Supplementary Information

Catenane versus Ring: Do both Assemblies of CS_2 Hydrolase Exhibit the same Stability and Catalytic Activity?

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1. Expression and purification

For a typical expression, 5 mL LB medium, supplemented with kanamycin (100 mg/L), was inoculated with a single colony of *E. coli* BL21(DE3) containing pET24bCS2hydsynth[1,2] and was incubated at 37 °C overnight (Innova 3100, New Brunswick Scientific Co., Inc., Edison, USA). This overnight culture was used to inoculate 1000 mL of LB medium supplemented with kanamycin (100 mg/L). The culture was grown at 37 °C 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 using a Sorvall RC 5B plus centrifuge with HB4 rotor (19679 g at 10 °C 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 °C followed by French press lysis (American Instrument Company, Silver Spring Maryland 20910). The lysates were incubated at 70 °C for 45 min to denature *E. coli* proteins and centrifuged at 15871 g for 5 min (Eppendorf 5424) to remove precipitated proteins.

Table S1: CS_2 hydrolase protein sequence:

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MVSEYIDSELKRLEDYALRRVKGIPNNRRLWVLTCMDERVHIEQSLGIQP
DDAHIYRNAGGIVTDDAIRSASLTTNFFGTKEIIVVTHTDCGMLRFTGEE
VAKYFISKGIKPTEVQLDPLLPAFRISSEEDFIKWFKFYEDLGVKSPDEM
ALKGVEILRNHPLIPKDVRITGYVYEVETHRLRKPNQIIYNETSKFEHGT
IVKE
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2. Separation of ring and catenane

All size exclusion chromatography (SEC) experiments for the purification of both assemblies of CS_2 hydrolase were performed on an Amersham Ettan LC system (GE Healthcare, Diegem, Belgium) equipped with a fraction collector.

2.1. Size exclusion chromatography with different buffers

The separation of the ring and catenane forms of CS_2 hydrolase by SEC using a Superdex 200 10/300 GL column (GE Healthcare) was optimized by testing different running buffers: (1) 20 mM HEPES pH 7.5; (2) 20 mM HEPES and 150 mM NaCl pH 7.5; (3) 20 mM NaH_2PO_4 pH 7.5; (4) 200 mM NaH_2PO_4 pH 7.5; (5) phosphate buffered saline pH 7.4 (see Figure S1).

2.2. *Preparative separation of ring and catenane*

Multiple injections (5-10) of 100 µL sample of ~5 mg/mL of CS_2 hydrolase were performed on Superdex 200 10/300 GL column (see Figure S2, blue line). The separation was performed at room temperature with a flow rate of 0.5 mL/min of 20 mM NaH_2PO_4 pH 7.5, while collecting 300 µL fractions. The fractions containing the CS_2 hydrolase ring and catenane were separately collected. The combined fractions were concentrated to 100 µL using centrifugal filtration (Millipore, Amicon Ultra-0.5 Centrifugal Filters, 10 kDa). Subsequently, both the concentrated ring (see Figure S2, red line) and catenane (see Figure S2, black line) were reinjected onto the column to obtain both assemblies in >95% purity. The concentration was determined via NanoDrop (ND-1000) using the theoretical extinction coefficient.

3. Analysis

3.1. SEC-MALLS

All analytical size exclusion chromatography – multi-angle laser light scattering (SEC-MALLS) experiments were performed on a Shimadzu LC-20A Prominence system (Shimadzu, 's Hertogenbosch, The Netherlands) using a Superdex 200 10/300 GL column in-line with a Wyatt DAWN HELEOS II light scattering detector using a laser operating at 658 nm, a Wyatt Optilab Rex refractive index detector and a Shimadzu SPD20A. 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 NaH_2PO_4, pH 7.5. For a typical analysis, 100 µL sample of 0.1 mg/mL was analysed on the column with a flow rate of 0.5 mL/min.

3.2. AF4-MALLS

The asymmetric-flow field-flow fractionation – multi-angle laser light scattering (AF4-MALLS) experiments were performed using a Shimadzu LC-20A Prominence system, with Shimadzu CTO20A injector, and a Wyatt Eclipse AF4 with the short channel with a regenerated cellulose 10 kDa membrane (Millipore) and wide spacer of 350 µm, connected to a Wyatt DAWN HELEOS II light scattering detector using a laser operating at 658 nm, a Wyatt Optilab Rex refractive index detector and a Shimadzu SPD20A. The channel was pre-equilibrated with running solution (phosphate buffered saline, pH 7.4) 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.

Unless noted otherwise, 30 µL samples of 0.1 mg/mL purified ring or catenane were incubated in 20 mM NaH_2PO_4 pH 7.5 at the indicated temperatures. To study the stability of the ring and catenane form of CS_2 hydrolase at 4 °C, samples were incubated in the fridge for 3 or 21 days. For the stability at 20 °C, samples were incubated at room temperature for 3 days. To study the potential equilibrium between the ring and the catenane form of CS_2 hydrolase at 60 °C, 70 °C and 80 °C, samples were incubated in an Eppendorf Thermomixer for 60 min. The conversion of the catenane to the ring (and vice versa) was

studied by incubation of 30 µL samples of 0.1 mg/mL at 70 °C for 1, 3, 5, 15, 60 and 240 min (catenane) and 30, 60, 240 min (ring) in an Eppendorf Thermomixer. After incubation, the protein samples were centrifuged for 2 min at 4 °C, 13k rcf. After preparation, the samples were kept on ice prior to injection. Typically, 25 µL sample of 0.1 mg/mL was analysed using the following flow rates and method:

0.50 mL/min detector flow, 1.50 mL/min focus flow, 0.20 inject flow

Duration (min)	Mode	Cross flow rate (mL/min)
2	Elution	3.0
1	Focus	-
2	Focus + inject	-
1	Focus	-
16	Elution	3.0
3	Elution	0.0
3	Elution + inject	0.0

The influence of protein concentration was analysed. Therefore, both assemblies of CS_2 hydrolase were incubated in three concentrations (ring: 0.05; 0.5; 5.0 mg/mL and catenane: 0.03; 0.3; 3.0 mg/mL) at two different temperatures for 15 min (60 °C and 70 °C) in an Eppendorf Thermomixer. After incubation, the protein samples were diluted to 0.1 mg/mL (for 0.3 – 5.0 mg/mL) or kept at 0.03 and 0.05 mg/mL and centrifuged for 2 min at 4 °C, 13k rcf. After preparation, the samples were kept on ice prior to injection. Typically, 2.5 µg of protein was analysed using the previously described flow rates and method.

For crowding experiments, purified ring or catenane (0.1 mg/mL) were incubated in the presence of 20% PEG6000 (Sigma Aldrich) in 20 mM NaH_2PO_4 (pH 7.5) for 60 min at 60 °C or for 15 min at 70 °C in an Eppendorf Thermomixer. After the incubation, the excess of PEG6000 was removed via centrifugal filtration (Millipore, Amicon Ultra-0.5 Centrifugal Filters, 50 kDa). 2.5 µg of protein was analysed using the previously described flow rates and method.

To investigate the influence of buffer conditions on the potential equilibrium between the ring and the catenane form of CS_2 hydrolase, the interconversion was studied by incubation of 30 µL samples of 0.1 mg/mL for 15 min at 70 °C in an Eppendorf Thermomixer in different buffer solutions: 20 mM NaH_2PO_4 pH 7.5; 20 mM NaH_2PO_4, 1 M NaCl, pH 7.5; PBS; 10x PBS. Typically, 2.5 µg of protein was analysed using the previously described flow rates and method.

The AF4-MALLS data was processed and analysed using Origin 8.5.1 SR2. First baselines were substracted and then peaks were integrated. In order to minimize the overestimation of the area of the overlapping ring and catenane peaks, the peaks were assumed to be symmetrical.

3.3. Native ESI-MS

Non-denaturing (native) electrospray ionization mass spectrometry (ESI-MS) was performed on Waters Q-TOF 2 mass spectrometer modified for ions with high *m/z* values. Samples in the original phosphate buffer at protein concentration of 0.7 mg/ml were subjected to heat incubation at 70 °C in a Stuart SBH 130DC block heater. Mass spectra were obtained at different time points after a quick buffer exchange for

200 mM ammonium acetate (pH = 7.5) using Bio Spin 6 microcolumns. Ion source pressures and voltages were optimized using a control sample to observe both the octamer and catenane complexes, and then kept constant during the experiments. Ions were electrosprayed using a capillary nanospray source with capillary voltage 1.55 kV, sample cone voltage 129 V, extractor cone voltage 106 V, the inlets pressure were 5.8×10^{-3} mBar (air) and the analyser pressure 1.1×10^{-4} mBar (argon). Mass spectra were calibrated with CsI cluster ions, molecular weights were calculated using Waters MassLynx software.

3.4. Denaturing ESI-MS

Protein mass characterization under denaturing conditions was performed by electrospray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF (Needle voltage: 800 V, orifice 1 voltage: 40 V, ring lens voltage: 10 V, orifice 2 voltage: 5 V, detector voltage: 2600 V, desolvating chamber temperature: 250 °C and orifice 1 temperature: 80 °C). Before analysis, 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.

3.5. Transmission electron microscopy

TEM grids (FCF200-Cu, EMS) were glow-discharged using a Cressington Carbon coater and power unit. 5 µL sample (0.1 mg/mL) was applied to the glow-discharged grid and was incubated for 1 minute. Then the sample was removed carefully using a filter paper and the grid was allowed to dry for at least 15 min. Subsequently, the grid was negatively stained by applying 5 µL of 2% uranyl acetate in water. The staining solution was removed after 15 seconds and the grid was again allowed to dry for at least 15 min. The samples were analyzed on a JEOL TEM 2100 microscope (JEOL, Japan).

3.6. Enzyme kinetics

CS_2 hydrolase activity in SEC purified ring and catenane fractions was determined by gas chromatography using a Chrompack CP9001 system employing a 40/60 Carbopack BHT 100 column. Enzyme activity was determined in 120 mL glass reaction bottles, containing 500 µL 20mM HEPES pH 7.0 (at 50 °C), sealed with rubber stoppers. Using a glass syringe, the desired volume of CS_2 gas, from a saturated CS_2 gas stock, was injected into a reaction bottle. The reaction bottle was then equilibrated for 10 min at 50 °C or at 45 °C under vigorous shaking (400 rpm).

After equilibration, exact CS_2 concentration and background COS and H_2S concentrations in the reaction bottle were determined by GC. The assay was then initiated by injecting 100 µL of 10 µg/mL CS_2 hydrolase in 20 mM HEPES pH 7.0 (at 50 °C or 45 °C) into the reaction bottle using a glass syringe. The reaction bottle was then incubated at 50 °C (or 45 °C) under vigorous shaking (400 rpm). 1 mL gas samples were taken from the reaction bottle using a glass syringe at 40, 80, 120 and 180 seconds after addition of the enzyme, and were then injected onto the GC system in order to determine H_2S and COS production. H_2S and COS production was determined at 10 substrate concentrations (10, 8, 6, 4, 2, 1,

0.75, 0.5, 0.25 or 0.12 mL of saturated CS_2 gas) in triplicate measurements conducted on 3 separate days for catenane and 3 separate days for ring samples. Kinetics studies performed at 45 °C were conducted in duplicate, on 2 separate days.

When experiments were performed in the presence of 100 μM KCN, 1 μM $CF_3SO_2NH_2$ or 0.35 μM $ZnCl_2$, the protein was pre-incubated with the selected compound for 1-2 hrs on ice under 6x concentrated conditions. Then, enzyme kinetics were studied as described above, at 50 °C and 10 mL saturated CS_2 gas.

Michaelis-Menten analyses provided values for catalytic parameters for the conversion of CS_2 into COS and H_2S. V_{max} and K_m values were obtained from V_0 values by non-linear regression, using Graphpad Prism 5. k_{cat} and k_{cat}/K_m values were subsequently calculated from obtained V_{max} and K_m values. For the experiments performed in the presence of KCN, $CF_3SO_2NH_2$ or $ZnCl_2$; V_{max} was determined using linear regression in order to obtain velocities under conditions where the protein was fully saturated with CS_2.

4. Supporting figures

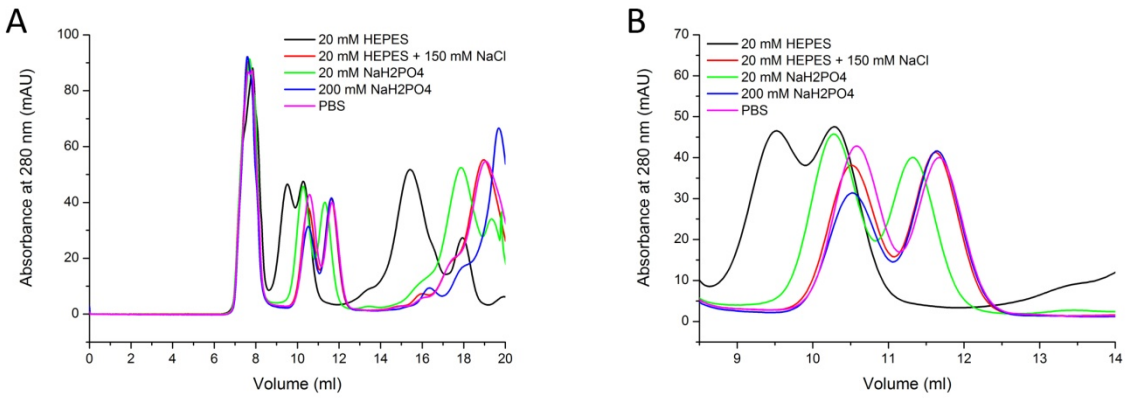

Figure S1. (A) SEC chromatograms during optimization of buffer condition for the separation of ring and catenane forms of CS_2 hydrolase. (B) Enlarged view on the elution of ring and catenane forms of CS_2 hydrolase

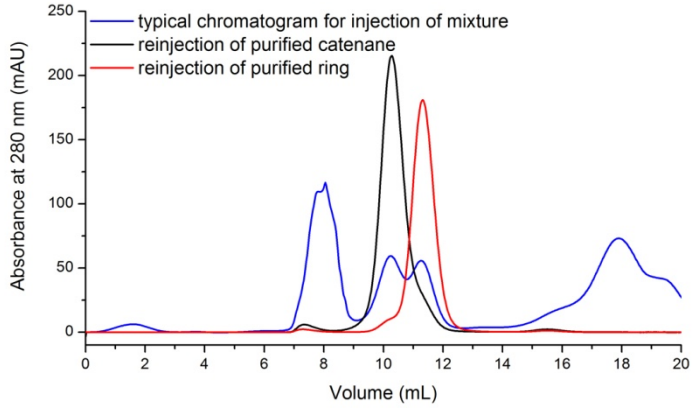

Figure S2. SEC chromatograms for purification of ring and catenane forms of CS_2 hydrolase.

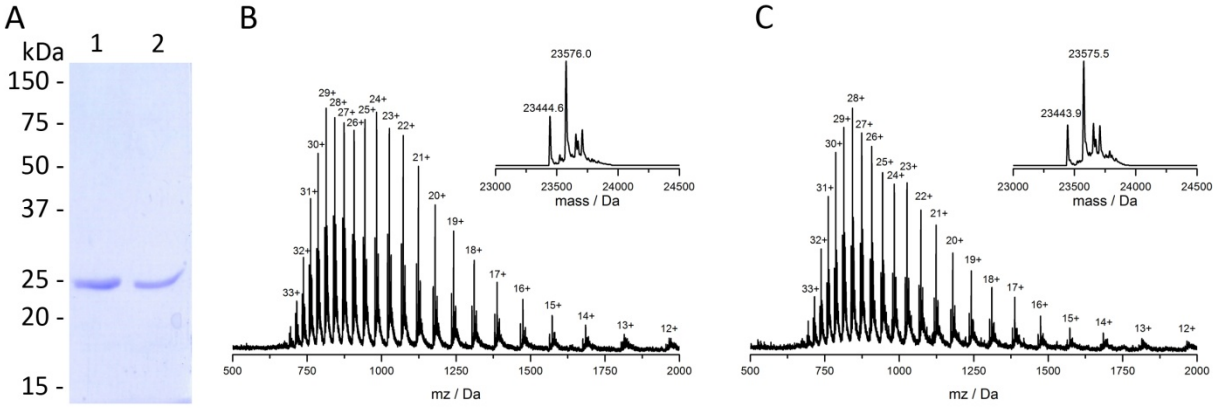

Figure S3. (A) SDS-PAGE analysis of purified catenane (lane 1) and ring (lane 2) forms of CS_2 hydrolase. Denaturing mass spectrometry for (B) catenane and (C) ring showing multiply-charged ion series. The insets depict the deconvoluted mass spectra, showing peaks that matched with CS_2 hydrolase monomers before and after processing of the N-terminal methionine.

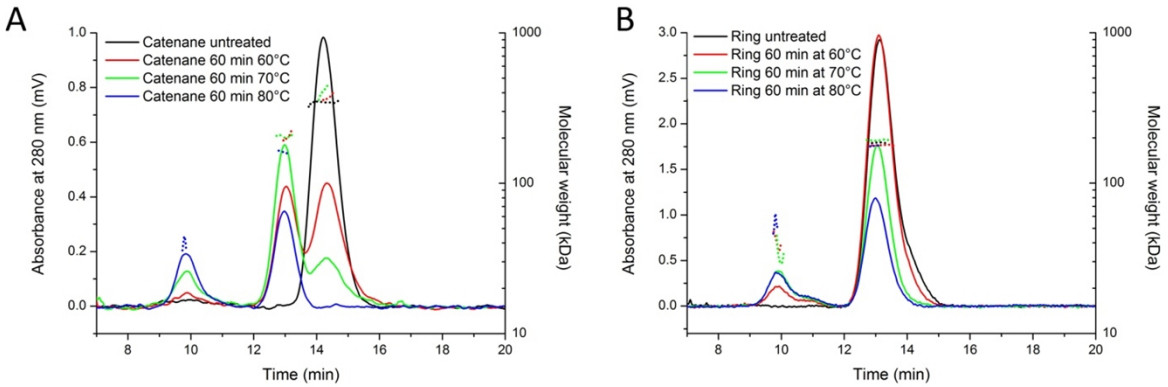

Figure S4. FFF-MALLS chromatograms showing both the absorbance at 280 nm and the molecular weight analyses of the exposure of the catenane (A) and the ring (B) to different temperatures for 60 minutes.

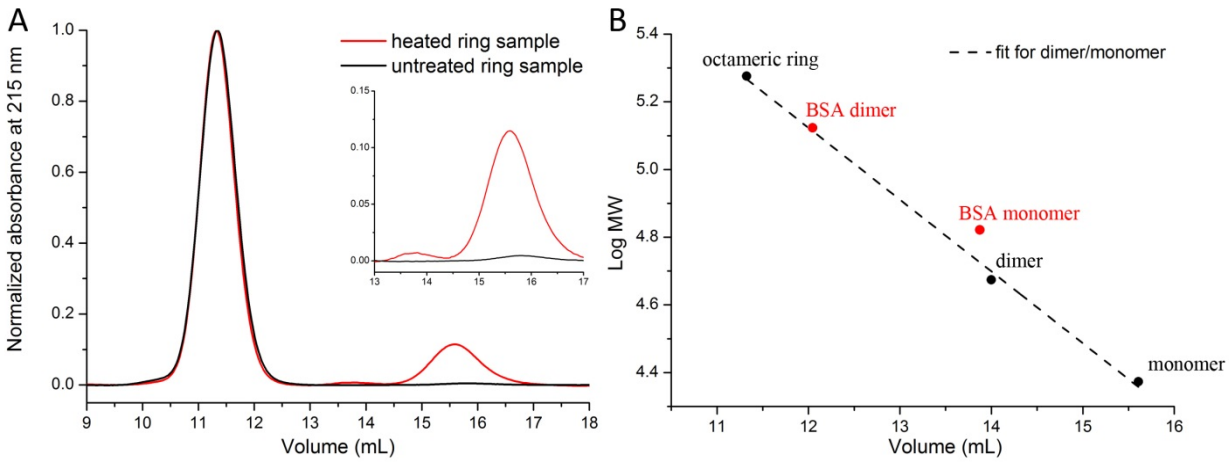

Figure S5. (A) SEC analysis of untreated ring and ring heated for 60 minutes at 70°C. The inset depicts the enlarged chromatogram to show the minor peak at 13.8 mL. (B) Dependence of the log Mw on the elution volume on column showed linear relationships for dimer/monomer (dashed line) forms of CS_2 hydrolase.

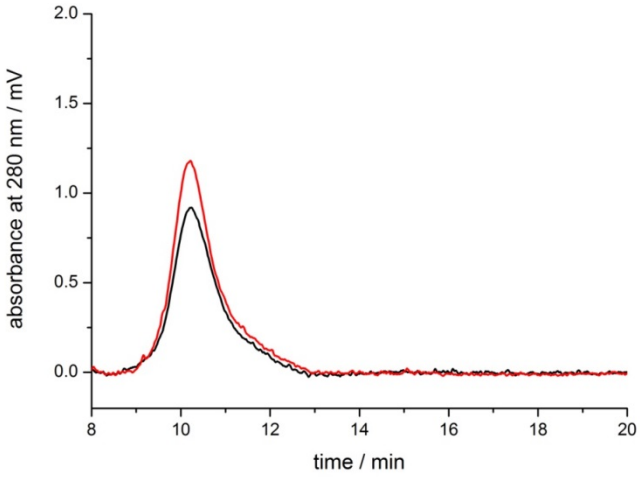

Figure S6. FFF data for the ring (red) and catenane (black) after heating for 30 minutes at 70 °C in 8M guanidine hydrochloride. Both peaks correspond to denatured monomeric CS_2 hydrolase.

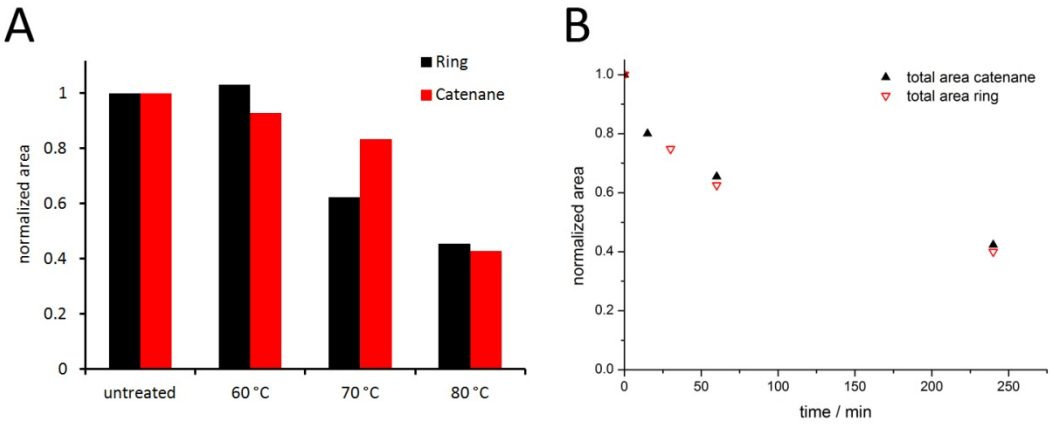

Figure S7. (A) Normalized overall area below the peaks (elution time from 8 until 18 minutes) in FFF chromatograms at various temperatures as shown in Figures 1E and 1F and (B) in Figures 3A and 3B for the incubation at 70 °C in sodium phosphate buffer (20 mM, pH 7.5). FFF showed that for both ring and catenane there is a loss of the protein over time at elevated temperature, as illustrated by the decreased overall area below the peaks in chromatograms. The disappearance of the overall amount of the protein could be attributed to protein degradation, precipitation and/or aggregation; it is likely that such forms of the protein are not visible in the chromatograms.

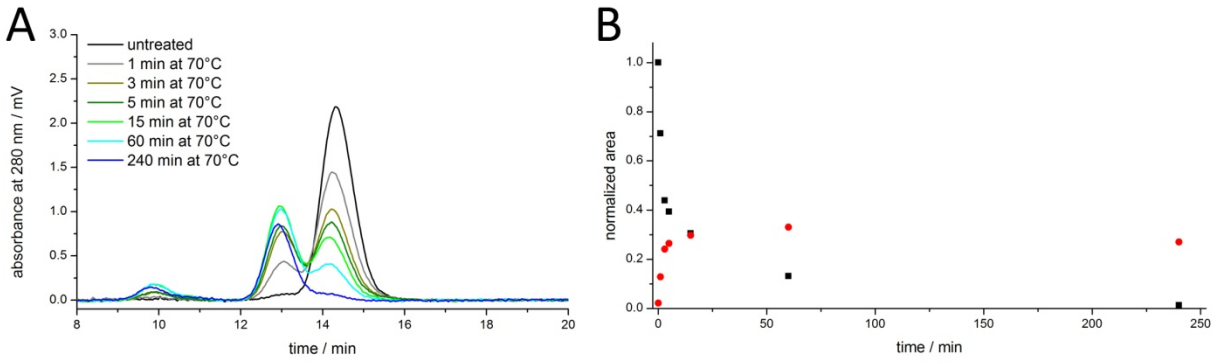

Figure S8. (A) FFF-MALLS chromatograms showing the time course of disassembly of the catenane of CS_2 hydrolase at 70 °C. (B) quantification of area under the peak for both the catenane (black squares) and the ring (red circles) as function of time.

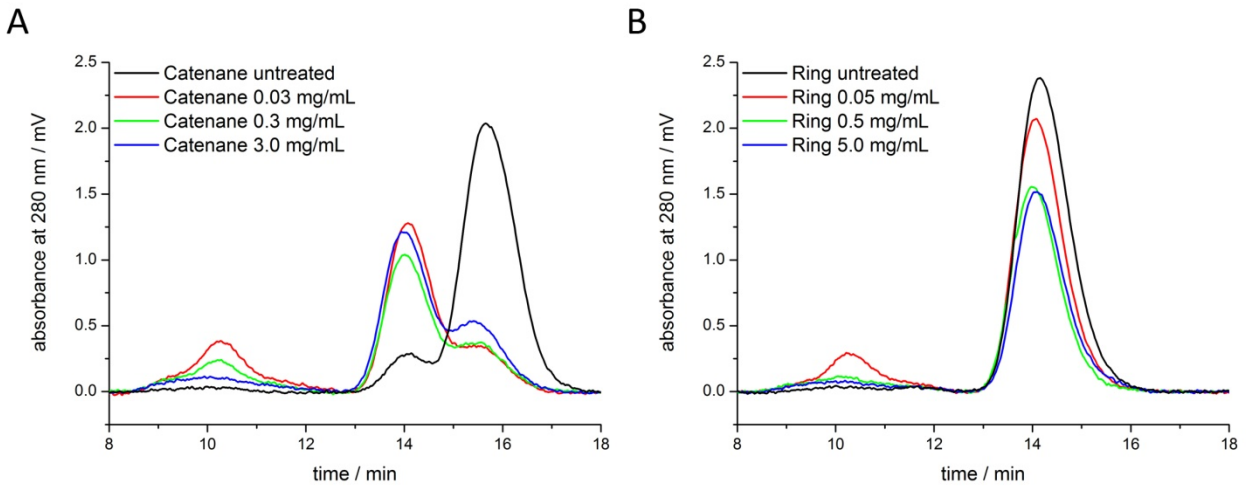

Figure S9. FFF data showing the stability at different concentration of (A) catenane and (B) ring forms of CS_2 hydrolase after 15 minutes at 70 °C.

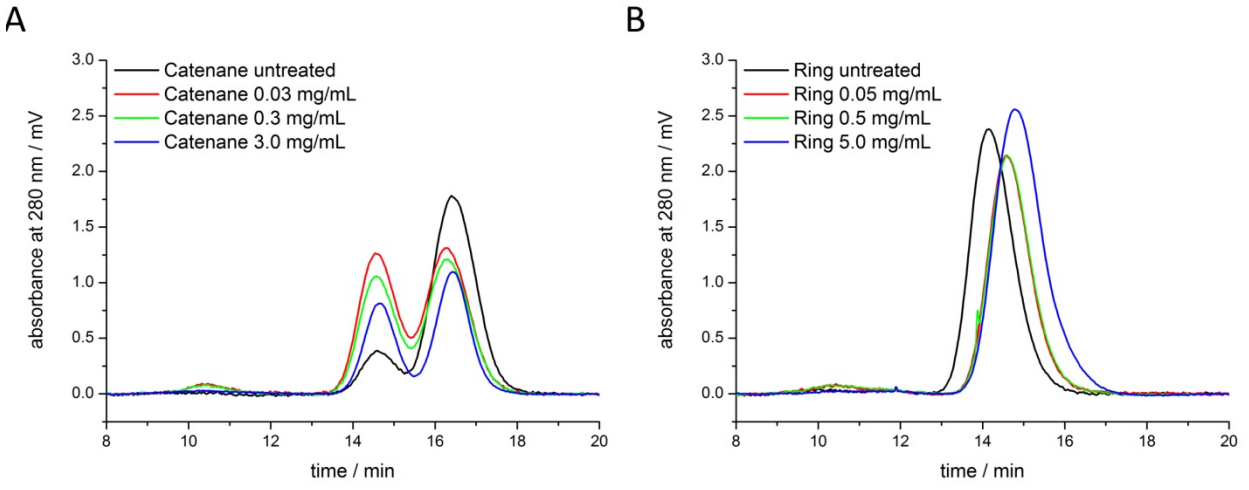

Figure S10. FFF data showing the stability at different concentration of (A) catenane and (B) ring forms of CS_2 hydrolase after 15 minutes at 60 °C.

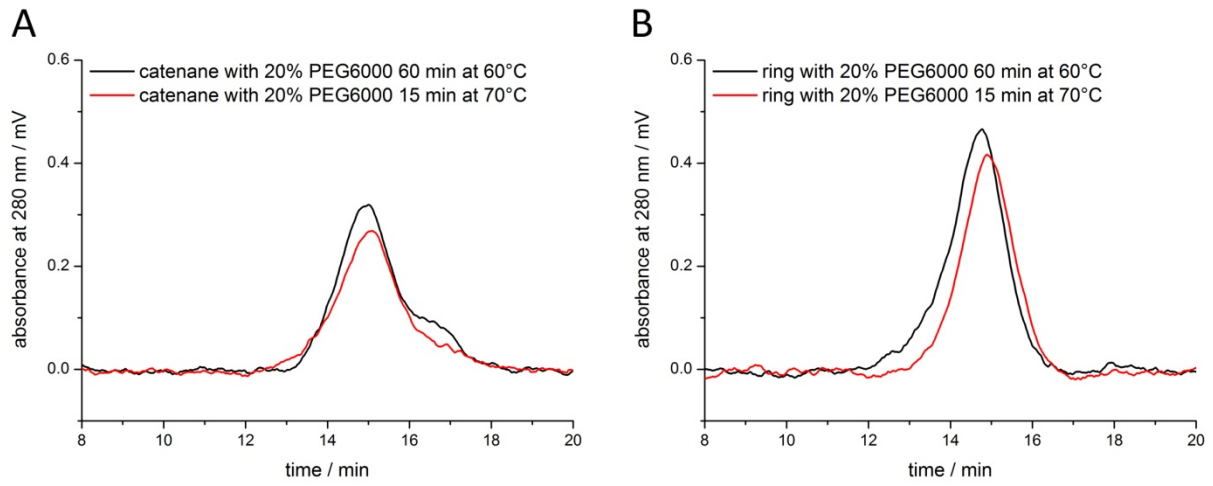

Figure S11. FFF data showing the stability of (A) catenane and (B) ring forms of CS_2 hydrolase in presence of 20% PEG6000 after 15 minutes at 70 °C (red line) and 60 minutes at 60 °C (black line).

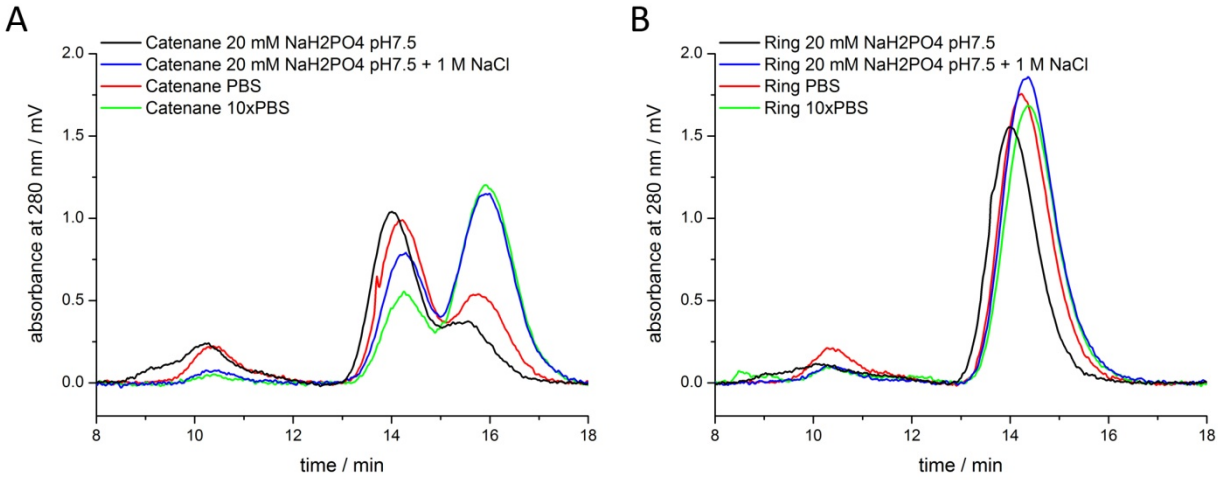

Figure S12. FFF data showing the stability of (A) catenane and (B) ring forms of CS_2 hydrolase in various buffers after 15 minutes at 70 °C.

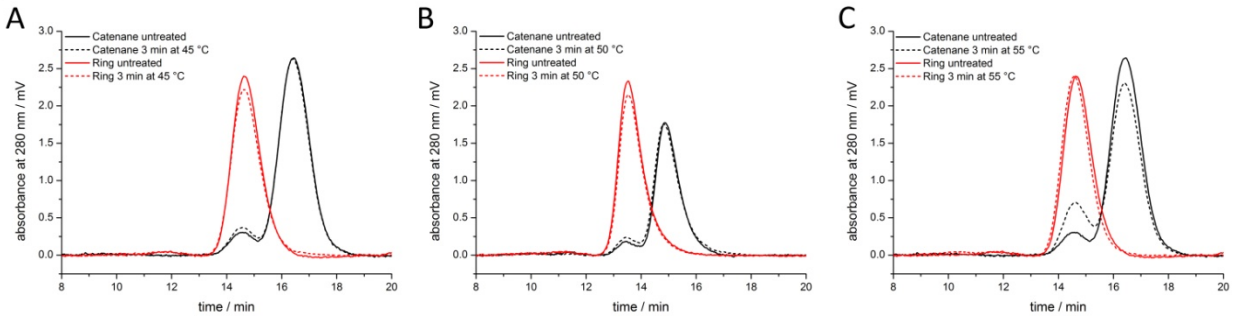

Figure S13. FFF analyses of the ring and the catenane assemblies of CS_2 hydrolase when heated for 3 minutes at (A) 45 °C, (B) 50 °C, and (C) 55 °C.

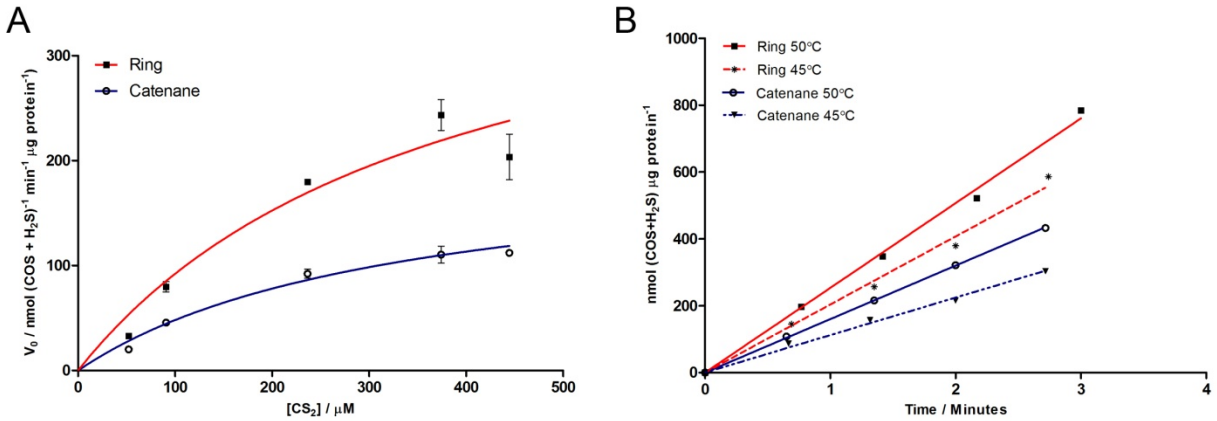

Figure S14. (A) Kinetic data for the conversion of CS_2 into COS and H_2S by the catenane (blue) and ring (red) forms of CS_2 hydrolase at 45 °C (Catenane: V_{max} = 207 nmol (COS + H_2S) min^{-1} µg $protein^{-1}$; k_{cat} = 81 s^{-1}; K_m = 330 µM; $k_{cat}/K_m = 2.45 \cdot 10^5$ s^{-1} M^{-1}; Ring: V_{max} = 440 nmol (COS + H_2S) min^{-1} µg $protein^{-1}$; k_{cat} = 172 s^{-1}; K_m = 378 µM; $k_{cat}/K_m = 4.55 \cdot 10^5$ s^{-1} M^{-1}) (Note: Full enzyme kinetics for both assemblies at 45 °C (presented here) and 50 °C (presented in Figure 4A) were performed on two different batches of CS_2 hydrolase derived from two different purifications). (B) Comparison of V_{max} values for the ring (red) and catenane (blue) at 50 °C and 45 °C in the presence of 10 mL of saturated CS_2. Both forms of CS_2 hydrolase are more active at 50 °C than at 45 °C (Note: These experiments were performed on the same batch of ring and catenanes (coming from the same purification)).

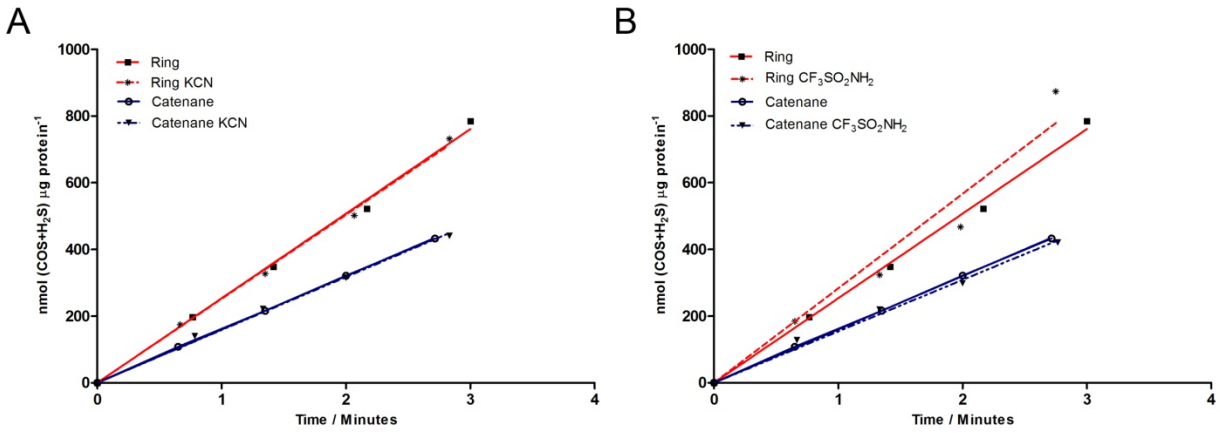

Figure S15. (A) The effect of 100 µM of KCN on the enzymatic activities of the ring (red) and catenane (blue) forms of CS_2 hydrolase at 50 °C in the presence of 10 mL of saturated CS_2. (B) The effect of 1 µM of $CF_3SO_2NH_2$ on the enzymatic activities of the ring (red) and catenane (blue) forms of CS_2 hydrolase at 50 °C in the presence of 10 mL of saturated CS_2.

5. References

(1) Smeulders, M. J.; Barends, T. R. M.; Pol, A.; Scherer, A.; Zandvoort, M. H.; Udvarhelyi, A.; Khadem, A. F.; Menzel, A.; Hermans, J.; Shoeman, R. L.; Wessels, H. J. C. T.; van den Heuvel, L. P.; Russ, L.; Schlichting, I.; Jetten, M. S. M.; op den Camp, H. J. M. *Nature* **2011**, *478*, 412.

(2) van Eldijk, M. B.; van Leeuwen, I.; Mikhailov, V. A.; Neijenhuis, L.; Harhangi, H. R.; van Hest, J. C.; Jetten, M. S.; Op den Camp, H. J.; Robinson, C. V.; Mecinović, J. *Chem. Commun.* **2013**, *49*, 7770.