

SUPPLEMENTARY INFORMATION

Mechanism of antibody-specific deglycosylation and immune evasion by *Streptococcal* IgG-specific endoglycosidases

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Supplementary Table 1. CryoEM data collection, refinement and validation statistics

	EndoS_{E235A}-Fc
PDB ID	8A64
Data collection	
Microscope	Titan Krios
Voltage (kV)	300
Detector	K2 Summit
Data collection software	EPU software (FEI)
Energy Filter	GIF Quantum
Magnification	130,000
Electron dose (e ⁻ /Å ²)	59.37
Pixel size (Å)	1.052
Defocus range (µm)	-1.3 to -2.5
Frames	60
Movie number	5546
Data processing	
Processing software	Relion 3.1.2/cryoSPARC v3.2.0
Number of extracted particles	339309
EMDB code	15205
Map resolution (Å) at: FSC=0.143 (unmasked/masked)	5.2/4.53
Map sharpening B factor (Å)	-330.0
Refinement	
Software	PHENIX v1.18.2
Models used (PDB code)	6EN3 & 1H3X
Atoms (Non-H)	6925
Protein residues	1354
Ligands	3
Bonds (RMSD)	
Length (Å)	0.009
Angles (°)	1.266
Mean B-factor	
Protein	196.28
Ligand	135.89
MolProbity score	2.79
Clashscore	20.58
Rotamer outliers (%)	5.9
Ramachandran plot	
Favored (%)	94.1
Allowed (%)	5.9
CC (volume)	0.74
Mean CC for ligands	0.64
CC (mask)	0.74

Supplementary Table 2. SAXS data collection and refinement parameters.

Data collection parameters	EndoS _{E235A} -Fc	EndoS _{2E186L} -Fc	EndoS	EndoS2	Fc
Instrument	B21 (DLS)	B21 (DLS)	B21 (DLS)	B21 (DLS)	B21 (DLS)
Wavelength (Å)	0.99	0.99	0.99	0.99	0.99
S range (Å ⁻¹)	0.002-0.420	0.002-0.420	0.002-0.420	0.002-0.420	0.002-0.420
Exposure time (s per frame)	0.56	0.56	0.56	0.56	0.56
Concentration range (μM)	55	55	55	55	55
Temperature (K)	298	298	298	298	298
Structure parameters					
<i>I</i> (0) (a.u.) ¹ (from <i>P</i> (<i>r</i>))	0.004	0.007	0.009	0.005	0.003
<i>R_g</i> (Å) (from <i>P</i> (<i>r</i>))	53.7	52.2	43.0	38.7	26.8
<i>I</i> (0) (a.u.) ¹ (from Guinier)	0.004	0.007	0.009	0.005	30.95
<i>R_g</i> (Å) (from Guinier)	53.6	52.2	42.9	38.7	26.8
<i>D_{max}</i> (Å)	182	176	148	125	80
Porod volume estimate (Å ³)	298375	324331	185228	168964	111242
Dry volume calculated from sequence (Å ³) ²	187759	170507	127075	109823	60706
Molecular mass determination³					
Molecular weight (kDa)	211.3	234.1	111.9	87.0	52.6
Calculated MW from sequence (kDa)/ Discrepancy (%): Monomeric proteins	-	-	105.0/6.5	90.7/4.1	50.1/4.9
Enzyme:substrate (1:1)	155.1/36.7	140.8/40	-	-	-
Enzyme:substrate (2:1)	260.1/26.5	231.5/1.1	-	-	-
OLIGOMER: χ^2 value	1.18	1.09	-	-	-
% of enzyme:substrate (1:1)	29	34	-	-	-
% of enzyme:substrate (2:1)	67	49	-	-	-
χ^2 value MONSA	2.4	0.9	1.8	1.9	1.6
Software employed					
Primary data reduction	SCATTER	SCATTER	SCATTER	SCATTER	SCATTER
Data processing	PRIMUS/ SCATTER	PRIMUS/ SCATTER	PRIMUS/ SCATTER	PRIMUS/SC ATTER	PRIMUS/S CATTER

¹ arbitrary units

² Dry volume determined using the server: <http://biotools.nubic.northwestern.edu/proteincalc.html>

³ Molecular weight determination by SAXSMoW2

Supplementary Table 3. Dissociation constants K_D , on- and off-rate constants k_{on} and k_{off} for CT, Rituximab-Fc, HM and Ca^{2+} binding to EndoS2_{E186L} and EndoBT_{D312A/E314L} in the presence of constant additives from fitting a two-state binding model to methyl-TROSY titration data using TITAN algorithm.

N°	Enzyme	Ligand	Additives ¹	K_D (μM)	k_{off} (s^{-1})	k_{on} ($\text{M}^{-1}\text{s}^{-1}$)
1	EndoS2 _{E186L}	CT	CaCl_2	111.2 ± 9.7	11.7 ± 1.9	$1.1 \times 10^5 \pm 2.0 \times 10^4$
2		Fc	CaCl_2	3.8 ± 1.0	4.5 ± 1.8	$1.2 \times 10^6 \pm 5.7 \times 10^5$
3		CaCl_2	CT	4100 ± 300	-	-
4		CaCl_2	Fc	-	-	-
5	EndoBT _{D312A/E314L}	HM	CaCl_2	0.4 ± 0.3	2.4 ± 1.1	$5.5 \times 10^6 \pm 4.8 \times 10^5$
6	EndoBT _{D312A/E314L}	CT	-	>755 (if present)	-	-

¹ Samples containing additives were prepared at saturating concentrations and diluted a maximum of 10% during the course of the titrations. Final additive concentrations were: 20 mM CaCl_2 , 830 μM CT, 56 μM Fc or 72 μM Fc aglycan in samples containing EndoS2_{E186L}, and 291mM CaCl_2 in samples containing EndoBT_{D312A/E314L}.

Supplementary Table 4. Precursors and quantities used for the preparation of [*U*-¹⁵N,²H] MIL^{proS}V^{proS} [¹³C,¹H₃]-methyl labeled endoglycosidases. A systematic review on precursors required for optimal methyl group isotope labelling in *E. coli* can be found somewhere else¹.

Labeled amino acid	Precursor	Quantity (mg/L)
Met-ε	L-Methionine (6- ¹³ C) ^a	250
Ile-δ1	2-ketobutyric acid (4- ¹³ C;3,3-D ₂)	60
Leu-δ2, Val-γ2 pro-(<i>S</i>)	2-hydroxy-2-(¹³ C)methyl-3-oxobutanoate (4-D ₃) ^b	120

Obtained from ^aAldrich, ^bNMR-Bio. 2-ketobutyric acid (4-¹³C;3,3-D₂) was synthesized in-house.

Supplementary Table 5. List of NMR experiments and experimental details used for the assignment of CT, CTn-1 and HM *N*-glycans. Experiments were acquired at 310 K.

Experiment	Pulse program from Bruker library	NS	TD	SW (ppm)	Carrier F2/F1 (ppm)	Observations
¹ H	zgesgp	64	32768	11.65	4.7	Water suppression with Squa100.1000 shaped pulse, 8 ms length to minimise suppression of anomeric signals ² .
[¹ H, ¹³ C]-Edited-HSQC	hsqcedetgpsisp2.2	32	2048x256	12.5x146	4.7/86	$J_{CH} = 145$ Hz
[¹ H, ¹ H]-NOESY	noesygp.php	16	2048x256	10x10	4.7/4.7	700 ms mixing time
[¹ H, ¹ H]-TOCSY	mlevgp.php19	16	2048x256	10x10	4.7/4.7	13 ms or 200 ms mixing times. Distance between nulls = 5.2 ppm
[¹ H, ¹³ C]-HMBC	hmbcgp1pndqf	128	2048x240	12x92	4.7/66	Long range $J_{CH} = 6$ Hz
[¹ H, ¹³ C]-HMQC-COSY	h2bcetgp13	328	2048x302	12x92	4.7/66	J_{CH} min - max = 129 - 190 Hz

Recovery delay was set to 1.5 – 2.3 s in all experiments.

Supplementary Table 6. Glycan $^1\text{H}/^{13}\text{C}$ chemical shifts of CT, CT_{n-1} and HM N-glycans (referenced to TSP). Experiments were acquired at 310 K.

Residue with residue number	C1/ H1	C2/ H2	C3/ H3	C4/ H4	C5/ H5	C6/ H6a;H6b	Acetyl-CH ₃	C α / H α	C β / H β a;H β b
CT									
Asn	-	-	-	-	-	-	-	53.8/ 3.98	38.0/ 2.94;2.86
GlcNAc 1	80.9/ 5.08	56.4/ 3.87	75.6/ 3.78	81.6/ 3.66	79.0/ 3.60	62.8/ 3.84;3.66	24.9/ 2.02	-	-
GlcNAc 2	104.0/ 4.66	57.8/ 3.79	74.7/ 3.80	82.2/ 3.74	77.3/ 3.63	62.8/ 3.89;3.76	25.0/ 2.09	-	-
Man 3	103.1/ 4.78	72.9/ 4.25	83.3/ 3.79	68.5/ 3.79	77.3/ 3.62	68.7/ 3.97;3.81	-	-	-
Man 4	102.3/ 5.14	79.3/ 4.20	72.2/ 3.91	70.1/ 3.51	75.8/ 3.63	64.4/ 3.92;3.64	-	-	-
Man 4'	99.8/ 4.94	79.2/ 4.12	72.2/ 3.91	70.1/ 3.51	75.8/ 3.63	64.4/ 3.92;3.64	-	-	-
GlcNAc 5/5'	102.3/ 4.60	57.6/ 3.75	74.9/ 3.75	81.5/ 3.74	77.5/ 3.59	62.8/ 3.99;3.86	25.1/ 2.06	-	-
Gal 6/6'	105.7/ 4.48	73.7/ 3.56	75.4/ 3.68	71.3/ 3.95	76.3/ 3.76	63.7/ 3.77;3.77	-	-	-
CT_{n-1}									
α -GlcNAc 2	93.2/ 5.21	56.4/ 3.90	72.5/ 3.93	82.7/ 3.91	77.1/ 3.62	62.1/ 3.73;3.73	24.6/ 2.06	-	-
β -GlcNAc 2	97.7/ 4.72	58.9/ 3.71	72.5/ 3.93	82.7/ 3.91	77.1/ 3.62	62.8/ 3.79;3.77	24.8/ 2.06	-	-
Man 3	103.2/ 4.77	73.0/ 4.24	83.2/ 3.77	68.2/ 3.79	77.1/ 3.62	68.6/ 3.96;3.79	-	-	-
Man 4	102.2/ 5.12	79.3/ 4.19	72.2/ 3.90	70.1/ 3.50	76.3/ 3.75	64.4/ 3.91;3.62	-	-	-
Man 4'	99.8/ 4.92	79.2/ 4.11	72.2/ 3.90	70.1/ 3.50	75.6/ 3.71	64.4/ 3.91;3.62	-	-	-
GlcNAc 5/5'	102.2/ 4.58	57.6/ 3.74	74.9/ 3.72	81.4/ 3.72	77.4/ 3.57	62.8/ 3.98;3.84	25.1/ 2.05	-	-
Gal 6/6'	105.7/ 4.47	73.8/ 3.54	75.3/ 3.66	71.3/ 3.93	78.1/ 3.72	63.7/ 3.75;3.75	-	-	-
HM									
Asn	-	-	-	-	-	-	-	53.8/ 3.99	38.0/ 2.93;2.88
GlcNAc 1	80.8/ 5.09	56.3/ 3.87	75.0/ 3.79	81.5/ 3.66	79.1/ 3.61	62.8/ 3.85;3.66	24.9/ 2.03	-	-
GlcNAc 2	104.0/ 4.63	57.8/ 3.79	75.0/ 3.79	81.9/ 3.74	77.2/ 3.61	62.8/ 3.89;3.74	25.0/ 2.08	-	-
Man 3	103.0/ 4.78	73.1/ 4.23	84.0/ 3.74	68.3/ 3.91	77.2/ 3.61	68.11/ 4.03;3.75	-	-	-
Man 4	103.5/ 5.34	81.3/ 4.12	72.8/ 4.01	69.7/ 3.77	76.1/ 3.82	63.8/ 3.90;3.77	-	-	-
Man 4'	102.4/ 4.88	81.6/ 3.90	81.5/ 3.90	67.8/ 3.88	74.2/ 3.85	68.7/ 3.95;3.80	-	-	-
Man 5	103.3/ 5.31	81.3/ 4.12	72.8/ 4.01	69.7/ 3.71	76.1/ 3.82	63.8/ 3.90;3.77	-	-	-
Man 5'	103.3/ 5.41	81.3/ 4.12	72.9/ 3.96	69.7/ 3.71	76.1/ 3.82	63.8/ 3.90;3.77	-	-	-
Man 5''	100.9/ 5.14	81.3/ 4.03	72.9/ 3.96	69.7/ 3.71	75.5/ 3.71	63.8/ 3.90;3.77	-	-	-
Man 6/6'/6''	105.0/ 5.07	72.8/ 4.09	73.2/ 3.86	69.6/ 3.67	76.0/ 3.78	63.8/ 3.90;3.77	-	-	-

Supplementary Table 7. Example of shell script for NMR data processing with NMRPipe

```
#!/bin/csh

foreach spec (1 3 5 7 9 11 12 13 14 15)
cd spec/$spec
bruk2pipe -in ./ser \
    -bad 0.0 -ext -aswap -AMX -decim 8330.666666666667 -dspfvs 20
-grpdly 67.9856719970703 \
    -xN          512 -yN          256 \
    -xT          256 -yT          128 \
    -xMODE       DQD -yMODE       States-TPPI \
    -xSW         2400.768 -ySW         3320.053 \
    -xOBS        600.230 -yOBS        150.931 \
    -xCAR        0.8578 -yCAR        19.7253 \
    -xLAB         1H -yLAB         13C \
    -ndim         2 -aq2D          TPPI \
    -out ./test.fid -verb -ov

nmrPipe -in test.fid \
| nmrPipe -fn EM -lb 1.0 -c 1.0 \
| nmrPipe -fn ZF -auto \
| nmrPipe -fn FT -auto \
| nmrPipe -fn PS -p0 62 -p1 0 -di -verb \
| nmrPipe -fn BASE -nw 5 -nl 0% 2% 98% 100% \
| nmrPipe -fn CS -rs 0Hz -sw \
| nmrPipe -fn EXT -x1 -0.3ppm -xn 2.4ppm -sw \
| nmrPipe -fn TP \
| nmrPipe -fn EM -lb 1.0 -c 1.0 \
| nmrPipe -fn ZF -auto \
| nmrPipe -fn FT -auto \
| nmrPipe -fn PS -p0 0 -p1 0 -di -verb \
| nmrPipe -fn BASE -nw 2 -nl 0% 2% 98% 100% \
| nmrPipe -fn CS -rs 0Hz -sw \
    -ov -out ../test-$spec.ft2
rm test.fid
cd ..
cd ..
end
```

Supplementary Note 1

Structural analysis of EndoS and EndoS2 in complex with the Fc region of Rituximab in solution by small angle X-ray scattering

To study the overall structure of EndoS and EndoS2 in complex with the Fc region of IgG1 in solution, we first performed in-line size-exclusion chromatography small-angle X-ray scattering (SEC-SAXS) (Supplementary Figs. 1 and 6; Supplementary Table 2). We purified the catalytically inactive EndoS_{E235A} and EndoS2_{E186L} variants and the Fc region of Rituximab, a chimeric IgG1 used to treat B cell lymphoma, by papain digestion. We mixed the EndoS_{E235A} or EndoS2_{E186L} with the Fc region substrate in a 1:5 ratio and we isolated the enzyme-substrate complexes (EndoS_{E235A}-Fc and EndoS2_{E186L}-Fc) from the free enzymes and substrate by SEC chromatography and conducted SAXS analyses of the individual proteins and the complexes (Supplementary Fig. 6). Comparison of the R_g shows an increase when EndoS_{E235A}-Fc ($R_g = 53.6 \text{ \AA}$) and EndoS2_{E186L}-Fc ($R_g = 52.2 \text{ \AA}$) complexes are formed compared to the unliganded EndoS_{E235A} ($R_g = 42.9 \text{ \AA}$) and EndoS2_{E186L} ($R_g = 38.7 \text{ \AA}$) (Supplementary Table 2). In addition, examination of the $P(r)$ -distribution shows that the maximum dimension, D_{max} , also increases when the enzyme-substrate is formed, from 148 $\text{\AA}$ to 181 $\text{\AA}$ in the case of EndoS and from 125 $\text{\AA}$ to 176 $\text{\AA}$ in the case of EndoS2. The resulting $P(r)$ function profile of both enzyme-substrate complexes exhibit a unimodal distribution of real-spaces distances that is characteristic of a globular protein, while the $P(r)$ function profiles of the unbound EndoS and EndoS2 are bimodal consistent with the overall “V” shape of the crystal structures of both enzymes (Supplementary Fig. 6). Thus, the change from a bimodal to a monomodal distribution in the enzyme-substrate complexes strongly suggests that the Fc might bind to the cleft of the EndoS or EndoS2, promoting a more compact, globular state. The experimental determination of the EndoS_{E235A}-Fc complex MW³ (Supplementary Table 2) shows a 36.7% and a 26.5 % discrepancy to the calculated MW of the enzyme-substrate complex with 1:1 and 2:1

stoichiometries, respectively. In the case of EndoS2_{E186L}-Fc complex, the experimental determination of the MW from the SAXS data shows a 40 % and 1.1 % discrepancy to the calculated MW of the enzyme-substrate complex with 1:1 and 2:1 stoichiometries (Supplementary Table 2). These results, together with the fact that the R_g values are decreasing across the SEC peak of the enzyme-substrate complexes (Supplementary Fig. 6) indicate that a mixture of complexes with different stoichiometries might be present in that SEC peak. Therefore, we used OLIGOMER⁴ to calculate the volume fractions of the complexes with different stoichiometry contributing to the scattering profile. In the case of the mixture EndoS_{E235A}-Fc, the results showed that the enzyme-substrate 2:1 and 1:1 complexes contributed 67% and 29% volume fractions, respectively, to the scattering. Similar ratio of volume fractions was found for the EndoS2_{E186L}-Fc mixture (Supplementary Table 2).

Supplementary Note 2

Extended NMR results

To shine more light on the interaction of endo- β -*N*-acetylglucosaminidases (ENGases) with their natural ligands, we have employed NMR-based chemical shift perturbation (CSP) experiments⁵. CSPs are usually extracted from uniformly ^2H , ^{15}N -labeled proteins applying ^1H , ^{15}N Transverse Relaxation Optimized Spectroscopy (TROSY) Heteronuclear Single Quantum Coherence (HSQC) spectroscopy. However, at a molecular weight of 97 kDa ^1H , ^{15}N HSQC-TROSY spectra of EndoS2 suffered from incomplete deuterium-hydrogen back exchange of backbone H^{N} . The difficulty becomes evident when comparing ^1H , ^{15}N HSQC-TROSY spectra of EndoS2 with those acquired from EndoBT-3987 glycosidase^{6,7}, the latter having a molecular weight of “only” 47 kDa (Supplementary Fig. 15A,B). We have previously shown that deuterium-hydrogen back exchange is crucial to extract reliable information from ^1H , ^{15}N HSQC-TROSY spectra⁸. Yet, all attempts to develop an efficient unfolding-refolding protocol for complete deuterium-hydrogen back exchange of backbone H^{N} in ENGases failed. Therefore, we turned to specific ^{13}C side chain methyl group labeling strategies^{1,9,10}, which enabled the acquisition of ^1H , ^{13}C Heteronuclear Multiple Quantum Correlation (HMQC) spectra, also known as methyl-TROSY¹¹. Such approach grants the extraction of precise CSPs upon, e.g., ligand binding and provides experimental access to binding thermodynamics and kinetics^{1,5}. Knowledge of the corresponding on- and off-rate constants would provide a link to the conformational changes associated with substrate binding as observed by cryoEM and H/D exchange,² assisting the mechanistic understanding of ENGases.

NMR lineshape analysis has been used in numerous instances for the evaluation of chemical shift titration experiments, yielding quantitative descriptions of chemical exchange processes such as conformational equilibria or binding of ligands to receptor proteins¹². A novel approach for analyzing CSP titration data from HSQC- and HMQC-type 2D NMR

spectra engages direct quantum mechanical simulation of a given pulse sequence, as it is implemented in the TITAN (TITration ANalysis) software package¹³. Here, we have used TITAN to analyze the kinetics of binding of EndoS2 to its substrate CT, Rituximab-Fc, the reaction product CT_{n-1} and Ca²⁺ ions. The interaction of the related EndoBT-3987 with HM was also studied and used as comparison.

The isotope labeling scheme selected for this study was [*U*-¹⁵N,²H] MIL^{proS}V^{proS} [¹³C,¹H₃]-methyl labelling (or simply MILV). Isotopically labeled ENGases were the catalytically inactive EndoS2_{E186L} and EndoBT_{D312A/E314L}, and their corresponding wildtype EndoS2 and EndoBT species^{2,6}. This labeling scheme rendered methyl-TROSY spectra with little to no signal overlapping and excellent signal-to-noise (Supplementary Fig. 15C,D). In addition, the selected methyl probes are uniformly distributed around the putative glycan binding pockets in both enzymes, granting the extraction of CSPs upon ligand titration (Fig. 6A; Supplementary Fig. 16A). ¹H, ¹⁵N-TRACT experiments¹⁴ were used to estimate rotational correlation times τ_c , affording values of 45.0 ns and 21.8 ns for EndoS2_{E186L} and EndoBT_{D312A/E314L}, respectively, in good agreement with their corresponding molecular weights (Supplementary Fig. 21). Taken together with the good signal dispersion observed in NMR spectra, τ_c values evidence the presence of folded protein in solution enabling protein-ligand interaction studies.

At the present time no resonance assignment is available for ENGases. Therefore, we avoided any attempt to use CSPs for epitope mapping. Instead, we focused our efforts on the quantification of the overall thermodynamic and kinetic binding parameters for the interaction with selected ligands. For such goal it is enough to identify a subset NMR signals reporting for the binding event of interest. Here, methyl signals associated with methyl groups in the vicinity of the glycan binding site were identified according to the following two-step exclusion criteria: a) Signals must show CSPs between the wild-type species and the corresponding enzymatically

inactive mutants (*i.e.* EndoS2 vs EndoS2_{E186L} and EndoBT-3987 vs EndoBT-3987_{D312A/E314L}). Such mutations were designed to render inactive enzymes while retaining the protein folding and protein dynamics associated with ligand recognition, as previously disclosed by crystallographic and H/D exchange MS studies^{2,6}. Therefore, observed CSPs can be linked to changes localized in the vicinity of the carbohydrate binding pockets, even in the event of long-range effects^{15,16}. b) Signals must exhibit CSPs after saturation with CT, CT_{n-1} and Rituximab-Fc (for EndoS2_{E186L}), or after saturation with HM (for EndoBT-3987_{D312A/E314L}). A visual description of ligands used in this study can be found in Fig. 6C, Supplementary Figs. 16B and 19). Fulfilling these criteria grants the selection of CSPs associated to ligand recognition, irrespective of whether they originate from the presence of a ligand in the vicinity, or from long-range effects. Only such methyl signals were selected for lineshape analysis with TITAN. Detailed examples from EndoS2_{E186L} and EndoBT_{D312A/E314L} can be scrutinized in Fig. 6B and in Supplementary Figs. 16C, respectively. Following this methodology, we could safely identify 10 methyl signals reporting for the binding of EndoS2_{E186L} to CT, CT_{n-1} and Rituximab-Fc, and another 10 signals reporting for the interaction of EndoBT_{D312A/E314L} with HM (signals 1-10 in Supplementary Fig. 22 and signals 11-20 in Supplementary Fig. 23, respectively). EndoS2 also possesses a calcium-binding pocket located in the β -sandwich (BS) domain (Fig. 6A). Signals exhibiting CSPs greater than 2 standard deviations upon titration with calcium chloride were selected as reporters for calcium binding.

Extraction of kinetic and thermodynamic parameters from methyl-TROSY spectra

Titration of catalytically dead ENGases with carbohydrates CT, CT_{n-1} or Rituximab-Fc produced CSPs in the slow-exchange regime in the NMR time-scale, leading to varying peak intensities during titration (Supplementary Figs. 24-29). On the other hand, titration of EndoS2 with CaCl₂ produced CSPs in the fast-exchange regime, which translates into shifts in peak

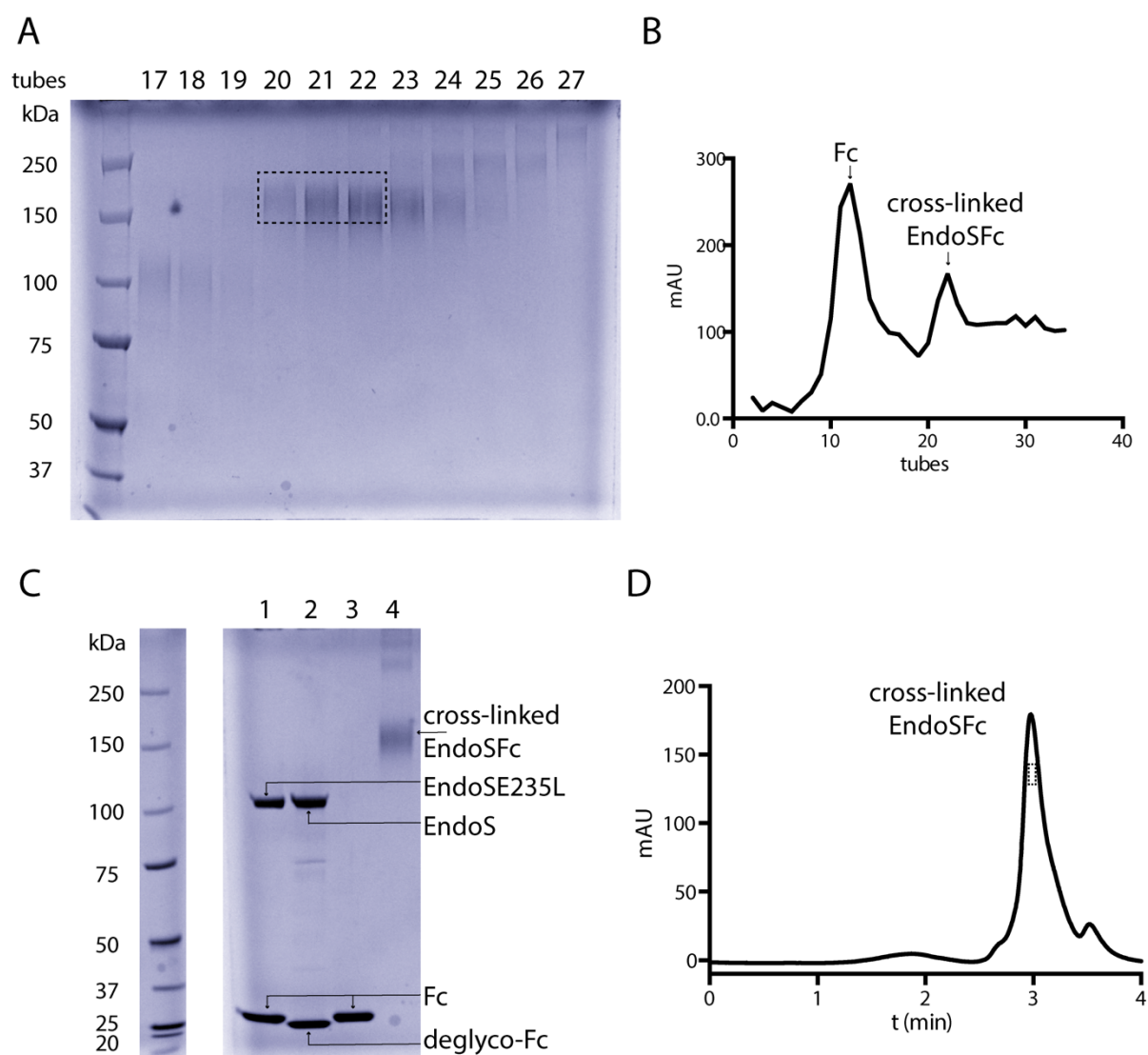
positions (Supplementary Fig. 30). Methyl-TROSY based titrations in slow exchange were subjected to lineshape analysis with TITAN software package. For each titration point, the corresponding methyl-TROSY spectrum was imported into MATLAB and then analyzed with the TITAN algorithm, applying a two-state ligand binding model. Quantitative lineshape analysis yielded dissociation constants K_D and on- and off-rate constants k_{on} and k_{off} . A representative example of lineshape fitting can be found in Fig. 6D.

In the main text, we have described the kinetics of binding of EndoS2 to its substrate CT, Rituximab-Fc and the reaction product CT_{n-1}. Here, we focus on the effect of calcium in the interaction of EndoS2 with CT and Rituximab-Fc. Calcium binding produced similar CSPs in EndoS2 and EndoS2_{E186L} species with virtually identical dissociation constants K_D , suggesting no influence of the mutation E186L on calcium recognition (Table 1, entries 6,7, Supplementary Fig. 31). It is worth noting however, that some signals showed significant CSPs during the titration of EndoS2_{E186L} with CT, CT_{n-1}, Rituximab-Fc and CaCl₂. Such promiscuity in CSPs suggests the presence of alternative calcium-binding sites or a cross-talking mechanism between the GH and BS domains, as it has already been proposed from functional studies with chimeric ENGases.² Here, we did not attempt any further analysis on such signals. Instead, they will be the subject of a future study, whenever a spectrum assignment is available for EndoS2. Titrations of CT and Rituximab-Fc were repeated in the presence of saturating concentrations of CaCl₂, producing almost identical results as in its absence. Similarly, no significant differences were observed for the titration of CaCl₂ in the presence of saturating concentrations of CT. No interaction between EndoS2_{D186L} and CaCl₂ was observed when the enzyme was pre-saturated with Rituximab-Fc. This observation is in good agreement with the cryoEM structure reported in this study, where no calcium ion is bound to EndoS2 in the complex EndoS2-Fc (Supplementary Table 3). It appears that calcium is not directly involved in the recognition of IgGs by EndoS2, although its exact role remains yet to be identified.

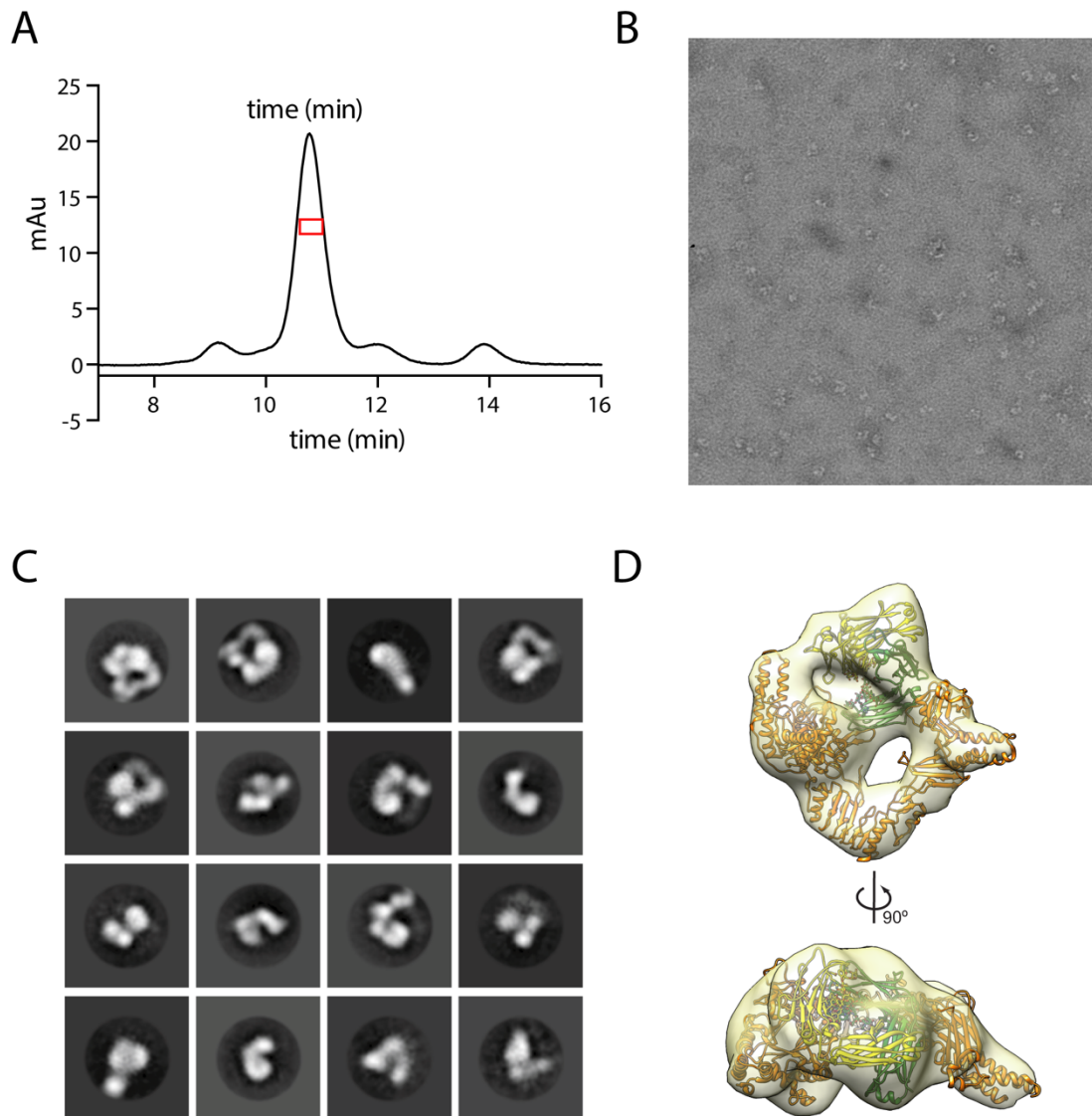
At this point, it is instructive to compare dissociation constants, on- and off-rate constants from ENGases with diverse specificities. EndoBT-3987 is a globular ENGase secreted by the gram-negative symbiont *Bacteroides thetaiotaomicron*, which displays high specificity for mammalian HM-type *N*-glycans. Titrations of EndoBT_{D312A/E314L} with HM in the absence and presence of CaCl₂ afforded dissociation constant, on- and off-rate constants in the same order of magnitude as those previously disclosed for EndoS2_{E186L} binding Rituximab-Fc. As expected, no affinity was observed for CT glycan, even at ~750 μ M glycan concentrations. (Table 1, entry 6, lineshape fittings for the titration of HM can be scrutinized in Supplementary Figs. 32 and 33).

Our study was complemented with the extraction of the catalytic rate k_{cat} and the Michaelis-Menten constant K_M from the catalytic cleavage of complex-type carbohydrates from Rituximab-Fc by EndoS2. Kinetic parameters were approximated using the Michaelis-Menten equation (5) (steady-state kinetics), hypothesizing a nearly constant concentration of enzyme-substrate complex during the reaction course. This assumption was based on the two orders of magnitude K_D difference observed for the binding of EndoS2 to Fc as compared to the reaction product CT_{n-1} ($K_D = 3.1 \mu\text{M}$ vs $K_D = 150.7 \mu\text{M}$, respectively). Concentration of enzymatically released carbohydrates was measured from ¹H spectra. Background signals were readily suppressed by a perfect CPMG T_2 -filter, which is a relaxation filter that avoids J -modulation¹⁷ (Supplementary Fig. 17). Cleaved carbohydrate was quantified from the integral of the composite signal at 2.08–2.10 ppm, which reports for *N*-acetylglucosamine and *N*-acetylneuraminic acid moieties (Supplementary Fig. 18A). Reaction time-courses were measured to close-to-reaction completion for five Rituximab-Fc initial concentrations (Supplementary Fig. 18B). The reaction rate was calculated from the fitting of initial velocity rates (v_0) to the equation of Michaelis-Menten (5) (Fig. 6E). Under these conditions, a catalytic rate k_{cat} of 5.9 s⁻¹ and a Michaelis-Menten constant K_M of 26.7 μ M were observed. It is

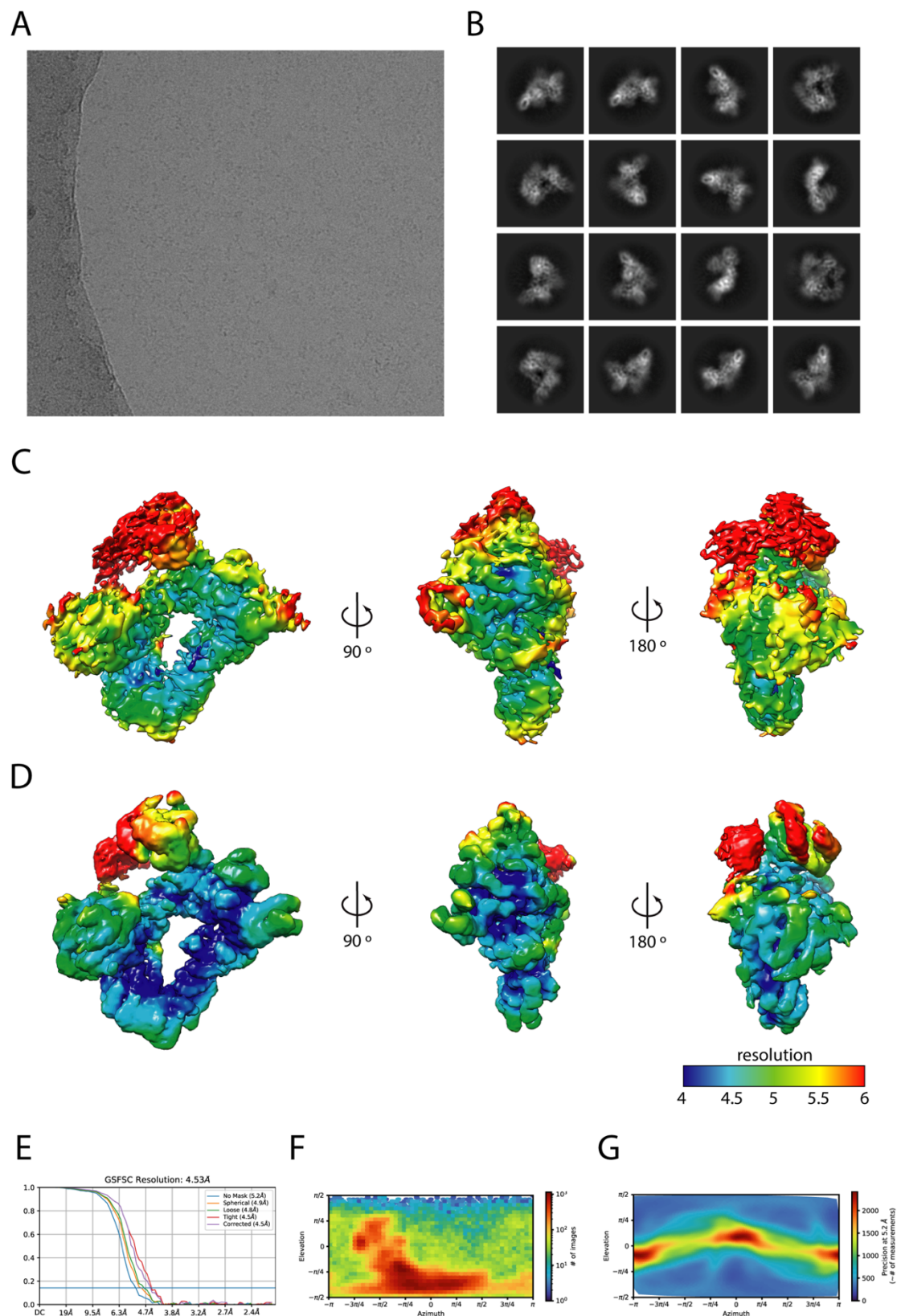
important to remark that K_M resembles K_D only when $k_{off} \gg k_{cat}$, according to Eq. 6. In this study this condition is not met ($k_{off} = 4.0 \text{ s}^{-1} \approx k_{cat} = 5.9 \text{ s}^{-1}$), indicating that the system is not in rapid equilibrium. Nevertheless, care has to be taken when combining results obtained from different EndoS2 species (wt and E186L), because non-identical protein-carbohydrate interactions can result in slightly different kinetic parameters. In conclusion, this study shows for the first time an overview of enzyme kinetics followed by EndoS2. We hope this information will be key for the future development of ENGases with improved activity and selectivity.



Supplementary Figure 1. Purification of the cross-linked EndoS_{E235A}-Fc complex. (A) SDS-PAGE analysis and (B) protein absorbance of fractions obtained by GraFix centrifugation. (C) SDS-PAGE showing the fractions of the EndoS_{E235A}-Fc complex concentrated from GraFix centrifugation and the enzyme and substrate components. (D) Superdex 200 Increase 10/300 GL profile showing the purified cross-linked EndoS_{E235A}-Fc complex.

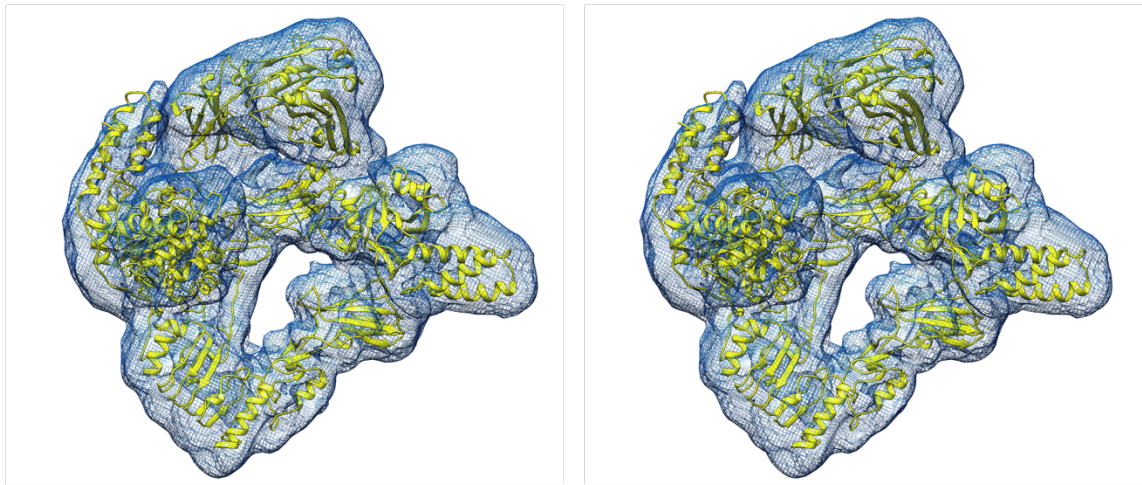


Supplementary Figure 2. The EndoS_{E235A}-Fc complex by NS. (A) Superdex 200 Increase 10/300 GL profile showing the purified cross-linked EndoS_{E235A}-Fc complex. (B) Micrograph detail. (C) Representative 2D classes. (D) 3D model reconstruction of EndoS_{E235A}-Fc complex. The EndoS_{E235A} (PDB code 6EN3) and Fc (PDB code 1H3X) X-ray crystal structures are fitted into the model using Chimera.

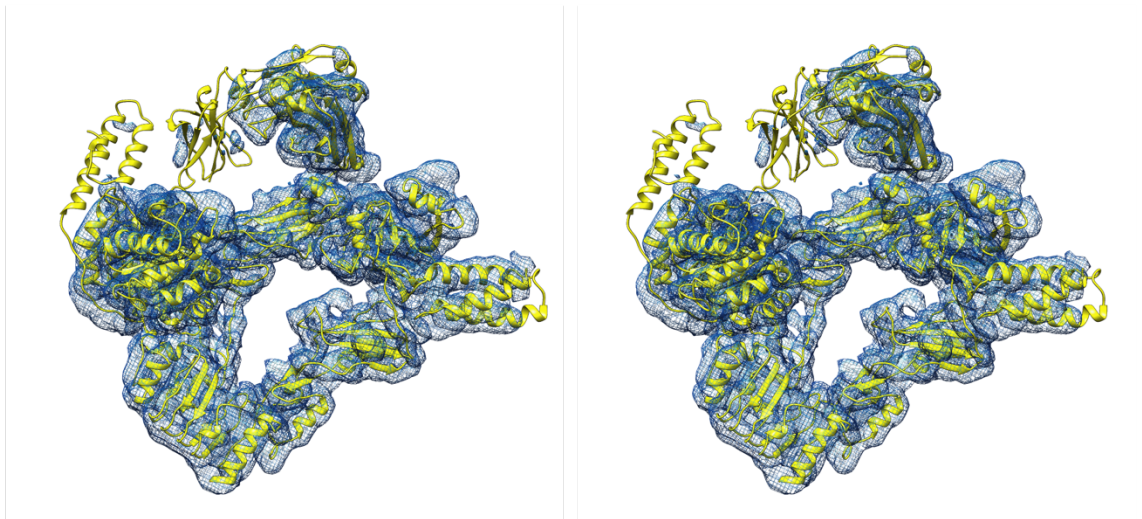


Supplementary Figure 3. The EndoS_{E235A}-Fc complex by cryoEM. (A) Micrograph detail. (B) Representative 2D classes. (C) Overall view of the single-particle reconstructions of EndoS_{E235A}-Fc complex using Cryosparc and (D) DeepEMhancer, colored according to local resolution. (E) FSC (F) direction distribution and (G) posterior precision plots of the EndoS_{E235A}-Fc complex.

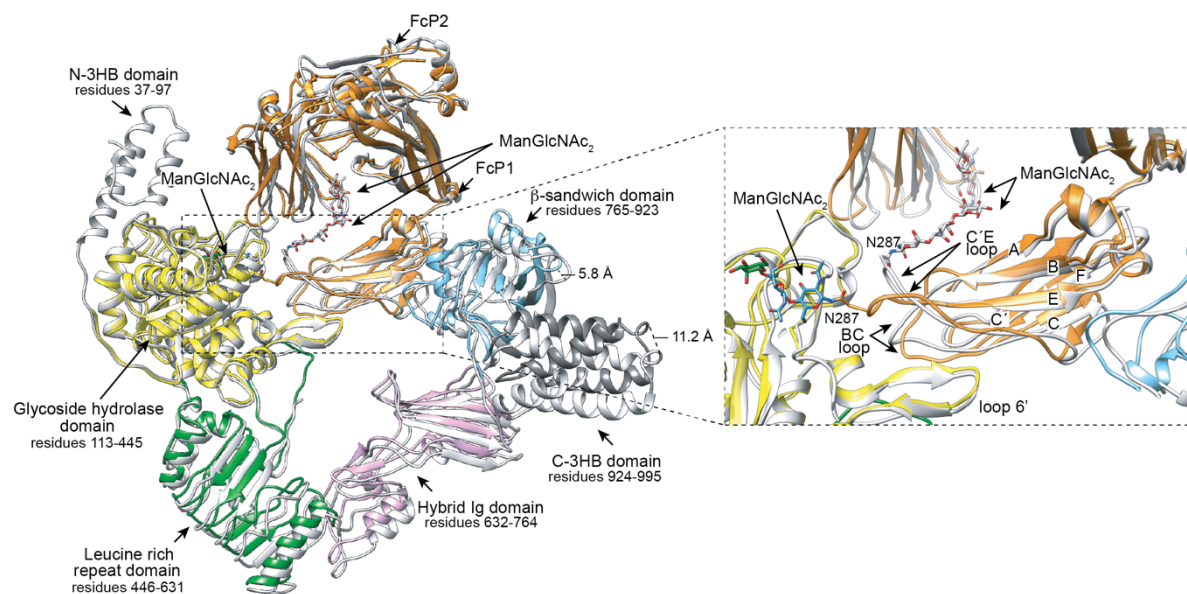
A

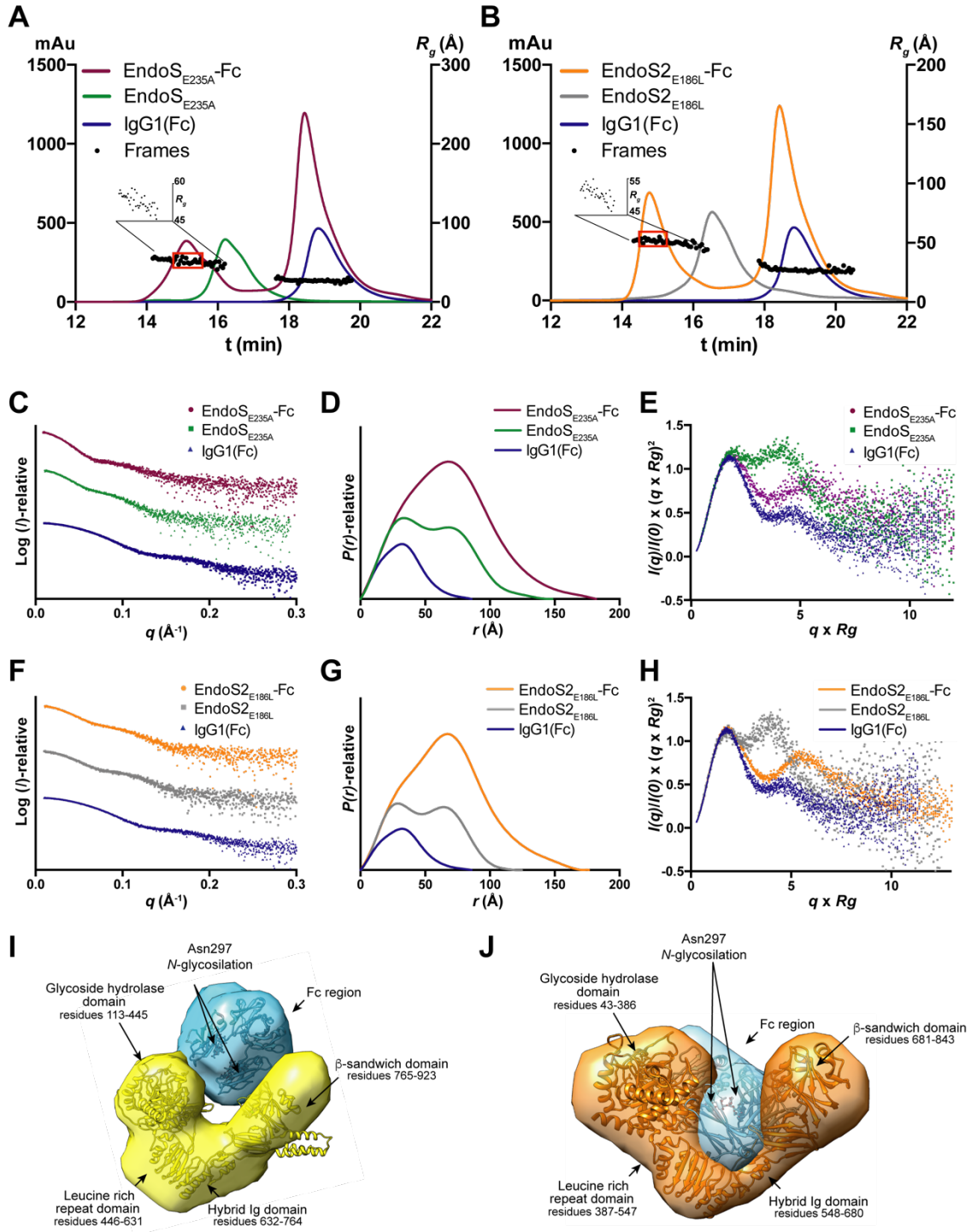


B



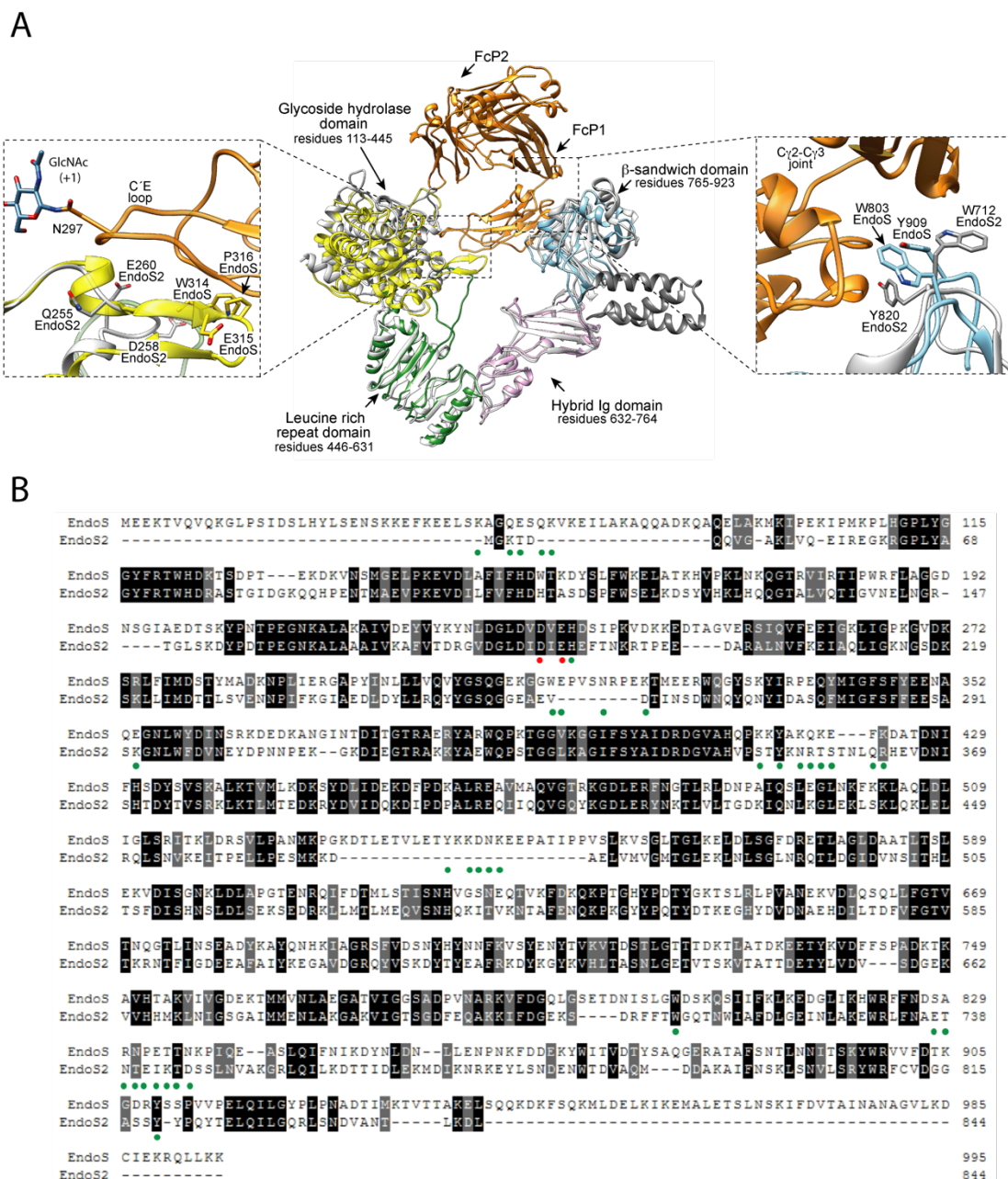
Supplementary Figure 4. Stereo views of the EndoSE235A-Fc complex models and cryosparc (**A**) and DeepEMhancer (**B**) maps.



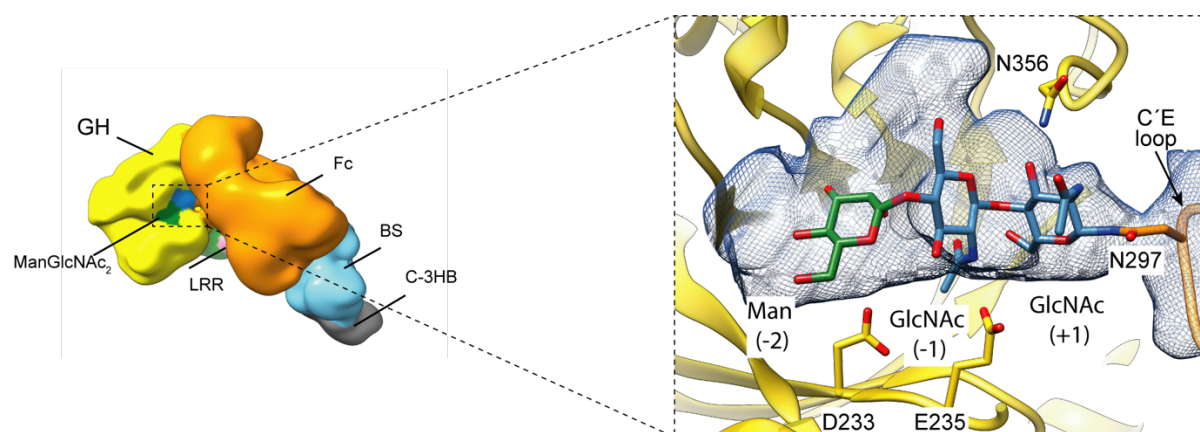


Supplementary Figure 6. SAXS analysis of the EndoS_{E235A}-Fc and EndoS2_{E186L}-Fc complexes. (A) In-line SEC-SAXS profile of EndoS_{E235A}, IgG1(Fc) and EndoS_{E235A}-Fc complex. The frames used for data analysis of the complex are highlighted with a red square. (B) SEC-SAXS profile of EndoS2_{E186L}, IgG1(Fc) and EndoS2_{E186L}-Fc complex. The frames used for data analysis of the complex are highlighted with a red square. (C) SAXS scattering curve of EndoS_{E235A}, IgG1(Fc) and EndoS_{E235A}-Fc. (D) $P(r)$ functions distributions of EndoS_{E235A}, IgG1(Fc) and EndoS_{E235A}-Fc complex. (E) Normalized Kratky plot EndoS_{E235A}, IgG1(Fc) and EndoS_{E235A}-Fc complex. (F) SAXS scattering curve of EndoS2_{E186L}, IgG1(Fc) and EndoS2_{E186L}-Fc. (G) $P(r)$ functions distributions of EndoS2_{E186L}, IgG1(Fc) and EndoS2_{E186L}-Fc complex. (H) Normalized Kratky plot EndoS2_{E186L}, IgG1(Fc) and EndoS2_{E186L}-Fc complex. (I) 3D domain model of EndoS_{E235A}-Fc complex. (J) 3D domain model of EndoS2_{E186L}-Fc complex.

EndoS2_{E186A}-Fc complex. **(I)** *Ab initio* modelling with MONSA of the envelope of EndoS_{E235A}-Fc and superimposition of EndoS (yellow) and Fc region of IgGa (blue) X-ray crystal structures. **(J)** *Ab initio* modelling with MONSA of the envelope of EndoS2_{E186A}-Fc and superimposition of EndoS2 (orange) and Fc region of IgGa (blue) X-ray crystal structures.



Supplementary Figure 7. Comparison of EndoS and EndoS2. (A) Cartoon representations of EndoS (colored by domains, PDB code 6EN3) and EndoS2 (grey, PDB code 6MDS) X-ray crystal structures. (B) Structure-based amino acid sequence alignment of EndoS and EndoS2. The catalytic residues and the residues that interact with the Fc region are highlighted in red and green, respectively.



Supplementary Figure 8. Electron density map of the *N*-glycan in the active site of the EndoSE235A-Fc cryoEM complex. Model of three residues of the CT *N*-glycan of the N297 of IgG1 in one of the possible conformations found in the binding site of EndoS. The alanine at position 235 is modelled as a glutamic acid like in the wild type enzyme.

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EndoS  MEEKTVQVQKGLPSIDSLHYLSENSKKEFKEELSKAGQESQKVKEILAKAQQADKQAQELAKMKPEKIPMKPLHGPLYG 115
EndoS2  -----MGKID-----QVQ-AKLVQ-EIREGKRGPLYA 68

EndoS  GYFRTWHDHTSDPT--EKKVNSMGEIPKEVDLAFIFHDHTKDYSLFWKELATKHVPKLNHQGTRVIRITIPNRFAGGD 192
EndoS2  GYFRTWHDRASTGIDGKQHFENTMAEMPEVDLAFVFDHTASDSFWSELKDSYVHKLHQQTALVQTIGVNELNGR- 147

EndoS  NSGIAEDTSKYPMTPPEGKALAKAIVDEYVYKYNLDGLDVEDHDSIPKVDKKEDTAGVERSIQVFEEIGKLGIPKGVDK 272
EndoS2  ---TGLSKDYPTTPPEGKALAAIVKAEVTDGRVDGLDIDEHEFTNKRTPE---DARALNVFKEIAQLIGKNGSDK 219

EndoS  SRLFIMDSITYMAKNFLIERGAPYINLLVQVYGSQGERGWEFVSNRPEKIMEERWQGYSKYIRPEQYMIGFSFYENNA 352
EndoS2  SKLLIMDTLSVENNPIFKGIAEDLDYLLRQVYGSQGERAEV-----DTINSDWQYQNYIDASQEMIGFSFEEESA 291

EndoS  QEGNLWYDINSRKDEDKANGINTDITGTRAEYARWQPMITGGVKGIFSYAIDRDGVAHQPKKYAKQKE--FQDATDNI 429
EndoS2  SQGNLWFDWNEYDPNNPEK--GKDIEGTRAKYARWQPMITGGVKGIFSYAIDRDGVAHVPSYKNRTSTNLQRHEVDNI 369

EndoS  FHSQYSSVSKALKTVMLKDKSYDLIDEKDFPDALREAVMAQVGTTRKGDLEFNCTLRDNPAIQSHGINKFKKLAQLDL 509
EndoS2  SHIDYTVSKKLTLMTEDEKRYDVIDQKDIIPDALREQIIQVQGVYKGDLERYNMTLVETGDIQNKGLKSLKQLLEL 449

EndoS  IGLSRITTKDRSVLPANMKPGKDTLETVLEYKKDNKEEFATIPPVS LKVSGLTGLKELDLSGFDRETLAGLDAATITSL 589
EndoS2  RQLSNVKEITPEELPESMKKD-----AELVMVGMTGLEKLNLSGLNRQTLTGIDVNSHTHL 505

EndoS  EKVDISGNRLDLPGTENROTFDMLSTISNVHGSNEQVVKFEKQKETGHPDYGKTSLLFVANEKVLDLQSLLFGTV 669
EndoS2  TSFDISHNSLDLSEKSEDRKLLMLMEQVSNHKKITVKTAFENQKEKGYYPQTYDTKEGYVDVDAEHDILITDFVFGTV 585

EndoS  INQGTLLINSEADWKAYQNHKTAGRSFVDSNVHYNNFKVSVENYTVKVTDSITLGTITDKTLATDKETVWVDFSPDKTK 749
EndoS2  TKRNIFIGDEEAGAIKKEGAVDGRQYVSKDITYEAFRKDYKGVVHLTASNLGPTVTSKVTATTDETYLVDV--SDGER 662

EndoS  AVHTAKVIVGDEKTMVNLAEAGAVIGGSADPVNARKVFDGQLGSETDNISLGNDSKQSIIEKLEKEDGLIKHNRFFNDSA 829
EndoS2  VVHHMKLNVGSGAIMMENLAKGAEVIGTSGDFEQAKKIFDGEK-----DRFFTNGQTNIWIAEDLSINLAKENRLEAET 738

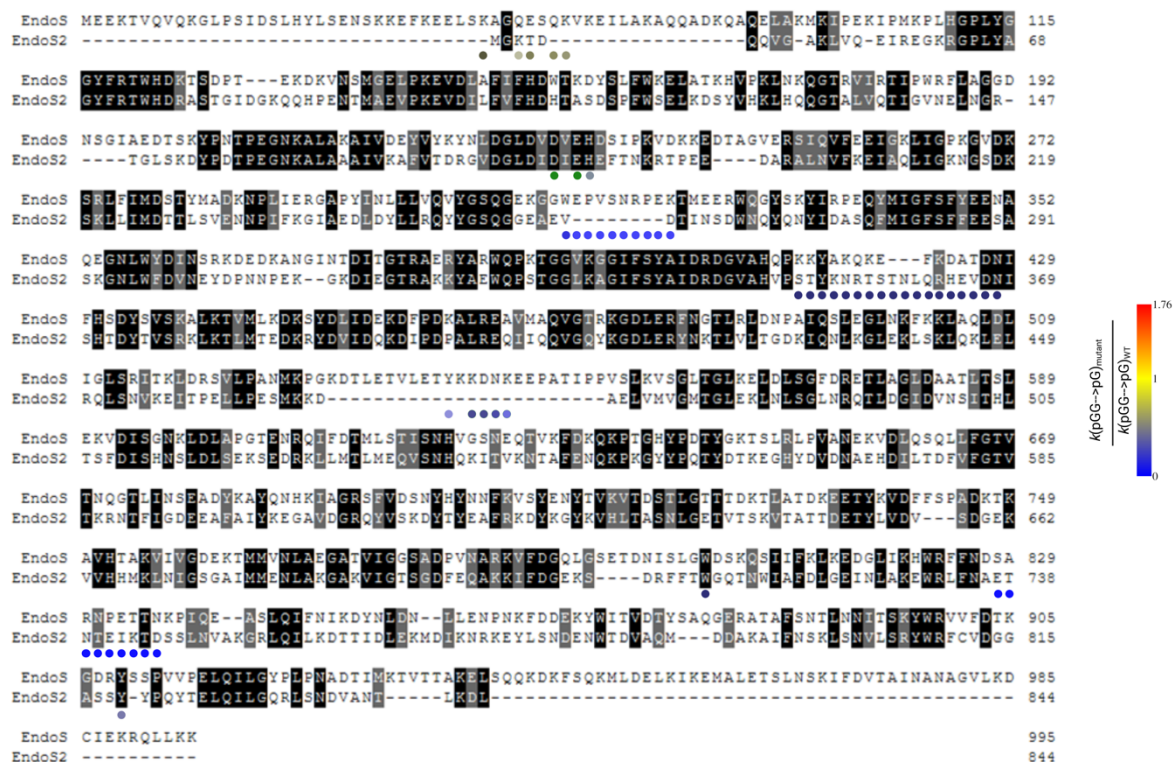
EndoS  RNPETTNKPIQE--ASLQIFNIKDYNLN--ILENPNKFDDKEYWITVDYSAQGERATAFSNTLNNTSKYWRVVDTK 905
EndoS2  NTEIKTDSSINVAKRLQILKDTTIDLEKMDKKNRKEYLSNENWTDVAM--DDAKAIFNSKESNVLSRYWRVFCVDDG 815

EndoS  GDRYSFVVPVELQILGYPLPNADIIMKIVTTAKELSQQKDKFSQKMLDELKIKEMALETSLNSKIFDVTAINANAGVLKD 985
EndoS2  ASSY-YPQYTELQILGQRLSNDVANI-----LKDI----- 844

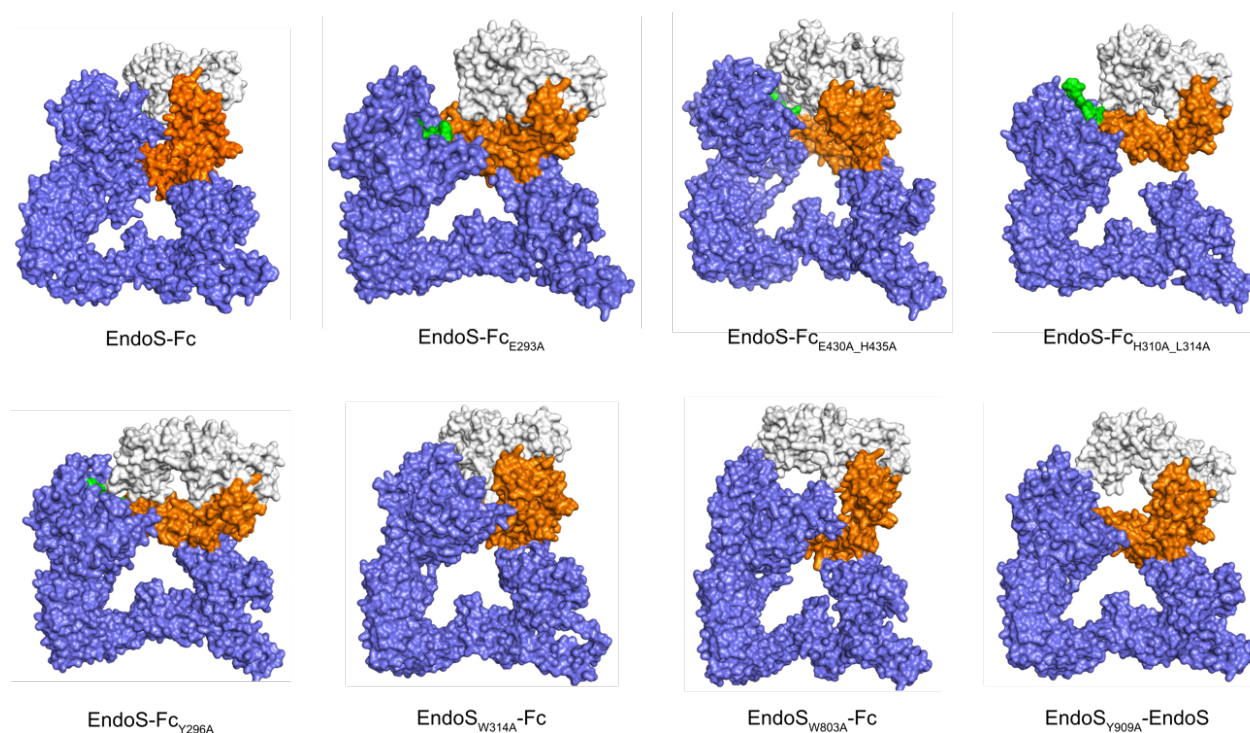
EndoS  CIEKRQLLKK 995
EndoS2  ----- 844

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Supplementary Figure 9. Alanine scanning mutagenesis of EndoS. Structure-based amino acid sequence alignment of EndoS and EndoS2. All the residues involved in the alanine scan are in red. The active site residues are green.

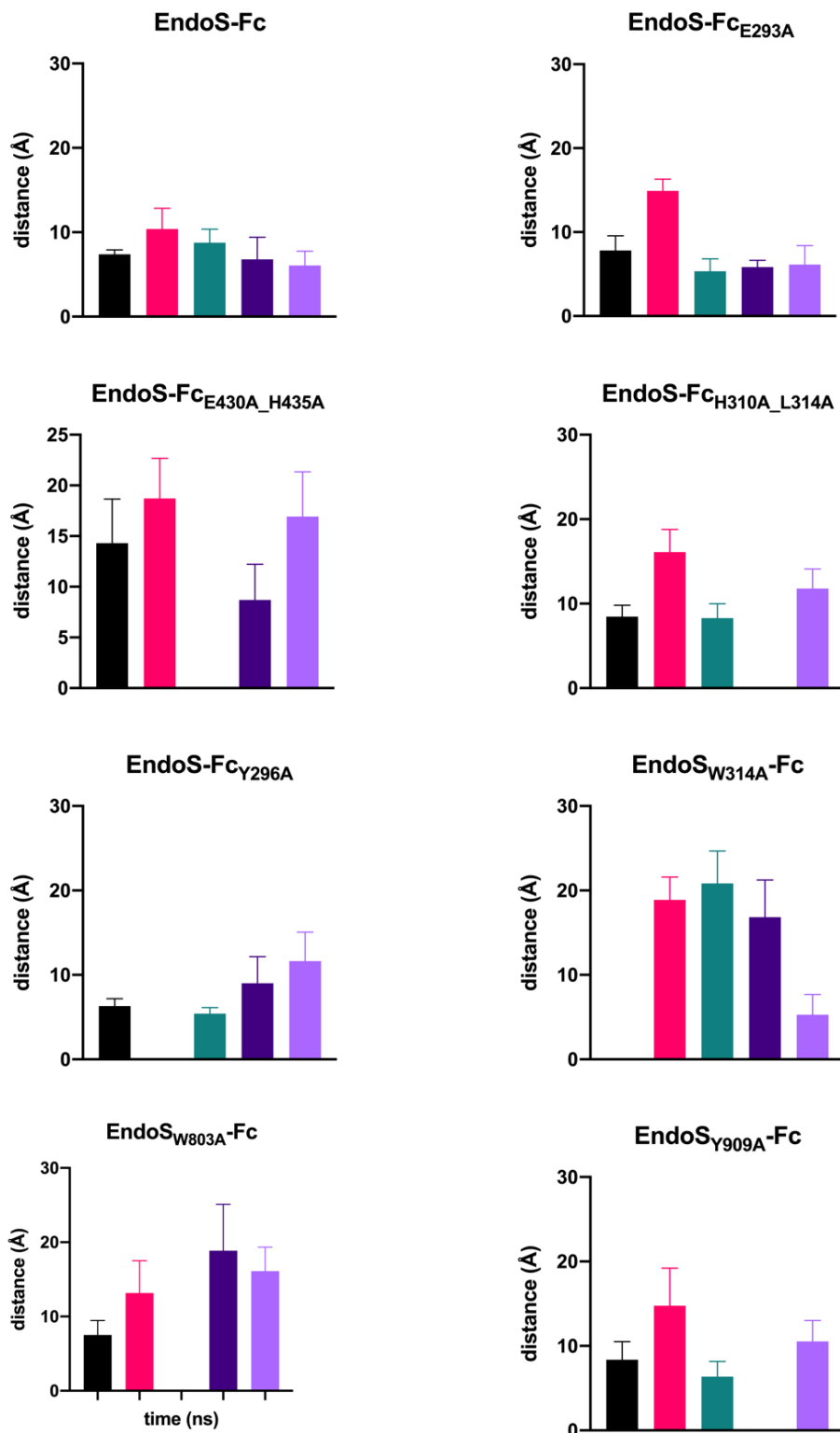


Supplementary Figure 10. Alanine scanning mutagenesis of EndoS. Structure-based amino acid sequence alignment of EndoS and EndoS2. The dots have been colored based on their kinetic rate as depicted in Fig. 3. The active site residues are green.

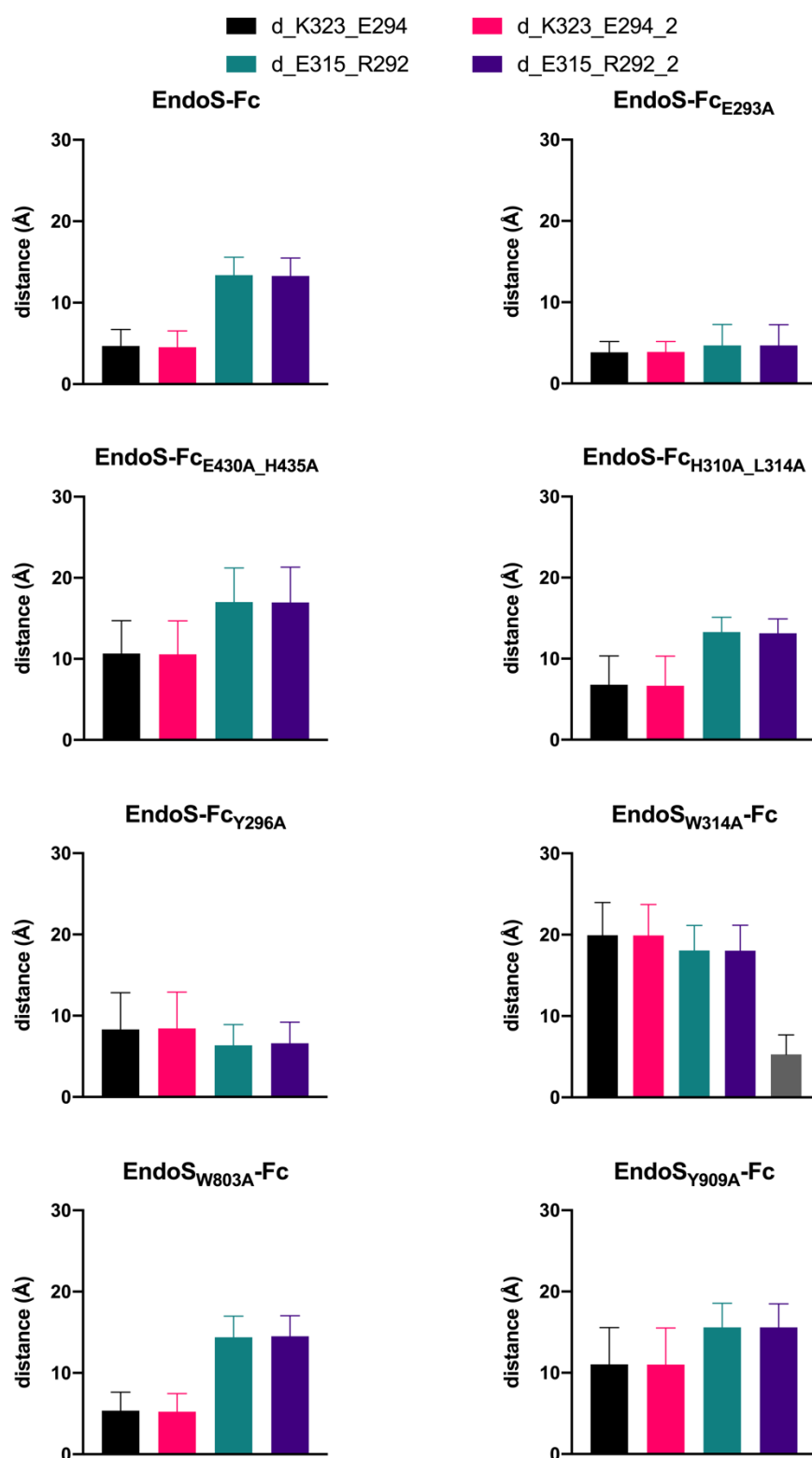


Supplementary Figure 11. Average structure derived from 1 μ s MD simulations of the different complexes simulated in this work. Only $C\alpha$ atoms of the proteins are considered for these calculations. The EndoS protein is shown as a purple surface and the Fc as white and orange surfaces.

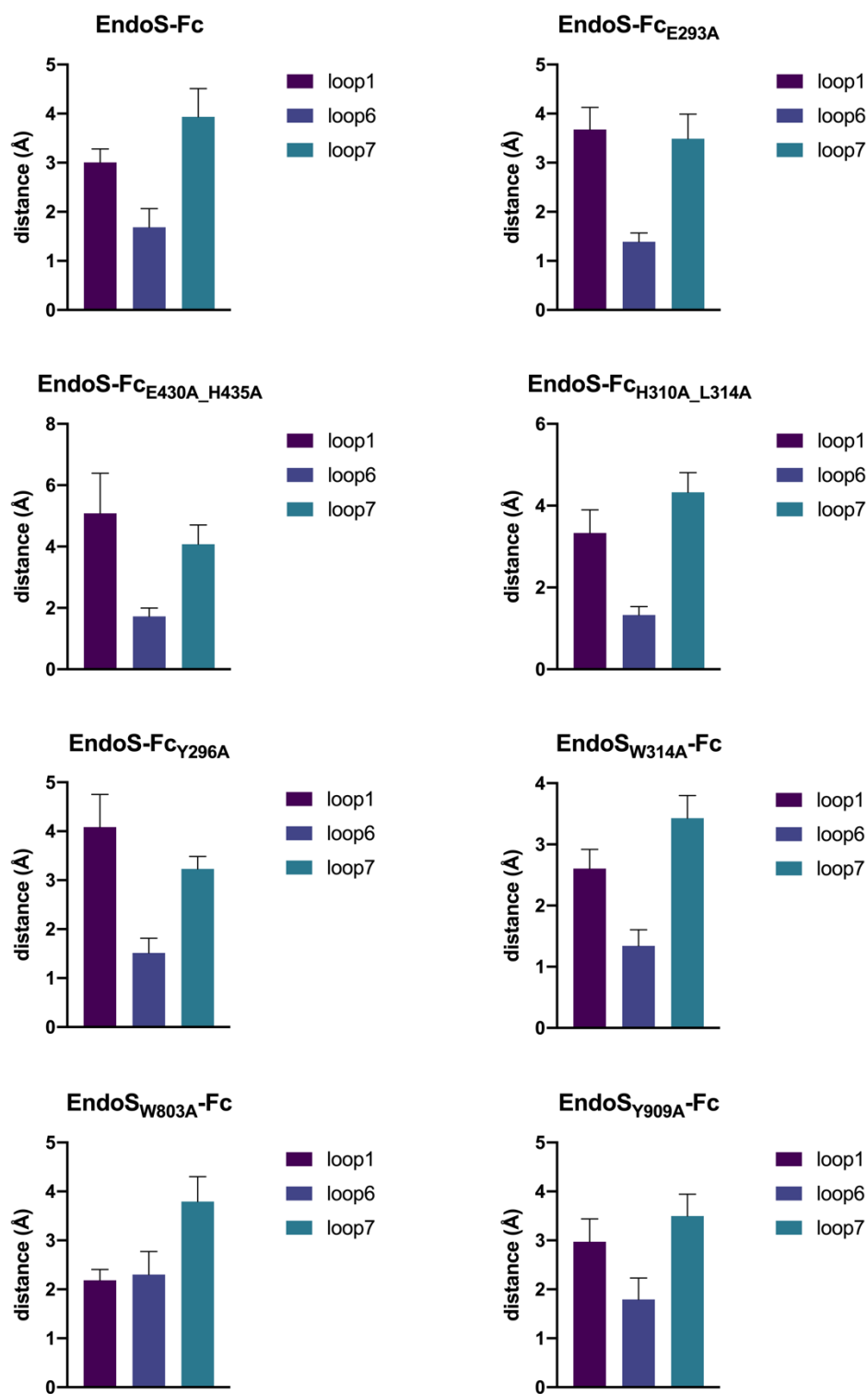
W314_P271
 H236_Y296
 W803_H435
 Y909_L314
 E354_S298



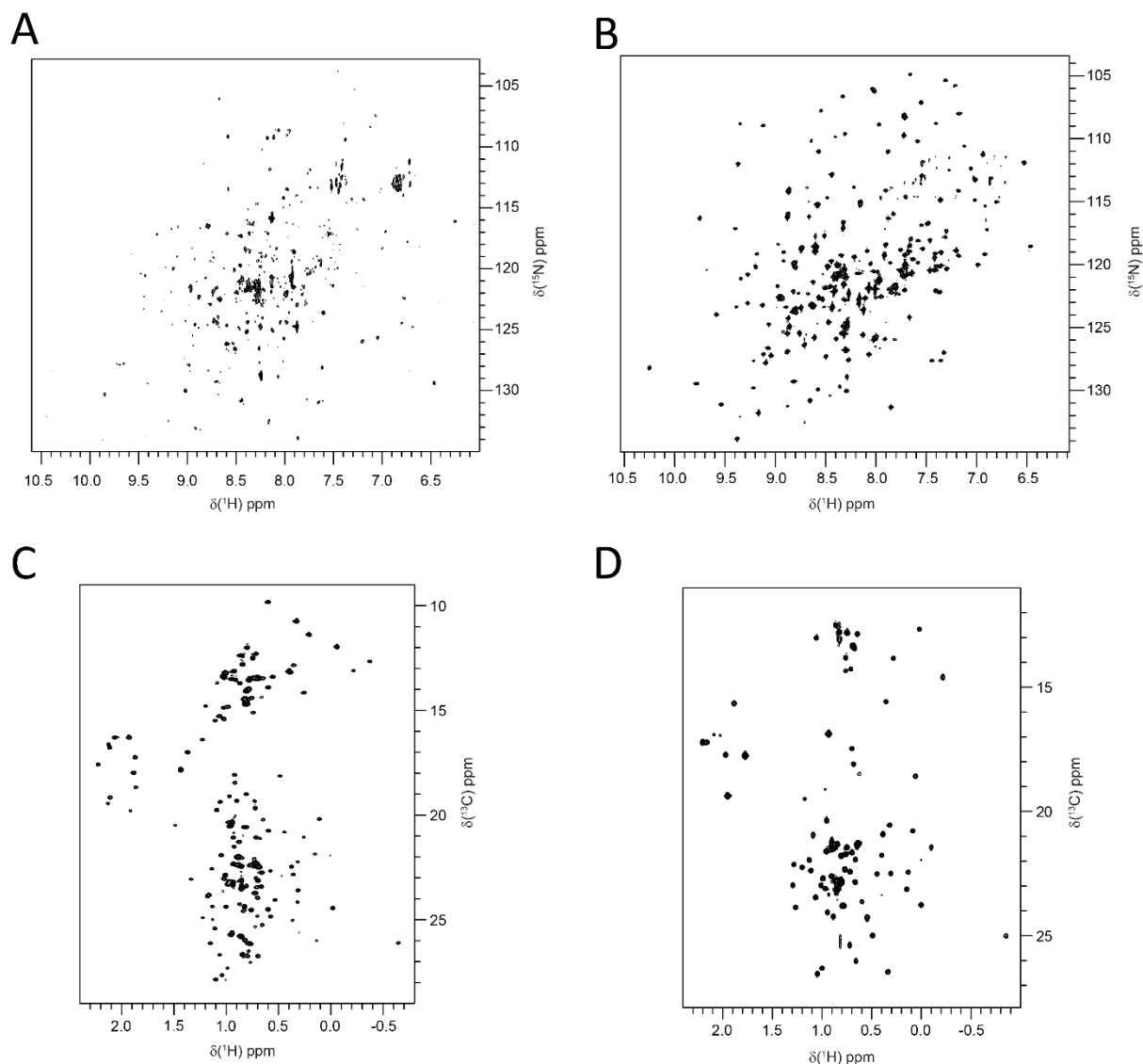
Supplementary Figure 12. Average distances derived from 1 μ s MD simulations between different residues of EndoS and Fc for the simulated complexes. For aromatic residues, the center of the aromatic ring of the side chain are considered for these calculations. In the case of L314, S298 and E354 C δ , the oxygen of the hydroxyl group and the oxygens of the carboxylate are considered, respectively, for the calculations. Errors are given as SD.



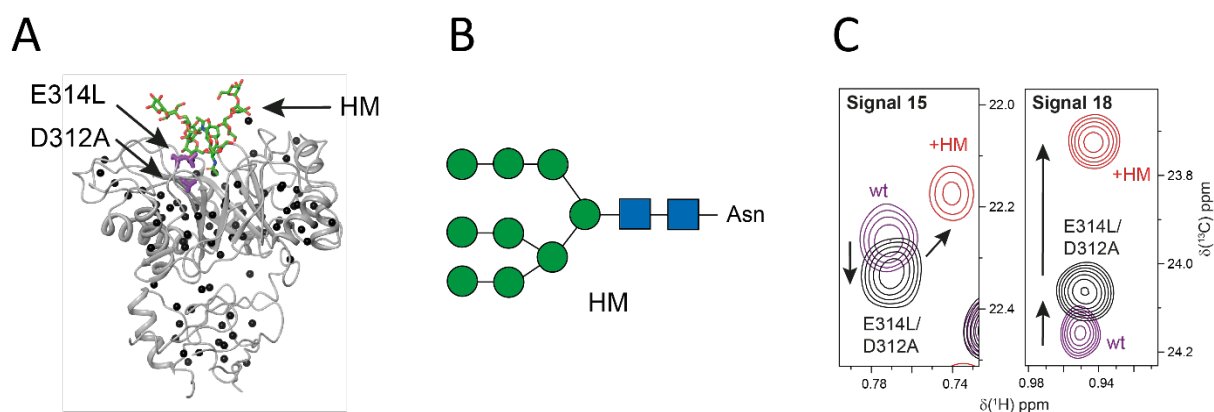
Supplementary Figure 13. Average distances derived from 1 μ s MD simulations for two salt-bridge interactions between EndoS and Fc for the simulated complexes. The side chain nitrogen of Lys and the oxygens of the carboxylate group of Glu are considered for these calculations. Errors are given as SD.



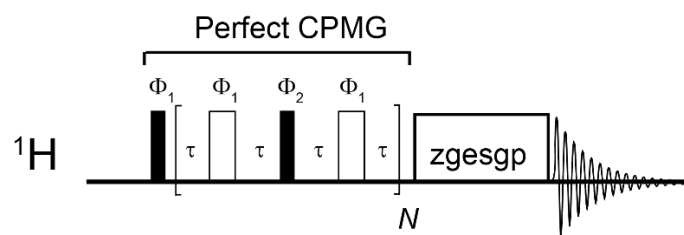
Supplementary Figure 14. RMSD value ($\pm$ SD) of the peptide backbone of loops 1, 6 and 7 derived from 1 μ s MD simulations for the simulated complexes is shown.



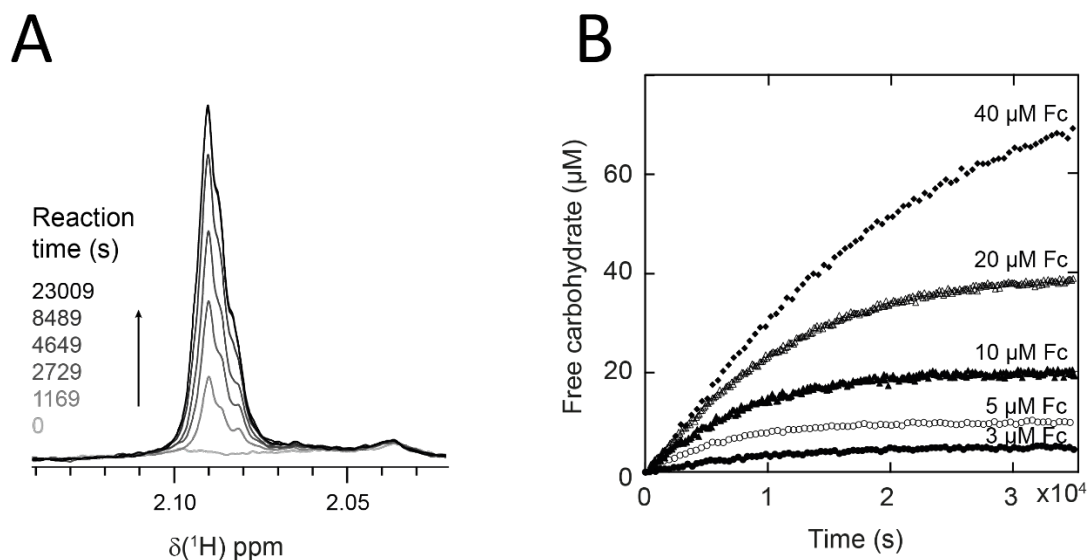
Supplementary Figure 15. Quality of NMR spectra of EndoS2_{E186L} and EndoBT_{D312A/E314L} samples. ¹H, ¹⁵N HSQC-TROSY spectra of [*U*-¹⁵N, ²H] MILV-[¹³C, ¹H₃]-methyl labeled sample of (A) EndoS2_{E186L} (97 kDa) at 71.5 μM protein concentration, and (B) EndoBT_{D312A/E314L} (47 kDa) at 74.4 μM protein concentration. Methyl-TROSY spectra of (C) EndoS2_{E186L} and (D) EndoBT_{D312A/E314L} at 51.5 μM and 100 μM protein concentrations are shown as comparison. All experiments were acquired at 298 K and 600 MHz.



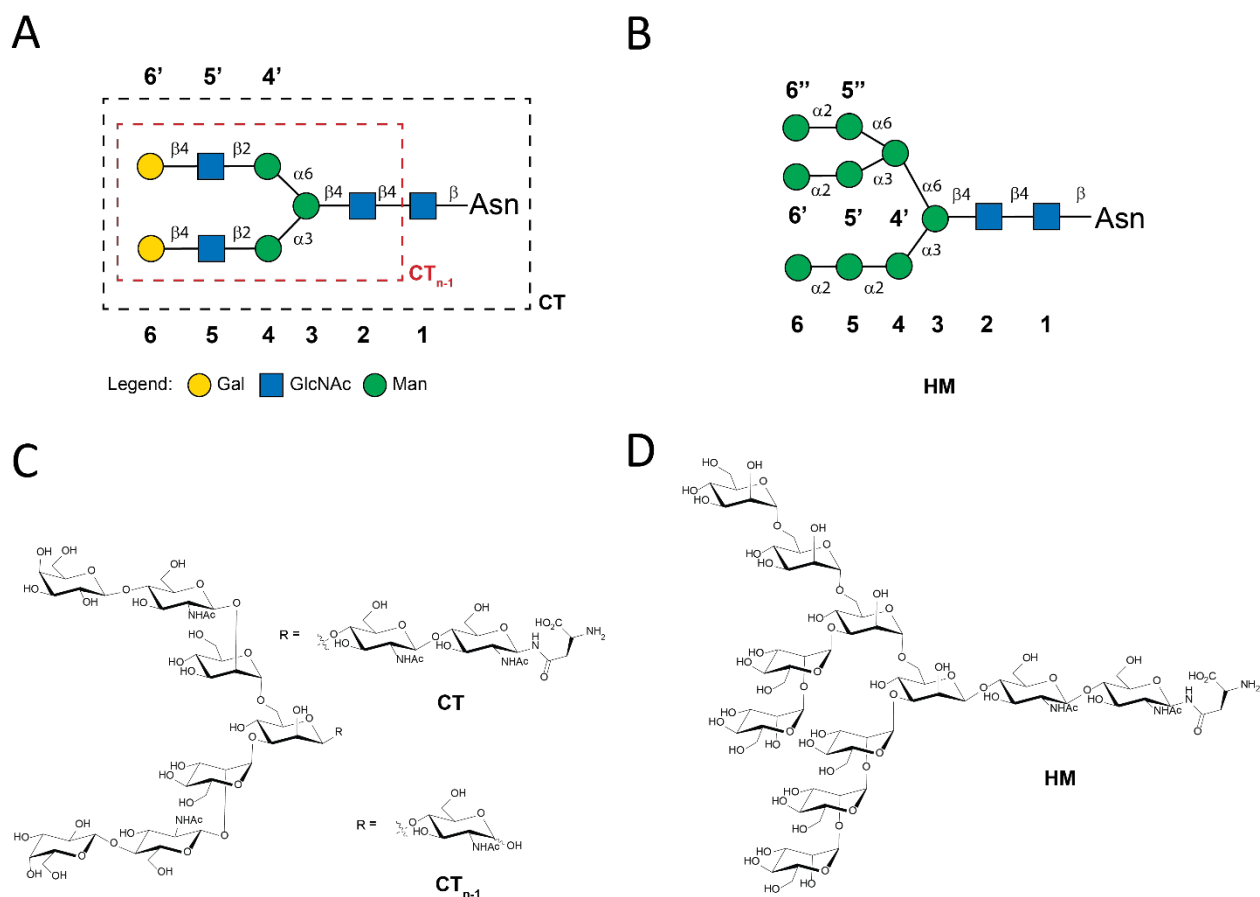
Supplementary Figure 16. NMR titration of EndoBT-3987_{D312A/E314} with high mannose-type *N*-glycan HM. (A) Crystal structure of EndoBT-3987_{D312A/E314} bound to HM glycan (PDB 6TCV). Mutated amino acids D312A and D314L are colored in purple, and HM glycan is shown as green sticks. Isotopically labeled methyl groups are indicated as black spheres. (B) Cartoon representation of HM. (C) Superimposition of selected regions of methyl-TROSY spectra showing the CSPs upon mutagenesis or at saturation concentrations of HM. Signals from EndoBT-3987 are shown in violet, and from EndoBT-3987_{D312A/E314L} in the absence (black) and in the presence of saturating concentrations of HM (red). Arrows indicate the extent of CSPs observed between the different protein states. All experiments were acquired at 600 MHz and 298 K



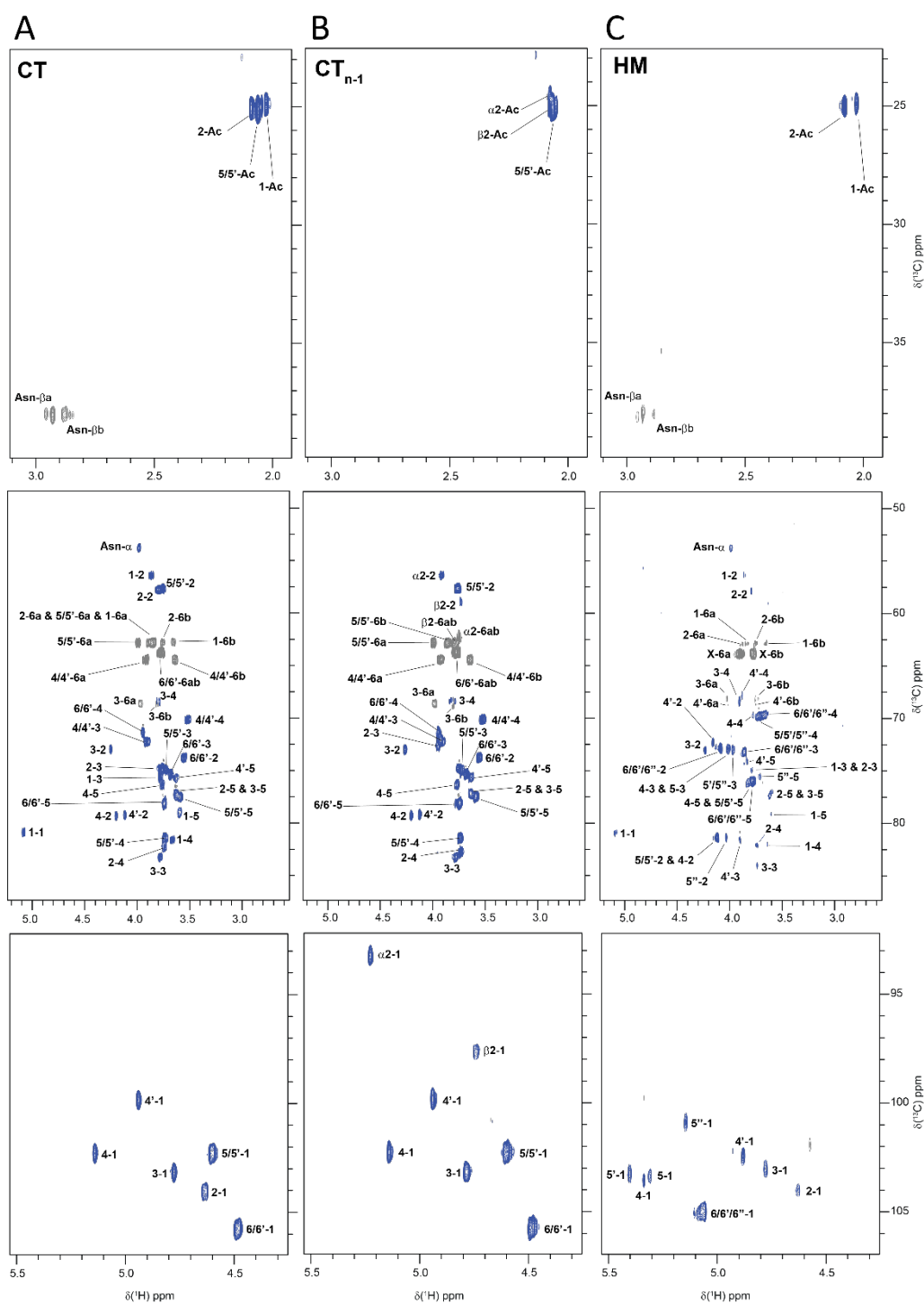
Supplementary Figure 17. Scheme showing the perfect CPMG building block. The initial 90° pulse in the standard Bruker pulse sequence zgesgp was substituted by the sequence shown above. Echo time τ was set to 0.3 ms and $N = 33$. Phase cycling during this block was as follows: $\Phi_1 = x$; $\Phi_2 = y$.



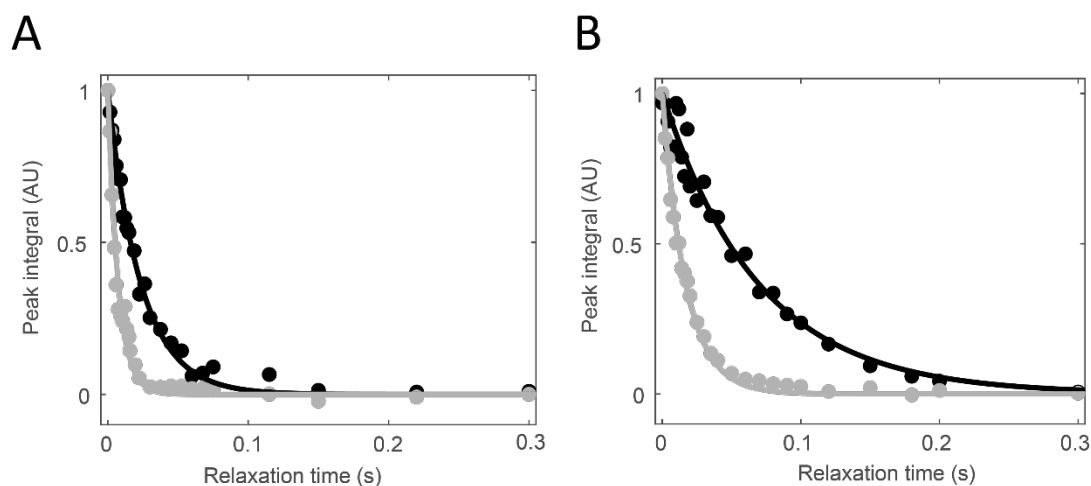
Supplementary Figure 18. Enzyme kinetics of EndoS2 and IgG1 Fc. (A) The composite signal at 2.08-2.11 ppm was used to monitor the enzymatic cleavage of IgG1 Fc complex *N*-glycans as a function of time. Spectra corresponds to 40 μM Fc concentration. (B) Time courses of *N*-glycan cleaved from IgG1 Fc after the addition of EndoS2 at different IgG1 Fc starting concentrations. Note that two glycans are released per IgG1 Fc unit. Data at times longer than 3.5×10^4 s were also included in the fit but are not shown for clarity. All experiments were acquired at 600 MHz and 298 K.



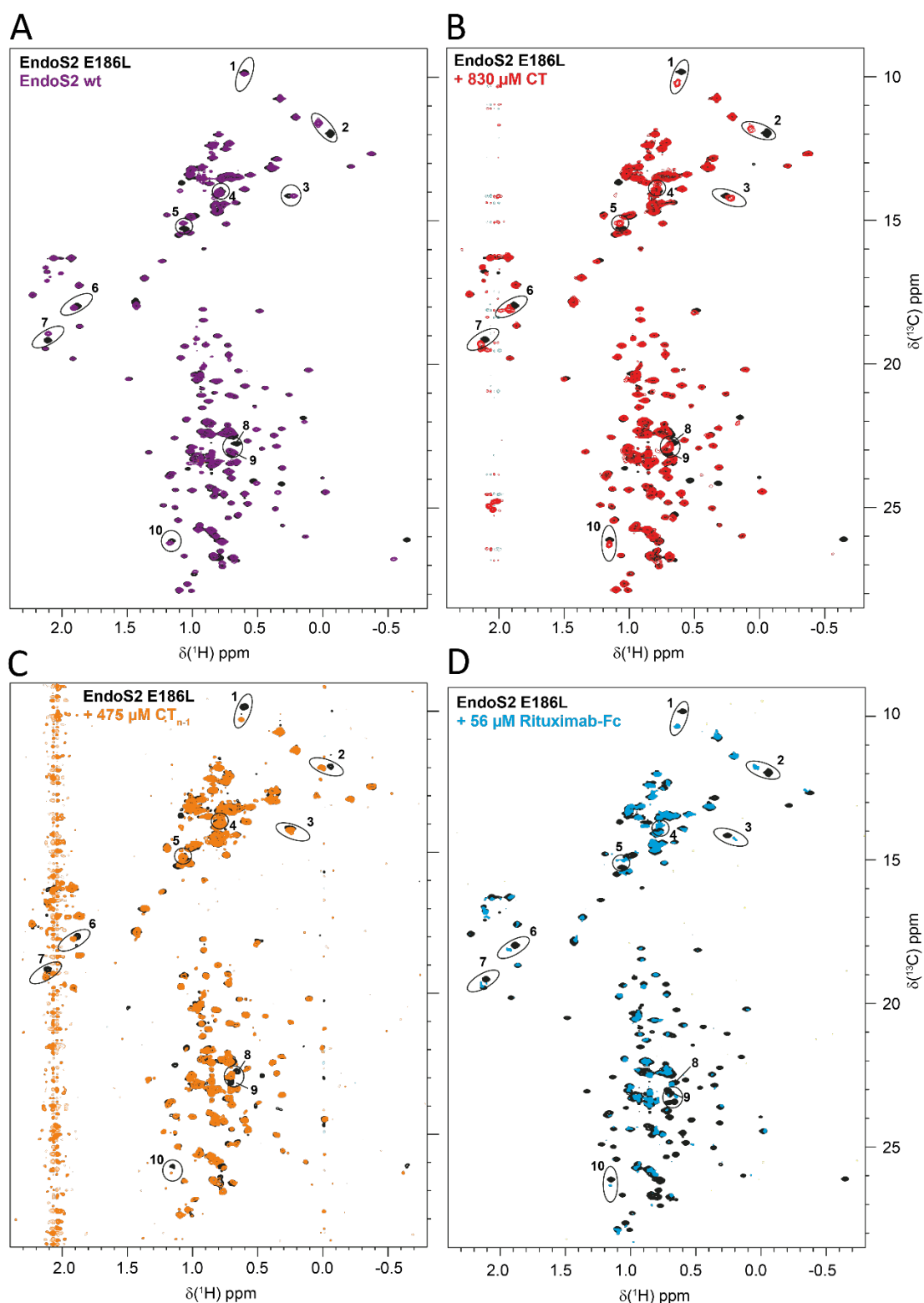
Supplementary Figure 19. Carbohydrates used for NMR titrations. Cartoon representation of (A) complex-type *N*-glycan CT and its reaction product after treatment with EndoS2 CT_{n-1}, and of (B) high mannose-type *N*-glycan HM. Their corresponding chemical structures are shown in (C) for CT and CT_{n-1}, and in (D) for HM. Abbreviations in the legend correspond to: Gal = galactose, GalNAc = *N*-acetylgalactosamine, Man = mannose and Asn = asparagine.



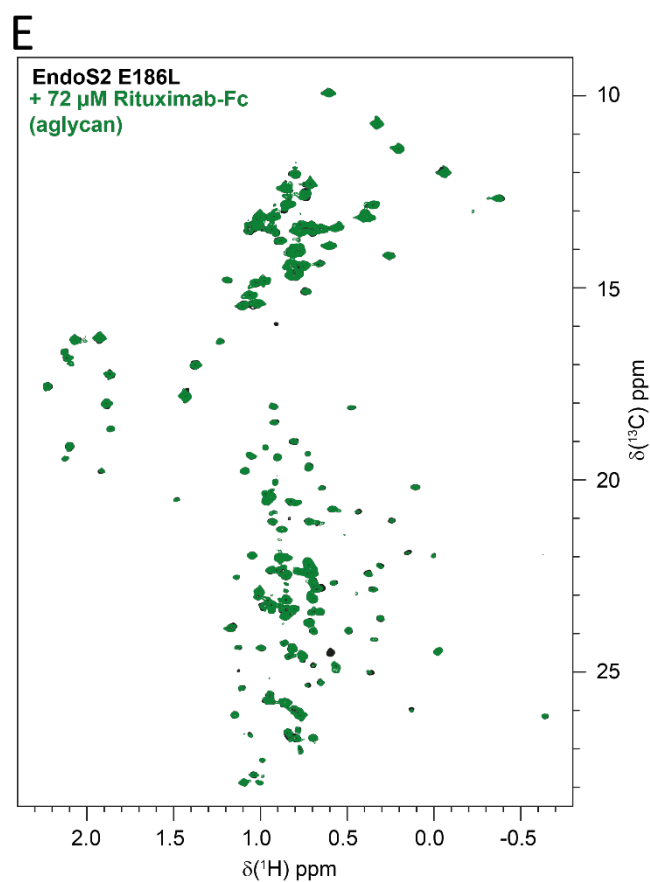
Supplementary Figure 20. ^1H and ^{13}C assignments of *N*-glycans used for NMR titrations. Sections of ^1H , ^{13}C -HSQC spectra showing the assignment of (A) CT, (B) CT_{n-1} and (C) HM. Spectra were acquired at 600 MHz and 310 K.



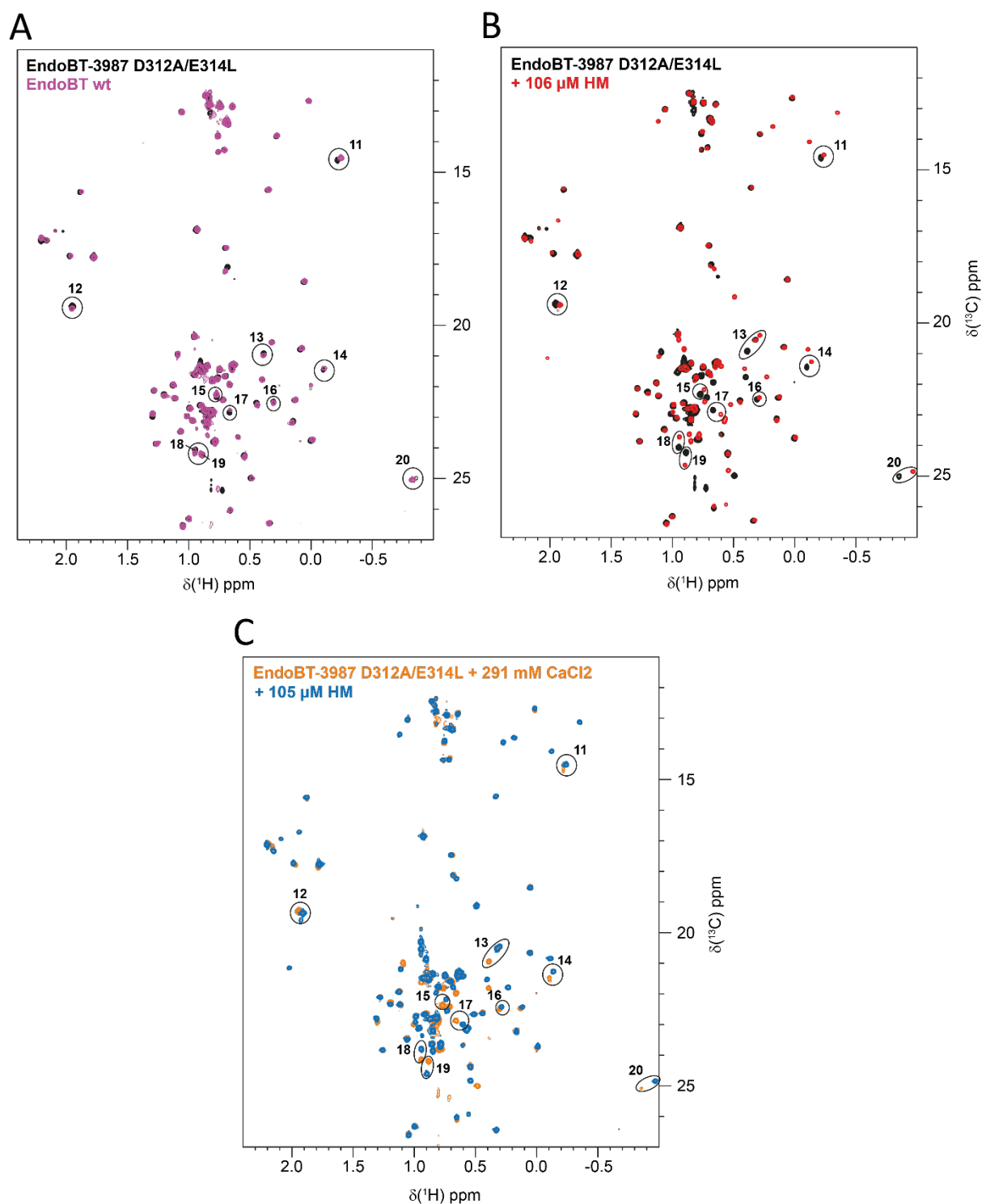
Supplementary Figure 21. $^1\text{H}, ^{15}\text{N}$ TRACT¹⁴ was used to estimate protein rotational correlation times τ_c , which indicates whether the protein sample is folded or unfolded in solution. Shown are the ^{15}N R_α (black) and R_β (grey) decay curves observed for (A) 71.5 μM of EndoS2_{E186L} and (B) 74.4 μM of EndoBT_{D312A/E314L} endoglycosidase mutants. Experiments were acquired at 600 MHz and 298 K.



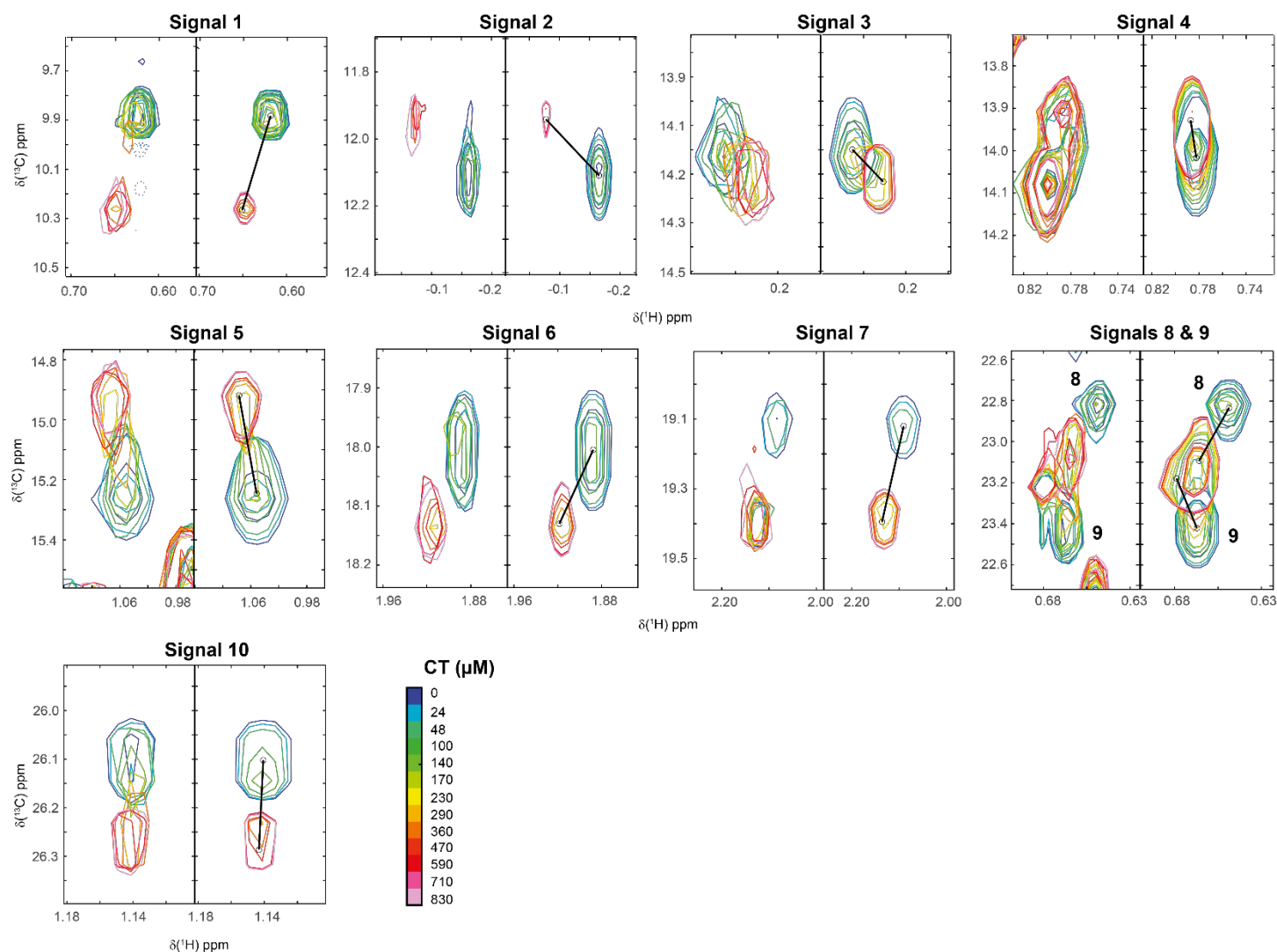
Supplementary Figure 22. CSPs observed for EndoS2 and EndoS2_{E186L}. Superimposition of methyl-TROSY spectra corresponding to MILV-labeled (A) EndoS2_{E186L} (black) and EndoS2 (violet), (B) EndoS2_{E186L} in the absence (black) and in the presence of saturating concentrations of CT (red), (C) EndoS2_{E186L} in the absence (black) and in the presence of close-to-saturation concentrations of CT_{n-1} (orange) and (D) EndoS2_{E186L} in the absence (black) and in the presence of saturating concentrations of Rituximab Fc (blue). Resonances highlighted in circles were used for lineshape analysis with TITAN. Spectra were acquired at 298 K and 600 MHz.



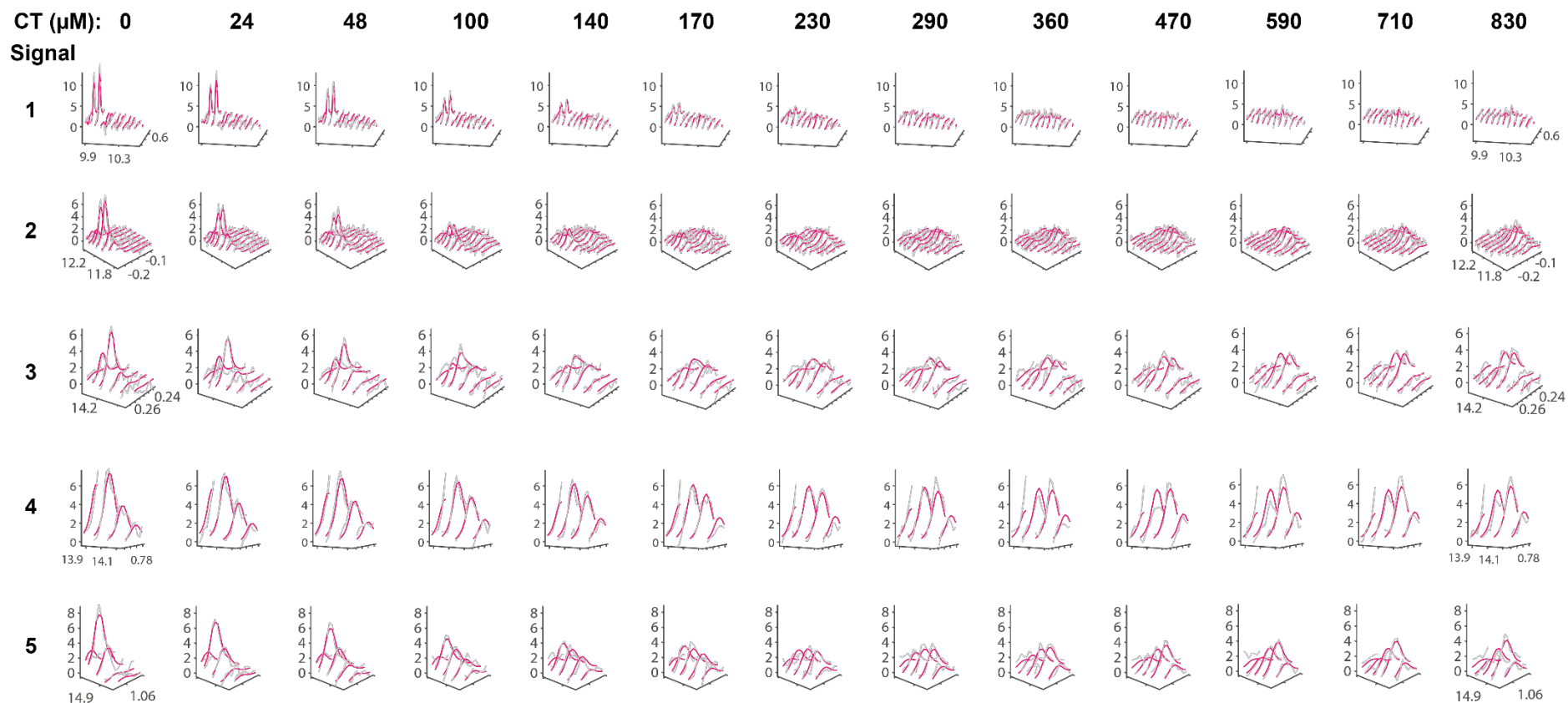
Supplementary Figure 22 - continued. (E) Superimposition of methyl-TROSY spectra corresponding to MILV-labeled EndoS2_{E186L} in the absence (black) and in the presence of 72 μ M of Rituximab-Fc aglycan (green). Spectra were acquired at 298 K and 600 MHz.



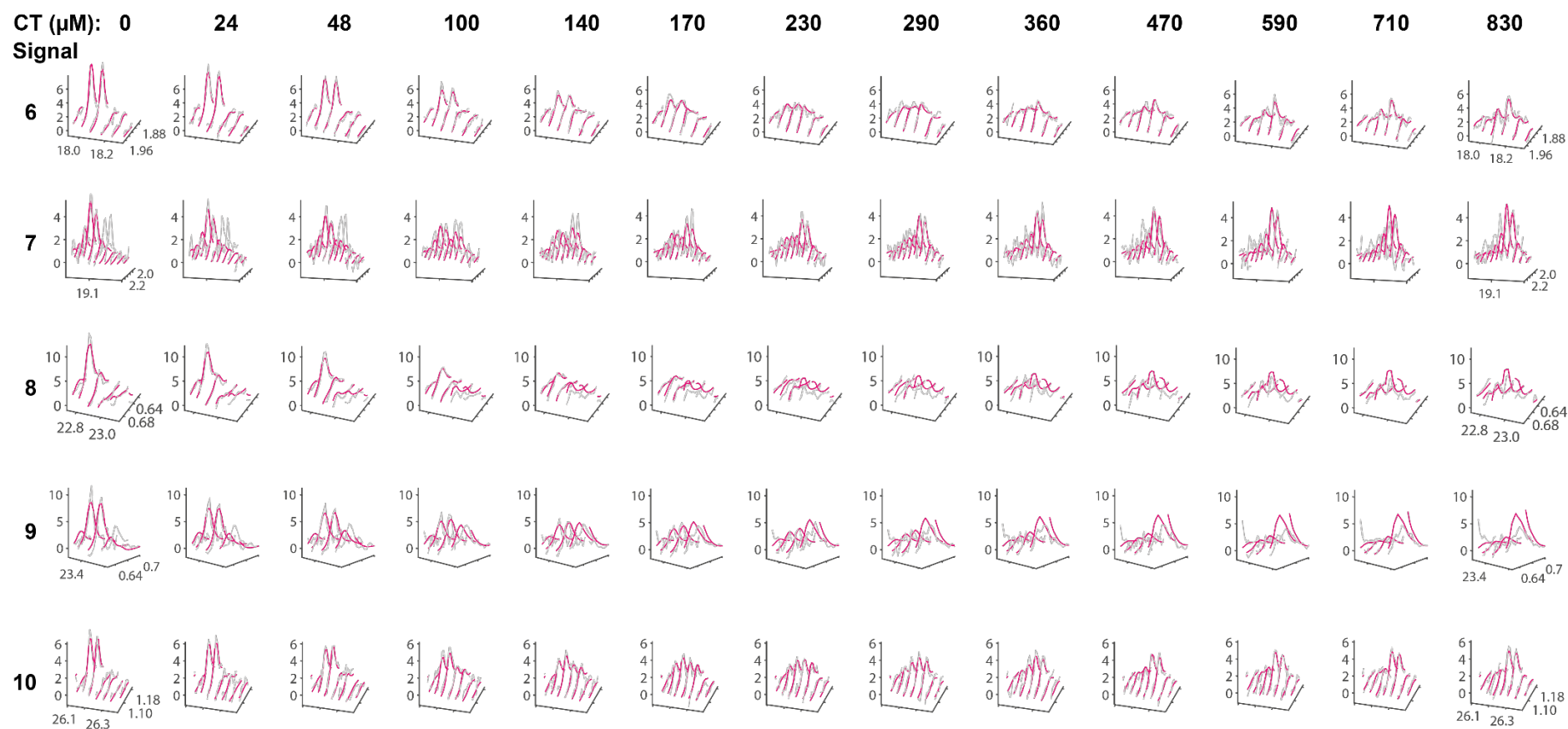
Supplementary Figure 23. CSPs observed for EndoBT-3987 and EndoBT-3987_{D312A/E314L}. (A) Superimposition of methyl-TROSY spectra of MILV-labeled EndoBT-3987 (purple) and EndoBT-3987_{D312A/E314L} (black). (B) Superimposition of methyl-TROSY spectra of MILV-labeled and EndoBT-3987_{D312A/E314L} in the absence (black) and in the presence (red) of saturating concentrations of HM. (C) Similar to (B) but under saturation concentrations of CaCl₂. Orange indicates no HM and blue saturation concentrations of HM. Resonances highlighted in circles were used for lineshape analysis with TITAN. Spectra were acquired at 298 K and 600 MHz.



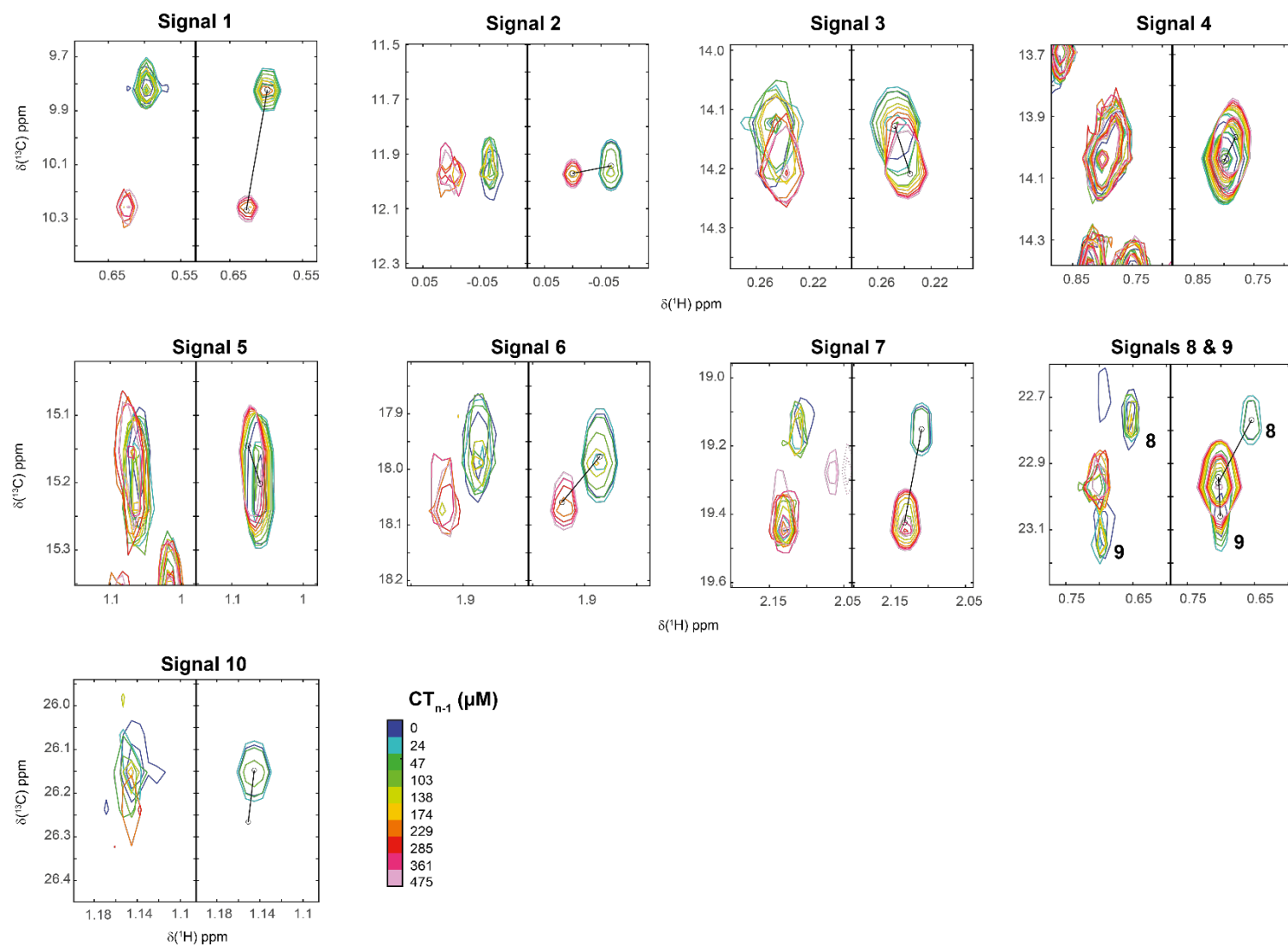
Supplementary Figure 24. Complete set of cross peaks used for the TITAN analysis of CT binding to MILV-labeled EndoS2_{E186L}. The left panel shows the sections from the methyl-TROSY experiment as measured. The right panel shows the simulated cross peaks using a simple two-states binding model.



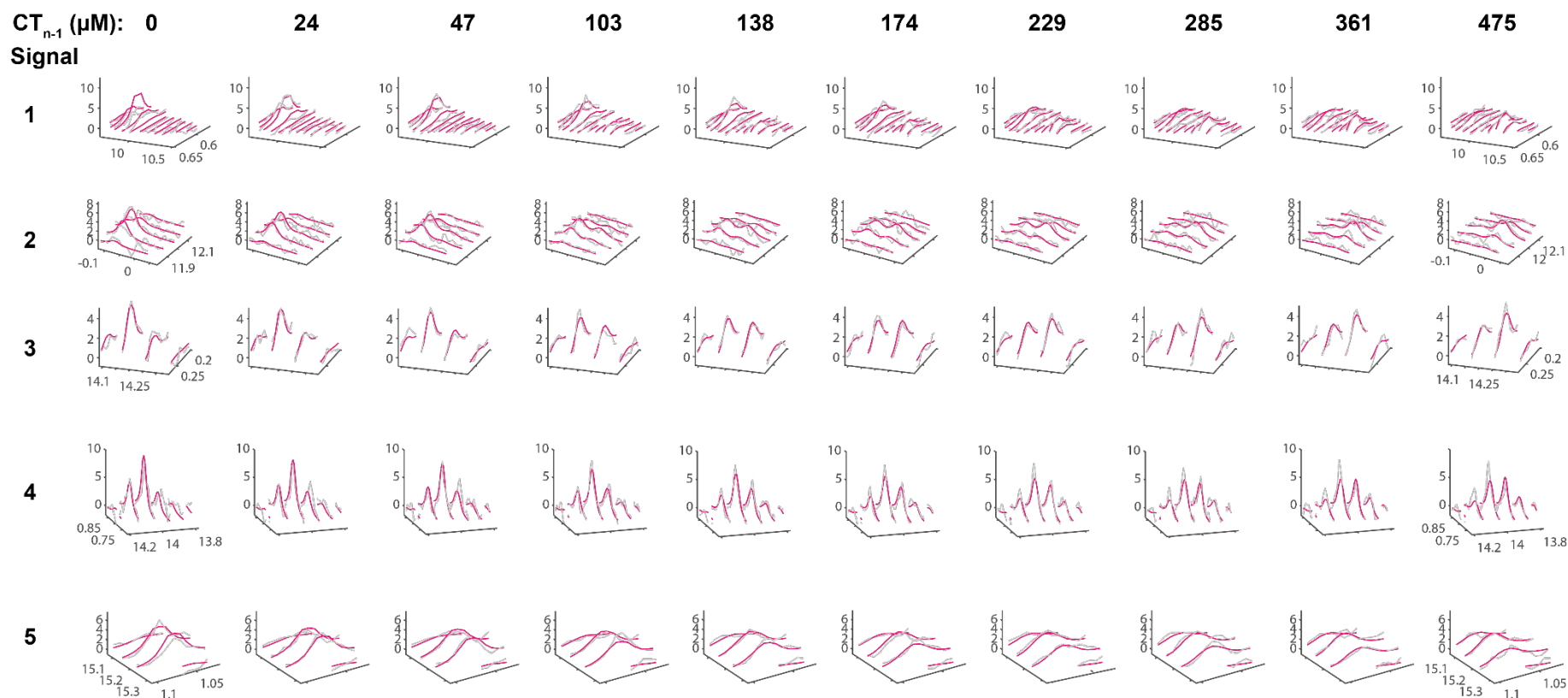
Supplementary Figure 25. 2D lineshape analysis of methyl-TROSY spectra of a MILV-labeled sample of EndoS2_{E186L} at increasing CT concentrations. Global lineshape analysis of a series of concentration dependent methyl-TROSY spectra using TITAN¹³ yielded a fitted set of 10 signals. It should be noted that in principle this analysis does not depend on any assignments. Experimental and fitted cross peak are shown in grey and magenta, respectively, reflecting the good quality of the fit. TITAN analysis results in a dissociation constant K_D of $106.9 \pm 6.7 \mu\text{M}$ and a dissociation rate constant k_{off} of $11.4 \pm 1.5 \text{ s}^{-1}$.



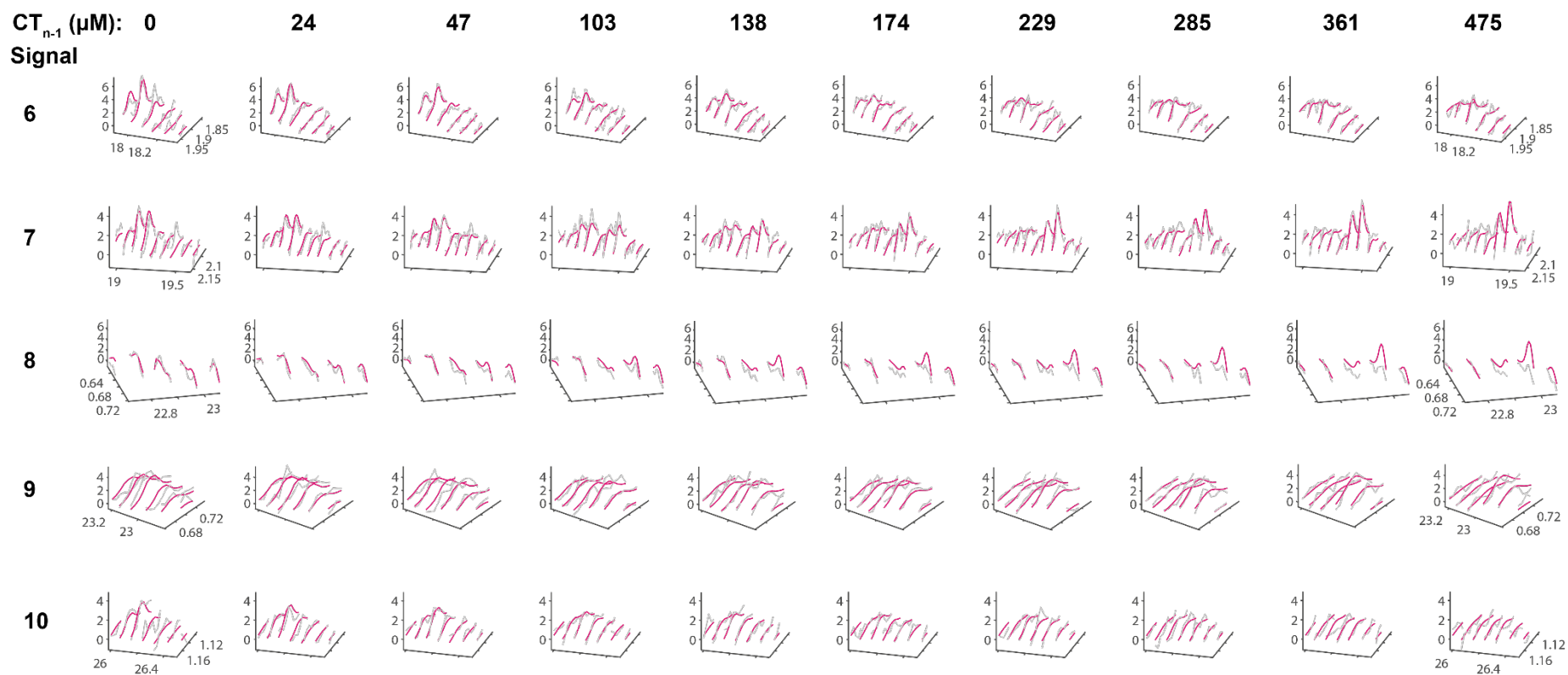
Supplementary Figure 25 - continued.



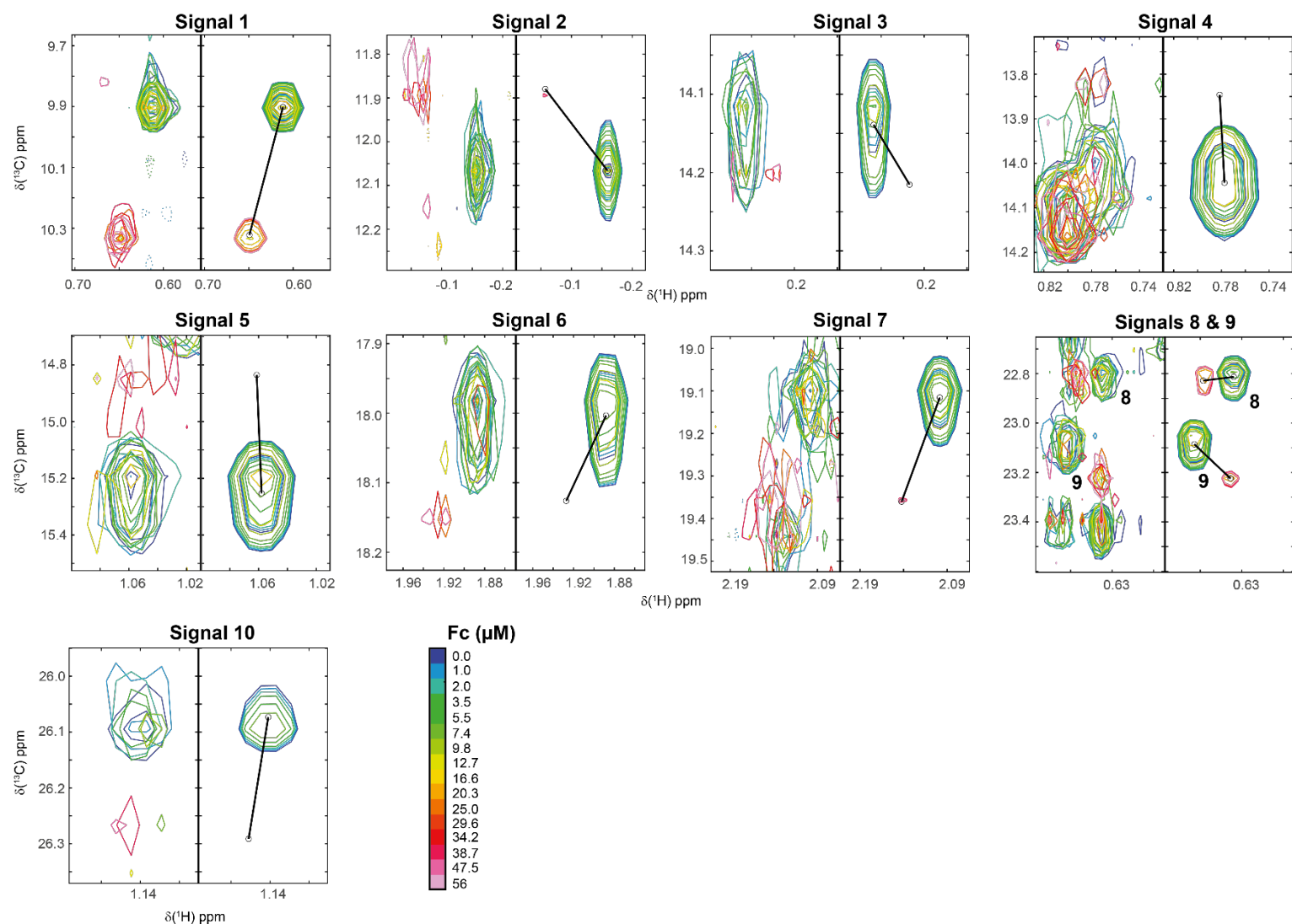
Supplementary Figure 26. Complete set of cross peaks used for the TITAN analysis of CT_{n-1} binding to MILV-labeled EndoS2_{E186L}. The left panel shows the sections from the methyl-TROSY experiment as measured. The right panel shows the simulated cross peaks using a simple two-states binding model.



Supplementary Figure 27. 2D lineshape analysis of methyl-TROSY spectra of a MILV-labeled sample of EndoS2_{E186L} at increasing CT_{n-1} concentrations. Global lineshape analysis of a series of concentration dependent methyl-TROSY spectra using TITAN¹³ yielded a fitted set of 10 signals. It should be noted that in principle this analysis does not depend on any assignments. Experimental and fitted cross peak are shown in grey and magenta, respectively, reflecting the good quality of the fit. TITAN analysis results in a dissociation constant K_D of $150.7 \pm 18.0 \mu\text{M}$ and a dissociation rate constant k_{off} of $9.4 \pm 3.7 \text{ s}^{-1}$.

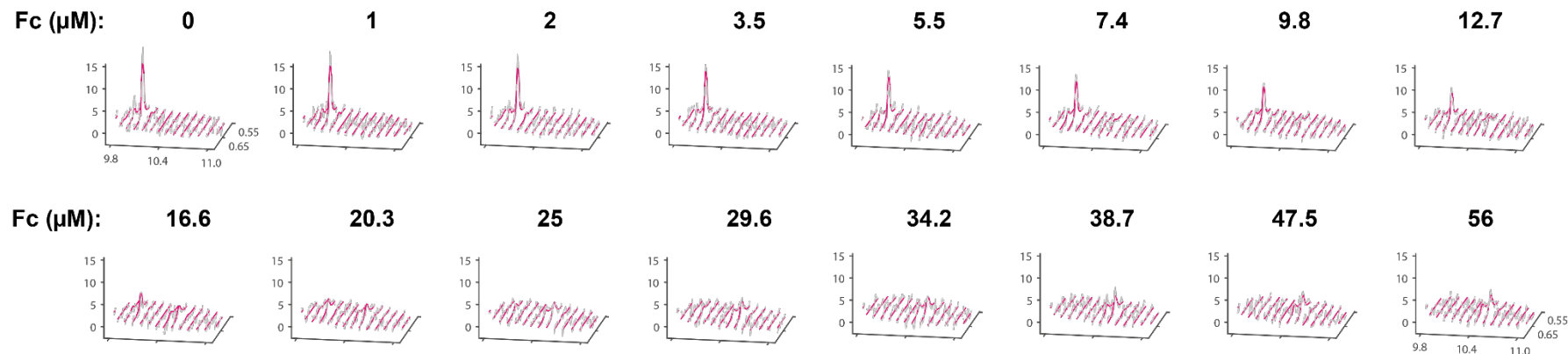


Supplementary Figure 27 - continued.

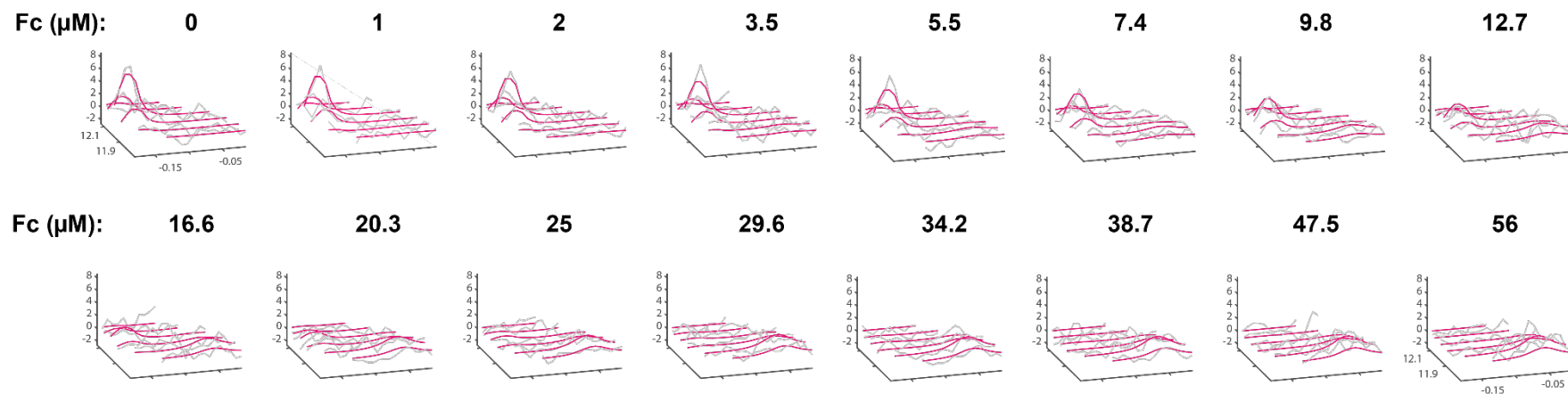


Supplementary Figure 28. Complete set of cross peaks used for the TITAN analysis of Rituximab-Fc binding to MILV-labeled EndoS2_{E186L}. The left panel shows the sections from the methyl-TROSY experiment as measured. The right panel shows the simulated cross peaks using a simple two-states binding model.

Signal 1



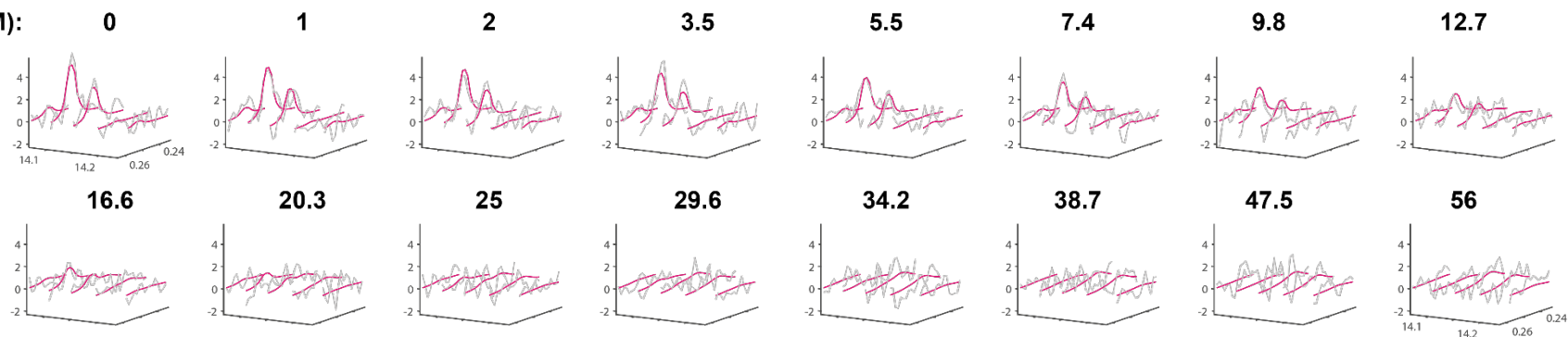
Signal 2



Supplementary Figure 29. 2D lineshape analysis of methyl-TROSY spectra of a MILV-labeled sample of EndoS2_{E186L} at increasing Rituximab-Fc concentrations. Global lineshape analysis of a series of concentration dependent methyl-TROSY spectra using TITAN¹³ yielded a fitted set of 10 signals. It should be noted that in principle this analysis does not depend on any assignments. Experimental and fitted cross peak are shown in grey and magenta, respectively, reflecting the good quality of the fit. TITAN analysis results in a dissociation constant K_D of $3.1 \pm 0.6 \mu\text{M}$ and a dissociation rate constant k_{off} of $4.0 \pm 1.5 \text{ s}^{-1}$.

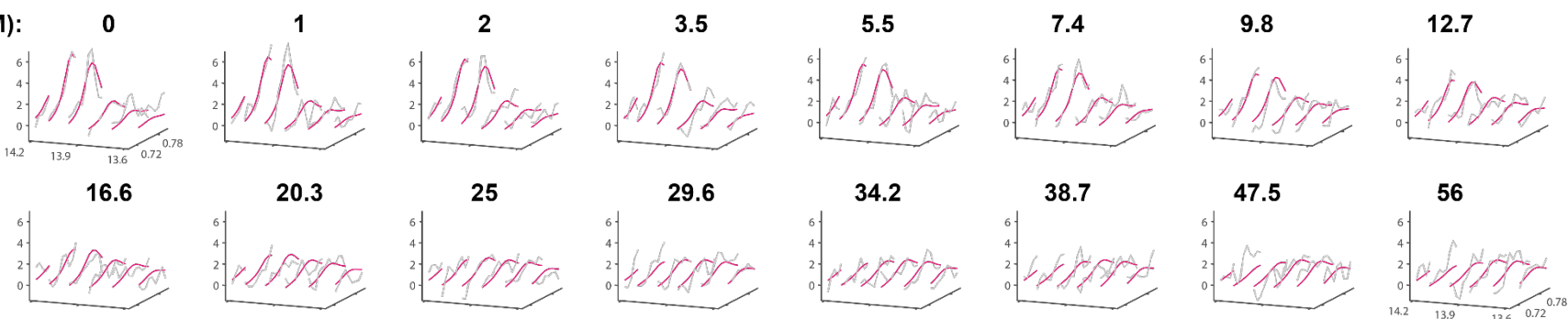
Signal 3

Fc (μM):



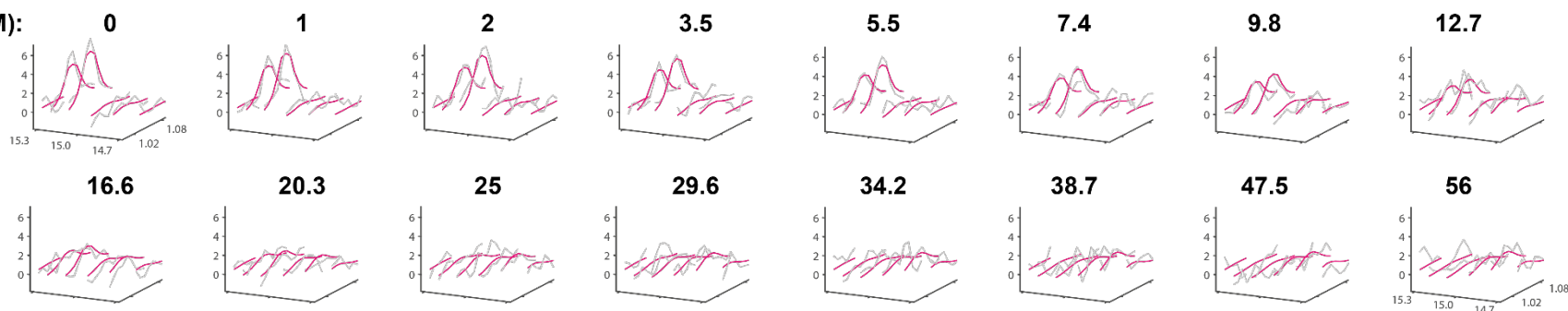
Signal 4

Fc (μM):

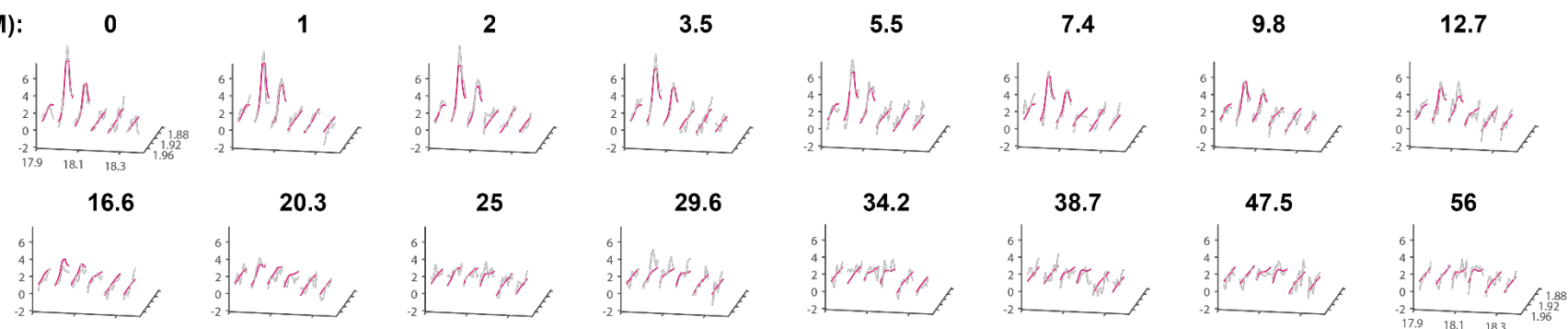
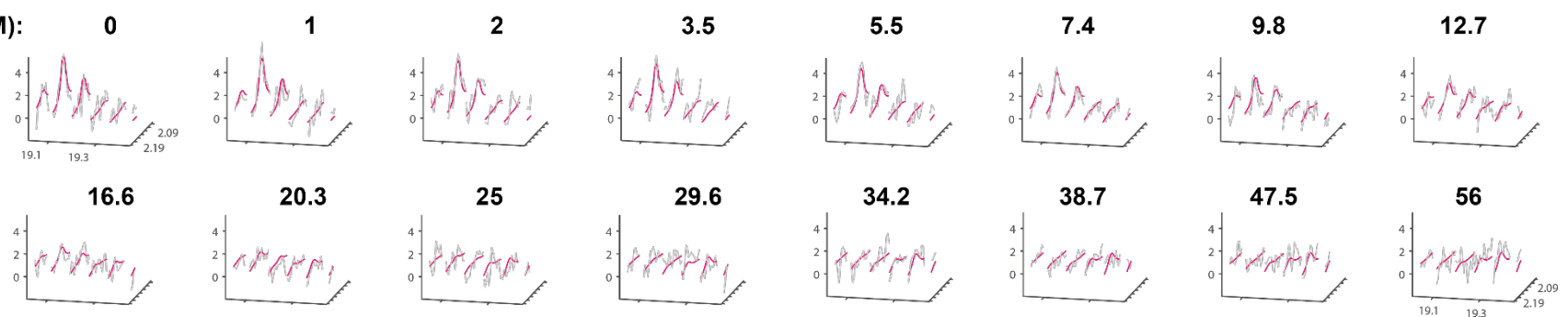
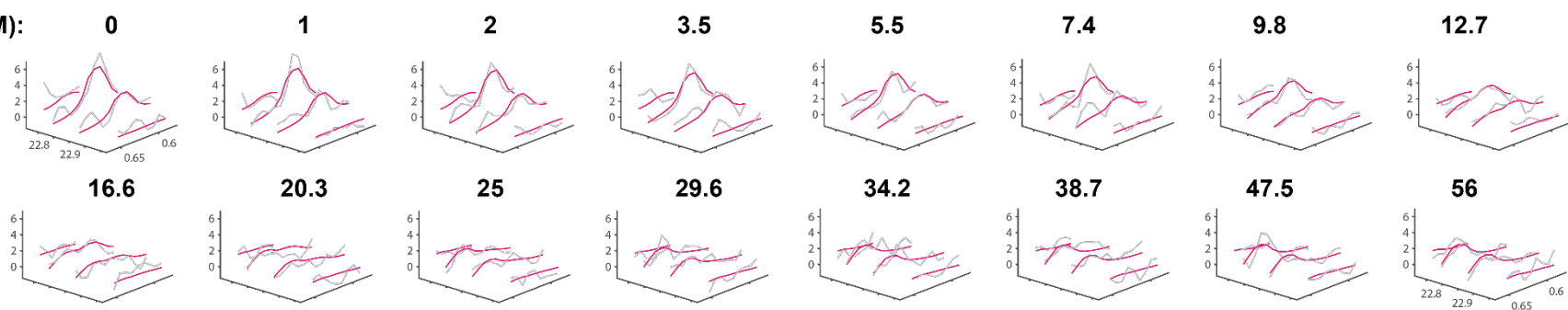


Signal 5

Fc (μM):



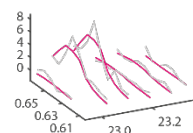
Supplementary Figure 29 - continued.

Signal 6**Fc (μM):****Signal 7****Fc (μM):****Signal 8****Fc (μM):****Supplementary Figure 29 - continued.**

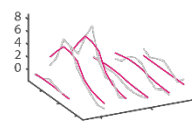
Signal 9

Fc (μM):

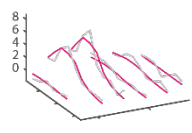
0



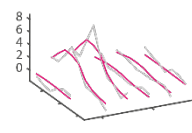
1



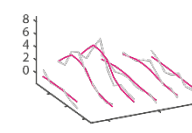
2



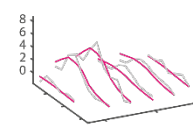
3.5



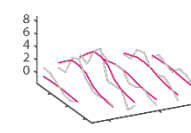
5.5



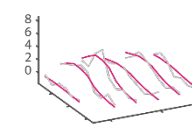
7.4



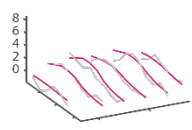
9.8



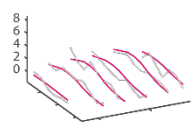
12.7



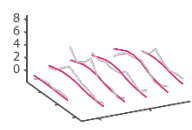
16.6



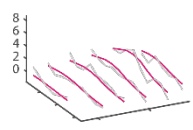
20.3



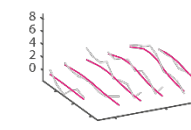
25



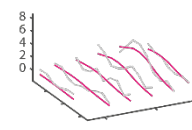
29.6



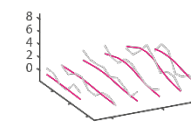
34.2



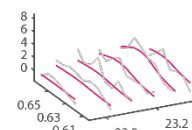
38.7



47.5



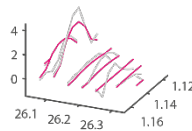
56



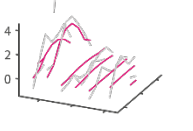
Signal 10

Fc (μM):

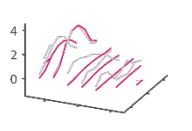
0



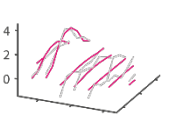
1



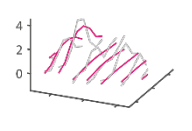
2



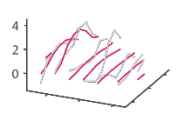
3.5



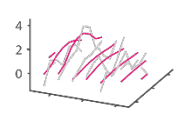
5.5



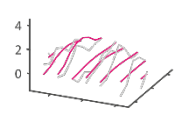
7.4



9.8



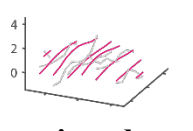
12.7



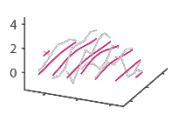
16.6



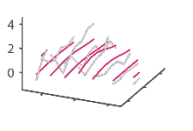
20.3



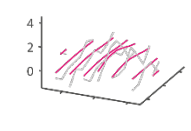
25



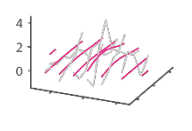
29.6



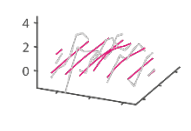
34.2



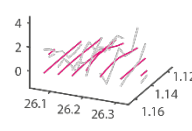
38.7



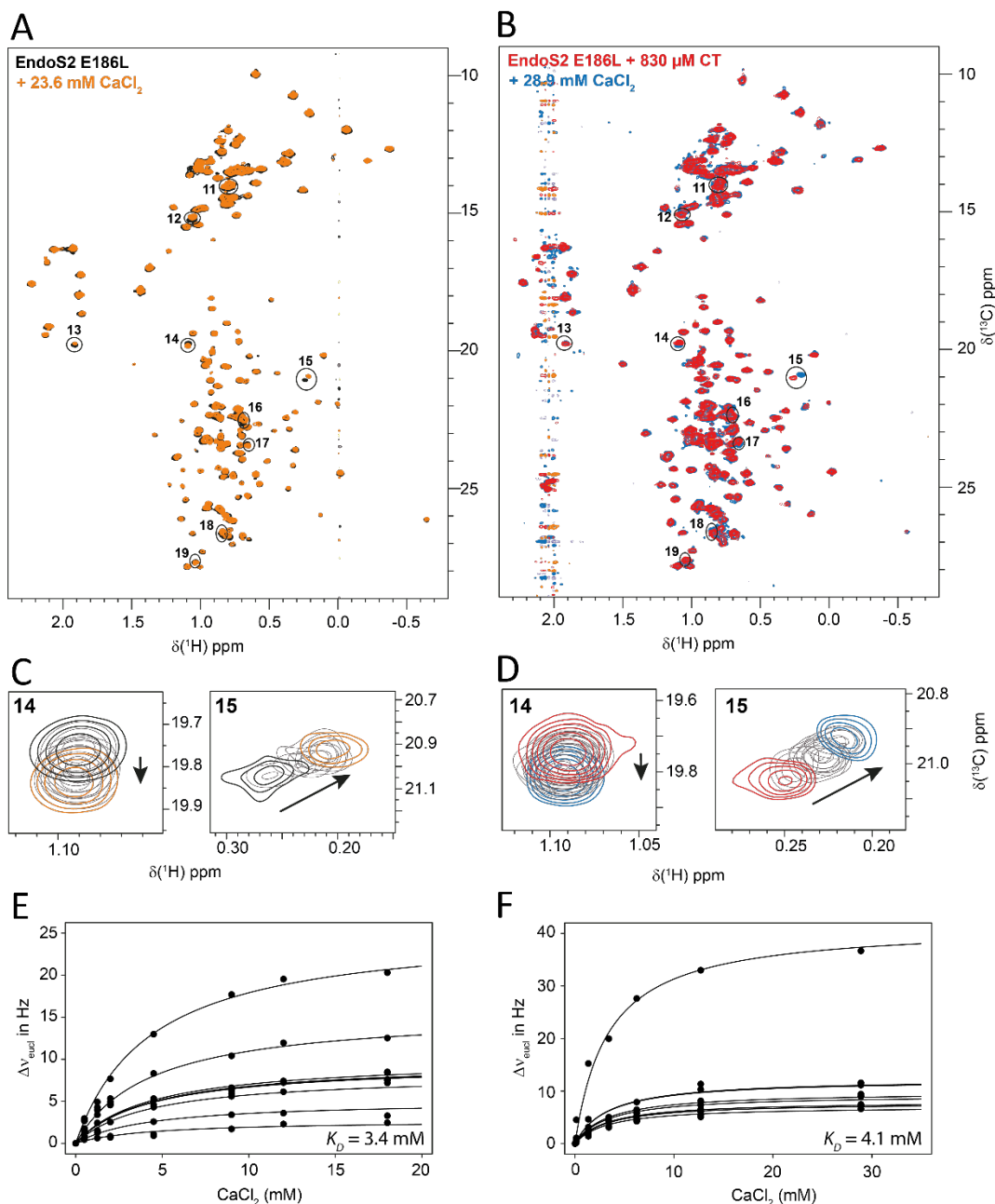
47.5



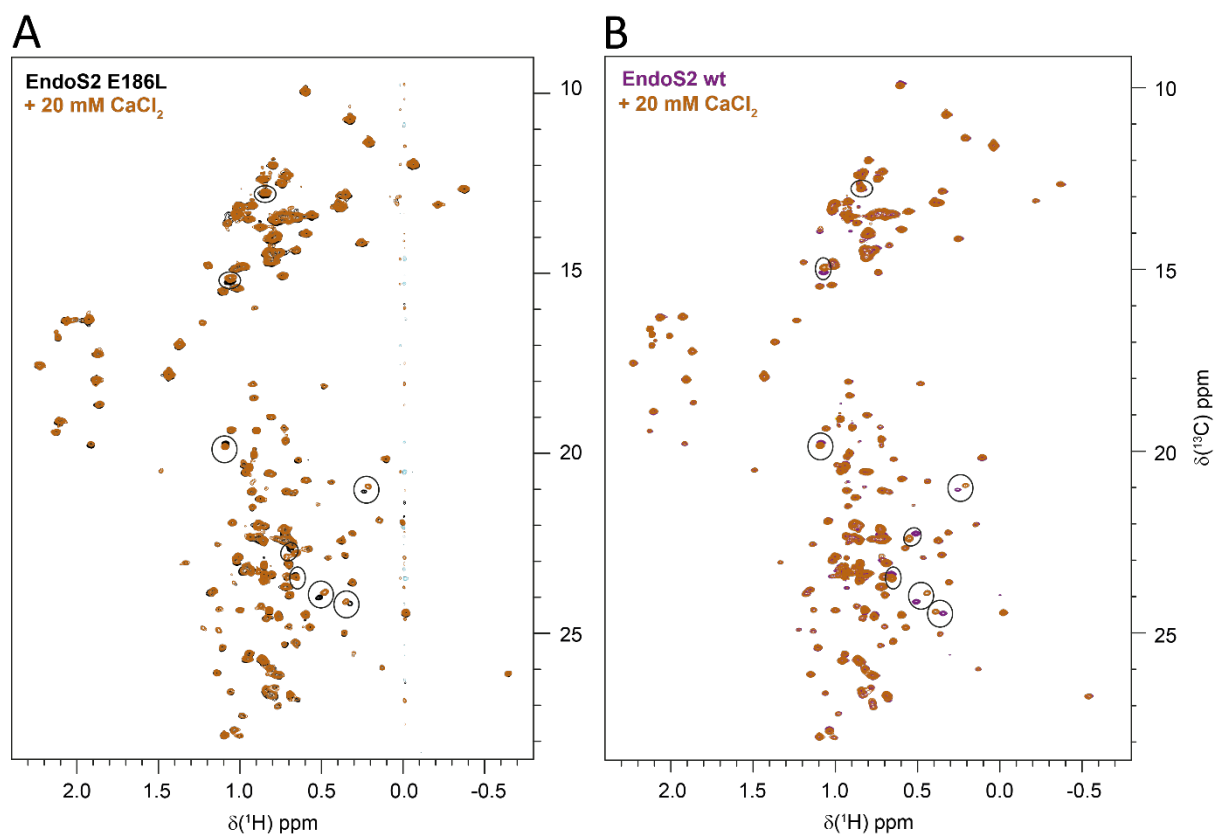
56



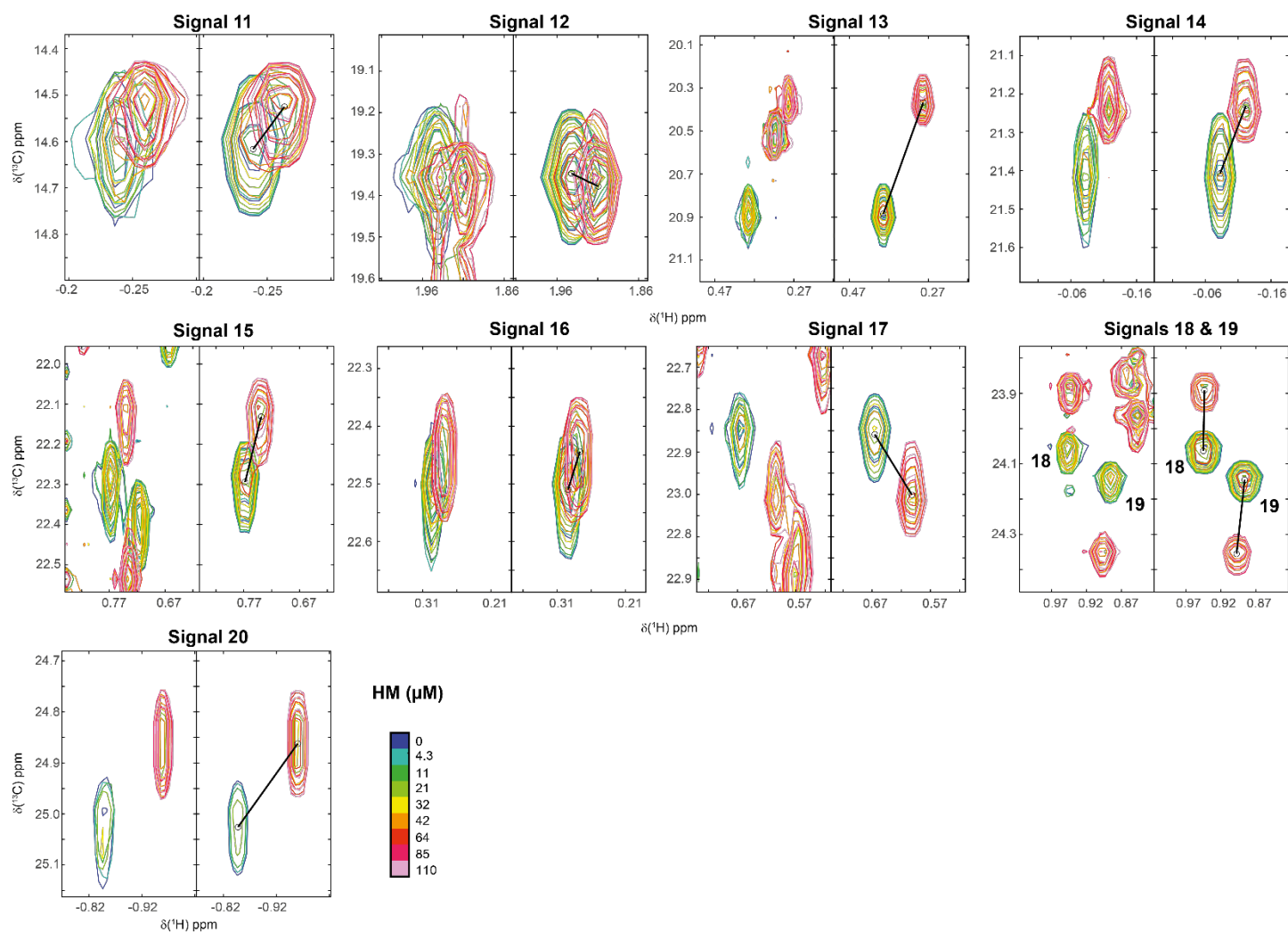
Supplementary Figure 29 - continued.



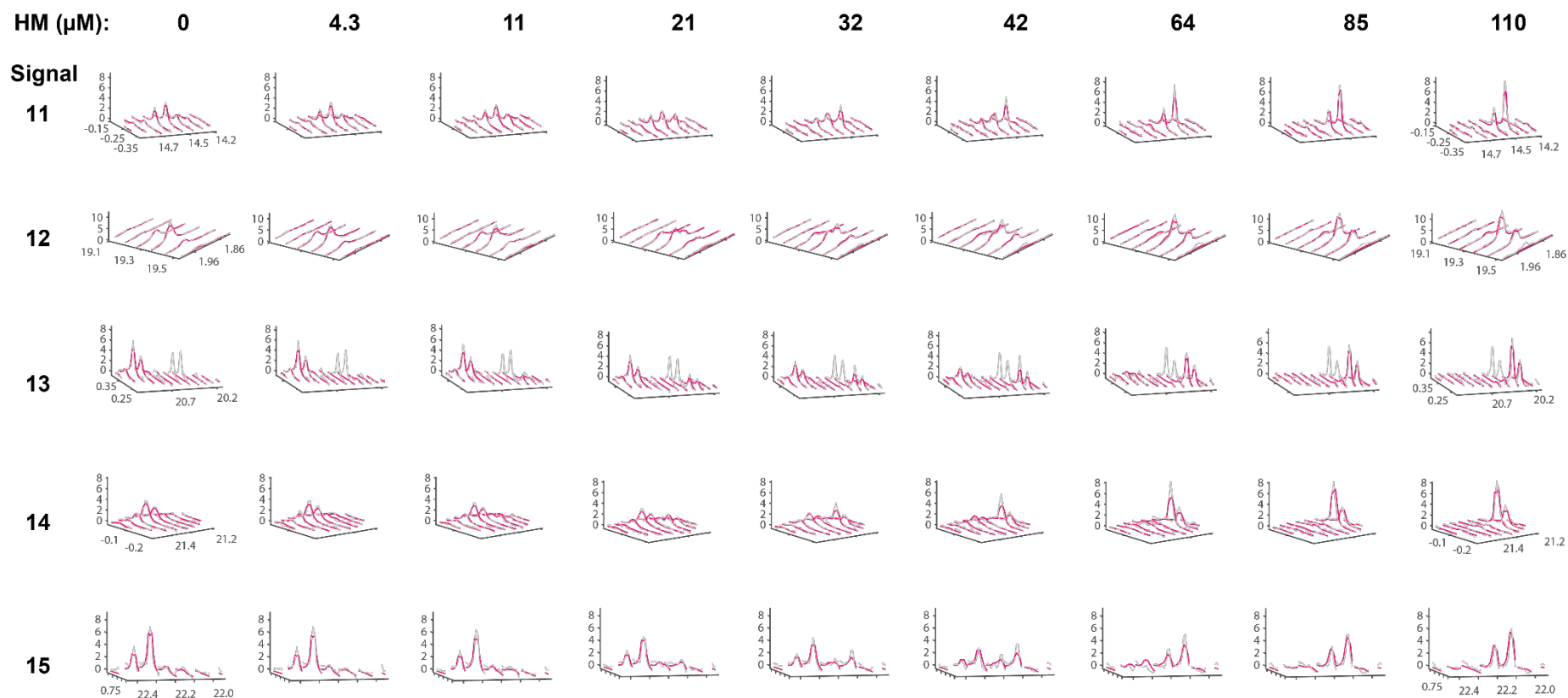
Supplementary Figure 30. Titration of EndoS2 and EndoS2_{E186L} with CaCl₂. Superimposition of methyl-TROSY spectra corresponding to the titration of MILV-labeled EndoS2_{E186L} with CaCl₂ (**A**) in the absence and (**B**) in the presence of saturating concentrations of CT. Spectra acquired in the absence and in the presence of saturating concentrations of Ca²⁺ are colored in (**A**) black and orange in, and (**B**) in red and blue, respectively. Resonances highlighted in circles were used to extract dissociation constants from CSPs. Exchange between calcium-free and calcium-bound protein forms are in fast exchange in the NMR time-scale, as can be seen for selected resonances in (**C**) the absence and (**D**) in the presence of saturating concentrations of CT. Dissociation constants K_D were calculated from the fitting of CSPs to the law of mass action. Binding isotherms corresponding to the titration of calcium (**E**) in the absence and (**F**) under saturation concentrations of CT. Spectra were acquired at 298 K and 600 MHz.



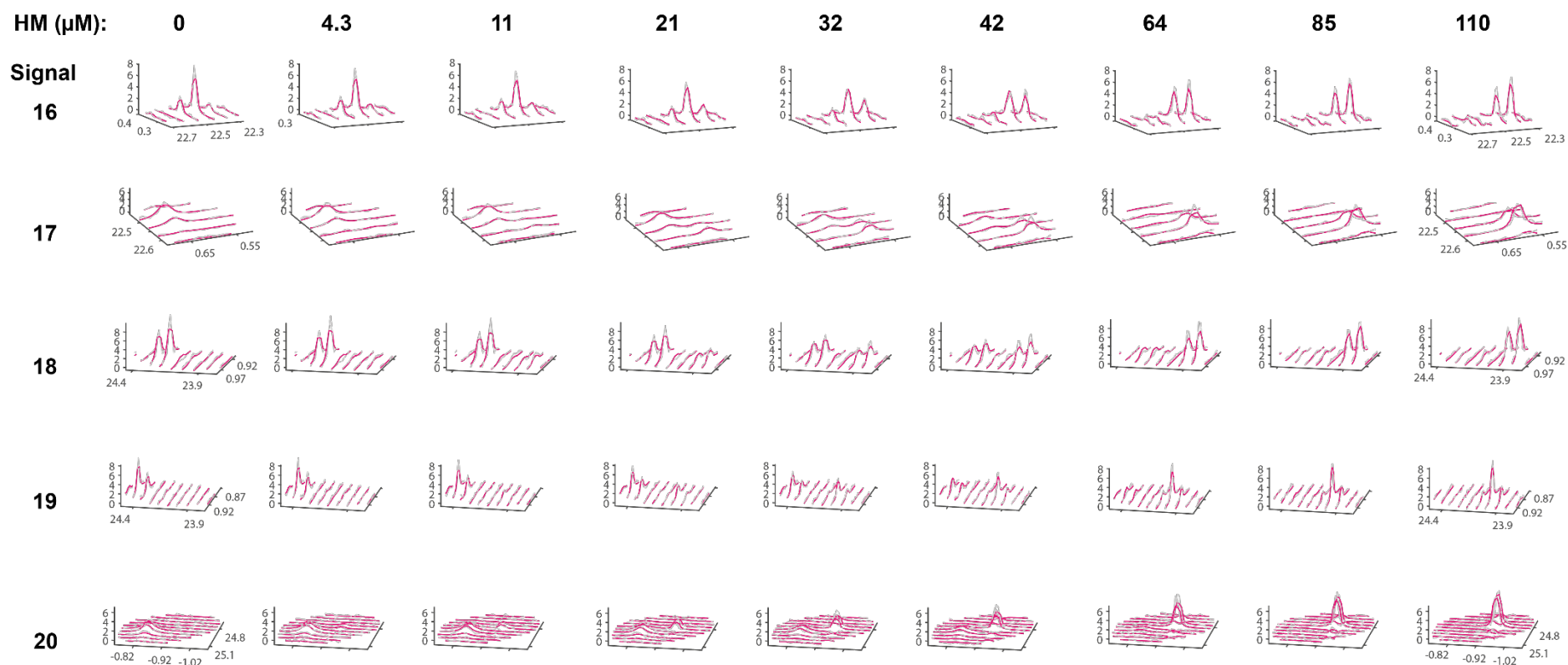
Supplementary Figure 31. Similar CSPs were observed for the binding of Ca^{2+} to EndoS2_{E186L} and to EndoS2. Superimposition of methyl-TROSY spectra corresponding to MILV-labeled (A) EndoS2_{E186L} in the absence (black) and in the presence of saturating concentrations of CaCl₂ (yellow), (B) and of EndoS2 in the absence (violet) and in the presence of saturating concentrations of CaCl₂ (yellow). Resonances highlighted in circles shift upon titration with CaCl₂. Spectra were acquired at 298 K and 600 MHz.



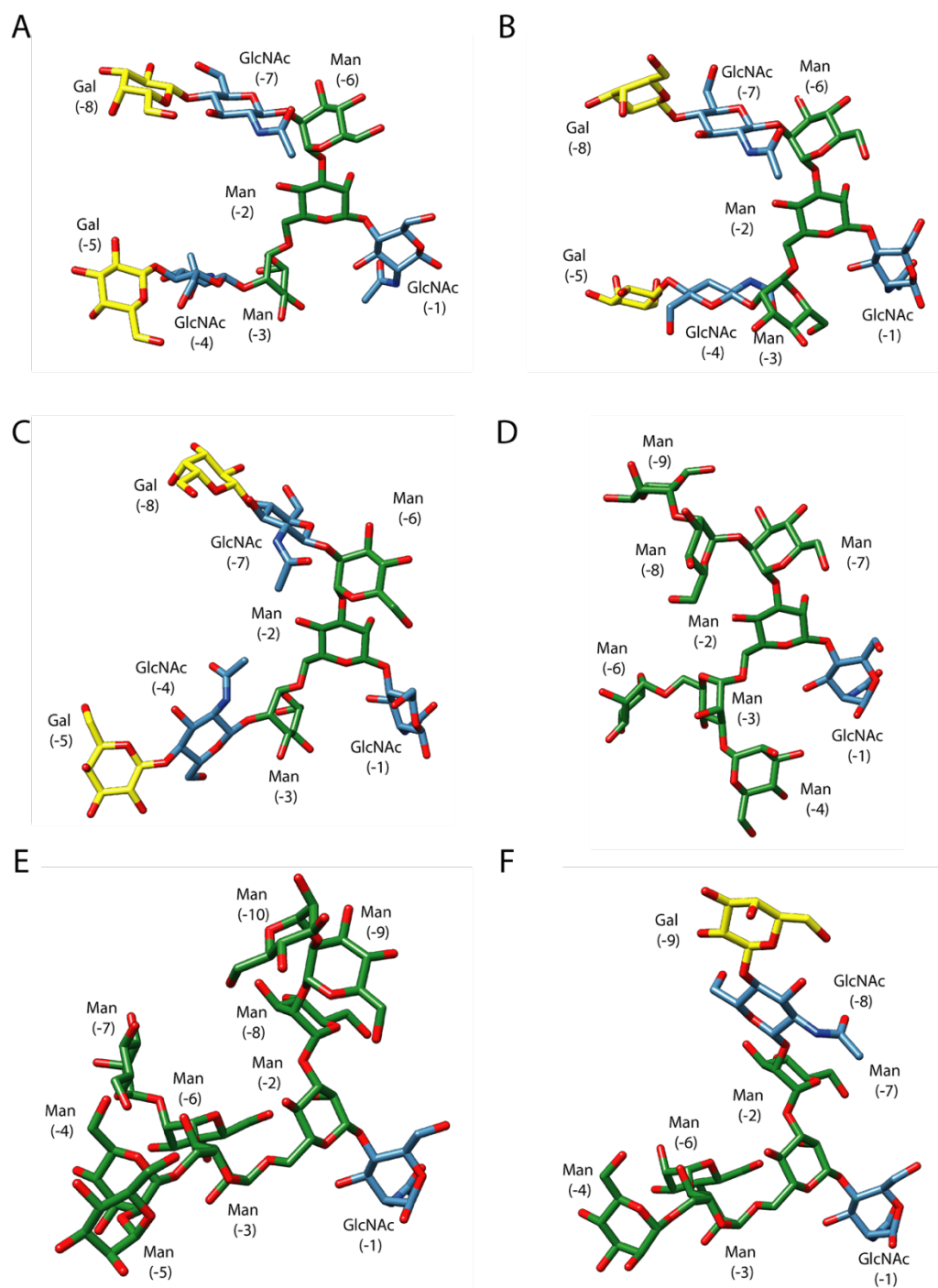
Supplementary Figure 32. Complete set of cross peaks used for the TITAN analysis of HM binding to MILV-labeled EndoBT_{D312A/E314L} in the absence of CaCl₂. The left panel shows the sections from the methyl-TROSY experiment as measured. The right panel shows the simulated cross peaks using a simple two-states binding model.



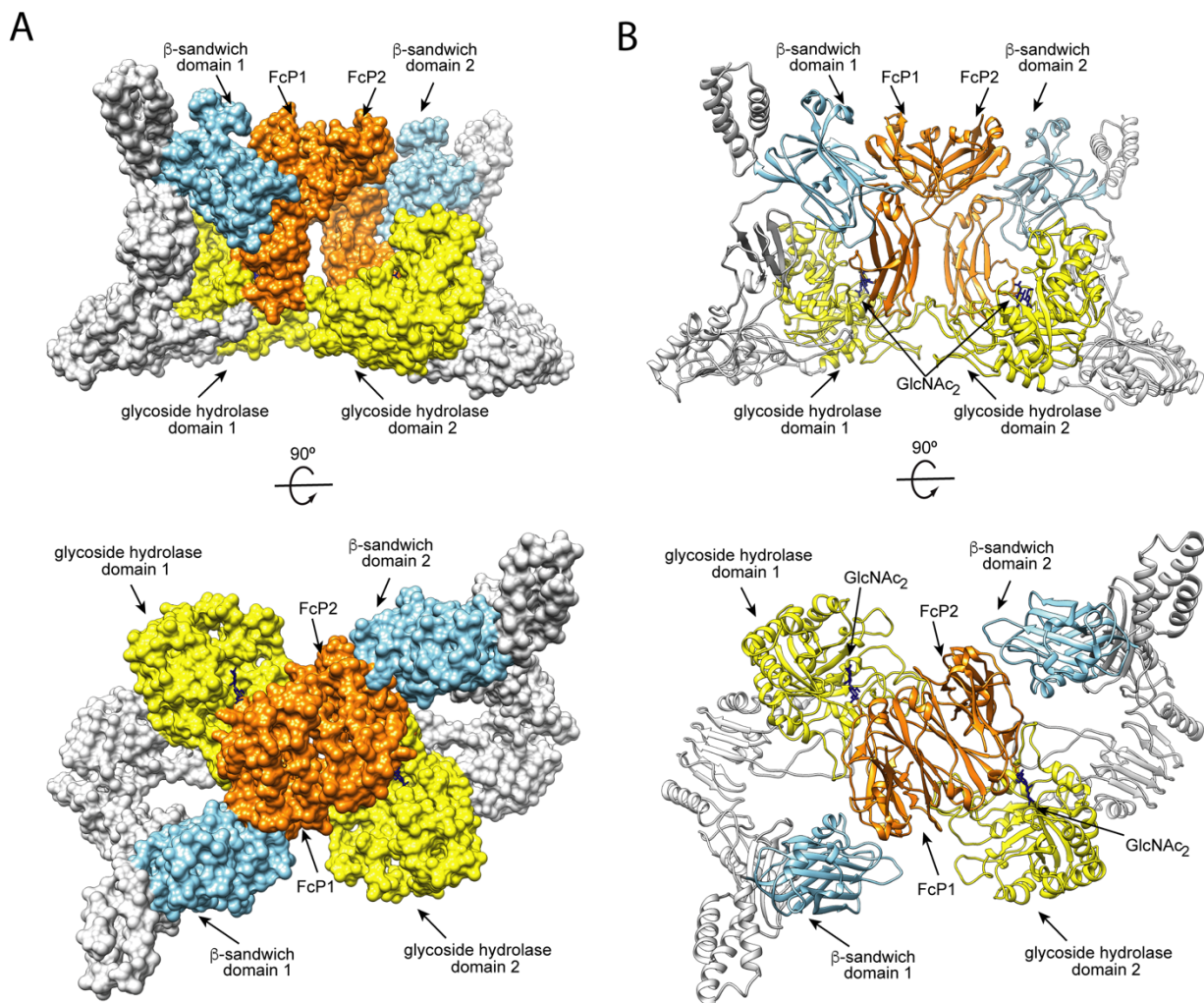
Supplementary Figure 33. 2D lineshape analysis of methyl-TROSY spectra of a MILV-labeled sample of EndoBT_{D312A/E314L} at increasing HM concentrations in the absence of CaCl₂. Global lineshape analysis of a series of concentration dependent methyl TROSY spectra using TITAN¹³ yielded a fitted set of 10 signals. It should be noted that in principle this analysis does not depend on any assignments. Experimental and fitted cross peak are shown in grey and magenta, respectively, reflecting the good quality of the fit. TITAN analysis results in a dissociation constant K_D of $1.7 \pm 0.9 \mu\text{M}$ and a dissociation rate constant k_{off} of $5.5 \pm 1.4 \text{ s}^{-1}$.



Supplementary Figure 33 - continued.



Supplementary Fig. 34. *N*-glycan conformations in the active site of ENGases. Distinct conformation of *N*-glycans found in the crystal structures of EndoS-CT (PDB code 6EN3) (**A**), EndoS2-CT (PDB code 6MDS) (**B**), EndoF3 (PDB code 1EOM) (**C**), EndoS2-HM (PDB code 6MDV) (**D**), EndoBT-3987-HM (PDB code 6T8L) (**E**) and EndoBT-3987-Hy (PDB code 7NWF) (**F**).



Supplementary Fig. 35. Model of the EndoS-Fc (2:1) complex. Two views of the surface (A) and cartoon representation (B) of the EndoS-Fc (2:1) based on our experimentally determined EndoS-Fc (1.1) cryoEM structure.

Supplementary Videos.

Supplementary Video 1. Conformational motions of the EndoS_{E235A}-Fc complex. Component 1

Supplementary Video 2. Conformational motions of the EndoS_{E235A}-Fc complex. Component 2

Supplementary Video 3. Conformational motions of the EndoS_{E235A}-Fc complex. Component 3.

Supplementary References

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