Supporting Information Demircioglu et al.

Fig. S1: Assembly of ER-Golgi SNARE components monitored by CD spectroscopy.

A. Structural change upon SNARE complex formation. CD spectra were collected either from single SNARE motifs (5 μM from each of Sed5 211-320 (Qa-), Bos1 51-221 (Qb-), Bet1 1-118 (Qc-) and Sec22 126-186 (R-SNARE) (1-5)) or after their overnight incubation in equimolar amounts. The CD spectrum shown in dashed line is the theoretical non-interacting spectrum, calculated from the observed CD spectra of the SNARE segments. The spectrum of the quaternary complex formed between SNARE segments is shown in black.

B. Assembly kinetics of ER-Golgi SNAREs followed by CD spectroscopy**.** Assembly kinetics of ER-Golgi SNAREs was observed by monitoring the change in the mean residue ellipticity at 222 nm over time. The measurement was started after addition of 2 μM Sed5p 211-320 to an equimolar mixture of the other SNAREs (Bos1 151-221, Bet1 1-118, Sec22 126-186). SNARE complex assembly is slow (not completed after 8 hours at 2 μM of each subunit), roughly comparable to the speed of assembly of neuronal complex when SNAP-25 helices are used in two different constructs (6).

Fig. S2: Interaction between the Habc domain and the SNARE motif of Sed5 monitored by nondenaturing gel electrophoresis.

Prior to separation by nondenaturing gel electrophoresis, the isolated Habc domain (Sed5 1-210) and the SNARE motif of Sed5 (Sed5 211-320) were incubated overnight in standard buffer containing 200 mM NaCl. Constant amounts of the SNARE motif (31.4 μM) were mixed with increasing amounts of the Habc domain (concentrations as indicated). Note that due to an isoelectric point of 9.6, the individual Habc domain is not visible in the nondenaturing gel.

Fig. S3: Sly1 does not affect the assembly rate of the isolated SNARE motif of Sed5 with its partner SNAREs.

Addition of Sly1 (11 μM) has no effect on the rate of SNARE complex formation in the presence of the SNARE segment of Sed5 (Sed5 211-320, 7 μM). SNARE complex formation was monitored by the increase in fluorescence anisotropy of fluorescent Sec22 (Sec22 126-186$^{C131\text{-}OG}$) as described in Fig. 1C.

Fig. S4: Isothermal titration calorimetry experiments that have been performed in this study.

All isothermal titrations were performed at 25 °C in PBS buffer. In each plot, the top panel displays the base-line corrected raw data in power versus time. The lower panel shows the integrated areas normalized to the amount of the injectant (kcal mol^{-1}) versus its molar ratio to the macromolecule(s) in the cell. The solid lines represent the best fit to the data for a single-site binding model. Thermodynamic parameters calculated from the fits are given in Table S1. All experiments have been performed in replicates, and a representative example is shown.

In most experiments, 15 μM of the different Sed5 variants were injected into 2 μM of Sly1. For the titration of Sed5 21-324 30 μM were used, however. For the titration of Sed5 1-21^{F10A} and Sed5 1-320^{F10A} 25 μM were injected into 1.5 μM of Sly1. Moreover, 25 μM of the SNARE complex containing the SNARE motif of Sed5 (Sed5 211-320) was titrated into 3.3 μM Sly1, whereas the purified SNARE complex containing the entire cytosolic domain of Sed5 (Sed5 1-320) was injected into Sly1 at lower concentrations (7.5 μM and 1 μM, respectively), since it was difficult to purify this SNARE complex in larger amounts. In general, for variants and complexes for which we only detected small or no heat changes higher protein concentrations were used.

Because the precise determination of the binding affinity using ITC becomes progressively difficult once it approaches and surpasses the nanomolar range, we used displacement titration in order to more precisely determine the affinity of full-length Sed5. For this experiment, we needed to displace a known binding partner from Sly1 with

varying amounts of Sed5. For this, we chose the N-peptide of the syntaxin Ufe1, which is involved in retrograde vesicle trafficking and known to bind to Sly1 (7-9). Replacement titrations were performed according to (10). 20 μM Sed5 1-320 or 40 μM Sed5 1-210 were injected into Sly1 (2 μM)/ Ufe1 1-21 (15 μM) mixes. For fitting of the competitive binding experiments, thermodynamic parameters of Sly1 (2 μM)/ Ufe1 1-21 (15 μM) binding (N, K_d and ΔH) were used as input. Upon ITC, we determined an affinity of 35.4 nM and an enthalpy of - 15.8 kcal/mole for the interaction of Sly1 and the N-peptide of Ufe1. The difference in enthalpy between binding of Sly1 to Sed5 1-320 and to the N-peptide of Ufe1 enabled us to titrate Sed5 to a premix of Sly1 and the Ufe1 N-peptide. This experimental strategy revealed an affinity of ≈ 0.17 nM for the interaction of Sly1 and Sed5 1-320. Unfortunately, this strategy could not be used for shorter Sed5 fragments, because their binding enthalpies were almost identical to the one of the Ufe1 N-peptide.

Notice that an ITC experiment, in which a preformed complex between the Habc and the SNARE motif was titrated into Sly1, did not provide a definitive result as the determined enthalpy (≈ 22.7 kcal/mole) and affinity (0.48 nM) were in between the affinities obtained for the Habc and for the full-length Sed5 molecule. It needs to be stressed, however, that because these affinities were obtained at the resolution limit of the ITC instrument, they have to be treated with caution.

We also tested whether Sly1 binds to an assembled SNARE complex. For this, we purified quaternary complexes containing the SNARE motifs of Bos1, Bet1, Sec22 and either the entire cytoplasmic region of Sed5 (Sed5 1-320) or the SNARE motif of Sed5 (Sed5 211-320). We found that the SNARE complex containing Sed5 1-320 bound with an enthalpy and affinity comparable to Sed5 fragments containing the intact N-terminal region, whereas no binding of the SNARE complex containing only the SNARE motif of Sed5 could be detected. This suggests that Sly1 does not bind with significant affinity to the core region of the completely assembled ER-Golgi SNARE complex.

Fig. S5: Sly1 forms a complex with the entire cytosolic portion but not with the isolated SNARE motif of Sed5.

A. Size exclusion chromatography elution profiles of Sly1 in blue, the entire cytosolic portion of Sed5 (1-320) in black, and an equimolar mixture in dark blue (14 µM each). The Sly1/Sed5 complex elutes at a higher molecular mass than the two monomers.

B. Elution profiles of Sly1 in blue, the SNARE motif of Sed5 (211-320) in red and an equimolar mixture in pink (35 µM each). The respective peak fractions were analyzed by SDS-PAGE and stained with Coomassie Blue.

Fig. S6: The remainder of Sed5 does not affect the binding strength of the Sed5 N-peptide to Sly1.

As in Fig. 3C about 50 nM of Oregon Green-labeled N-peptide of Sed5 (Sed5 1-22$^{C22\text{-}OG488}$) was first mixed with saturating amounts of Sly1 (~300 nM). Then, an excess of the unlabeled N-peptide (1-21, ≈ 5 µM) was added and the dissociation of the N-peptide/Sly1 complex was monitored (orange curve). When the experiment was carried out in the presence of 15 µM of the remaining portion of the cytosolic domain of Sed5 (21-324), no significant change in the off-rate of the N-peptide/Sly1 complex was observed (blue curve), suggesting that the Sed5 21-324 does not influence the binding strength of the N-peptide. A similar result was obtained by titrations of Sly1 to Sed5 1-22$^{C22\text{-}OG488}$ in the absence or presence of Sed5 21-324 (data not shown).

Table S1: Thermodynamic parameters obtained by all ITC experiments in this study

Cell	Syringe	K_d (nM)	ΔH (kcal/ mole)	n
Sly1	Sed5 1-320	0.30 ± 0.11	-25.6 ± 0.16	1.01
Sly1	Sed5 1-210	0.24 ± 0.35	-18 ± 0.16	1.00
Sly1	Sed5 1-21	1.47 ± 0.45	-16 ± 0.13	0.98
Sly1	Sed5 21-324	-	-	-
Sly1	Sed5 211-320	-	-	-
Sly1	Sed5 1-320^{F10A}	234 ± 72	-8.98 ± 0.63	1.09
Sly1	Sed5 1-21^{F10A}	-	-	-
Sly1	Sed5 1-320^{I290A}	0.93 ± 0.24	-21.1 ± 0.12	1.04
Sly1	Sed5 1-210/ 211-320 complex	0.48 ± 0.49	-22.7 ± 0.20	0.92
Sly1	Quaternary SNARE complex containing Sed5 1-320	0.23 ± 0.30	-20.3 ± 0.22	0.88
Sly1	Quaternary SNARE complex containing Sed5 211-320	-	-	-
Sly1	Ufe1 1-21	34.4 ± 5.2	-15.8 ± 0.23	1.02
Sly1/Ufe1 1-21	Sed5 1-320	0.17 ± 0.09	-21.9 ± 0.37	0.85
Sly1/Ufe1 1-21	Sed5 1-210	-	-	-
Sed5 211-320	Sed5 1-210	2700 ± 400	+6.13 ± 0.34	0.98

All isothermal calorimetric experiments were performed at 25 °C in PBS buffer. The errors reported are the numbers obtained from fitting the data of a single ITC run. The experimental ITC data for each of the interactions are shown in Fig. S4.

References

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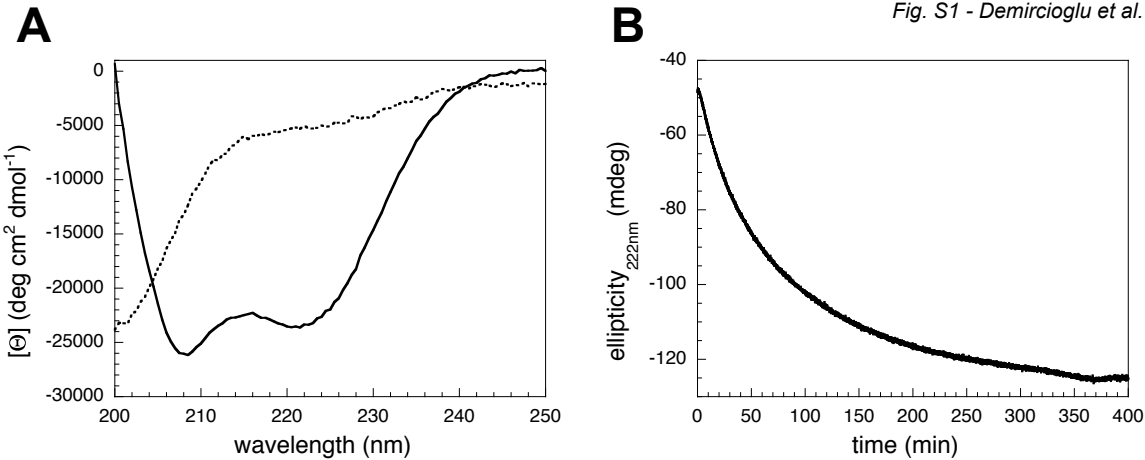
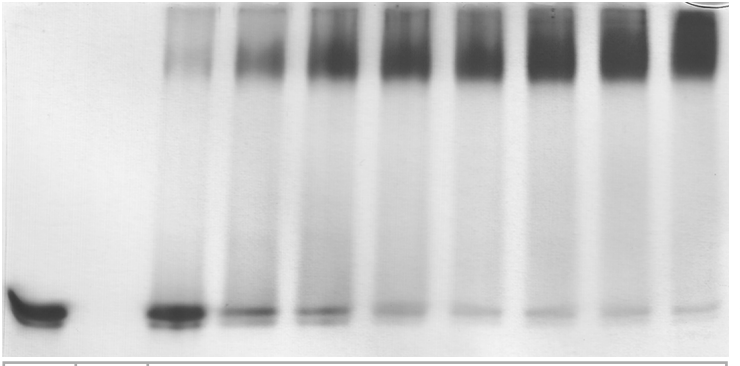


Fig. S2 - Demircioglu et al.



31.4	0	31.4								[μM] Sed5 211-320
0	137.1	17.1	34.2	51.4	68.6	85.7	102.9	120	137.1	[μM] Sed5 1-210

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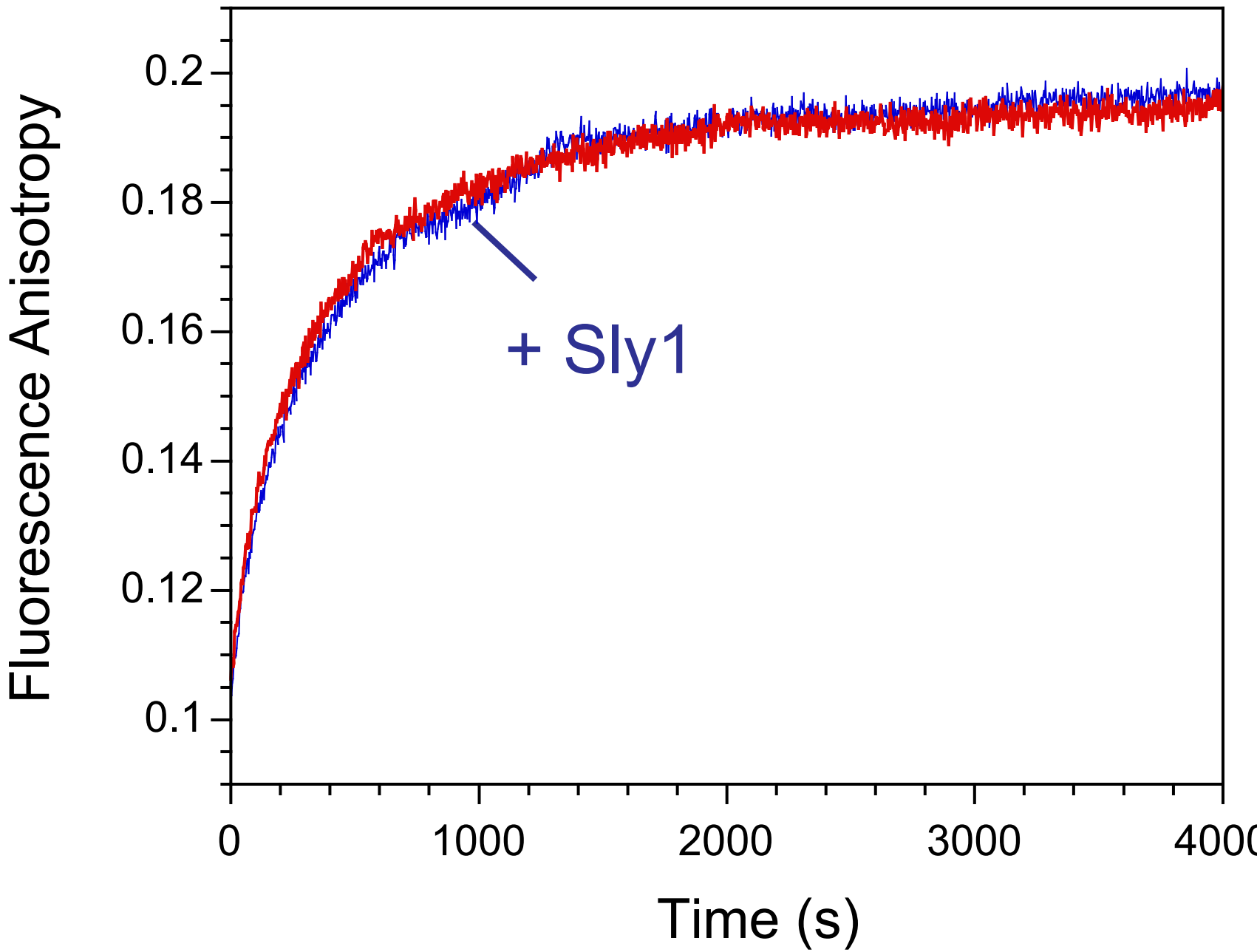

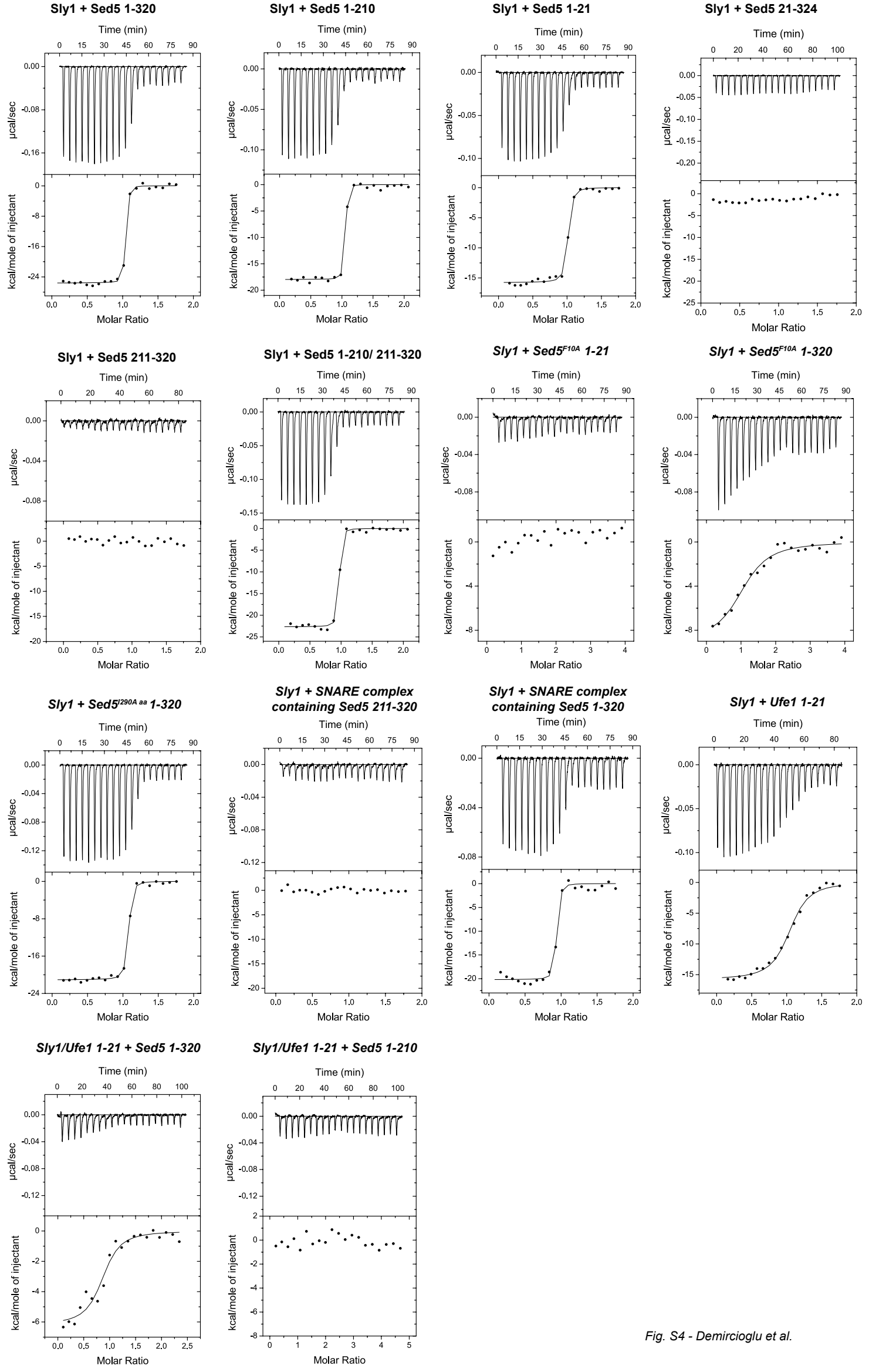

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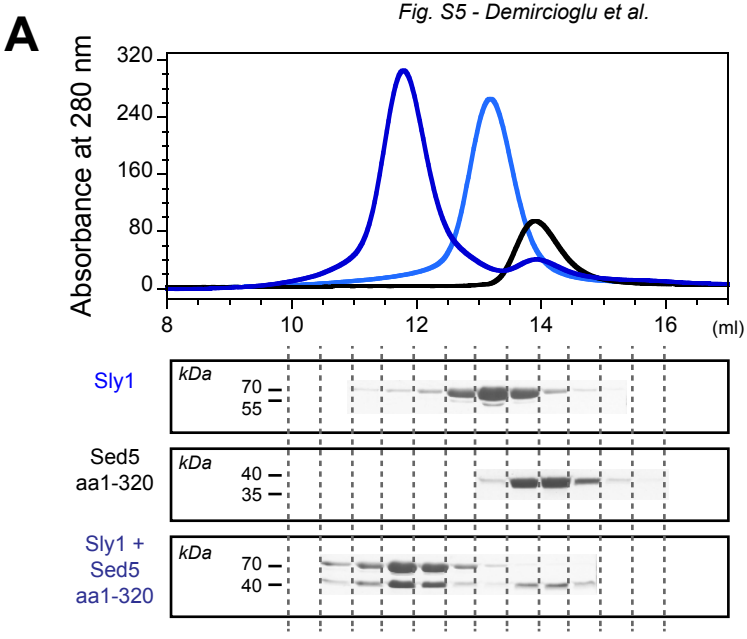

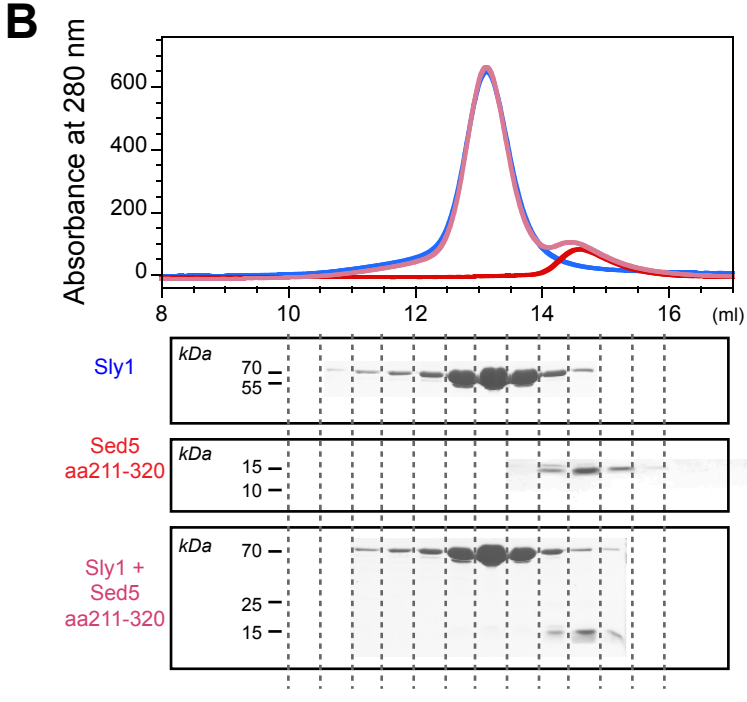

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