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Supplementary Information

Evidence that the catenane form of CS_2 hydrolase is not an artefact

Mark B. van Eldijk,[a] Iris van Leeuwen,[a,b] Victor A. Mikhailov,[c] Lotte Neijenhuis,[a,b] Harry R. Harhangi,[b] Jan C. M. van Hest,[a] Mike S. M. Jetten,[b] Huub J. M. Op den Camp,[b] Carol V. Robinson[c] and Jasmin Mecinović*[a]

[a] Institute for Molecules and Materials, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands. Fax: +31 24 3653393; Tel: +31 24 3652381; E-mail: j.mecinovic@science.ru.nl

[b] Department of Microbiology, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands.

[c] Chemistry Research Laboratory, University of Oxford, Mansfield Road, OX1 3TA Oxford, United Kingdom.

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1. Expression and purification of CS_2 hydrolase

For a typical expression, 5 mL LB medium, supplemented with ampicillin (100 mg/L), was inoculated with a single colony of *E. coli* BL21(DE3) containing pET24bCS2hydsynth[1] and was incubated at 37 ºC overnight (Innova 3100, New Brunswick Scientific Co., Inc., Edison, USA). This overnight culture was used to inoculate 1000 mL of LB medium supplemented with ampicillin (100 mg/L). The culture was grown at 37 ºC and protein expression was induced during logarithmic growth (OD_{600} = 0.4-0.6) by addition of 0.5 mM IPTG (Sigma-Aldrich) final concentration. After 24 h of expression, the cells were harvested by centrifugation in using a HB4 rotor and Sorvall RC 5B plus centrifuge (19679 g at 10 ºC for 10 min).

The cell pellet was resuspended in HEPES (20 mM, pH 7.5) and incubated with lysozyme (2 mg/mL) and protease inhibitor cocktail (Roche) for 30 min at 37 ºC followed by French press lysis (American Instrument Company, Silver Spring Maryland 20910). The lysates were incubated at 70 ºC for 45 min to denature *E. coli* proteins and centrifuged at 15871 g for 5 min (Eppendorf 5424) to remove precipitated proteins.

Further purification of the CS_2 hydrolase was performed by SEC using a Superdex 200 10/300 GL column from GE Health. The purification was carried out at room temperature on an Amersham Ettan LC system (GE Healthcare, Diegem, Belgium) equipped with a fraction collector. The fractions containing CS_2 hydrolase were combined and concentrated using centrifugal filtration (Millipore, Amicon Ultra-0.5 Centrifugal Filters, 10 kDa). Then the concentration was determined via NanoDrop (ND-1000) using the theoretical extinction coefficients.

2. Analytical size exclusion chromatography

All analytical SEC measurements were performed at 25 ºC on a Shimadzu LC-20A Prominence system (Shimadzu, ‘s Hertogenbosch, The Netherlands) using a Superdex 200 10/300 GL column from GE Healthcare. For a typical analysis, 100 µL sample was analysed on the column with a flow rate of 0.5 mL/min.

Concentration range

A dilution series of CS_2 hydrolase in 20 mM HEPES was prepared starting from 8.0 mg/mL and then the samples were incubated overnight at 4 ºC.

Another concentration range was prepared via concentrating to different volumes using centrifugal filtration (Millipore, Amicon Ultra-0.5 Centrifugal Filters, 50 kDa). These results are shown in Figure S2.

Different buffers

For SEC analysis in different buffers (20 mM HEPES pH7.5 and 20 mM Tris pH7.5), buffer-exchange was performed via centrifugal filtration (Millipore, Amicon Ultra-0.5 Centrifugal Filters, 50 kDa). Prior to analysis the samples were incubated overnight at 4 ºC.

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3. Multi-angle laser light scattering

The size exclusion chromatography - multi angle laser light scattering (SEC-MALLS) experiments were conducted at 25 ºC using the Superdex 200 10/300 GL column in-line with a Wyatt DAWN HELEOS II light scattering detector using a laser operating at 658 nm and a Wyatt Optilab Rex refractive index detector. Overnight flushing of the system was performed to pre-equilibrate followed by normalisation using Bovine Serum Albumin. Weight-averaged molecular weight calculations were performed using ASTRA 6.0.6.13, using a dn/dc value of 0.1850 for 20 mM HEPES, pH 7.5.

4. Native PAGE

By modification of previously described procedure:[1] 6% native PAGE gel, with a nativeMark™ unstained protein standard (Novex).

5. Mass spectrometry

Non-denaturing

Non-denaturing (native) electrospray ionization mass spectrometry (ESI-MS) was performed on Waters Q-TOF 2 and Ultima Q-TOF mass spectrometers modified for ions with high m/z values.[2] Samples in the original buffer (20 mM HEPES, pH=7.5) were buffer-exchanged for 200 mM ammonium acetate, pH=7.5 using Bio-Spin 6 microcolumns, and diluted further in ammonium acetate to investigate the effect of protein concentration. Ion source pressures and voltages were optimized to observe both the octamer and catenane complexes, and then kept constant during the experiments. Mass spectra were calibrated with CsI cluster ions, molecular weights were calculated using Waters MassLynx software.

Denaturing

Protein mass characterization under denaturing conditions was performed by electrospray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF. Samples were dialysed against MilliQ via centrifugal filtration (Millipore, Amicon Ultra-0.5 Centrifugal Filters, 10 kDa). Deconvoluted mass spectra were obtained using MagTran 1.03b2.

6. Supplemental figures

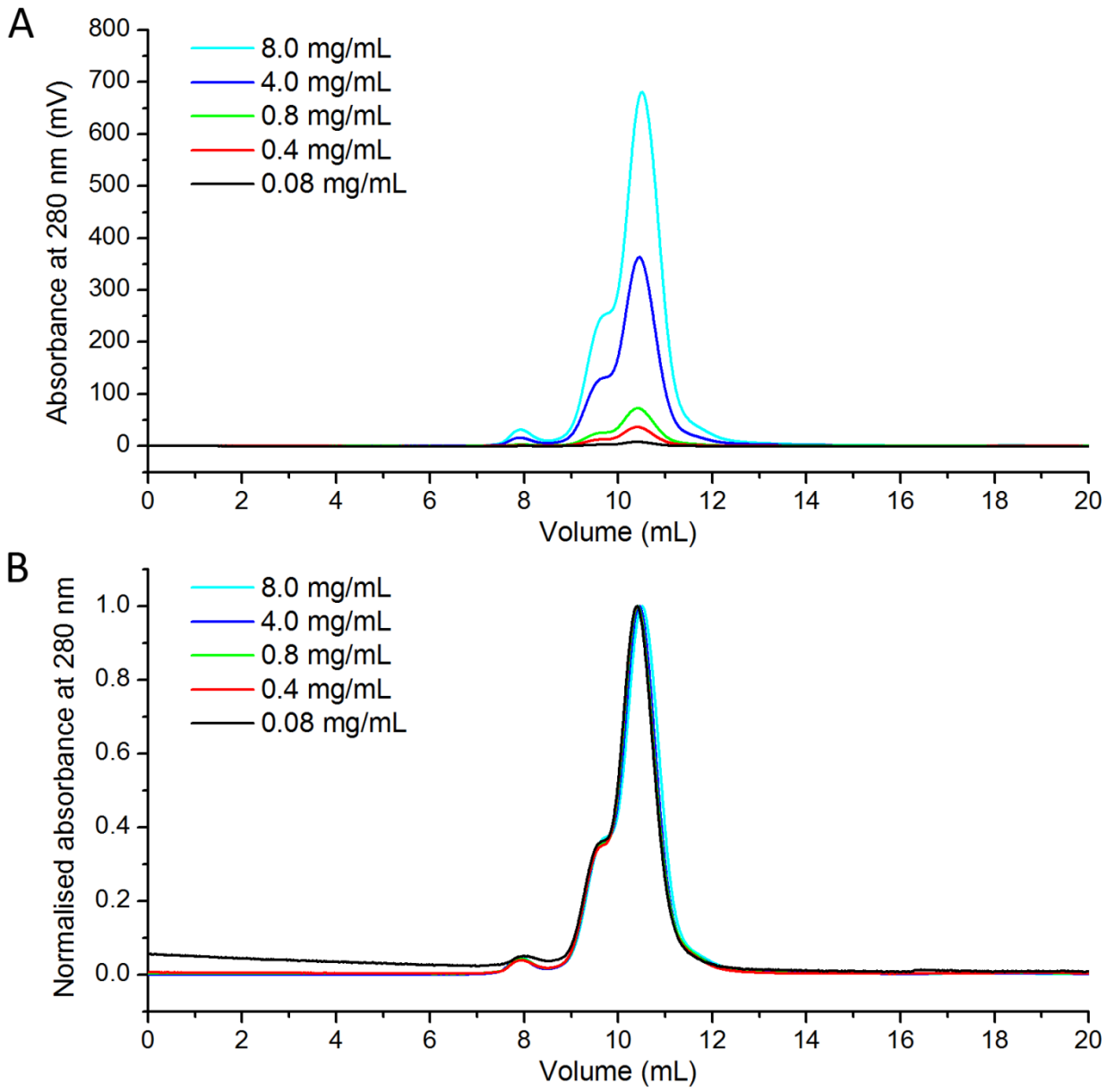

Fig. S1 a) SEC chromatograms of CS_2 hydrolase showing complete elution (0-20 mL) at 8.0 mg/mL, 4.0 mg/mL, 0.8 mg/mL, 0.4 mg/mL and 0.08 mg/mL; b) normalised chromatograms.

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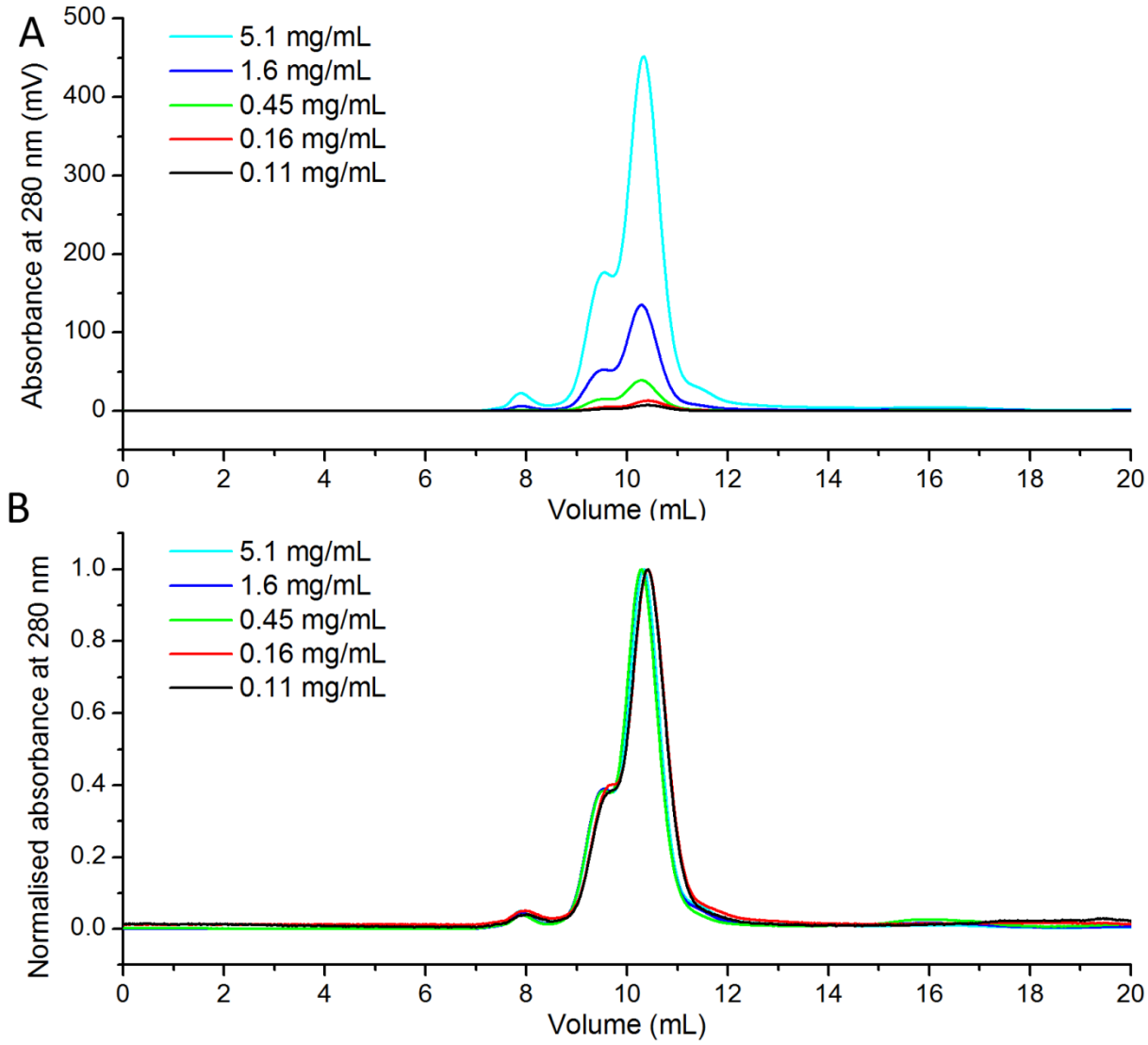

Fig. S2 a) SEC chromatograms of CS_2 hydrolase at 5.1 mg/mL, 1.6 mg/mL, 0.45 mg/mL, 0.16 mg/mL and 0.11 mg/mL; b) normalised chromatograms.

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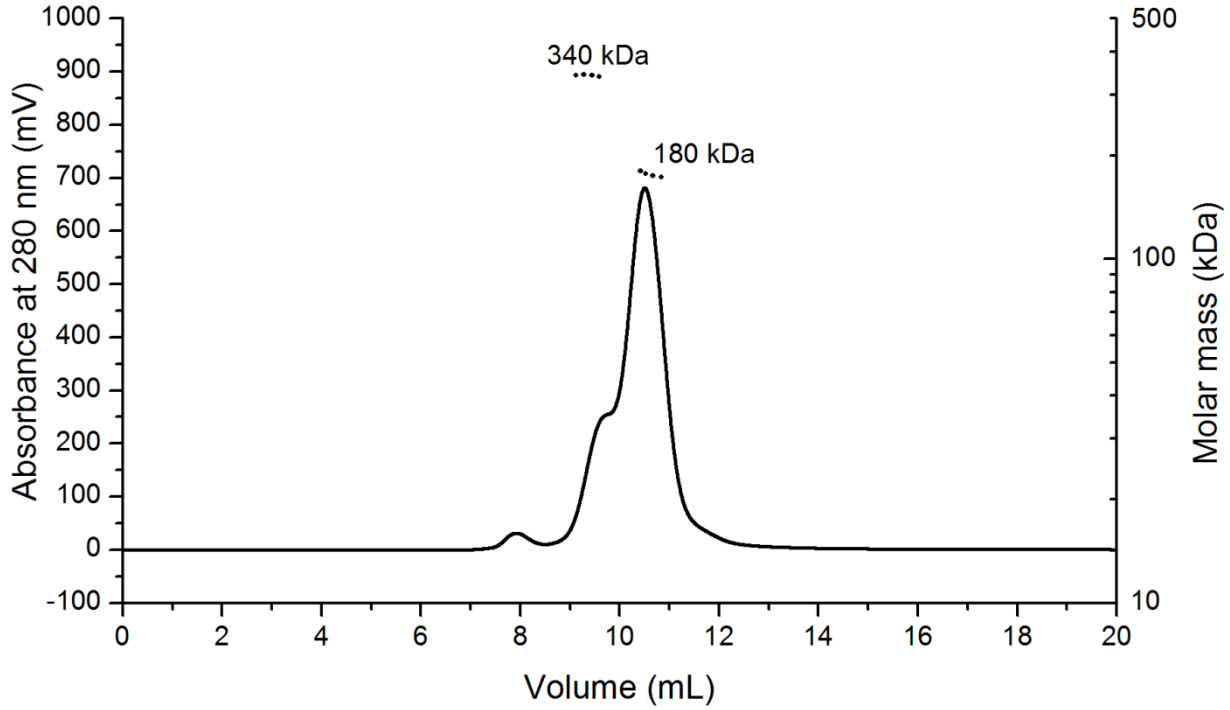

Fig. S3 SEC-MALLS chromatogram of CS_2 hydrolase at 8.0 mg/mL.

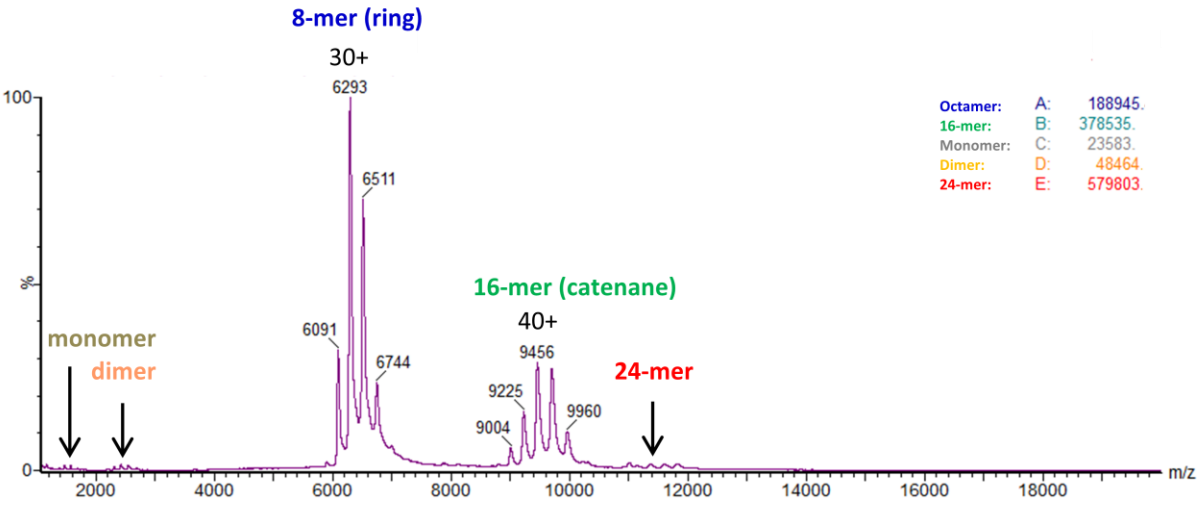

Fig. S4 Non-denaturing ESI-MS of CS_2 hydrolase showing that the octameric ring and the hexadecameric catenane are dominant forms. Signals that correspond to the 24-mer disappear upon dilution, this indicates that the complex is non-specific.

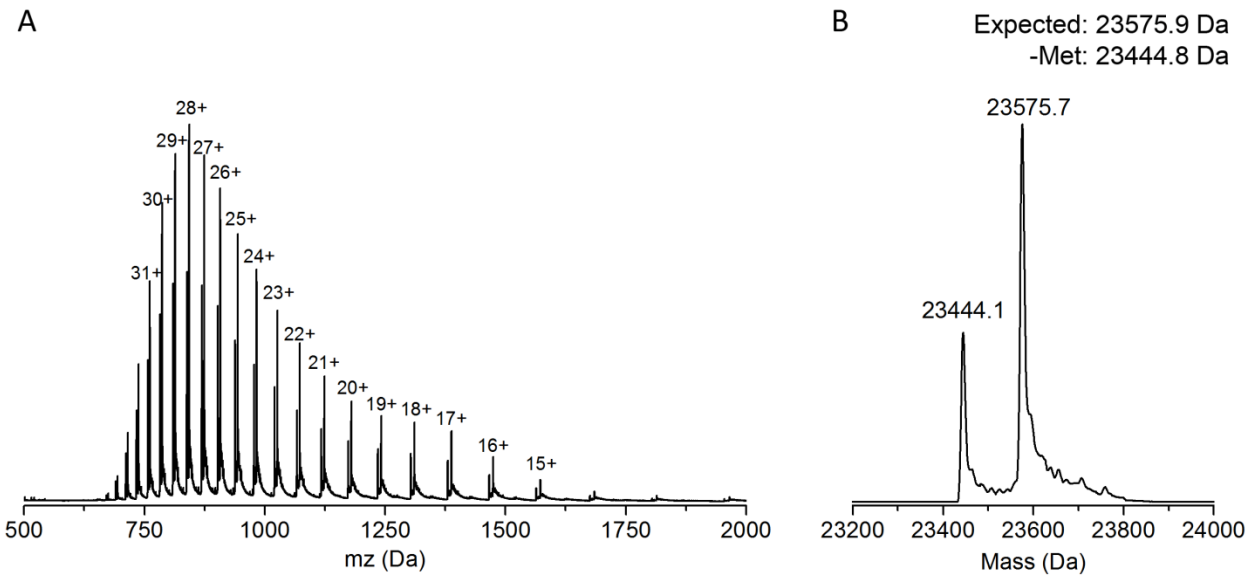

Fig. S5 ESI-TOF mass spectrometry of CS_2 hydrolase under denaturing conditions a) multiply charged ion series; b) deconvoluted total mass spectrum showing peaks of 23575.7 Da and 23444.1 Da matching with CS_2 hydrolase before and after processing of the N-terminal methionine, respectively.

7. References

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2. F. Sobott, H. Hernandez, M. G. McCammon, M. A. Tito and C. V. Robinson, *Anal Chem*, 2002, **74**, 1402-1407.