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Catenane versus ring: do both assemblies of CS\textsubscript{2} hydrolase exhibit the same stability and catalytic activity?†

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Catenane structures, in which two or more rings are mechanically interlocked, have historically occupied one of the central places in the field of supramolecular chemistry. In contrast to synthetic small-molecule catenanes, examples of naturally-occurring catenanes are scarce. Here, we report thermodynamic and enzymatic studies on CS\textsubscript{2} hydrolase, which exists in solution as a mixture of unique hexadecameric catenane and octameric ring forms. A combination of field-flow fractionation coupled to multi-angle laser light scattering (FFF-MALLS) and native mass spectrometric analyses revealed that the catenane form is converted into the ring form at elevated temperatures, whereas the ring does not assemble into the catenane under the same conditions. Measurements of the enzyme kinetics for the conversion of CS\textsubscript{2} into COS and H\textsubscript{2}S showed that the ring form of CS\textsubscript{2} hydrolase possesses higher enzyme efficiency (per monomer) than the catenane form, whereas the catenane form is overall more active (per assembly).

Introduction

Small-molecule supramolecular structures, including catenanes, rotaxanes and molecular knots, have received astonishing recognition by the chemical community in the past decades.\textsuperscript{1–9} Catenane structures, in which two rings are mechanically interlocked, can be synthesized in a straightforward fashion employing sequential template-directed procedures or a dynamic combinatorial approach. In contrast to numerous examples of small-molecule catenanes, biological catenanes have rarely been observed in nature.\textsuperscript{10} The catenane form of DNA has been known for about fifty years,\textsuperscript{11–12} whereas only a few proteins with the uncommon catenane quaternary structure have been recently characterized.\textsuperscript{13–20} Limited studies on ‘non-covalent’ catenane proteins, however, suggested that the interlocked structures occur artificially, which is typically caused by the high concentration of the protein or the precipitant during analysis.\textsuperscript{13,14}

We have recently provided experimental support for the existence of the hexadecameric catenane and the octameric ring assemblies of CS\textsubscript{2} hydrolase from hyperthermophilic Acidianus archaean.\textsuperscript{21} CS\textsubscript{2} hydrolase is a zinc-dependent enzyme that converts CS\textsubscript{2} into CO\textsubscript{2} and H\textsubscript{2}S (via intermediate COS).\textsuperscript{16,22} It is, perhaps, the only ‘real’ catenane protein discovered to date in which two rings are maintained via weak non-covalent interactions; its catenane form was observed in the gas phase, in the crystal state, and in solution at very dilute protein concentrations. Due to these special (and from a prevalent point of view unique) characteristics among protein assemblies, we are particularly interested in understanding the underlying supramolecular chemistry of CS\textsubscript{2} hydrolase.

Our motivation for this work was: (i) to investigate whether the catenane and the ring assemblies of CS\textsubscript{2} hydrolase are in dynamic equilibrium; (ii) to compare the catenane and the ring stabilities at various temperatures; and (iii) to link how the quaternary structure of CS\textsubscript{2} hydrolase affects the enzymatic activity (i.e. do the catenane and ring forms possess the same catalytic parameters).

Results and discussion

Purification of the catenane and ring forms of CS\textsubscript{2} hydrolase

In order to investigate whether the ring and catenane forms of CS\textsubscript{2} hydrolase are in equilibrium, we first developed an efficient separation procedure for these two assemblies. Previous studies demonstrated that an adequate separation of both forms is very challenging by size exclusion chromatography (SEC).\textsuperscript{21} Further optimization of buffer conditions, however, showed that in
phosphate buffer the separation of the ring and catenane forms was sufficient to obtain these two protein assemblies in good purities (>95%) and satisfactory quantities (1–2 mg), required for further biochemical and biophysical analyses (Fig. 1A, see ESI Fig. S1–S3†). SEC coupled to a multi-angle laser light scattering detector (SEC-MALLS) provided molecular weight values of the CS₂ hydrolase oligomers: 360 kDa for the catenane form and 190 kDa for the ring form (Fig. 1A). Because SEC-MALLS analyses typically require a substantial amount of protein for a reasonable detection, we subsequently started using AF4-MALLS-UV (asymmetric flow field-flow fractionation coupled to a MALLS, differential refractive index and UV detector; hereafter referred to as FFF-MALLS). FFF-MALLS allowed us to detect very small amounts (typically 2.5 µg of protein sample at a concentration of 0.1 mg mL⁻¹ was injected into the channel) of both assemblies. In agreement with SEC-MALLS results, FFF-MALLS analyses afforded a good separation of the ring and catenane forms of CS₂ hydrolase and also confirmed the molecular weight of the protein assemblies (Fig. 1B). It is noteworthy that the larger catenane elutes in front of the ring using SEC, whereas, as expected, the smaller ring elutes before the catenane using FFF (Fig. 1A and B).

**Transmission electron microscopy analysis**

Further confirmation for the existence of the ring and catenane topologies of the purified samples was provided by transmission electron microscopy (TEM). TEM data showed clear ring-shaped structures for the samples of the putative octameric ring (Fig. 2). The observed square-shaped rings with a central hole and a diameter of about 10 nm are in good agreement with the crystallographic structure of the ring form of CS₂ hydrolase. TEM data for the catenane sample of CS₂ hydrolase evidently showed that the protein assembly does not exhibit a ring-shaped structure (Fig. 2). Although the resolution of TEM is low, the structures of the putative hexadecameric catenane obtained by TEM are compatible with the catenane topology in which two rings possess a compact and perpendicular orientation. A diameter of 13 nm for the catenane-like structures obtained by

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**Fig. 1**  Purified catenane and ring forms of CS₂ hydrolase using SEC-MALLS (A) and FFF-MALLS (B). FFF analyses of the stability of the catenane and the ring at 4 °C (C) and 20 °C (D). FFF analyses of the catenane (E) and the ring (F) after exposing them to different temperatures for 60 minutes.

**Fig. 2**  Uranyl acetate-stained TEM micrographs of the purified ring (A) and catenane (B) forms of CS₂ hydrolase.
TEM is comparable to the length obtained by the crystal structure of the catenane form of CS₂ hydrolase.\textsuperscript{16}

**Interconversion between the catenane and the ring**

Protein assemblies that are stabilised by multiple non-covalent interactions have a potential to undergo a dynamic interconversion between different oligomerisation states.\textsuperscript{24,25} Because monomers in the ring and catenane structures of CS₂ hydrolase interact with neighbouring monomers solely via non-covalent interactions, it was of special interest to explore whether the ring and catenane assemblies are in equilibrium. The potential equilibrium between the catenane and the ring was studied using FFF. FFF analyses of the pure ring and catenane samples of CS₂ hydrolase revealed that the proteins remained unchanged when kept in phosphate buffer (20 mM, pH 7.5) between 3 and 21 days at 4 °C (Fig. 1C). A small degree of degradation of the catenane sample was observed at 20 °C within 3 days, whereas the ring was stable under the same conditions (Fig. 1D). At elevated temperatures, interestingly, the catenane disassembled into the ring form, whereas the ring did not convert into the catenane form (Fig. 1E). Approximately 30\% and 80\% of the catenane was converted to the ring when heated for 1 hour at 60 °C and 70 °C, respectively (Fig. 1E). Notably, no catenane remained in the sample after 1 hour incubation at 80 °C; the newly observed peaks corresponded to the octameric ring, and smaller monomeric and dimeric products which elute at 10 and 11 minutes, respectively (Fig. S4–S6†). In contrast to the catenane, the ring remained stable for 1 hour at 60 °C, whereas at 70 °C and 80 °C it partially degraded or denatured into monomers and dimers (Fig. 1F). FFF showed that for both ring and catenane, there is a loss of protein over time at elevated temperatures, as illustrated by the decreased overall area below the peaks in the chromatograms (Fig. S7†). The disappearance of the overall amount of 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. FFF data illustrated that most of the catenane was converted into the ring form within 15 min at 70 °C, and that virtually all of the catenane disappeared after 4 hours (Fig. 3A and C and S8†). The ring was found to be more resistant to prolonged incubation at 70 °C (Fig. 3B). The conversion of the catenane into the ring was observed at 0.03–3.0 mg mL\textsuperscript{–1}, whereas no conversion of the ring into the catenane was detected in the range of 0.05–5.0 mg mL\textsuperscript{–1} (Fig. S9 and S10†). Moreover, crowding conditions using PEG6000 did not have any effect on the interconversion of both assemblies (Fig. S11†). An increased ionic strength of the buffer resulted in a lower conversion of the catenane into the ring after 15 minutes at 70 °C, presumably by stabilising the hydrophobic interactions between the monomers of CS₂ hydrolase (Fig. S12†). Overall, these results imply that the ring and the
Catenane structures of CS₂ hydrolase are not in equilibrium in relatively diluted solutions at low temperatures. At high temperatures, however, the equilibrium between the catenane and the ring is completely on the side of the ring; only the catenane form of CS₂ hydrolase disassembled into the ring form, whereas we never observed the opposite conversion of the ring form of CS₂ hydrolase into the catenane form. Based on these results, we conclude that among the two structures of CS₂ hydrolase, the ring is the thermodynamically more stable form, whereas the catenane is a kinetically trapped form.

We have previously shown that CS₂ hydrolase is amenable to non-denaturing electrospray ionisation mass spectrometry (ESI-MS) analyses. Thus, SEC-purified ring and catenane samples of the highest purity were subjected to ESI-MS for the identification of both assemblies of CS₂ hydrolase. Native ESI-MS data of the untreated catenane sample confirmed the existence of the hexadecameric catenane (about 90% pure), with only small signals of the octameric ring (about 10%) (Fig. 3D); it is very likely that the catenane kept at 4°C for a few days prior the MS experiments slowly degraded into the ring, as demonstrated by FFF-MALLS (see above). The untreated ring sample of CS₂ hydrolase was observed to only exist as the octameric ring species (>99% pure) (Fig. 3E). The measured molecular weights for the purified catenane and ring samples were 379.4 kDa and 189.6 kDa, respectively (compared to calculated $M_m$ 378.2 kDa for the catenane and 189.1 kDa for the ring).

Starting from the pure catenane and ring complexes of CS₂ hydrolase, the potential conversion of the catenane to the ring (and vice versa) was also studied by ESI-MS. In agreement with the FFF-MALLS experiments, the catenane form of CS₂ hydrolase underwent a progressive conversion into the ring form in the sodium phosphate buffer (20 mM, pH 7.5) at 70°C. Fig. 3D and F illustrate the progress of the conversion of the catenane form of CS₂ hydrolase into the ring form.
(m/z ~ 9500) into the ring (m/z ~ 6500) over 3.5 hours at 70 °C. In contrast, the ring form of CS₂ hydrolase remained stable when heated at 70 °C up to 3.5 hours (Fig. 3E). The ESI-MS data clearly showed that there has not been any conversion of the ring into the catenane at elevated temperatures in the phosphate buffer, whereas the catenane form of CS₂ hydrolase disassembled into the ring form under the same conditions. Overall, the non-denaturing mass spectrometry results are compatible with the FFF-MALLS solution data; both techniques provided strong evidence for the conversion of the catenane into the ring, and the lack of conversion of the ring into the catenane at high temperatures.

Enzymatic activity of the catenane and the ring

An apparent stability of the catenane and ring oligomers and the lack of equilibrium between these two assemblies at low temperatures subsequently allowed us to investigate whether both forms of CS₂ hydrolase exhibit similar catalytic properties for the conversion of CS₂ into COS and H₂S (Table 1). The activity of both forms of the enzyme was measured at 50 °C within the first 3 minutes using gas chromatography (Fig. 4A). Note that the ring and catenane assemblies were observed to be stable for 3 minutes at 45 °C and 50 °C, but not at 55 °C, as confirmed by FFF-MALLS (Fig. S13†). As expected, both the ring and catenane forms of CS₂ hydrolase were found to be catalytically active. The quaternary structure of the protein, interestingly, had an observable effect on the enzymatic properties of CS₂ hydrolase. Michaelis–Menten analyses for both assemblies showed that the octameric ring and hexadecameric catenane forms of CS₂ hydrolase exhibit different maximal velocity (V_max) values for the conversion of CS₂ into COS and H₂S. Consequently, the value for the rate constant (k_cat) for the ring was about 1.5-fold higher than the k_cat for the catenane. The K_M values for the ring and catenane structures were observed to be indistinguishable (193 μM for the catenane vs. 177 μM for the ring). Based on the values of the enzyme efficiency (k_cat/K_M), we conclude that the ring form of CS₂ hydrolase is catalytically more active (about 1.64-fold) than the catenane form (per mass or per monomer). Similarly, the enzyme efficiency for the ring was about 1.85-fold higher than for the catenane at 45 °C, again demonstrating that the ring is the more active form (per monomer) (Fig. S14†). Nonetheless, the overall activity of the catenane is about 1.22-fold and 1.08-fold higher than the activity of the ring (per mol or per assembly) at 50 °C and 45 °C, respectively.

The addition of ZnCl₂ did not increase the V_max for the catenane and the ring, demonstrating that there is no apo form in the initial protein samples (Fig. 4B). Hence, the difference in the observed kinetic parameters does not arise from variations in the distribution of the zinc cofactor in the active sites of the catenane and the ring. Furthermore, KCN and CF₃SO₂NH₂, both known to competitively inhibit homologous carbonic anhydrase, were found to be inactive in inhibiting both assemblies of CS₂ hydrolase (Fig. S15†). These results indicate that the narrow and apolar entrances of the active sites of the catenane and ring forms are very sensitive to polarity and the size of the substrate analogues.

A closer view of the X-ray structures (PDB ID: 3TEN, 3TEO) of the ring and catenane topologies of CS₂ hydrolase might provide a rationale for the observed difference of the catalytic parameters. The entrance of the ring’s eight active sites is completely accessible for the CS₂ substrate. On the contrary, the access of CS₂ to four of the sixteen active sites of the catenane can be partially blocked due to the packed perpendicular arrangement of the two interlocked rings; one ring can block the entrance into the ‘internal’ active sites of the other ring of the catenane for the substrate. The bottom zoomed view in Fig. 5 illustrates the catenane active sites that can be partially inaccessible for CS₂. The inability of the small KCN and CF₃SO₂NH₂ to inhibit CS₂ hydrolase suggests that the narrow entrances to the active sites control the entry of the small molecule substrates or inhibitors. In this respect, it is possible that the second ring of the catenane form provides, at least in part, an additional access barrier for the CS₂ substrate.

Conclusions

In conclusion, we have demonstrated that the octameric ring form of CS₂ hydrolase is thermodynamically favoured over its hexadecameric catenane form. A combination of FFF-MALLS and native ESI-MS experiments revealed that the catenane can disassemble into the ring at elevated temperatures, whereas the ring does not assemble into the catenane. The ring exhibits higher catalytic efficiency than the catenane form for the conversion of CS₂ into COS and H₂S. Although the biological significance of our results is currently unclear, we do not exclude an evolutionary advantage of the unique quaternary catenane structure of CS₂ hydrolase over its ring structure. When comparing the enzyme efficiency per oligomer (i.e. per mol of assembly), the catenane is more active than the ring, because it contains 16 active sites (the ring only contains 8). The catenane architecture also allows the protein to occupy a smaller space than the same amount of the ring; this way, the cell can accommodate large amounts of CS₂ hydrolase required for the organism’s viability.

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Notes and references