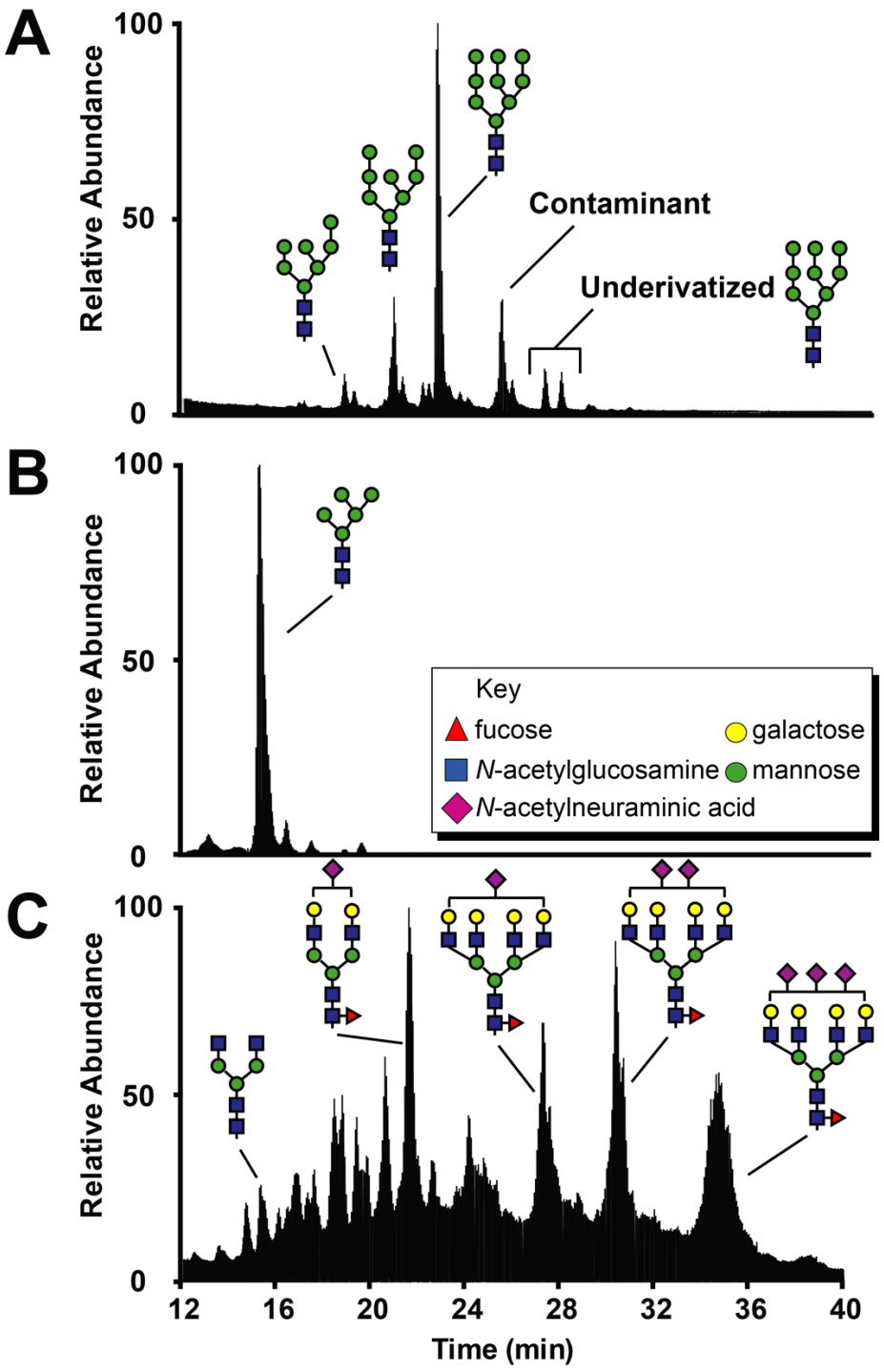
Space group	C 2
Protein chains per asymmetric unit	3
Unit Cell Parameters	
a, b, c (Å)	124.1, 48.8, 134.9
α, β, γ (°)	90.0, 103.8, 90.0
Resolution (Å)	40.21 - 2.256 (2.336 - 2.256)
Total reflections	148774 (14090)
Unique reflections	37344 (3601)
Multiplicity	4.0 (3.8)
Completeness (%)	98 (100)
Mean I/σ	12.63 (1.78)
CC1/2	0.996 (0.679)
Wilson B-factor	38.68
R_{merge}	0.0974 (0.7649)
R_{work}	0.192
R_{free}	0.237
Number of non-hydrogen atoms	5299
protein	4760
carbohydrate	285
solvent	254
Average B-factor	27.88
protein	26.83
carbohydrate	45.00
solvent	28.39
RMS bond lengths (Å)	0.013
RMS bond angles (°)	1.78
Ramachandran statistics (%; ProCheck)	
most favored region	90.9
additionally allowed region	8.9
generously allowed	0.2
disallowed	0.0

*Values in parentheses are for the highest resolution shell

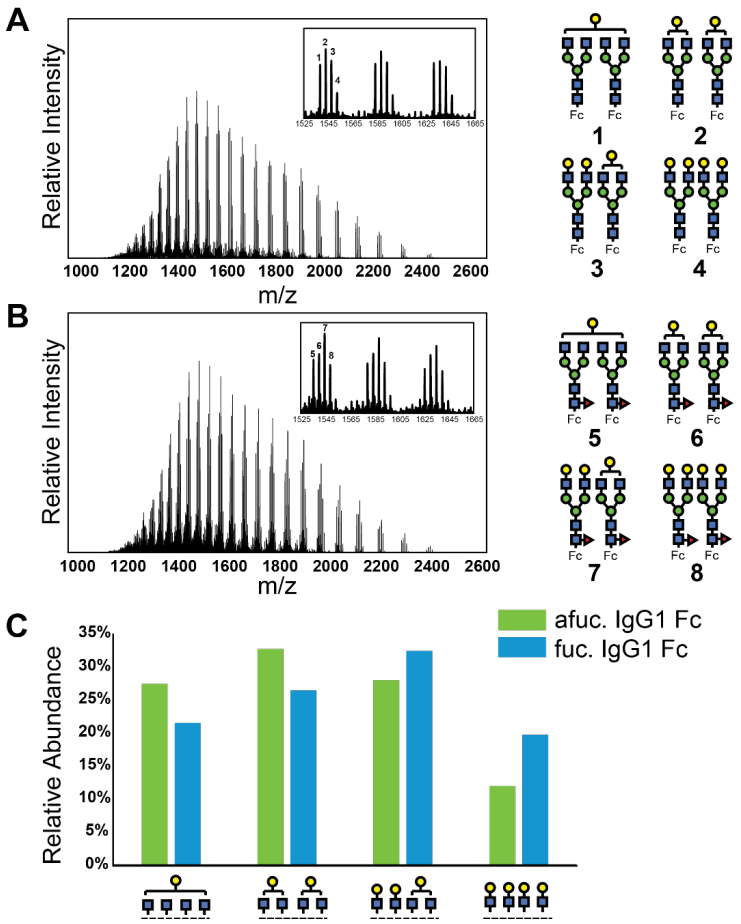
Supplemental Table 2. Summary of simulation statistics. Values represent the percentage of each conformation sampled in each simulation

			Simulation			
Conformation a (Φ/Ψ)	**pentapeptide**	**cd16a**	**afuc. IgG1 Fc + CD16a**	**fuc. IgG1 + CD16a**	**aMD afuc. IgG1 Fc + CD16a**	**aMD fuc. IgG1 Fc + CD16a**
1	100.00	100.00	100.00	100.00	100.00	100.00
other	0.00	0.00	0.00	0.00	0.00	0.00
Conformation b (Φ/Ψ)	**pentapeptide**	**cd16a**	**afuc. IgG1 Fc + CD16a**	**fuc. IgG1 + CD16a**	**aMD afuc. IgG1 Fc + CD16a**	**aMD fuc. IgG1 Fc + CD16a**
1	99.02	100.00	100.00	100.00	99.98	98.04
2	0.98	0.00	0.00	0.00	0.00	0.00
other	0.00	0.00	0.00	0.00	0.02	1.96
Conformation c (Φ/Ψ)	**pentapeptide**	**cd16a**	**afuc. IgG1 Fc + CD16a**	**fuc. IgG1 + CD16a**	**aMD afuc. IgG1 Fc + CD16a**	**aMD fuc. IgG1 Fc + CD16a**
1	73.12	84.63	98.76	99.92	71.66	61.60
2	24.54	8.73	0.00	0.00	0.00	0.00
3	2.35	6.63	1.22	0.07	28.20	37.80
other	0.00	0.01	0.02	0.01	0.14	0.60
Conformation d (Φ/Ψ)	**pentapeptide**	**cd16a**	**afuc. IgG1 Fc + CD16a**	**fuc. IgG1 + CD16a**	**aMD afuc. IgG1 Fc + CD16a**	**aMD fuc. IgG1 Fc + CD16a**
1	79.14	85.92	78.72	95.97	68.24	66.62
2	18.32	9.51	17.59	2.89	16.52	10.36
3	1.23	3.68	2.83	1.07	11.30	19.00
4	1.30	0.87	0.82	0.07	3.90	3.96
other	0.01	0.02	0.04	0.00	0.04	0.06
Conformation d (Ψ/Ω)	**pentapeptide**	**cd16a**	**afuc. IgG1 Fc + CD16a**	**fuc. IgG1 + CD16a**	**aMD afuc. IgG1 Fc + CD16a**	**aMD fuc. IgG1 Fc + CD16a**
1	27.70	4.24	33.56	65.25	25.24	45.32
2	0.88	0.57	9.40	0.01	3.08	4.58
3	1.78	4.17	13.41	4.18	19.38	16.10
4	0.46	0.01	0.01	0.00	0.54	1.44
5	51.00	80.82	36.03	27.55	38.46	25.86
6	18.18	10.16	7.42	3.01	13.28	6.70
other	0.01	0.02	0.18	0.00	0.02	0.00
Conformation e (Φ/Ψ)	**pentapeptide**	**cd16a**	**afuc. IgG1 Fc + CD16a**	**fuc. IgG1 + CD16a**	**aMD afuc. IgG1 Fc + CD16a**	**aMD fuc. IgG1 Fc + CD16a**
1	98.62	99.27	99.17	98.78	84.16	85.98
2	0.69	0.73	0.82	1.22	15.72	13.86
3	0.69	0.00	0.00	0.00	0.00	0.00
other	0.00	0.00	0.02	0.00	0.12	0.16
Conformation f (Φ/Ψ)	**pentapeptide**	**cd16a**	**afuc. IgG1 Fc + CD16a**	**fuc. IgG1 + CD16a**	**aMD afuc. IgG1 Fc + CD16a**	**aMD fuc. IgG1 Fc + CD16a**

1	91.93	91.44	90.86	80.52	75.60	75.44
2	1.38	1.43	1.62	0.70	15.38	13.84
3	6.69	7.12	7.51	18.79	8.92	10.72
other	0.00	0.01	0.00	0.00	0.10	0.00
Conformation f (Ψ/Ω)	**pentapeptide**	**cd16a**	**afuc. IgG1 Fc + CD16a**	**fuc. IgG1 + CD16a**	**aMD afuc. IgG1 Fc + CD16a**	**aMD fuc. IgG1 Fc + CD16a**
1	16.33	8.56	6.61	5.94	39.66	31.26
2	11.17	9.63	12.27	12.22	26.12	38.30
3	66.03	74.93	73.70	63.12	25.92	21.04
4	4.28	5.22	5.98	9.98	4.52	5.82
5	2.19	1.59	1.42	8.74	3.74	3.54
other	0.00	0.06	0.02	0.00	0.04	0.04
Conformation g (Φ/Ψ)	**pentapeptide**	**cd16a**	**afuc. IgG1 Fc + CD16a**	**fuc. IgG1 + CD16a**	**aMD afuc. IgG1 Fc + CD16a**	**aMD fuc. IgG1 Fc + CD16a**
1	97.92	98.75	99.11	98.88	61.56	71.20
2	0.70	0.07	0.36	0.19	1.68	1.44
3	1.38	1.04	0.53	0.93	36.74	27.30
other	0.00	0.14	0.00	0.00	0.02	0.06

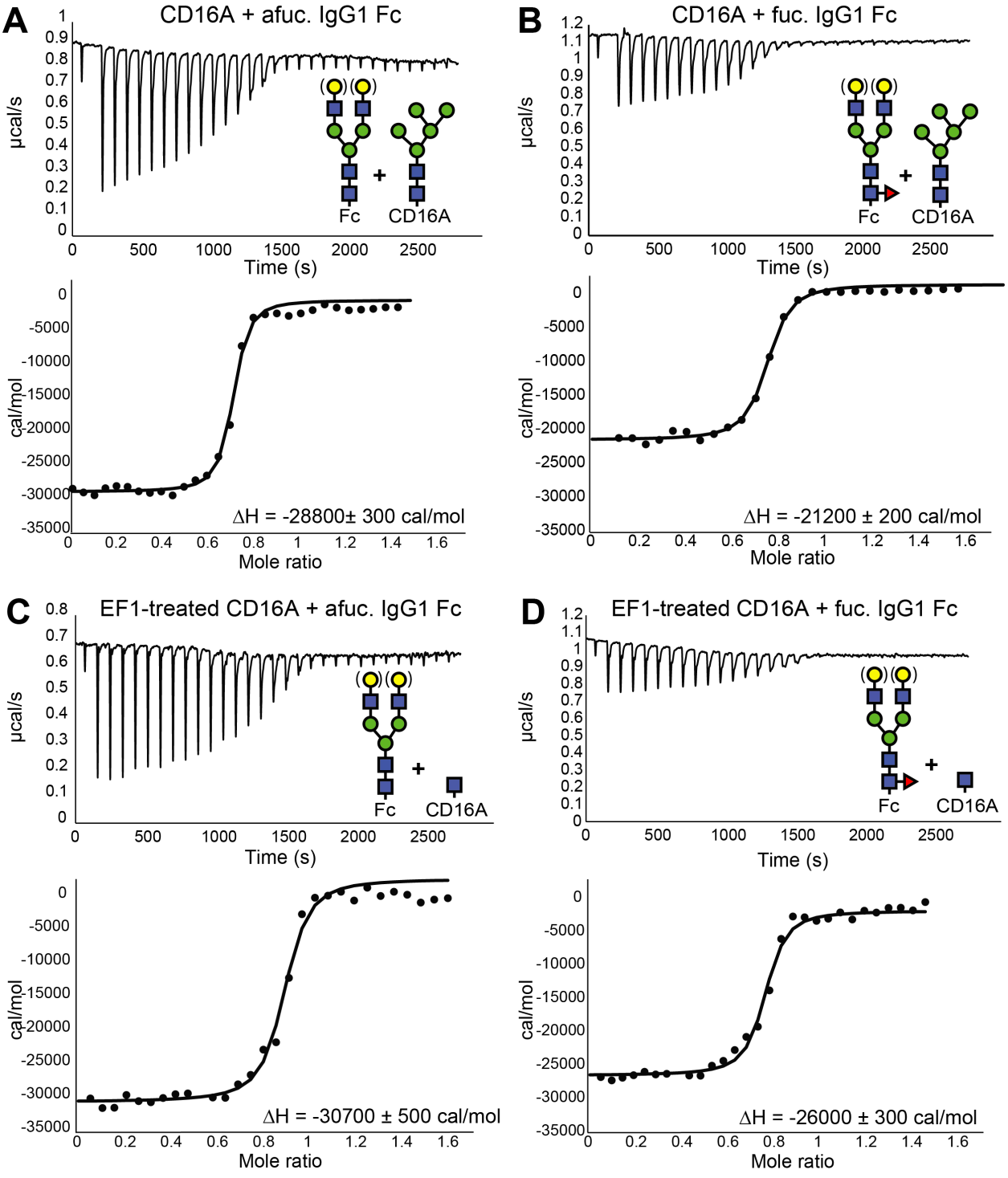


Supplemental Figure 1. Glycan distribution of CD16A used in this study. Each chromatogram represents the total ion current (TIC) of each HILIC-MS/MS run. (a) CD16a expressed from HEK293F cells treated with kifunensine. (b) CD16A expressed from HEK293S (*lec1*$^{-/-}$) cells. (c) CD16a expressed from HEK293F cells. Cartoon diagrams represent the predominant species; isobaric species were not distinguished.

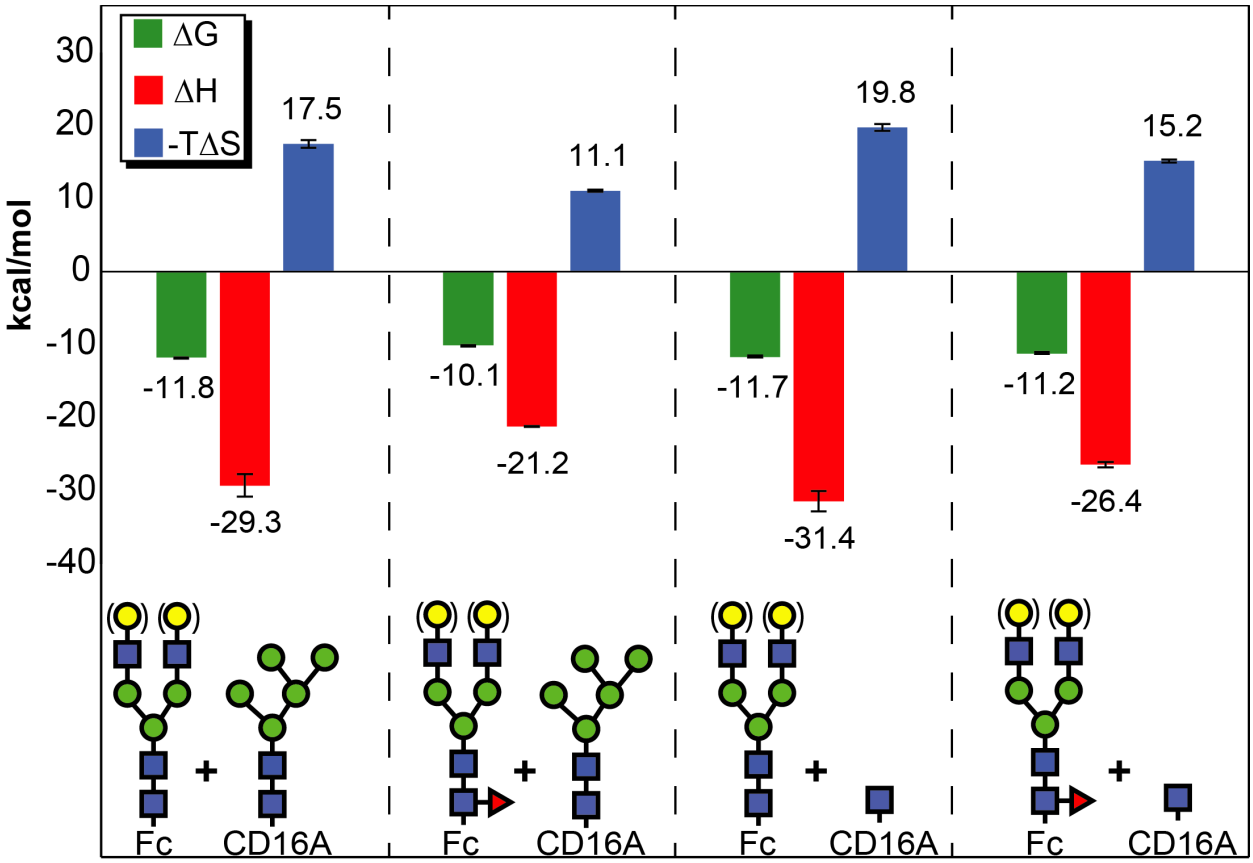


	Observed Mass (amu)	intensity (%)	# HexNAc	# Hexose	# Deoxyhexose	Calculated Mass (amu)	Delta (amu)
afucosylated	53965.9	100.0	4	5	0	53960.9	5.0
	53803.7	85.7	4	4	0	53798.8	4.8
	54127.9	84.5	4	6	0	54122.9	5.0
	54290.0	34.2	4	7	0	54285.0	5.1
fucosylated	55559.5	100.0	4	6	2	55554.4	-5.1
	55397.2	83.7	4	5	2	55392.3	-4.8
	55234.9	69.2	4	4	2	55230.3	-4.6
	55721.6	60.4	4	7	2	55716.4	-5.1

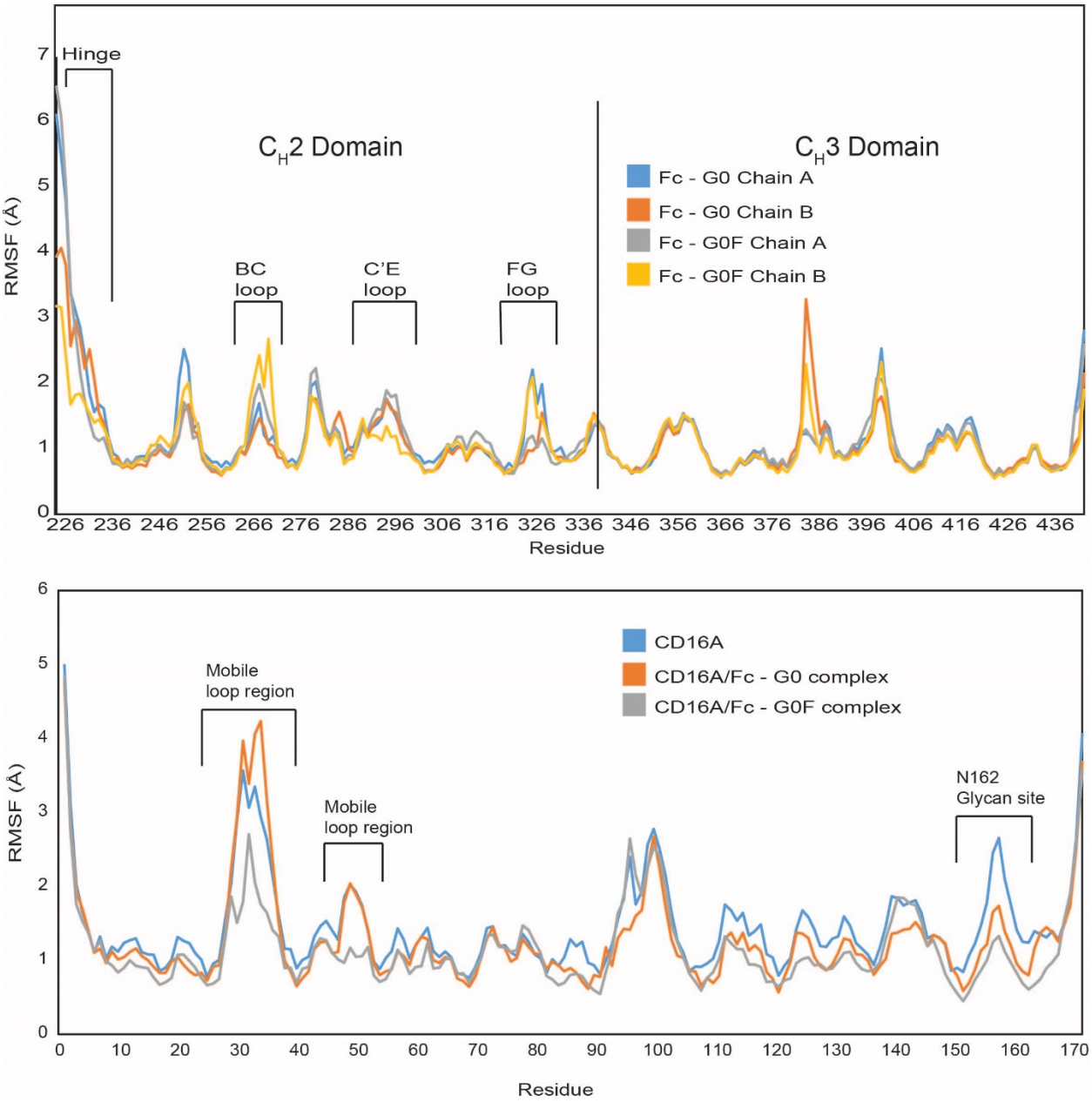
Supplemental Figure 2. Glycan distribution of HEK293F-expressed IgG1 Fc. ESI-MS-based quantification of the relative distribution of glycans present in the IgG1 Fc samples. Protein expression generated either (a) afucosylated or (b) fucosylated IgG1 Fc. (c) A comparison of relative IgG1 Fc N-glycan galactosylation from the different expressions. (table) Analysis of deconvoluted ESI-MS spectra. Limited Fc proteolysis of Fc hinge residues preceding the linking disulfides and at the C-terminal tails occurs during expression as previously characterized.



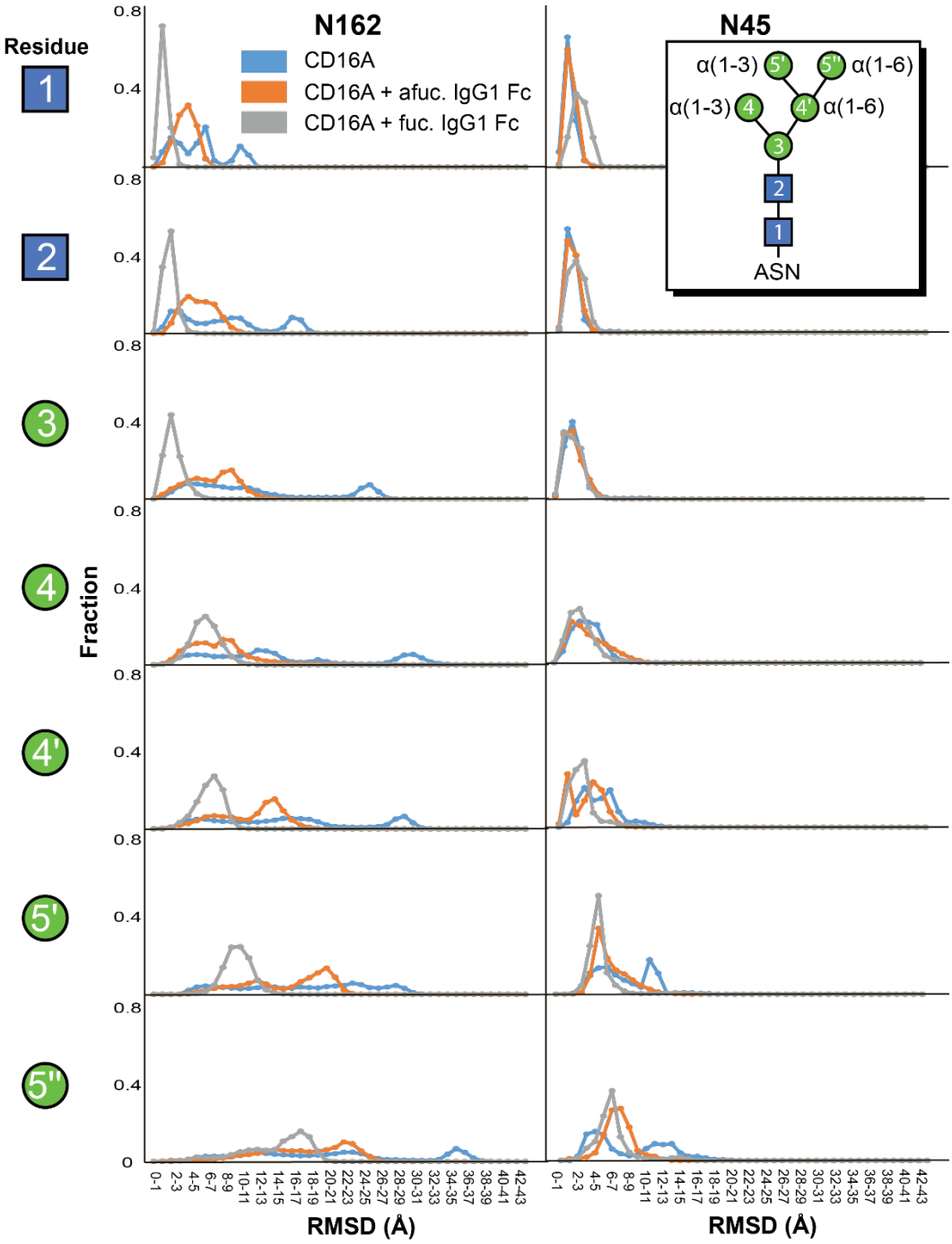
Supplemental Figure 3. Representative ITC binding isotherms for CD16A N–glycoforms binding IgG1 Fc. Equilibrium dissociation constants and enthalpy derived from direct fitting of the ITC data are indicated. Errors are representative of the least–squares fitting.



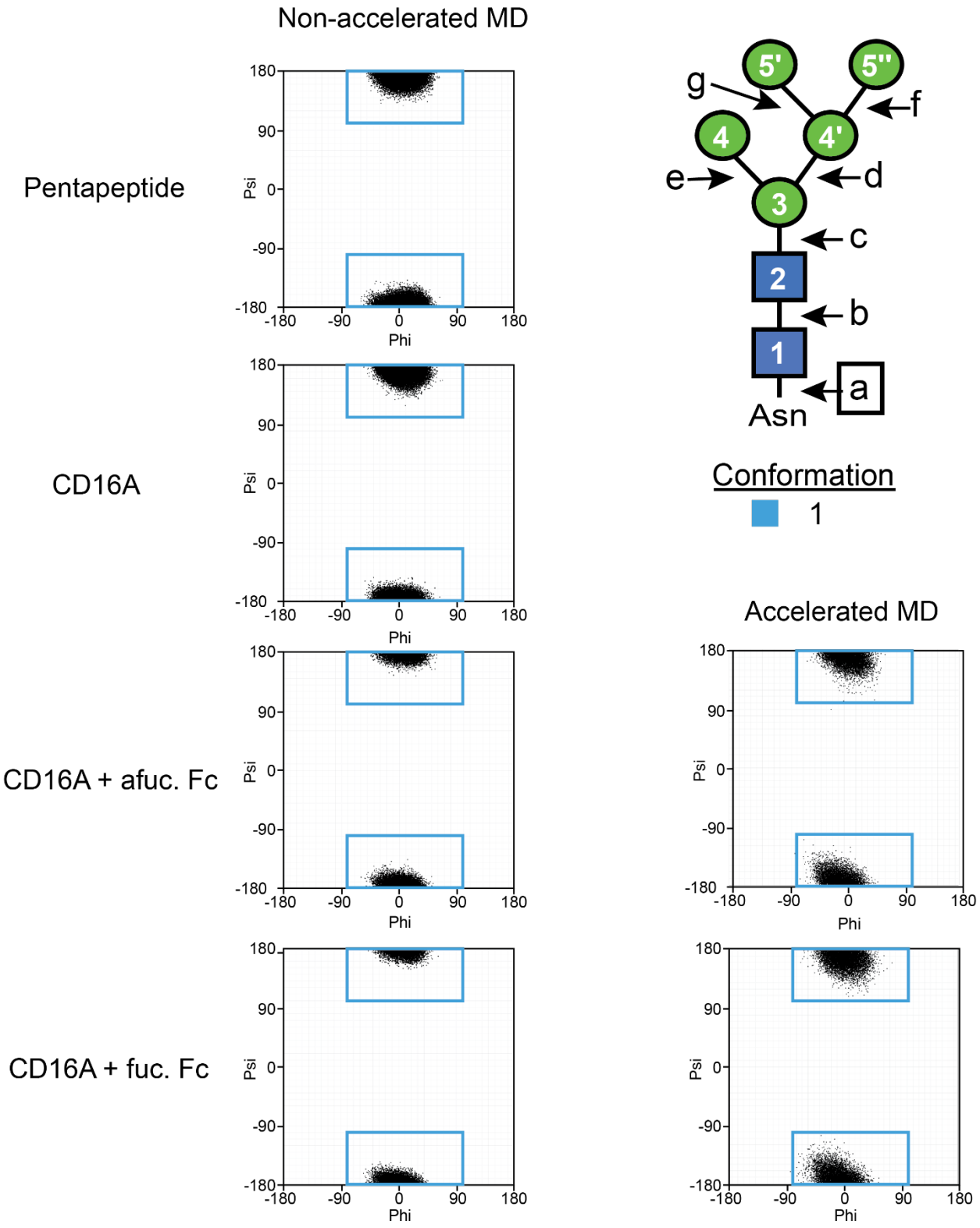
Supplemental Figure 4. Truncating the CD16A N–glycans relieves the enthalpic penalty of IgG1 Fc fucosylation in CD16A binding. Endoglycosidase F1 treatment truncated CD16A N–glycans (the two interactions shown at the *right*). Glycosidase truncation of the CD16A N–glycans minimally affected binding to afucosylated Fc with a 2.1 kcal/mol increase in the magnitude of ΔH and a 2.3 kcal/mol increase in (–TΔS). Binding between fucosylated Fc with CD16A showed a 5.2 kcal/mol increase in ΔH magnitude and a 4.1 kcal/mol increase in (–TΔS) following CD16A treatment. IgG1 Fc fucosylation reduces the magnitude of both ΔH and (–TΔS) upon binding CD16A with full–length N–glycans (8.1 and 6.4 kcal/mol, respectively). Truncating the CD16A glycans to a single (1)GlcNAc residue decreased the effect of fucose on the magnitude of ΔH and –TΔS (5.0 and 4.6 kcal/mol, respectively). A combination of free energy of binding (ΔG) measured by SPR and enthalpy (ΔH) from ITC at 25 °C provided an estimate of entropy (ΔS) using the equation ΔG = ΔH - TΔS. Error bars are ± std. dev.



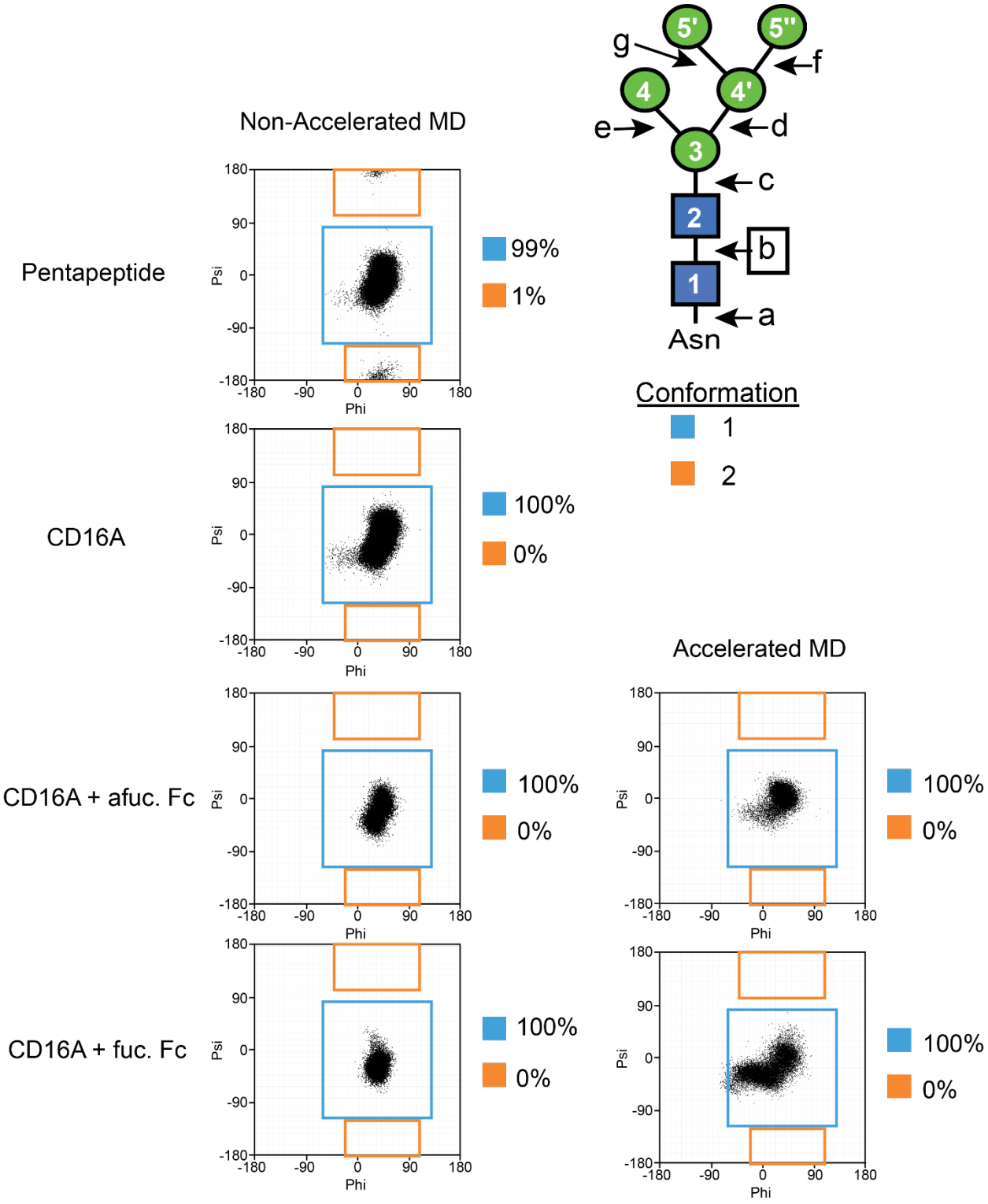
Supplemental Figure 5. RMSF plots of the various polypeptide backbones show that fucosylation has a limited effect on Fc and CD16A backbone motion. RMSF data was calculated from the Cα atoms over the course of the simulation using the first frame as reference.



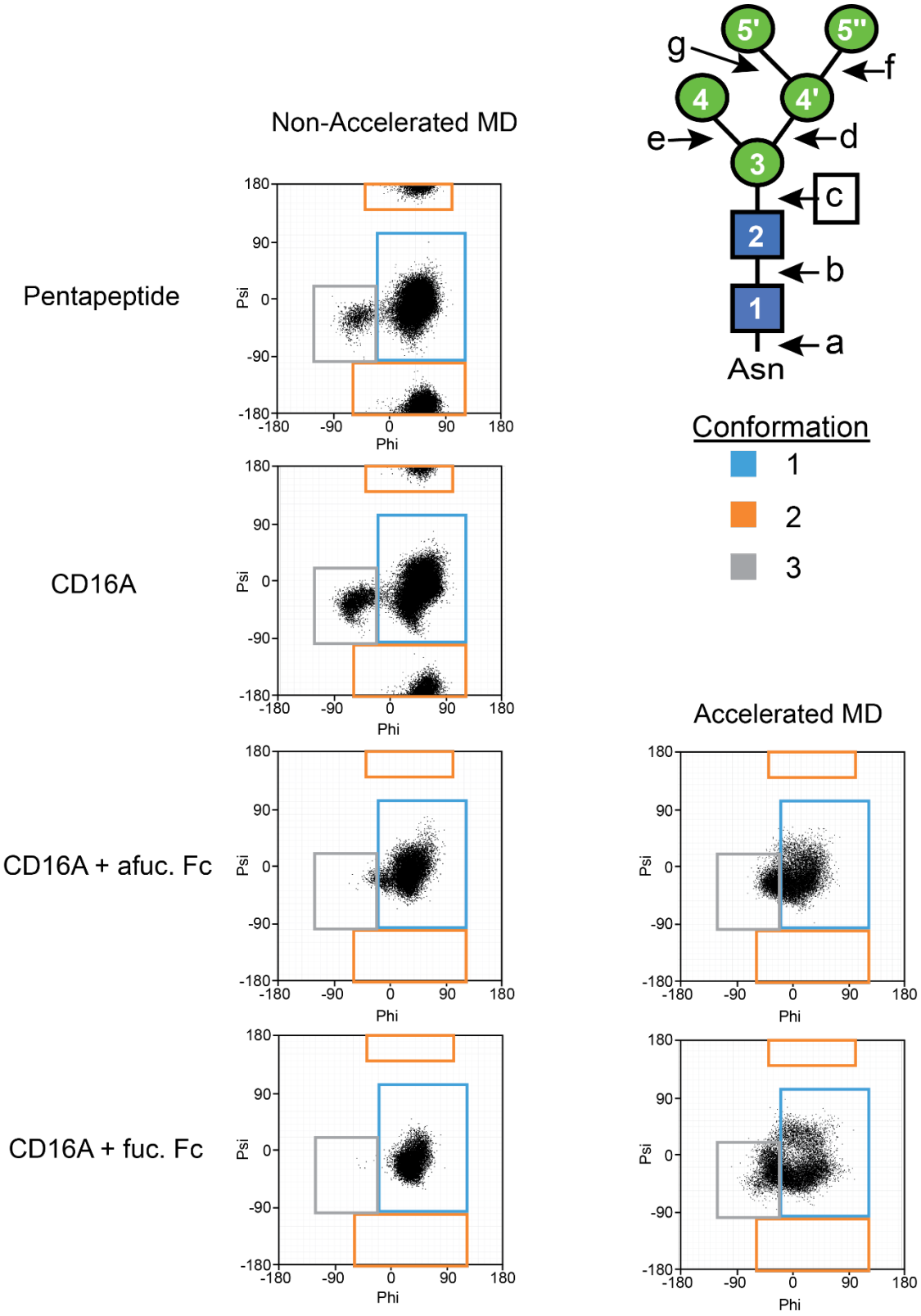
Supplemental Figure 6. The CD16A N162-glycan samples less space in complex with fucosylated Fc than afucosylated Fc. Histograms of RMSD per residue from extensive 1 μs computational simulations of CD16A N38Q/N74Q/N169Q alone (*blue*), in complex with fucosylated IgG1 Fc (*orange*) or in complex with afucosylated IgG1 Fc (*grey*) glycans show large differences. RMSD was calculated using the C1 atom of each residue over the course of the simulation with the first frame serving as a reference.



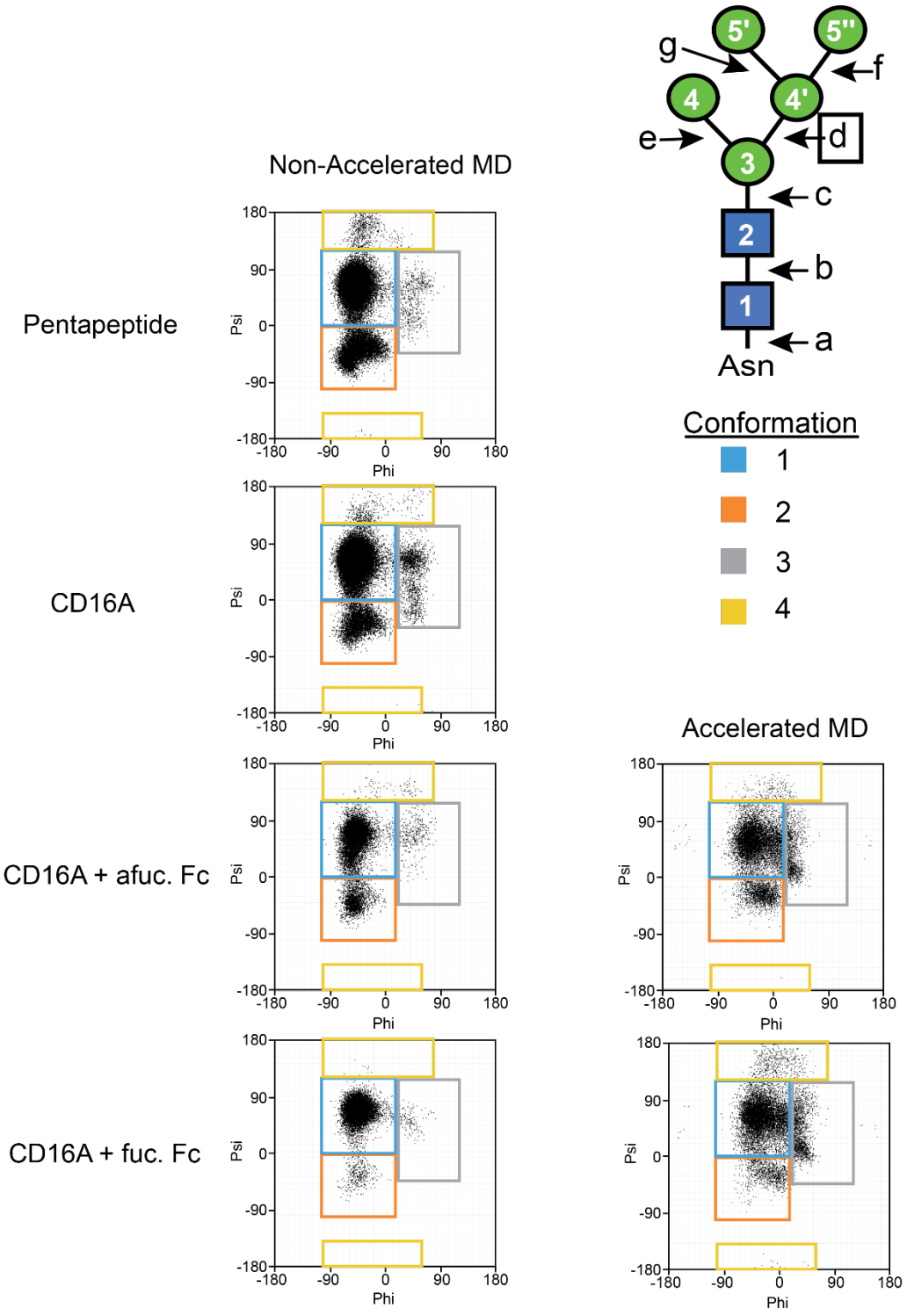
Supplemental Figure 7-1. Conformations sampled in MD simulations for the β(1-*N*)GlcNAc glycosidic linkage (denoted "a").



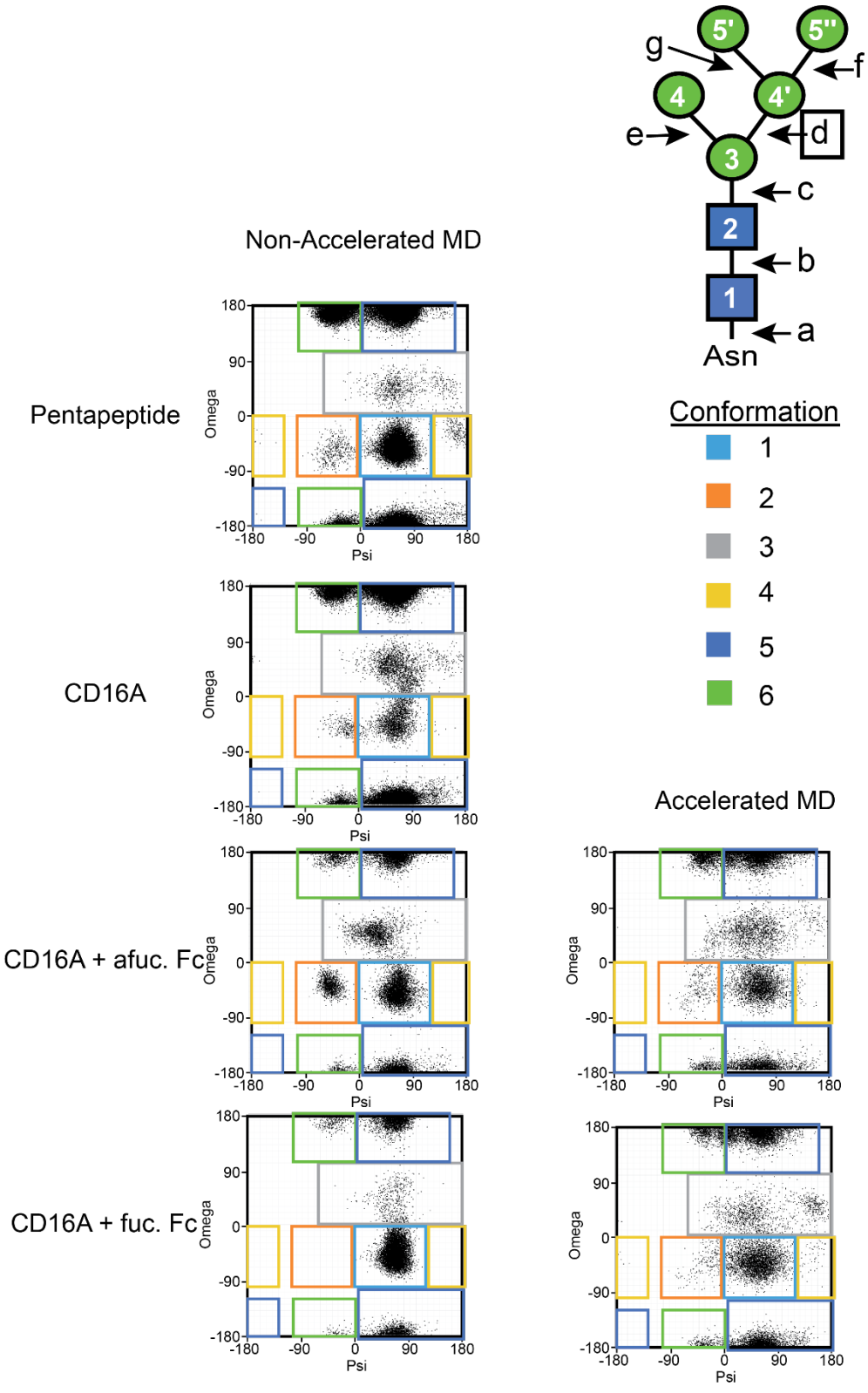
Supplemental Figure 7-2. Conformations sampled in MD simulations for the β(1-4)GlcNAc glycosidic linkage (denoted "b").



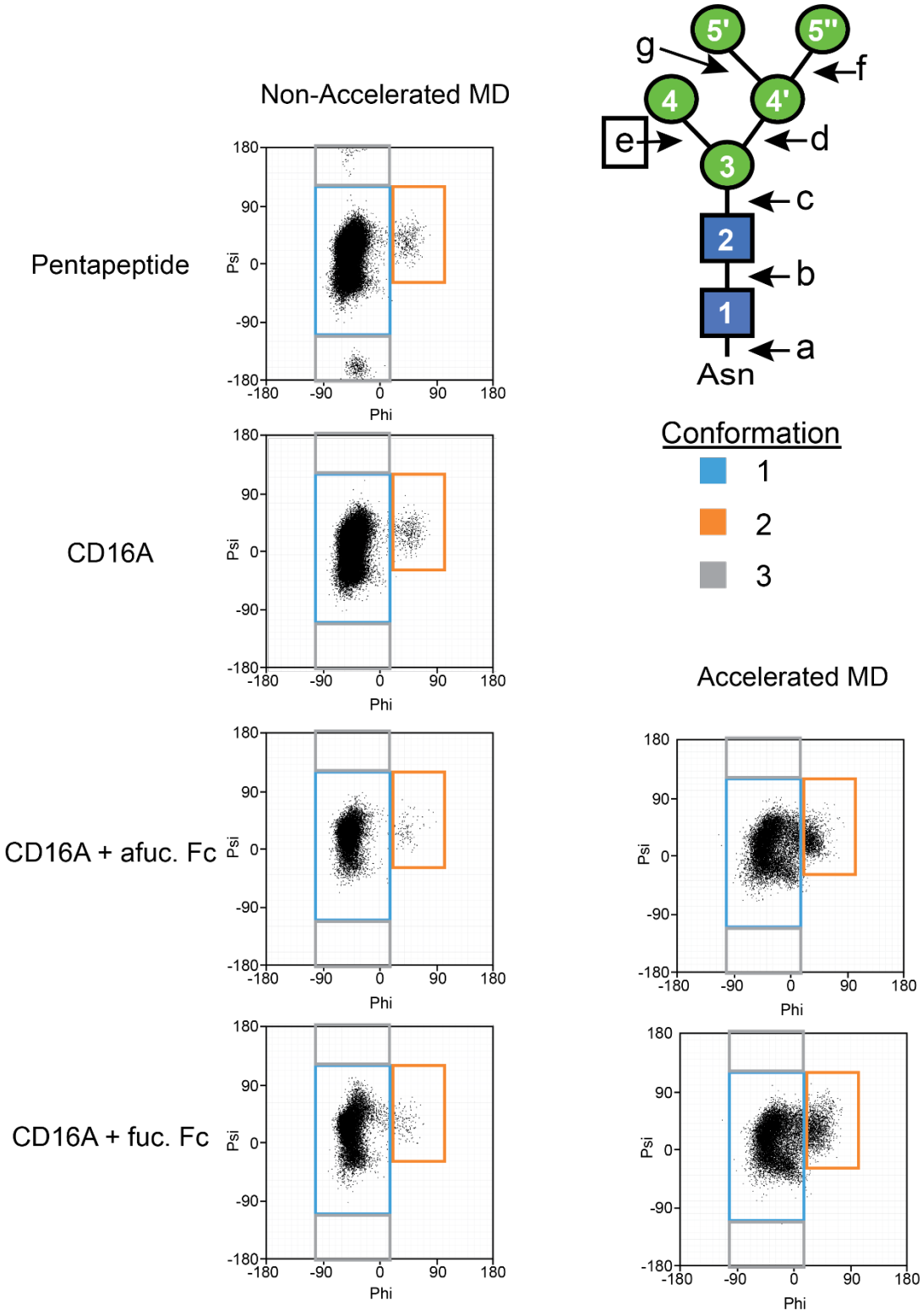
Supplemental Figure 7-3. Conformations sampled in MD simulations for the β(1-4)Man glycosidic linkage (denoted "c").



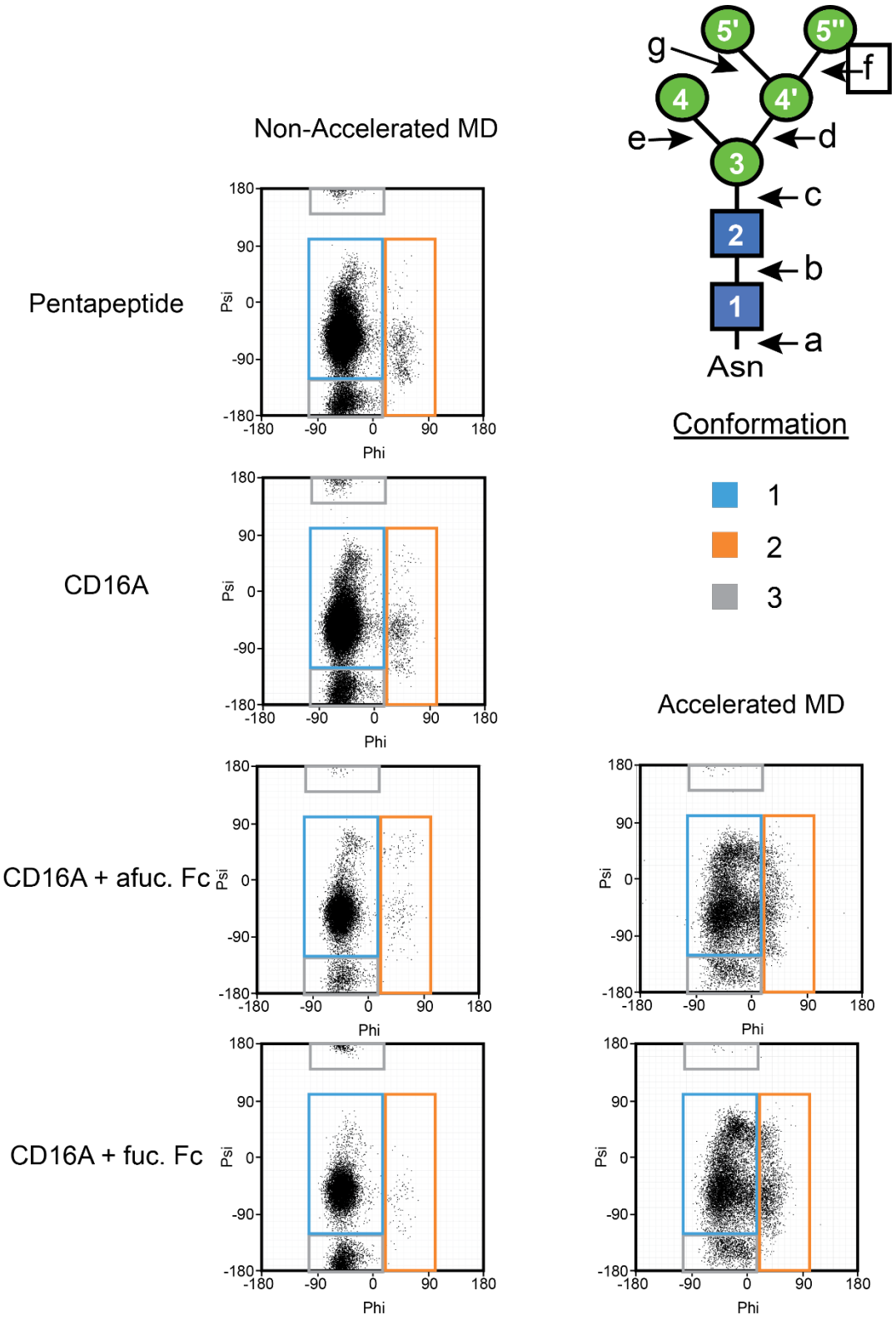
Supplemental Figure 7-4. Conformations sampled in MD simulations for the α(1-6)Man glycosidic linkage (Φ/Ψ; denoted "d").



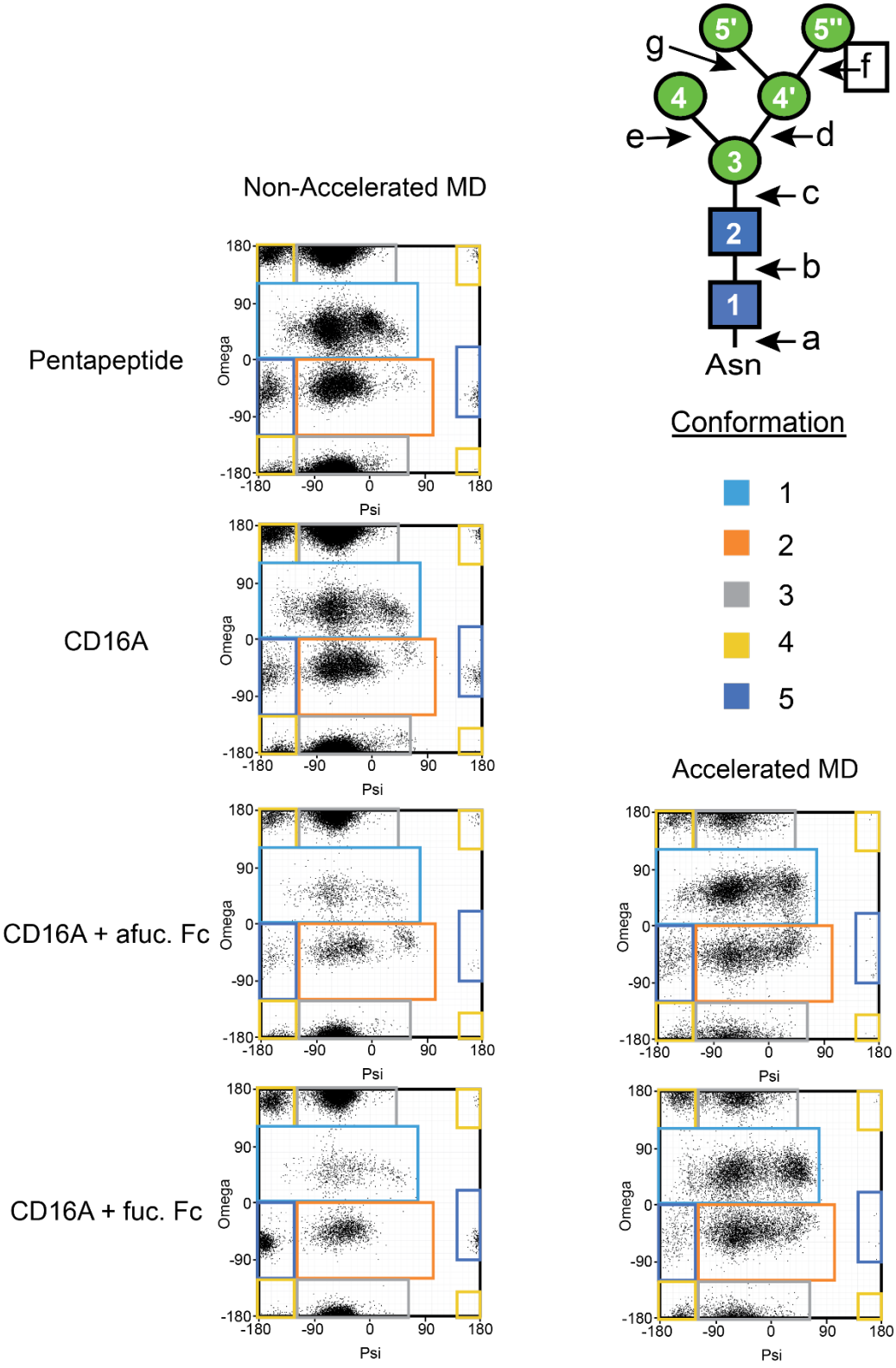
Supplemental Figure 7-5. Conformations sampled in MD simulations for the α(1-6)Man glycosidic linkage (Ψ/Ω; denoted "d").



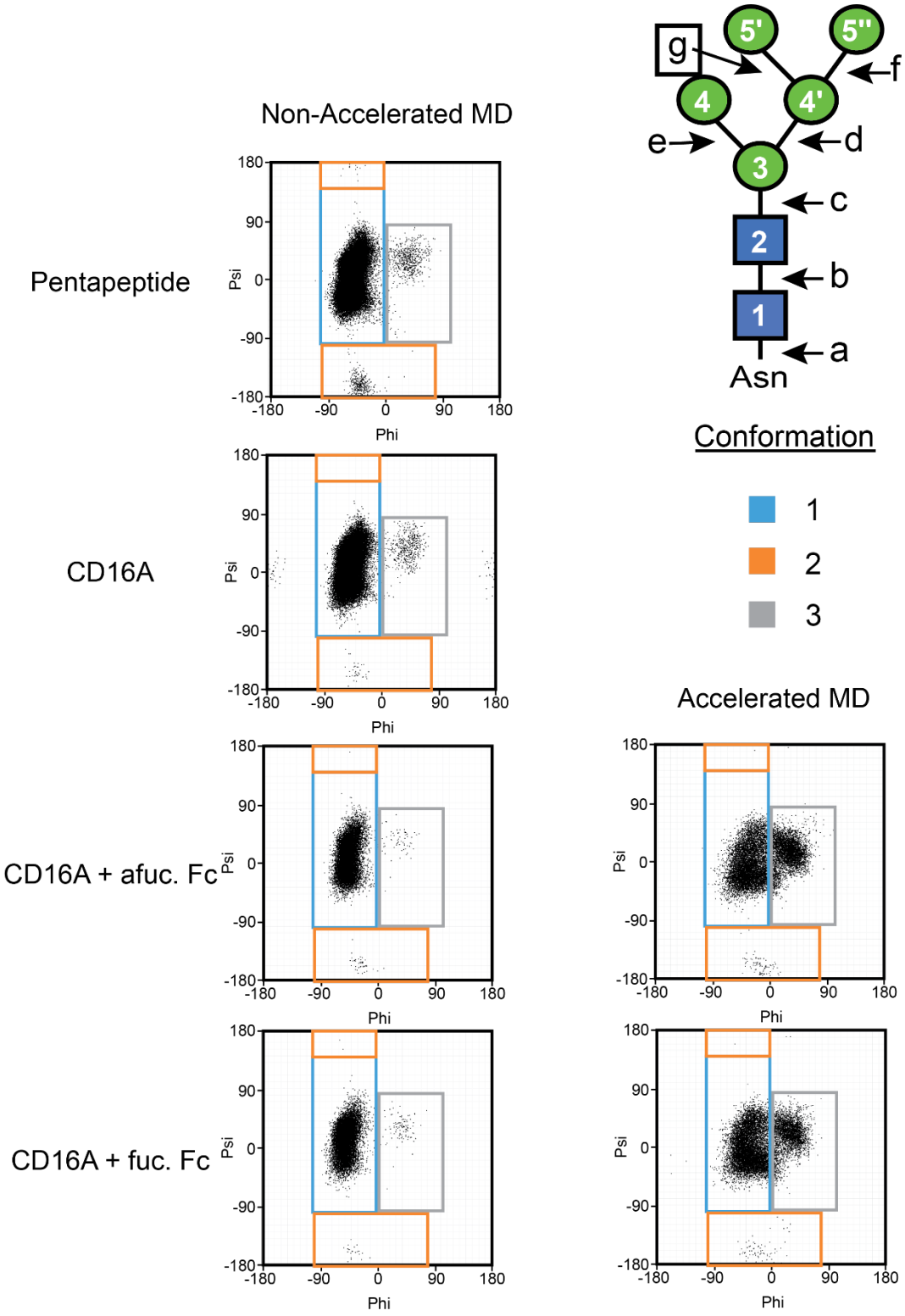
Supplemental Figure 7-6. Conformations sampled in MD simulations for the α(1-3)Man glycosidic linkage (denoted “e”).



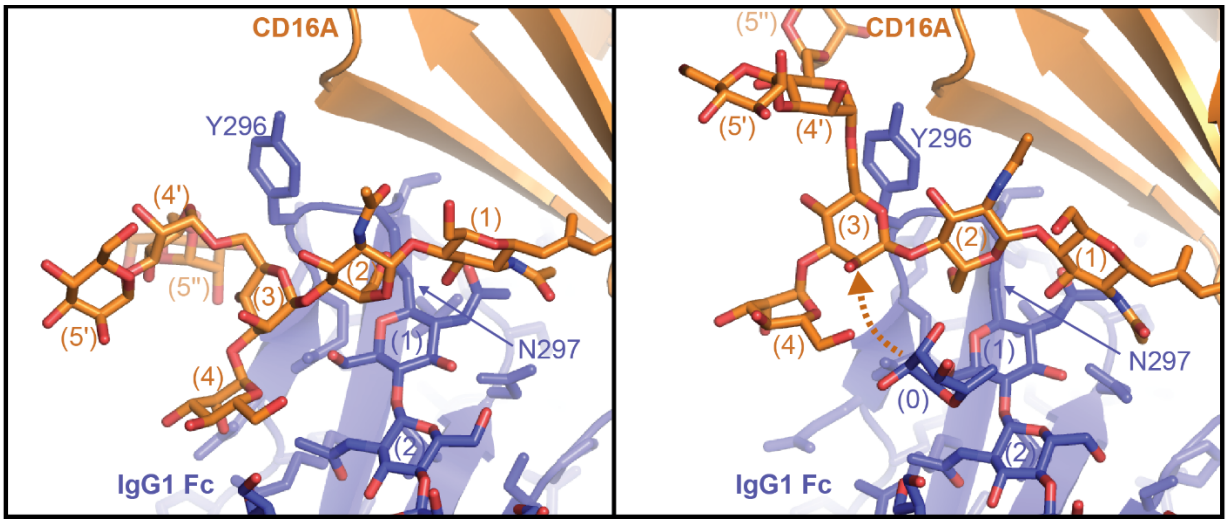
Supplemental Figure 7-7. Conformations sampled in MD simulations for the α(1-6)Man glycosidic linkage (Φ/Ψ; denoted "f").



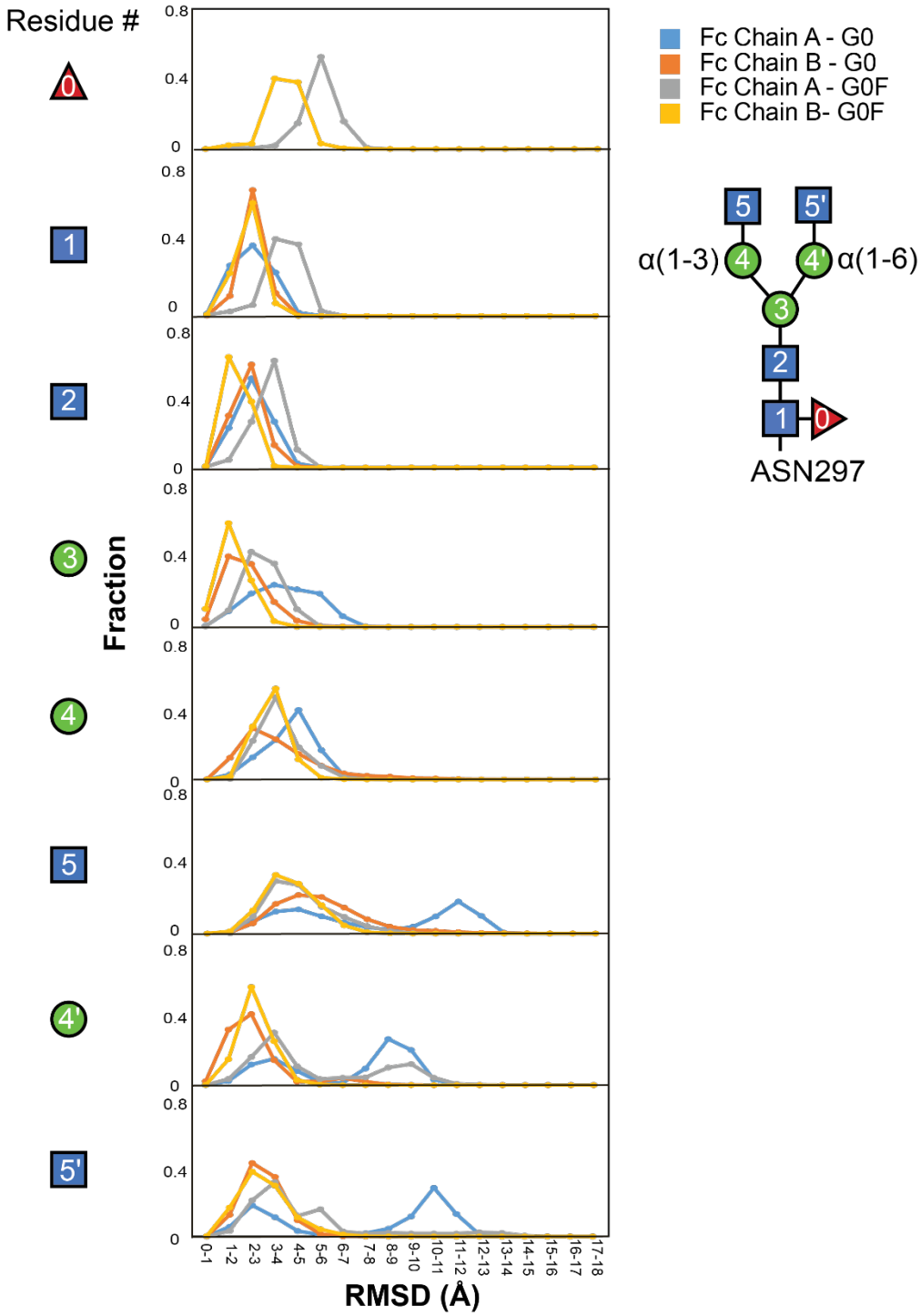
Supplemental Figure 7-8. Conformations sampled in MD simulations for the α(1-6)Man glycosidic linkage (Ψ/Ω; denoted "f").



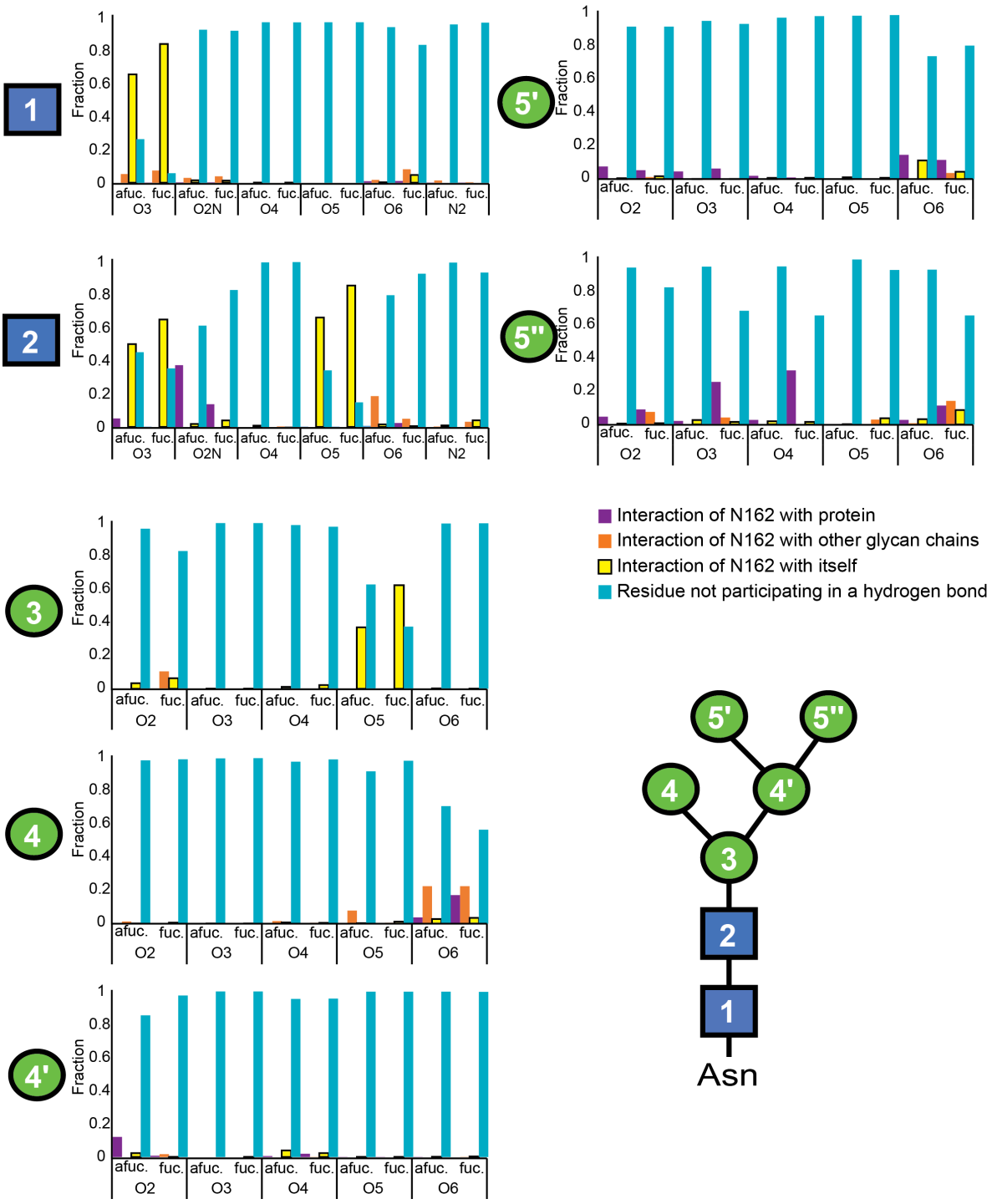
Supplemental Figure 7-9. Conformations sampled in MD simulations for the α(1-3)Man glycosidic linkage (denoted "g").



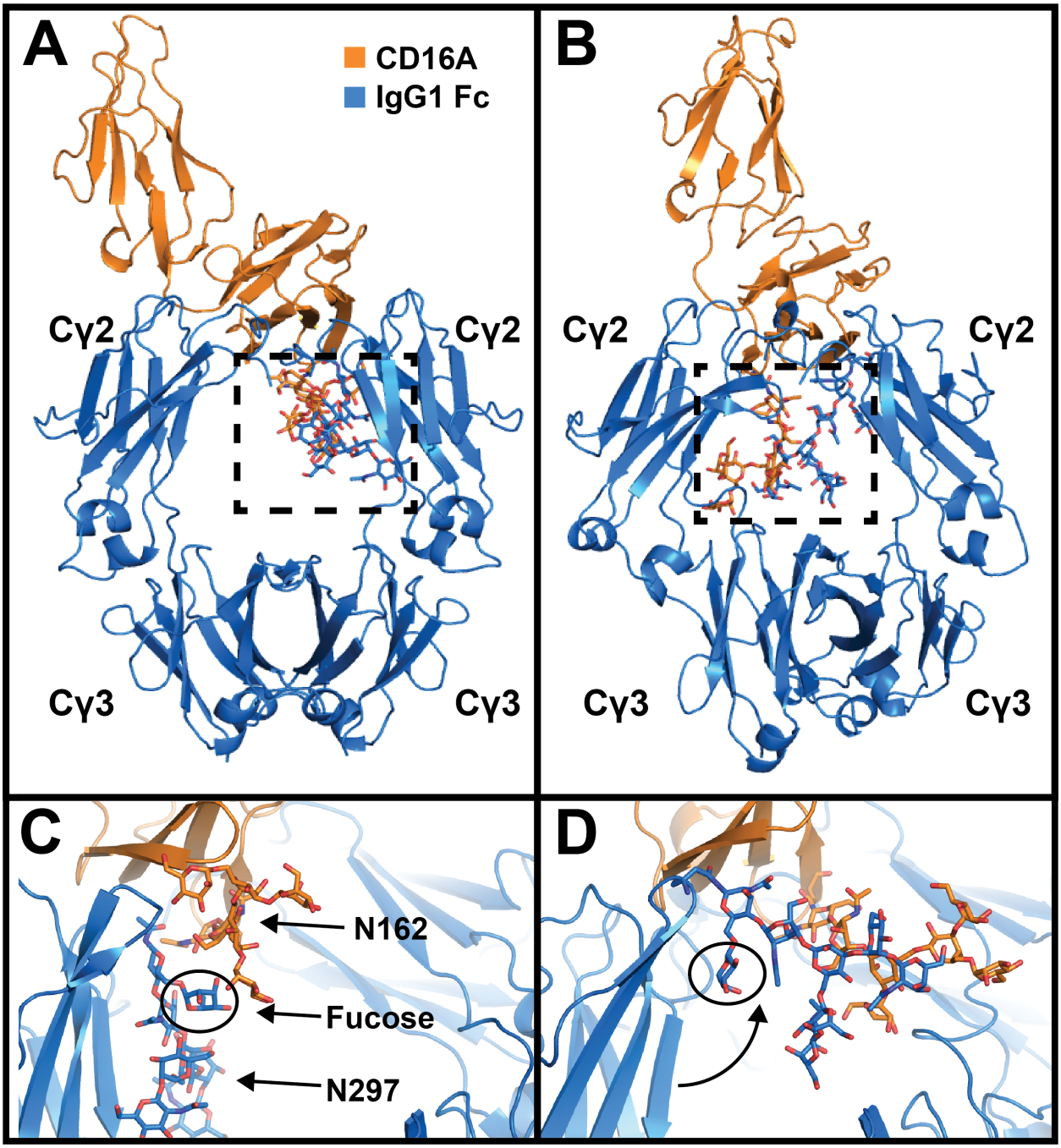
Supplemental Figure 8. All-atom simulation shows antibody fucosylation perturbs conformations sampled by the CD16A Asn162-glycan. The *left* panel shows the interaction with afucosylated IgG1 Fc, and fucosylated IgG1 Fc (residue (0)) is shown in the *right* panel. Fucosylation pushes the Asn162-glycan (*orange* sticks) away from the IgG1 Asn297-glycan (*blue* sticks). This movement restricts the conformation of the Asn162-glycan by bringing it in proximity to IgG1 Tyr296 and the CD16A polypeptide backbone.



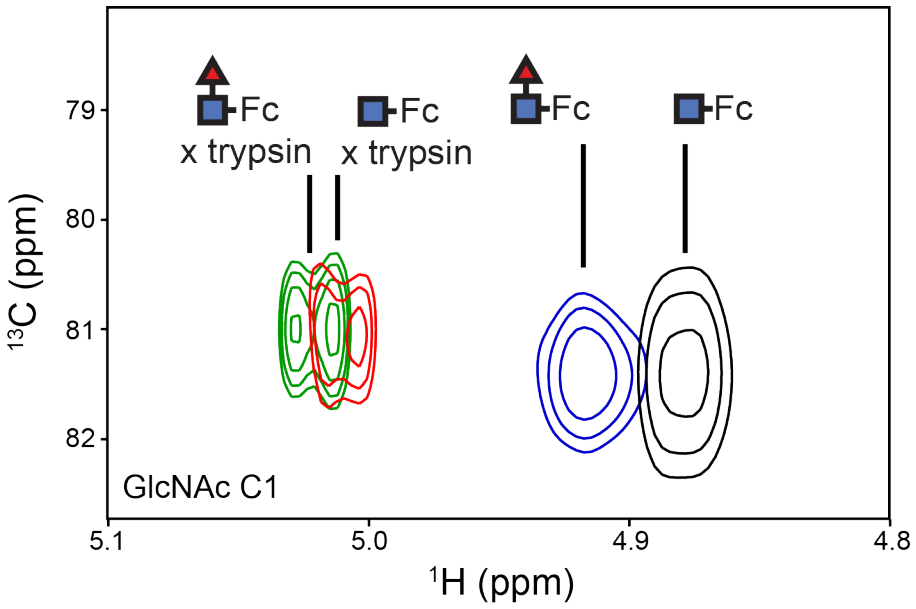
Supplemental Figure 9. The RMSD plot of the IgG1 Fc N-glycan residues shows fucosylation minimally impacts Fc N297-glycan motion. In these simulations, the C'E loop of chain A interacts with CD16A residue 128-131. RMSD was calculated using the C1 atom of each residue over the course of the simulation with the first frame serving as a reference.



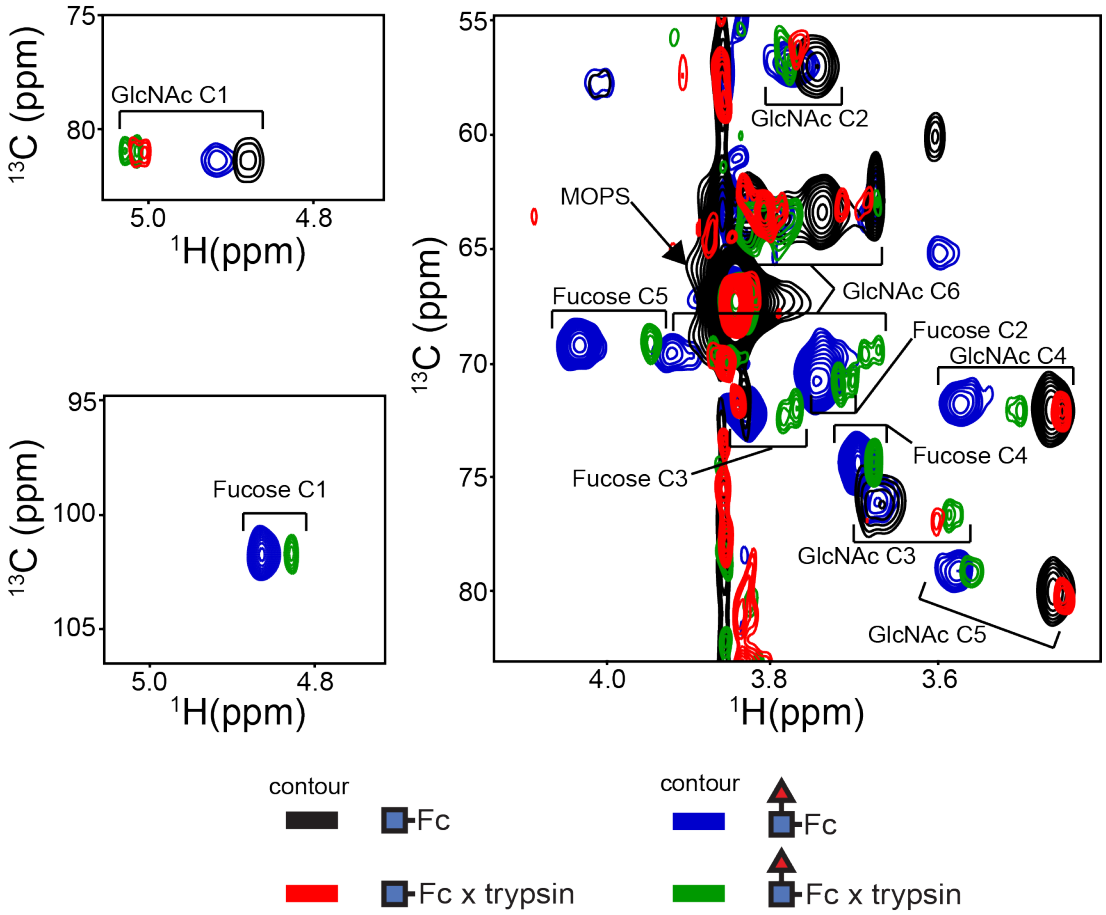
Supplemental Figure 10. H-Bonds formed between CD16A N162-glycan residues and other atoms. Glycan-glycan H-bonds (denoted with orange bars) are formed only transiently in 250 ns simulations of the IgG1-CD16A complex. Atom names are denoted on the x-axes of the plots for each N-glycan residue. Simulations using afucosylated IgG1 Fc ("afuc.") or fucosylated IgG1 Fc ("fuc.") are likewise denoted in the x-axes.



Supplemental Figure 11. aMD induced global changes to the IgG1 Fc:CD16a complex. AMD simulation of the IgG1 Fc:CD16a complex simulation snapshot from the 5001-12500 frame region of supplemental figure 11. Cγ3 domains show severe distortion into unknown conformations not described previously.



Supplemental Figure 12. ^{1}H-^{13}C HSQCs of the IgG1 Fc Asn297 (1)GlcNAc H1 show changes in the observed resonance frequency as a result of fucosylation. The effect of protein structure was removed by trypsin-catalyzed cleavage (shown in the *red* and *green* contours).



Supplemental Figure 13. Complete ^{1}H-^{13}C HSQC spectra of the IgG1 Fc N297 (1)GlcNAc show changes in the observed resonance frequency as a result of fucosylation.