Supporting Information

Characterizing heterogeneous mixtures of assembled states of the tobacco mosaic virus using charge detection mass spectrometry

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General Methods and Instrumentation

Unless noted otherwise, all reagents were obtained from commercial sources and used without further purification. Tyrosinase isolated from *Agaricus bisporus* (abTyr) was purchased from Sigma-Aldrich (St. Louis, MO). NAc-a-endorphin was purchased from GenScript (Piscataway, NJ). Protein expression media and all buffers were prepared using doubly distilled water obtained from a NANOpure purification system (Barnstead, USA).

Steady-state Spectroscopy. Protein concentration was determined by UV/Vis analysis on a Nanodrop 1000 instrument (Nanodrop, USA) by monitoring absorbance at 280 nm.

Denaturing Mass Spectrometry (MS). Proteins and their bioconjugates were analyzed using an Agilent 1260 series liquid chromatograph that was connected in-line with an Agilent 6530C Quadrupole Time-of-Flight (QTOF) LC/MS system (Santa Clara, CA). Protein samples were run with a Proswift RP-4H column (Dionex, USA). Protein mass reconstruction was performed on the charge ladder with Agilent Mass Hunter software, Qualitative Analysis Version B.10.0, Build 10.0, Agilent Technologies Inc.© 2020 (Agilent, USA).

High Performance Liquid Chromatography (HPLC). HPLC was performed on Agilent 1100 Series HPLC Systems (Agilent, USA). Sample analysis for all HPLC experiments was achieved with an in-line diode array detector (DAD) and in-line fluorescence detector (FLD). Size exclusion chromatography (SEC) was performed using a Polysep-GFC-P-5000 column (4.6 x 250 mm) (Phenomenex, USA) at 1.0 mL/min using a mobile phase of 10 mM sodium phosphate buffer, pH 7.2.

Dynamic Light Scattering (DLS). DLS was performed on a Zetasizer Nano Series (Malvern Instruments, UK). Measurements were taken in triplicate at protein concentrations of 0.2-1.0 mg/mL in 10 mM sodium phosphate buffer, pH 7.2, at 25°C.

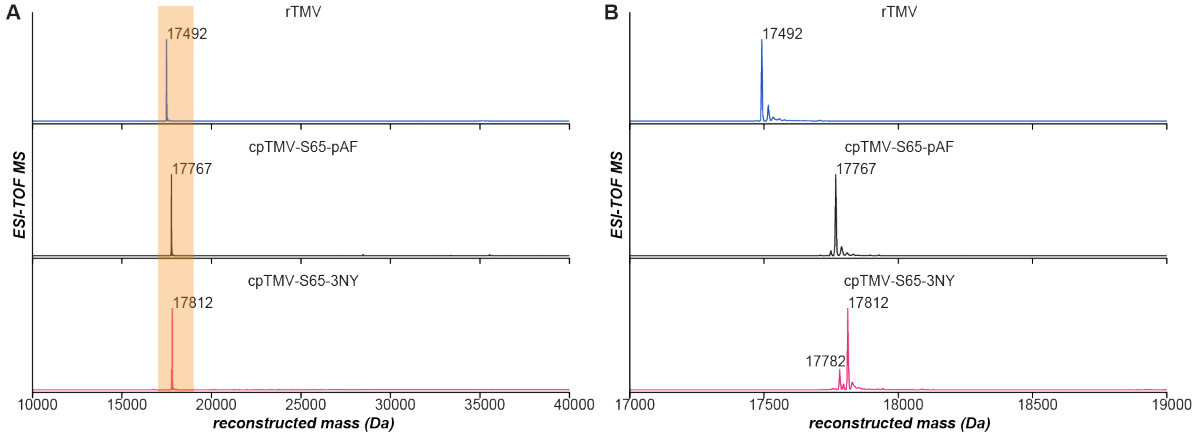

Figure S1. Denaturing mass spectra of TMV mutants assembling into odd-numbered disk stacks or 16-monomer disks. (A) The mass spectra of rTMV (expected MW: 17493 Da), cpTMV-S65-pAF (expected MW: 17767 Da), and cpTMV-S65-3NY (expected MW: 17813 Da) are shown, indicating high purity and homogeneity of each sample. The orange bar indicates an enlarged window that is depicted in (B). (B) An enlarged window of the same spectra in (A) shows a small proportion of the reduced version of cpTMV-S65-3NY in which the nitrophenol is spontaneously reduced to an aminophenol (expected MW: 17783 Da). This mass difference would not be expected to significantly affect the assembled mass of a 34-monomer complex (at an expected mass of 605 kDa for a 34-monomer complex of 17783 Da monomers versus an expected mass of 606 kDa for a 34-monomer complex of 17812 Da monomers.

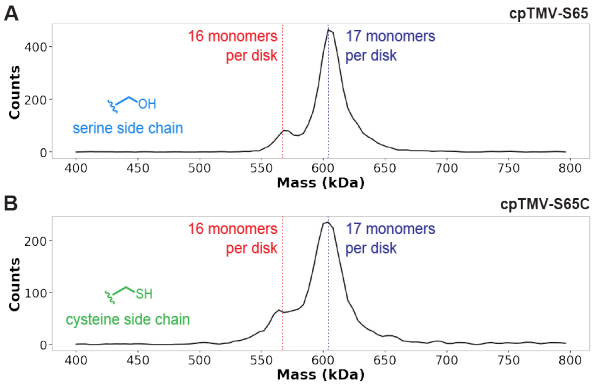

Figure S2. Ratio of 16:17-mers of cpTMV point mutants at position S65. (A) A CDMS histogram of cpTMV with the native serine residue at position S65 shows a small proportion of 16-monomer disks alongside 17-monomer disks in a 2-disk stack. (B) A CDMS histogram of cpTMV-S65C also shows a small proportion of 16-monomer disks in a 2-disk stack.

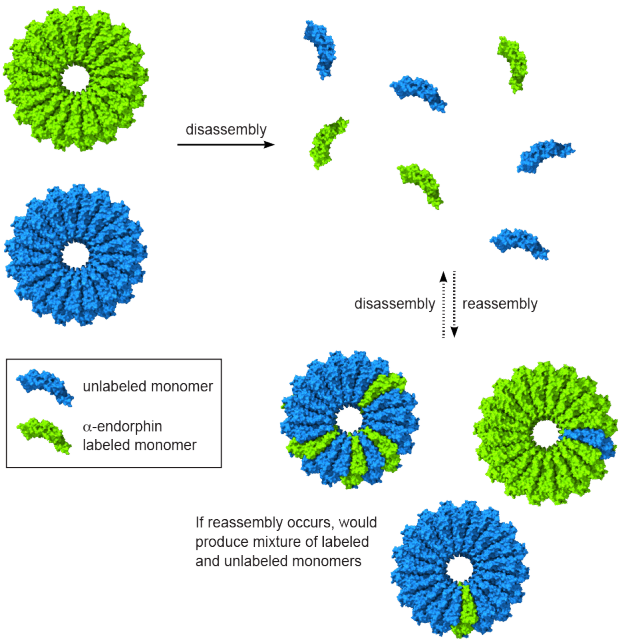

Figure S3. A schematic of the experiment mixing labeled and unlabeled cpTMV populations. This experiment was used to determine whether disassembly of cpTMV disks into monomers is reversible under a specific set of conditions (in this work, incubating the protein at room temperature in solution at a concentration of 0.5 mg/mL in 100 mM ammonium acetate buffer). One cpTMV disk population at a higher molecular weight, labeled with endorphin (shown in chartreuse) was mixed with an equivalent concentration of unlabeled cpTMV, at a lower molecular weight (shown in blue). If complete or incomplete disassembly and reassembly occured in solution, disks containing mixtures of unlabeled and labeled disks would be expected to form. If disassembly is irreversible, only uniform disks would be expected to be observed.

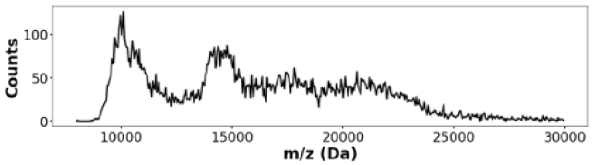

Figure S4. Histogram of m/z values measured by CDMS of unmodified cpTMV-S23C-S65-pAF mixed with α-endorphin modified cpTMV 3 d post-mixing.

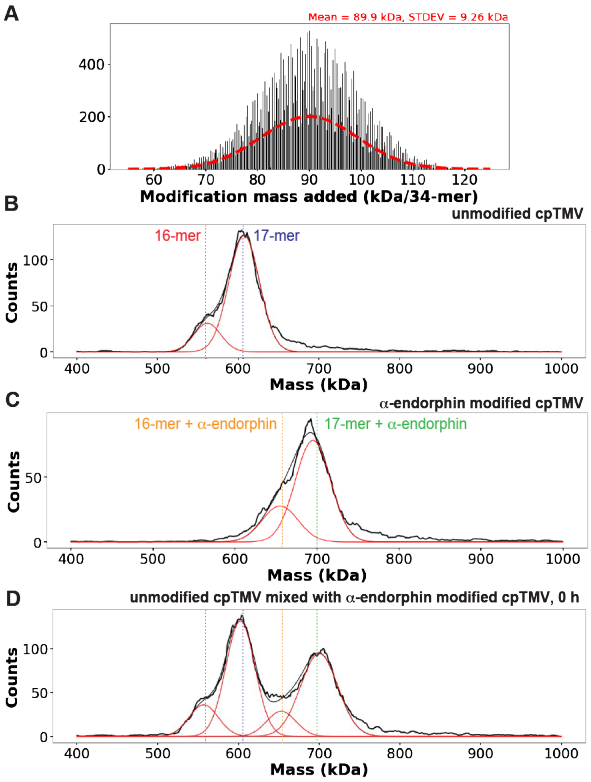

Figure S5. Predicted masses based on % modification and truncation levels of unlabeled and labeled cpTMV-S23C-S65-pAF (Figure 4b). (A) A synthetic mass histogram and overlaid smoothed curve demonstrating that the mean difference between modified and unmodified monomers should be 89.9 kDa. (B) An overlay of unmodified cpTMV-S23C-S65-pAF CDMS histogram (black) and predicted distributions (red). (C) An overlay of α-endorphin-labeled cpTMV-S23C-S65-pAF CDMS histogram (black) and predicted distributions (red). (D) An overlay of mixed unlabeled and α-endorphin-labeled cpTMV-S23C-S65-pAF CDMS histogram (black) and predicted distributions (red).

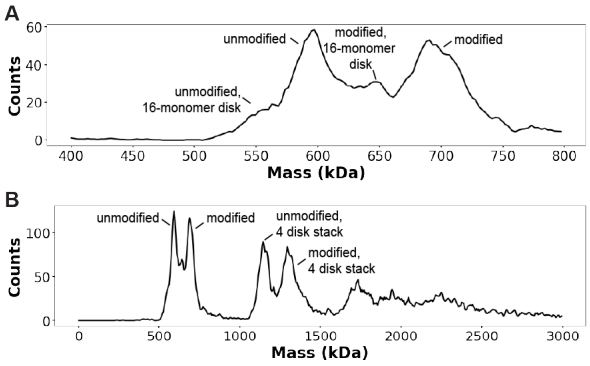

Figure S6. An additional 3 hour timepoint for the experiment mixing labeled and unlabeled cpTMV populations. To determine whether cpTMV monomers were exchanged, two populations of cpTMV-S23C-S65-pAF, one modified with α-endorphin and the other unmodified, were mixed (corresponding to Figure 4.) (A) A mass histogram with a window showing only the double disk species 3 h after mixing displays two distinct populations that do not equilibrate over time. (B) A mass histogram with an extended window 3 h after mixing also displays two distinct populations at multiple stacking stoichiometries that do not equilibrate over time.

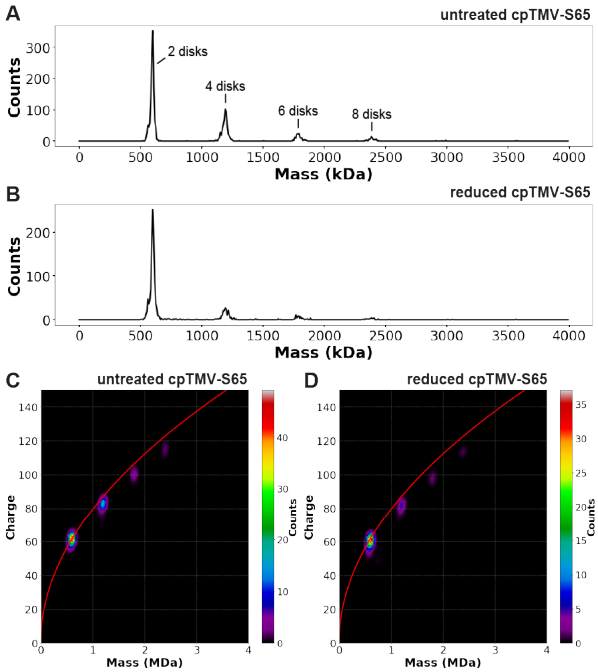

Figure S7. The effect of reduction of cpTMV without an engineered cysteine residue on assembly state. A comparison of cpTMV-S65 without a cysteine mutation at position 65 was made under identical conditions as those used for the oxidation and reduction of cpTMV-S65C shown in Figure 5. (A) A mass histogram of cpTMV-S65 shows even-numbered disk stacks, with a lower proportion of higher molecular weight stacks compared to cpTMV-S65C. (B) After reduction with 1 mM tris(2-carboxyethyl)phosphine (TCEP), most of the higher MW species remain. A mass versus charge diagram comparing cpTMV-S65 (C) before and (D) after reduction with TCEP shows minimal differences between the two conditions, with neither condition showing multiple species with the same mass but different charge observed for cpTMV-S65C.

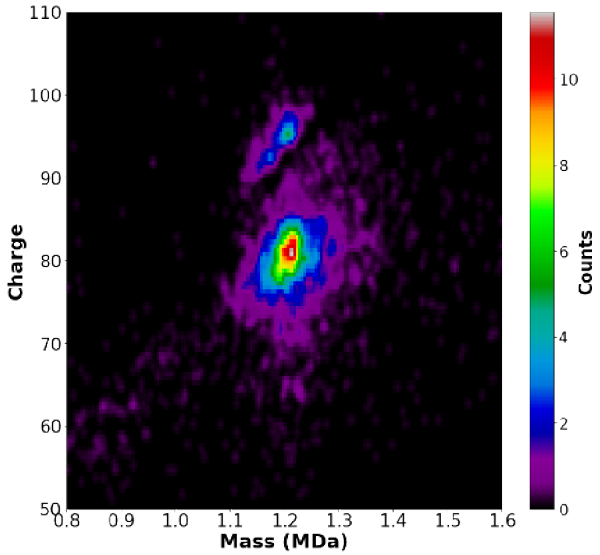

Figure S8. Expanded region of the CDMS mass spectrum of untreated cpTMV-S65C (Figure 4E) in the region where the two different 4-disk charge distributions are observed.

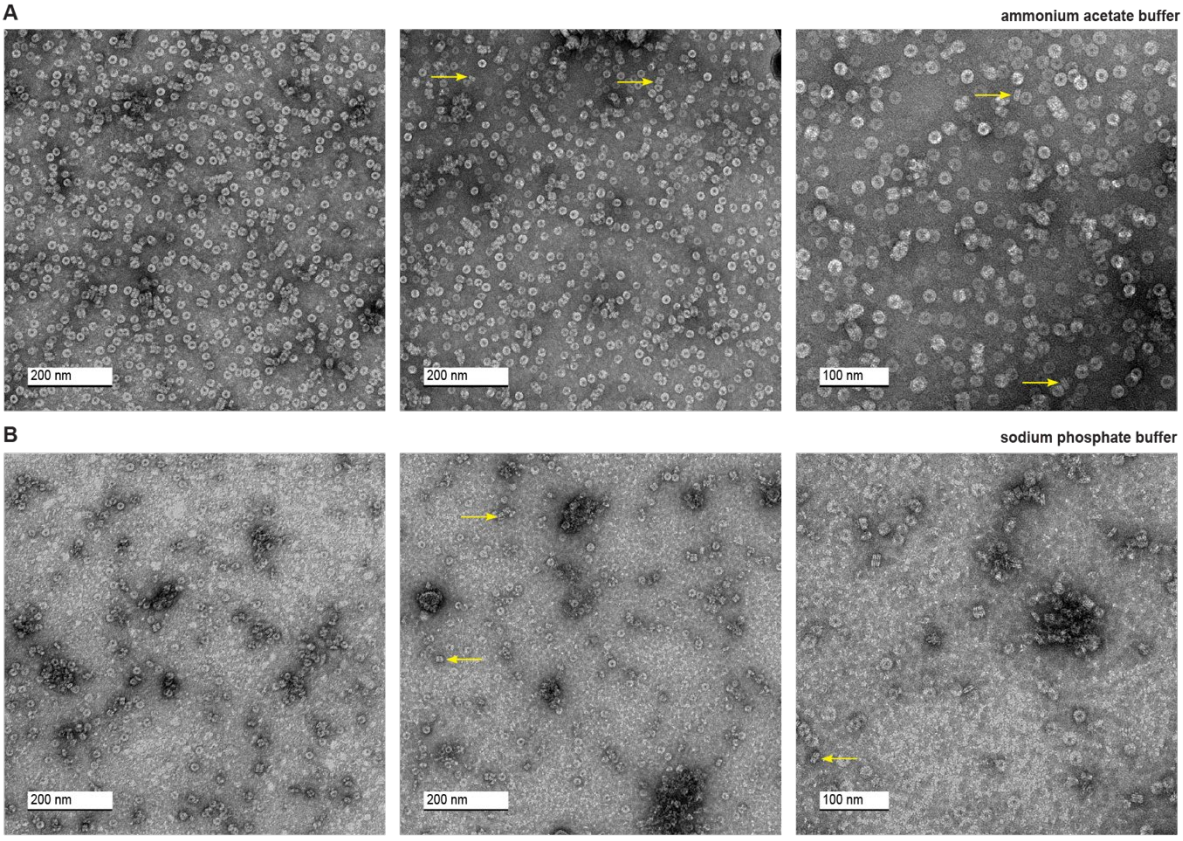

Figure S9. Additional TEM images of rTMV. (A) TEM images of rTMV in 100 mM ammonium acetate show face down views of rTMV and some examples of short stacks of disks. (B) TEM images of rTMV in 10 mM sodium phosphate buffer pH 7.2 show both face and side views of short stacks of disks. Several examples of odd-numbered stacks of disks are identified with yellow arrows.