Molecular basis of broad spectrum N-glycan specificity and processing of therapeutic IgG monoclonal antibodies by Endoglycosidase S2.

Erik H. Klontz[1,2,3,#], Beatriz Trastoy[4,#], Daniel Deredge[5], James K. Fields[1,2,3], Chao Li,[6] Jared Orwenyo[6], Alberto Marina[4], Robert Beadenkopf[1], Sebastian Günther[1,7], Jair Flores[1], Patrick L. Wintrode[5], Lai-Xi Wang[6], Marcelo E. Guerin[4,8,*], Eric J. Sundberg[1,2,9,*]

[1]Institute of Human Virology, [2]Department of Microbiology & Immunology, [3]Program in Molecular Microbiology & Immunology, University of Maryland School of Medicine, Baltimore, MD 21201 USA

[4]Structural Biology Unit, CIC bioGUNE, Bizkaia Technology Park, 48160 Derio, Spain

[5]Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, MD 21201 USA

[6]Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA

[7]Photon Science, Deutsches Elektronen-Synchrotron, Hamburg, Germany

[8]IKERBASQUE, Basque Foundation for Science, 48013, Bilbao, Spain.

[9]Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, USA

Running head: *Diverse N-glycan specificity of EndoS2*

[*]To whom correspondence should be addressed: Eric J. Sundberg: ESundberg@som.umaryland.edu and Marcelo E. Guerin: mrcguerin@cicbiogune.es

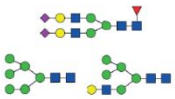
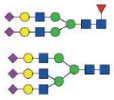
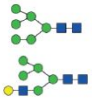
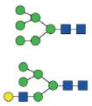
[#]These authors contributed equally to this work

Supplementary Tables

Table S1. Data collection and refinement statistics. Overall values are reported with highest resolution shell in parentheses.

Data Collection	EndoS2 unliganded	EndoS2-CT	EndoS2-HM
Resolution range	38.89 - 2.75 (2.85 -2.75)	39.71 - 2.5 (2.59 - 2.5)	29.46 - 2.5 (2.59 - 2.5)
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit cell (a, b, c, α, β, γ)	89.17, 105.83, 259.28, 90, 90, 90	89.38, 105.51, 259.77, 90, 90, 90	88.97,105.353, 257.30, 90, 90, 90
Total reflections	389940 (37833)	312202 (31674)	507462 (51943)
Unique reflections	64288 (6088)	75959 (7820)	84391 (8314)
Multiplicity	3.2 (3.0)	4.1 (4.1)	6.0 (6.2)
Completeness (%)	93.9 (95.7)	88.0 (93.0)	100 (100)
Mean I/sigma(I)	6.1 (1.6)	8.41 (1.21)	10.01 (1.37)
Wilson B-factor	40.89	43.96	45.53
R_{merge}	0.149 (0.712)	0.140 (1.21)	0.143 (1.21)
R_{meas}	0.197 (0.942)	0.159 (1.37)	0.157 (1.32)
CC1/2	0.975 (0.363)	0.993 (0.488)	0.995 (0.519)
CC*	0.994 (0.694)	0.998 (0.81)	0.999 (0.827)
Refinement			
Reflections used in refinement	60280 (6056)	75877 (7800)	84382 (8314)
Reflections used for R-free	3042 (300)	3796 (364)	4298 (426)
R_{work}	0.210 (0.309)	0.193 (0.295)	0.198 (0.281)
R_{free}	0.264 (0.373)	0.242 (0.348)	0.248 (0.333)
CC(work)	0.941 (0.618)	0.966 (0.728)	0.956 (0.733)
CC(free)	0.896 (0.565)	0.942 (0.702)	0.926 (0.666)
Number of non-hydrogen atoms	12631	13077	13060
Macromolecules	12349	12459	12514
Ligands	2	198	175
Protein residues	1573	1574	1574
Waters	283	420	372
RMS(bonds)	0.009	0.009	0.009
RMS(angles)	1.11	1.21	1.16
Ramachandran favored (%)	96	96	96
Ramachandran allowed (%)	3.1	3.8	3.5
Ramachandran outliers (%)	0.9	0.2	0.5
Rotamer outliers (%)	1.4	1.2	1.2
Average B-factor for macromolecules	38.22	48.70	52.65
Average B-factor for carbohydrates	N/A	66.34	69.01
PDB Code	6E58	6MDS	6MDV

Table S2. Structural homology of EndoS2 with GH18 family of endoglycosidases

Enzyme	Source	Glycan structure hydrolyzed	Protein substrate	Z-score	RMSD (Å)	PDB CODE
EndoS2	*Streptococcus pyogenes (serotype M49)*		IgG(1-4) Fc AGP (alpha-1-acid glycoprotein)	-	-	-
EndoS	*Streptococcus pyogenes (serotype M1)*		IgG1 Fc	44.9	1.9	6EN3
$EndoF_3$	*Elizabethkingia meningoseptica*		No specific substrate known	21.6	2.5	1EOM
EndoT	*Hypocrea jecorina*		No specific substrate known	19.0	3.1	4AC1
EndoH	*Streptomyces plictus (griseus)*		No specific substrate known	16.7	3.2	1C3F
$EndoF_1$	*Elizabethkingia meningoseptica*		No specific substrate known	14.4	3.3	2EBN
EndoBT	*Bacteroides thetaiotaomicron*		No specific substrate known	12.9	3.4	3POH

Fuc GlcNAc Man Gal Neu5Ac

Table S3. Summary of constructs

Description	Sequence	Template	Primers (5' to 3')	Method
$EndoS_{WT}$	-	pGEXndoS (GenBank entry: AF296340)	1)GGAATTCCATATGGAGGAGAAGACTGTTCAG GTTCAGAAAGGATTACCTTCTATCGATA 2)CGCGGATCCCAGTTTTTTTAGCAGCTGCCTTT TCTCAATACAATCTTTCAAAACTCCA	Restriction/ligation (NdeI, BamHI)
$EndoS2_{WT}$	-	pGEXndoS2 (GenBank entry: ACI61688.1)	1)GGAATTCCATATGGGAAAGACAGATCAGCAG GTTGGTGCTAAATTGGTACAGGAAATCC 2)CGCGGATCCCAGATCCTTCAGCGTATTAGCG ACATCATTTGATAAACGTTGTCCGAGG	Restriction/ligation (NdeI, BamHI)
$EndoS2_{R72A/Q87A/H88A}$	-	$EndoS2_{WT}$	1)GCTTCAACAGGAATAGATGGTAAACAGGCTG CTCCAGAAAATACTATGGCTGAGGTCCCAAAA GAAG 2)CTATTCCTGTTGAAGCACGATCATGCCATGTA GCAAAATAACCAGCATATAGTGGTCCGCGTTTT CCTTC	PCR-based site-directed mutagenesis
$EndoS2_{D108A}$	-	$EndoS2_{WT}$	1)GTTGATATCTTATTTGTTTTTCATGCGCATACA GCTTCAGATAGTCCATTTTG 2)CAAAATGGACTATCTGAAGCTGTATGCGCAT GAAAAACAAATAAGATATCAAC	PCR-based site-directed mutagenesis
$EndoS2_{H109A}$	-	$EndoS2_{WT}$	1)GATATCTTATTTGTTTTTCATGACGCTACAGC TTCAGATAGTCCATTTTGG 2)CCAAAATGGACTATCTGAAGCTGTAGCGTCA TGAAAAACAAATAAGATATC	PCR-based site-directed mutagenesis
$EndoS2_{N142A/E143A/T148A}$	-	$EndoS2_{WT}$	1)TGCTTTAAATGGACGTGCTGGTTTATCTAAAG ATTATCCTGATACTCCTGAGGGGAAC 2)ACGTCCATTTAAAGCAGCAACACCAATTGTCT GAACAAGTGCCGTTCCCTGTTG	PCR-based site-directed mutagenesis
$EndoS2_{E188A}$	-	$EndoS2_{WT}$	1)GGACTAGATATTGATATTGAGCACGCATTTAC GAACAAAAGAACACCTG 2)CAGGTGTTCTTTTGTTCGTAAATGCGTGCTCA ATATCAATATCTAGTCC	PCR-based site-directed mutagenesis
$EndoS2_{T190A/N191A/R193A}$	-	$EndoS2_{WT}$	1)TTGCTGCTAAAGCTACACCTGAAGAAGATGC TCGTGCTCTAAATGTTTTTAAAG 2)GTAGCTTTAGCAGCAAATTCGTGCTCAATATC AATATCTAGTCCATCGACACC	PCR-based site-directed mutagenesis
$EndoS2_{Q250A/Y252A}$	-	$EndoS2_{WT}$	1)TTCTTAGAGCTTATGCTGGTTCACAAGGTGG AGAAGCTGAAGTGGATACTATAAAC 2)GCATAAGCTCTAAGAAGATAATCAAGATCTTC CGCTATCCCTTTAAATATTGG	PCR-based site-directed mutagenesis
$EndoS2_{S285A/E288A/E289A/N295A}$	-	$EndoS2_{WT}$	1)CTGCTTCTGCGTCCAAAGGGGCTTTATGGTT TGATGTTAACGAATACGACCCTAACAATCCTG 2)TTGGACGCAGAAGCAGCAAAAAACGCGAATC CAATCATGAACTGGCTAGCATCAATATAATTCT G	PCR-based site-directed mutagenesis
$EndoS2_{EndoS\ loop\ 3/4\ swap}$	$EndoS2_{43-97}$—$EndoS_{184-199}$—$EndoS2_{109-145}$—$EndoS_{236-252}$—$EndoS2_{158-787}$	<u>Reaction 1:</u> $EndoS2_{WT}$ <u>Reaction 2:</u> $EndoS2_{loop\ 3\ swap}$	<u>Reaction 1:</u> 1)TTGACAAAAAAGAAGATACAGCAGGCGTAGA ACGTGCTCTAAATGTTTTTAAAGAGATTGCGCA GTTAATAGG 2)TCTTCTTTTTTGTCAACTTTTGGAATACTATCG TGCTCAATATCAATATCTAGTCCATCGACACCA CGATCAG <u>Reaction 2:</u> 1)TGGGGGTGATAACAGTGGTATTGCAGAAGAT TCTAAAGATTATCCTGATACTCCTGAGGGGAAC AAAGC	FastCloning[1]

			2)ACTGTTATCACCCCCAGCTAGGAAACGCCATGGAATTGTCTGAACAAGTGCCGTTCCCTGTTGATG	
EndoS with EndoS2 CBM	$EndoS_{37-767}$—$EndoS2_{639-787}$—$EndoS_{921-995}$	<u>Reaction 1a:</u> $EndoS_{WT}$ <u>Reaction 1b:</u> $EndoS2_{WT}$	<u>Reaction 1a:</u> 1)AATATACCGAACTCCAAATTTTAGGTTATCCGTTACCTAACGCCGACACTATC 2)GCCCCTTTTGCCAAATTAACCATCATGGTTTTTTTCGTCACCAACAATCACTTTAG <u>Reaction 1b:</u> 1)ATTTGGCAAAAGGGGCTAAAGTGATTGGTACATCTGGGGACTTTGAGC 2)TGGAGTTCGGTATATTGAGGGTAATAAGAGCTAGCTCCACCATCTACAC	FastCloning[1]
EndoS with EndoS2 GH	$EndoS2_{42-386}$—$EndoS_{446-995}$	<u>Reaction 1a:</u> $EndoS2_{WT}$ <u>Reaction 1b:</u> $EndoS_{WT}$	<u>Reaction 1a:</u> 1)ATATACATATGGGAAAGACAGATCAGCAGGTTGGTGCTAAATTGG 2)TCAATCAGATCATAGCGTTTGTCTTCGGTCATCAATGTTTTTAATTTTCG <u>Reaction 1b:</u> 1)GCTATGATCTGATTGATGAGAAAGATTTCCCAGATAAGGCTTTGC 2)TTTCCCATATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTG	FastCloning[1]
EndoS with EndoS2 CBM and GH	$EndoS2_{42-386}$—$EndoS_{446-764}$—$EndoS2_{639-787}$—$EndoS_{921-995}$	<u>Reaction 1a:</u> $EndoS2_{WT}$ <u>Reaction 1b:</u> EndoS with EndoS2 CBM	<u>Reaction 1a:</u> 1)ATATACATATGGGAAAGACAGATCAGCAGGTTGGTGCTAAATTGG 2)TCAATCAGATCATAGCGTTTGTCTTCGGTCATCAATGTTTTTAATTTTCG <u>Reaction 1b:</u> 1)GCTATGATCTGATTGATGAGAAAGATTTCCCAGATAAGGCTTTGC 2)TTTCCCATATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTG	FastCloning[1]
$EndoS2_{W712A}$	-	$EndoS2_{WT}$	1)TCCAGTTAGTTTGTCCCGCAGTGAAGAATCTATCTGACTTTTCAC 2)GTGAAAAGTCAGATAGATTCTTCACTGCGGGACAAACTAACTGGA	PCR-based site-directed mutagenesis
$EndoS2_{F710S}$	-	$EndoS2_{WT}$	1)CAGTTAGTTTGTCCCCAAGTGCTGAATCTATCTGACTTTTCACC 2)GGTGAAAAGTCAGATAGATTCAGCACTTGGGGACAAACTAACTG	PCR-based site-directed mutagenesis
$EndoS2_{Y820S}$	-	$EndoS2_{WT}$	1)CGGTATATTGAGGGCTATAAGAGCTAGCTCCACCATCTAC 2)GTAGATGGTGGAGCTAGCTCTTATAGCCCTCAATATACCG	PCR-based site-directed mutagenesis
$EndoS2_{F710S/Y820S}$	-	$EndoS2_{F710S}$	1)CGGTATATTGAGGGCTATAAGAGCTAGCTCCACCATCTAC 2)GTAGATGGTGGAGCTAGCTCTTATAGCCCTCAATATACCG	PCR-based site-directed mutagenesis
$EndoS2_{E186L}$	-	$EndoS2_{WT}$	1)CTTTTGTTCGTAAATTCGTGCGCAATATCAATATCTAGTCCATCGAC 2)GTCGATGGACTAGATATTGATATTGCGCACGAATTTACGAACAAAAG	PCR-based site-directed mutagenesis

Supplementary Figures

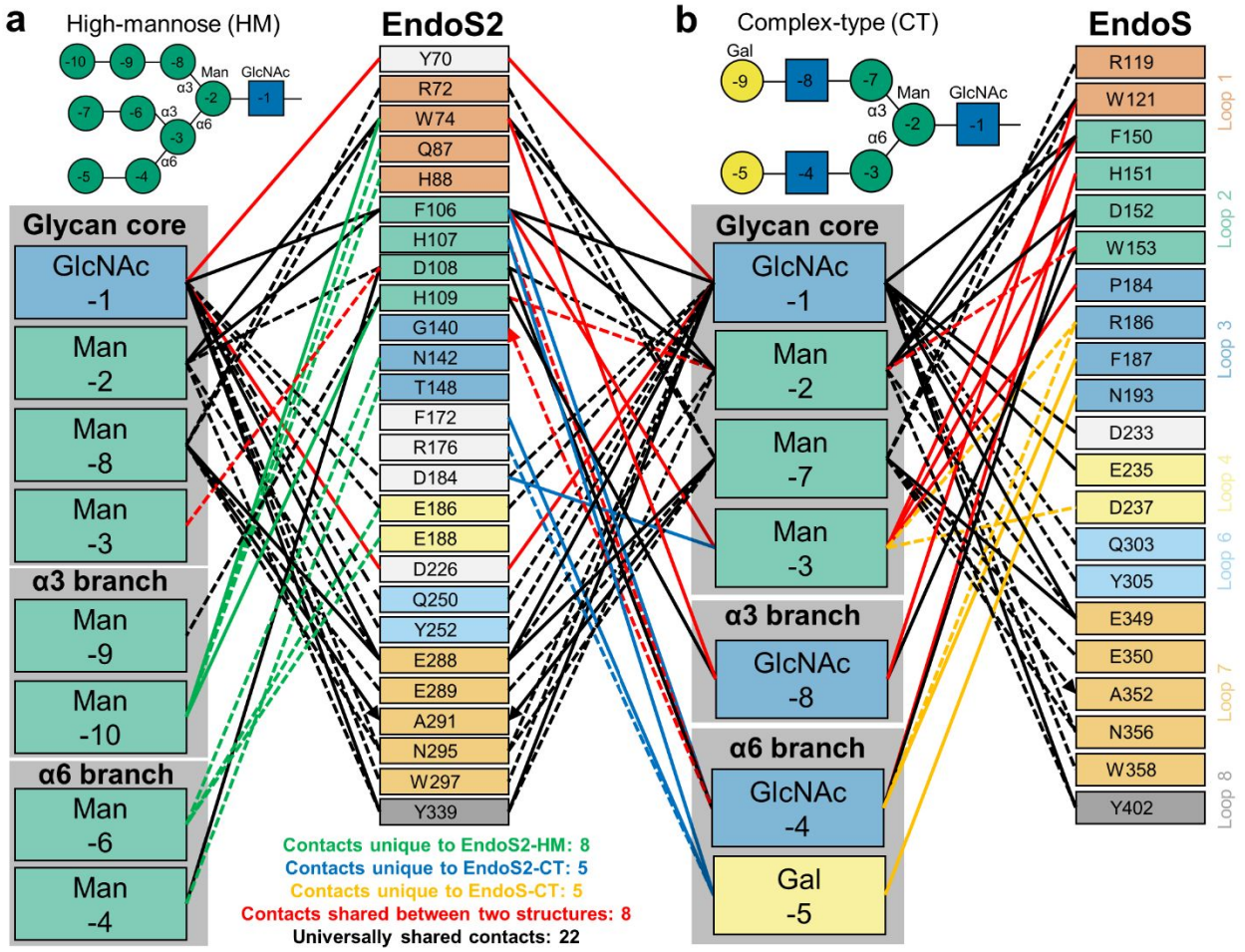
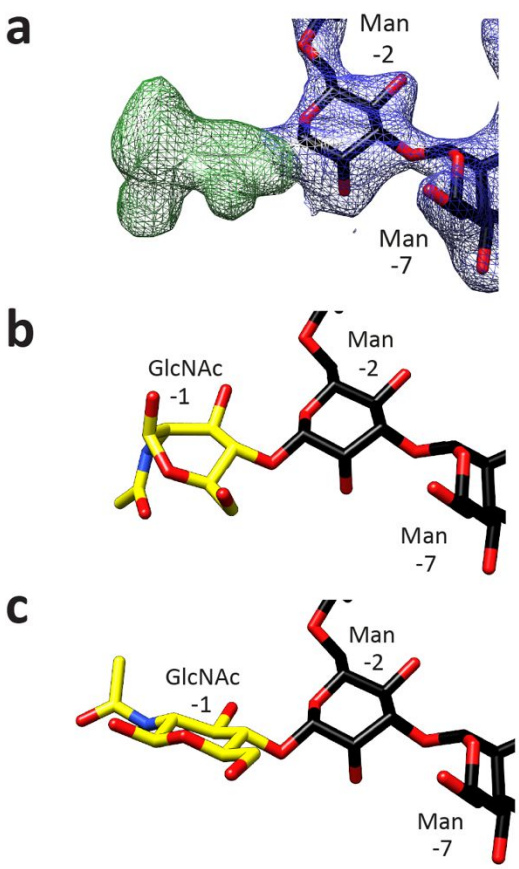


Figure S1. Diagram of contacts between EndoS, EndoS2, and substrates. A. All contacts made between EndoS2 and high-mannose glycan; **B.** All contacts made between EndoS2, EndoS and complex-type glycan. Solid lines represent van der Waals and hydrophobic interactions, while dashed lines represent hydrogen bonds and ionic interactions. Arrows represent contacts made with the enzyme backbone. Black lines are universally conserved contacts; red lines are shared between two structures; yellow lines are unique to EndoS-CT; blue lines are unique to EndoS2-CT; green lines are unique to EndoS2-HM. Enzyme residues are colored according to the loop they reside in.



d

Enzyme	Source	PDB CODE
ChtI	*Ostrinia furnacalis*	3WL1[1]
Chi60	*Moritella marina*	4HME[2]
YKL-39	*Homo sapiens*	4P8V[3]
CHTI	*Homo sapiens*	4WK9[4]
ChiW	*Paenibacillus sp.*	5GZV[5]
NCTU2	*Bacillus cereus*	3N17[6]
ChiA	*Serratia marcescens*	1K9T
ChiD	*Serratia marcescens*	4PTM
ChiB	*Serratia marcescens*	1UR8[7]
Chi3L1	*Bubalus bubalis*	4MTV
SGP-40	*Capra hircus*	2AOS
Chitinase	*Ostrinia furnacalis*	5WVH
AcMNPV	*ACNPV*	5DEZ

Figure S2. Conformation of the GlcNAc (-1) in the EndoS2-HM complex structure. (a) Final electron density maps ($2mF_o$-DF_c contoured at 1σ [blue] and mF_o-DF_c at 3σ [green]) corresponding to the HM glycan. **(b)** Boat conformation of the GlcNAc -1 found in the crystal structure of EndoS2-HM complex **(c)** Chair conformation of the GlcNAc **(d)** Representative enzymes of the family GH18[2-8] that present a "skew-boat" conformation in the enzyme-product X-ray crystal structures. A substrate-assisted mechanism with retention of the anomeric configuration has been described for the enzymes of the GH18 family[9-13]. First, the protonation of the anomeric oxygen by an acidic residue (E186 in EndoS2) facilitates the nucleophilic attack of the carbonyl group of the 2-acetamide group of the GlcNAc (-1) to the anomeric carbon forming an oxazoline intermediate. Secondly, a water molecule, deprotonated by the same residue that acts as an acid in the previous step, hydrolyses the oxazoline intermediate. During the catalytic cycle diverse conformational changes of the GlcNAc (-1) has been described[14]. The observed "skew-boat" conformation of GlcNAc (-1) observed in the EndoS2-HM structure corresponds to the step after the nucleophilic attack of the water molecule and before releasing the glycan product.

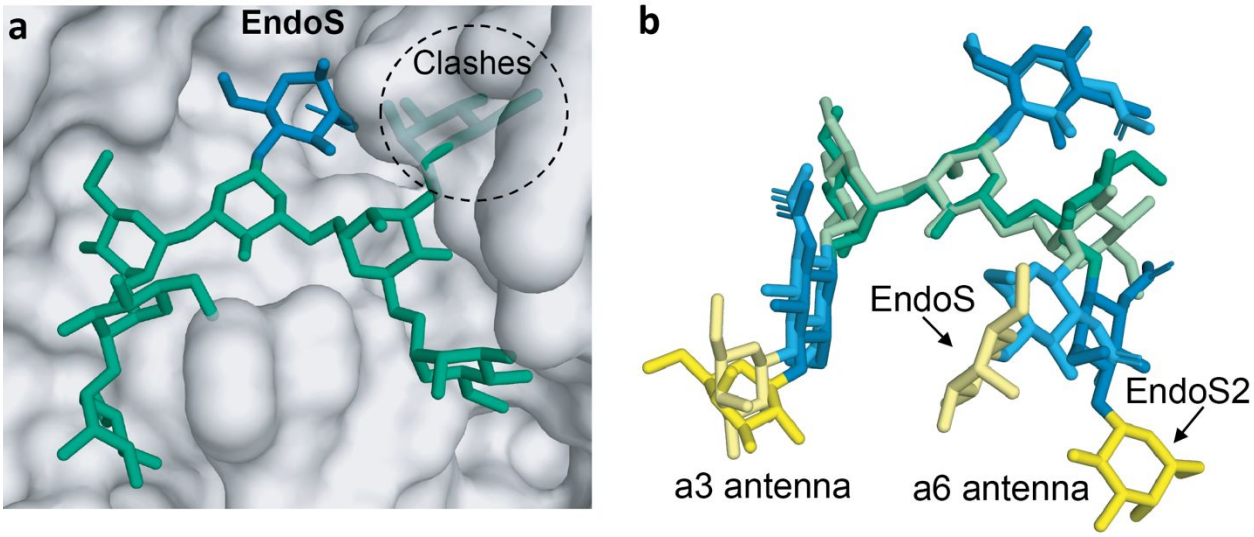


Figure S3. Structural comparison of EndoS2 and EndoS active sites. (a) Overlay of the high-mannose glycan from EndoS2 modelled into the EndoS active site (PDB 6EN3), highlighting clashes formed between the α6 antenna and loops 3 and 4 of EndoS. **(b)** Overlay of the complex-type glycans bound in the EndoS versus EndoS2 co-crystal structures, highlighting extreme similarity in the core and α3 antenna, with slight differences in α6 antenna.

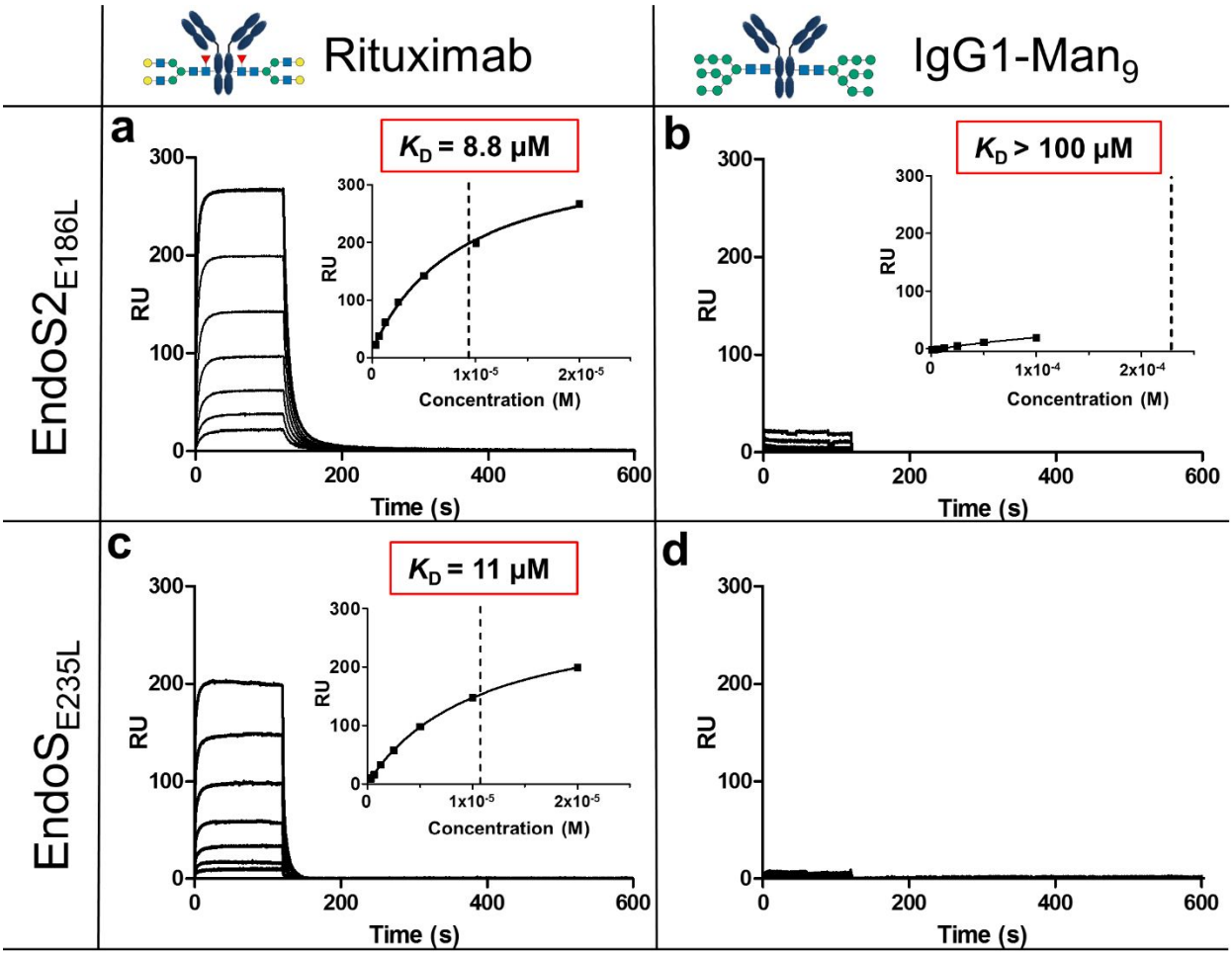
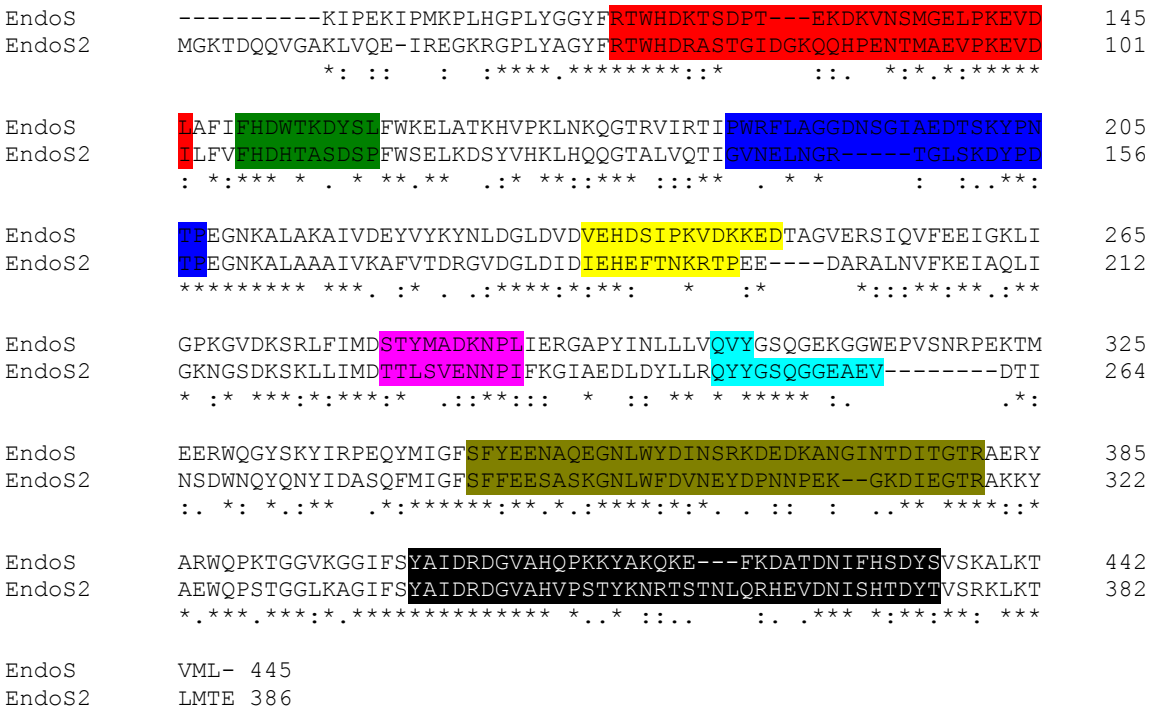


Figure S4. Surface plasmon resonance of catalytically dead EndoS and EndoS2 with complex-type and high-mannose substrates. (a) EndoS2 with Rituximab (IgG1-CT) and **(b)** IgG1-Man_9. **(c)** EndoS with Rituximab and **(d)** IgG1-Man_9.



<u>Figure S5.</u> Sequence alignment of EndoS and EndoS2 GH domains. GH domain loops are colored as followed: loop 1, red; loop 2, green; loop 3, blue; loop 4, yellow; loop 5, pink; loop 6, cyan; loop 7, olive; loop 8/9, black. Alignment made using Clustal Omega.

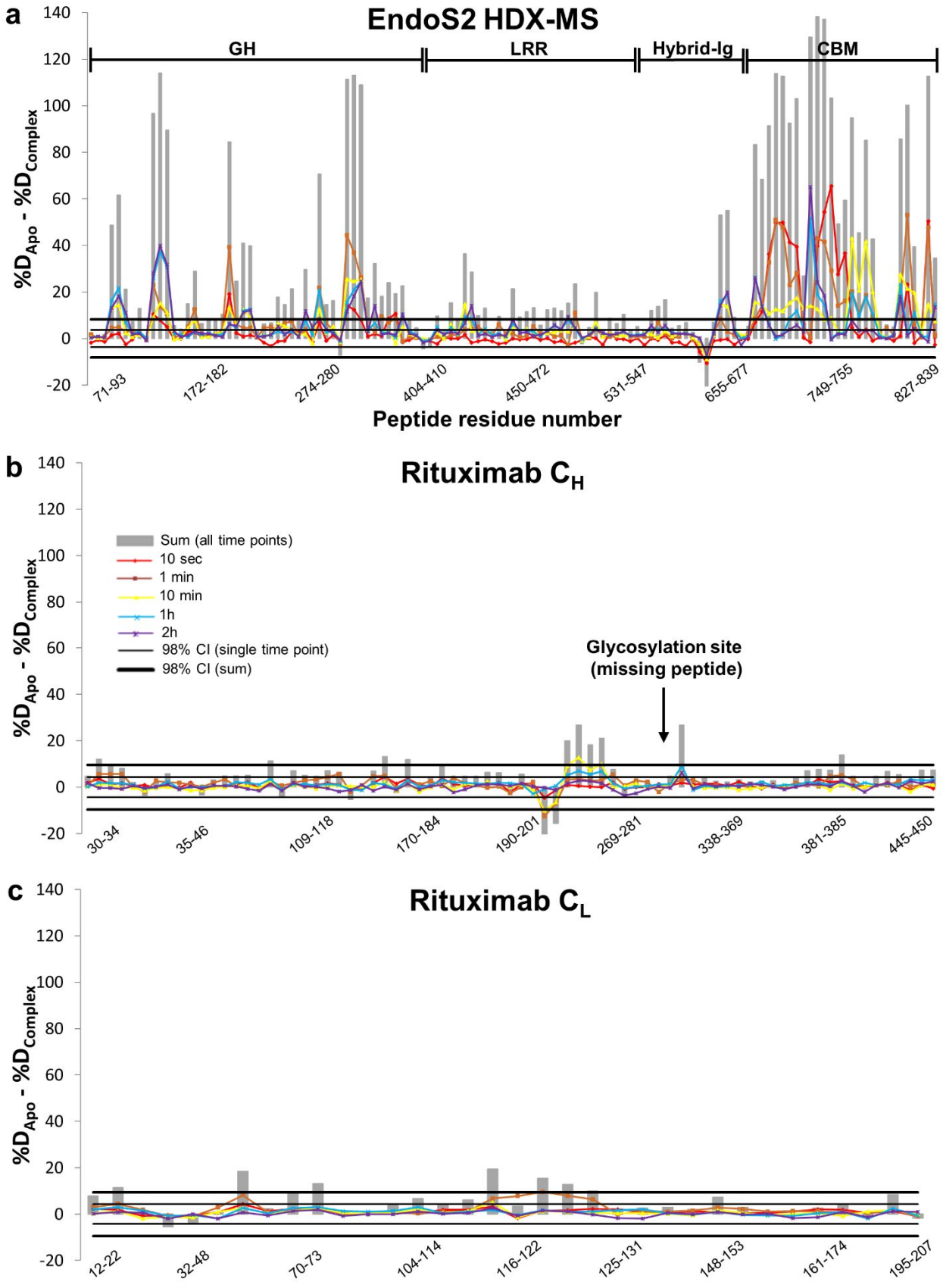

a
EndoS2 HDX-MS
GH
LRR
Hybrid-Ig
CBM
%D_Apo - %D_Complex
Peptide residue number
71-93
172-182
274-280
404-410
450-472
531-547
655-677
749-755
827-839
b
Rituximab C_H
Sum (all time points)
10 sec
1 min
10 min
1h
2h
98% CI (single time point)
98% CI (sum)
Glycosylation site
(missing peptide)
30-34
35-46
109-118
170-184
190-201
269-281
338-369
381-385
445-450
c
Rituximab C_L
12-22
32-48
70-73
104-114
116-122
125-131
148-153
161-174
195-207

Figure S6. Hydrogen-deuterium exchange mass spectrometry. (a) Differences in percent deuteration between peptides from EndoS2$_{E186L}$ in the unliganded and IgG1-complexed state. Glycoside hydrolase (GH), leucine-rich repeat (LRR), hybrid-Ig, and carbohydrate-binding module (CBM) domains are annotated. (**b**) Differences in deuteration between peptides from IgG1 heavy chain and **(c)** light chain in the unliganded and EndoS2$_{E186L}$-complexed state. Data could not be obtained for the annotated glycosylation site. (**a-c**) Individual peptides are plotted on the x-axis from N- to C-terminus based on the sequence number of the first residue in the peptide. For each peptide, differences in percent deuteration at individual time points are color coded according to the legend, with the sum of the differences over all time points plotted as grey bars. 98% confidence intervals for individual time points and sums are plotted as thin and thick black lines, respectively.

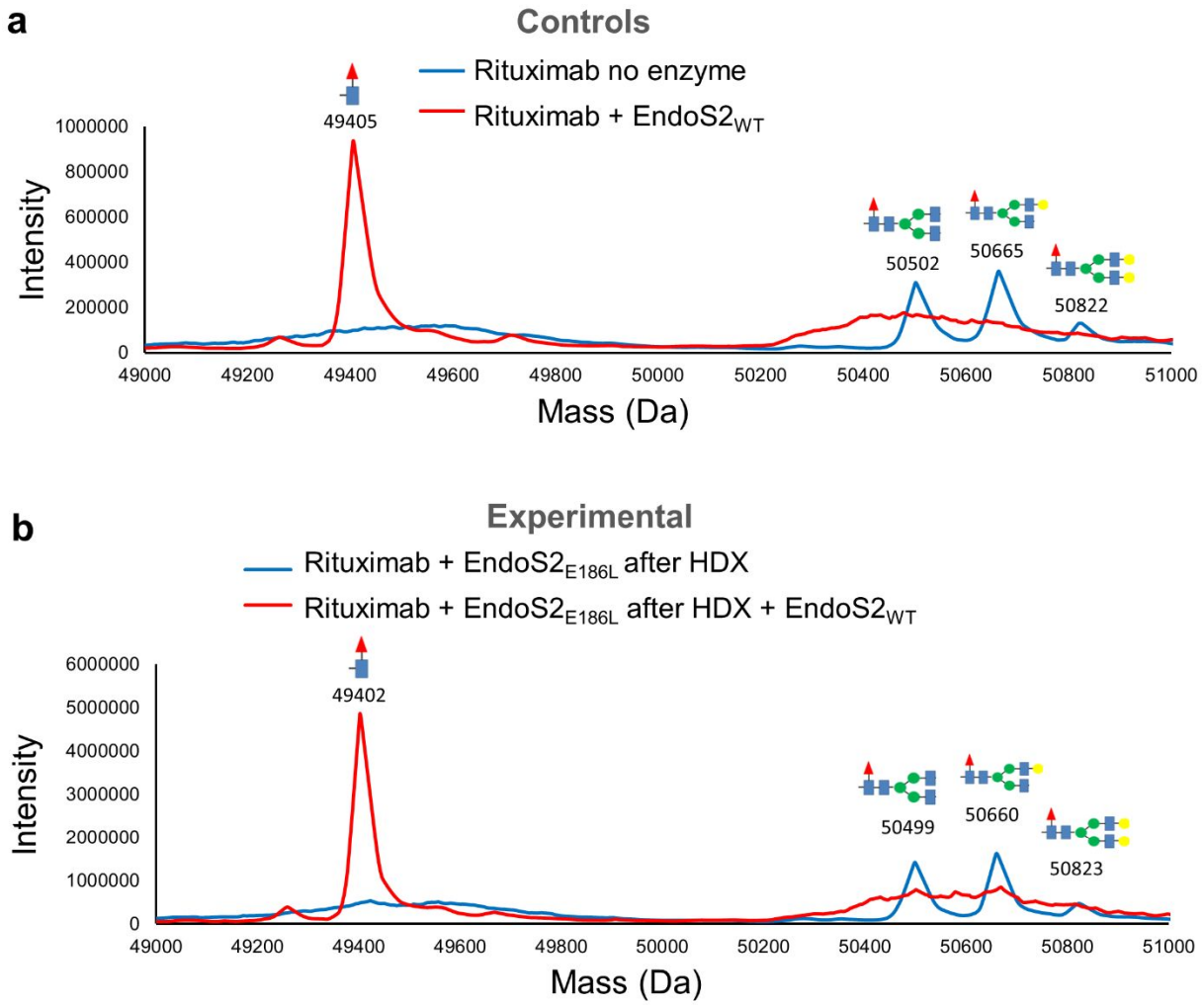


Figure S7. Mass spectrometry of Rituximab glycosylation. (a) Rituximab before (blue) and after (red) treatment with EndoS2$_{WT}$. **(b)** Samples that were subjected to hydrogen-deuterium exchange with catalytically inactive EndoS2$_{E186L}$ were analyzed for deglycosylation (blue). The same samples after addition of EndoS2$_{WT}$ (red).

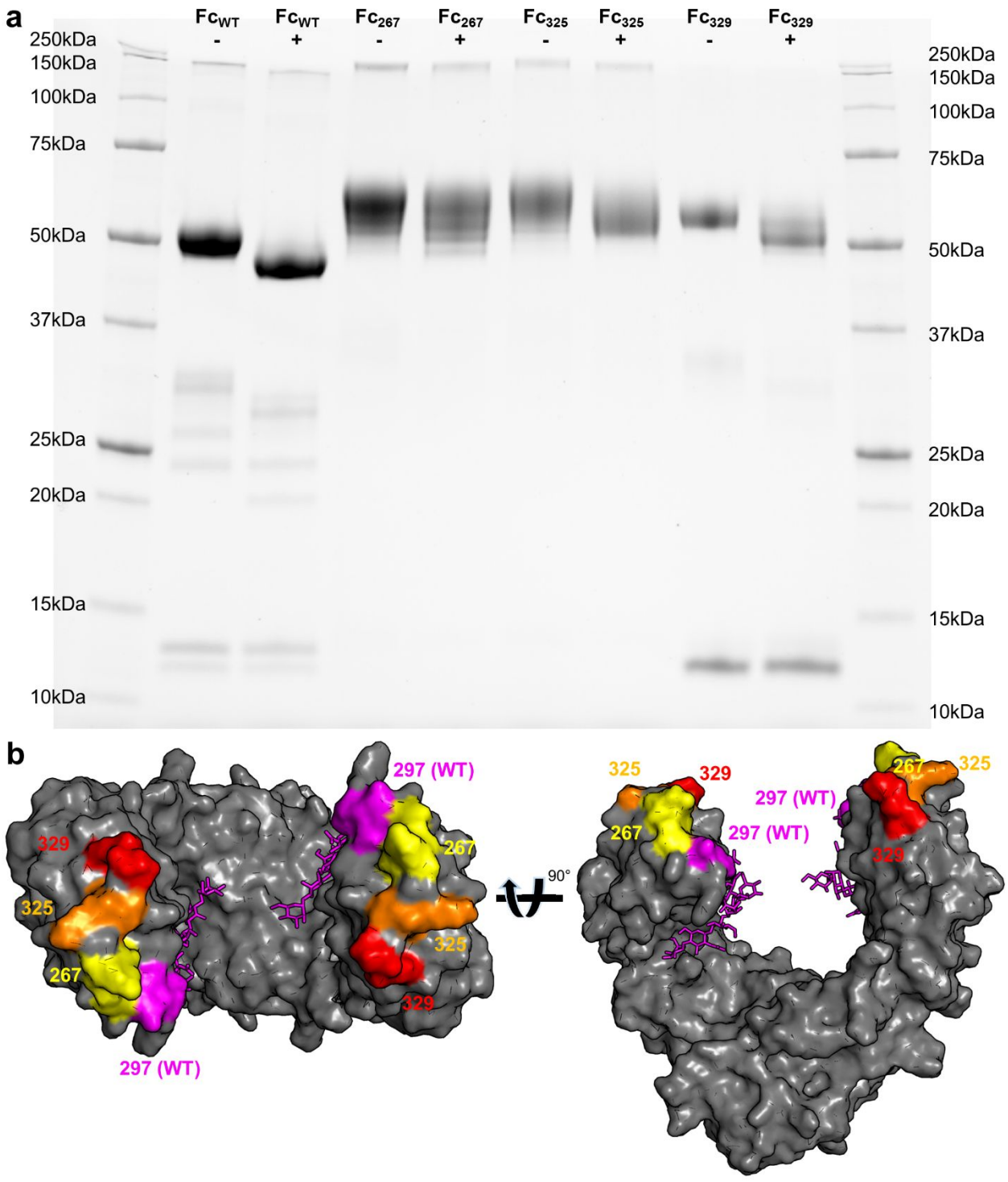


Figure S8. EndoS2 activity on hyper-glycosylated Fc mutants. (a) One additional glycosylated site was added to wild-type IgG1 Fc at position 267, 325, or 329, and treated with either PBS (-) or $EndoS2_{WT}$ (+) for one week. **(b)** Glycosylation sites are mapped onto the crystal structure of IgG1 Fc (PDB 5JII)[15] with the wild-type N297 site displayed in magenta, and artificial sites displayed in yellow (N267), orange (N325) and red (N329).

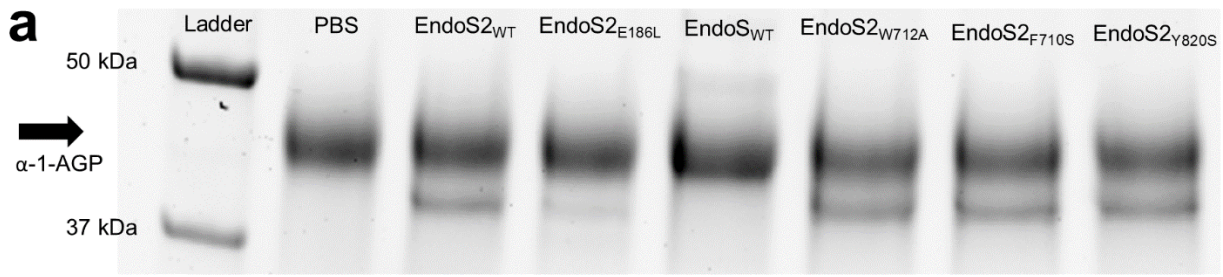


Figure S9. EndoS2 activity on α-1-AGP. (a) 8 µg α-1-AGP was mixed with 4 µg enzyme in 20 µL PBS for 48 hours at 37°C, and analyzed by SDS-PAGE.

Supplementary References

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