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The rosettazyme: A synthetic cellulosome

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ABSTRACT

Cellulose is an attractive feedstock for biofuel production because of its abundance, but the cellulose polymer is extremely stable and its constituent sugars are difficult to access. In nature, extracellular multi-enzyme complexes known as cellulosomes are among the most effective ways to transform cellulose to useable sugars. Cellulosomes consist of a diversity of secreted cellulases and other plant cell-wall degrading enzymes bound to a protein scaffold. These scaffold proteins have cohesin modules that bind conserved dockerin modules on the enzymes. It is thought that the localization of these diverse enzymes on the scaffold allows them to function synergistically. In order to understand and harness this synergy smaller, simplified cellulosomes have been constructed, expressed, and reconstituted using truncated cohesin-containing scaffolds.

Here we show that an 18-subunit protein complex called a rosettasome can be genetically engineered to bind dockerin-containing enzymes and function like a cellulosome. Rosettasomes are thermostable, group II chaperonins from the hyperthermo-acidophilic archaeon *Sulfolobus shibatae*, which in the presence of ATP/Mg^{2+} assemble into 18-subunit, double-ring structures. We fused a cohesin module from *Clostridium thermocellum* to a circular permutant of a rosettasome subunit, and we demonstrate that the cohesin–rosettasomes: (1) bind dockerin-containing endo- and exo-gluconases, (2) the bound enzymes have increased cellulose-degrading activity compared to their activity free in solution, and (3) this increased activity depends on the number and ratio of the bound glucanases. We call these engineered multi-enzyme structures rosettazymes.

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With the growing interest in biofuels and the focus on cellulosic biomass as a feedstock that does not directly compete with food crops, there are strong incentives to improve cellulose-degrading enzyme systems (Bayer et al., 2007; Himmel et al., 2007; Service, 2007; Lynd et al., 2008). In addition to finding improved cellulases, efforts are underway to harness or construct functional cellulosomes or cellulosome-like structures (Cho et al., 2004; Bayer et al., 2007; Cha et al., 2007; Heyman et al., 2007; Himmel et al., 2007; Mingardon et al., 2007a,b; Lynd et al., 2008). The cellulosome of the anaerobic bacterium, *Clostridium thermocellum*, is an extracellular structure with an estimated molecular mass of >2 MDa (Bayer et al., 2004; Doi and Kosugi, 2004). It consists of scaffoldin (CipA), a 196 kDa, multi-module protein with one cellulose-binding module and nine type I cohesin modules separated by flexible peptide linkers of 20–40 amino acids. Enzymes with conserved type I dockerin modules can attach to this array of cohesins and in the complete genome sequence of *C. thermocellum*, a total of 71 genes were identified with dockerin modules, including cellulases, hemicellulases, pectinases, chitinases, glycosidases, and esterases (Zverlov et al., 2005a,b). A type II dockerin on scaffoldin is used to anchor the complex to cell-surface proteins containing a type II cohesin module.

In an effort to understand the relationships between cellulosome structure and enzymatic activity, truncated cellulosomes, sometimes called "mini-cellulosomes," have been constructed with two or three cohesin modules (Fierobe et al., 2002, 2005; Arai et al., 2007; Cha et al., 2007; Mingardon et al., 2007a,b). Combinations of cellulases associated with mini-cellulosomes are more active than these same cellulases free in solution. These efforts served as an inspiration for the present work in which we designed and constructed a robust synthetic cellulosome using the 18-subunit, self-assembling protein complex known as a rosettasome combined with four dockerin-containing cellulases from *C. thermocellum*. Since we began this work, a similar approach has been reported in which a cohesin module fused to each of the 12 subunits of a protein complex from aspen trees (SP1) was shown to bind a sin-

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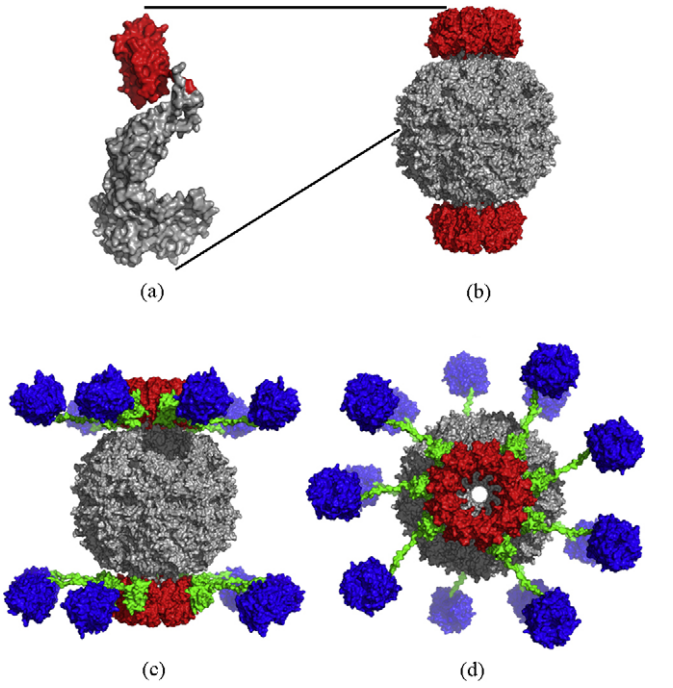

Fig. 1. Illustration of the rosettasome–cohesin and a rosettazyme: (a) a circular permuted rosettasome subunit homology model (grey) combined with the crystal structure of a cohesin module from CipA (red); (b) the assembled rosettasome–cohesin double rings with lines indicating the position of a subunit; (c and d) two views of an assembled rosettazyme with an example of a dockerin-containing cellulase, CelA (blue) with linker and dockerin (green), arranged around the double ring, occupying seven of the nine cohesins on each ring, (c) side view and (d) end view. Enzymes in this illustration were placed in reasonable conformations, not rigorously modeled. Images were made using Pymol (DeLano, 2008).

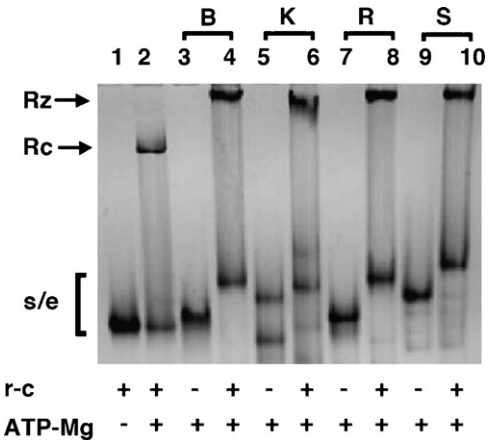

Fig. 2. Native PAGE of the rosettasome–cohesin subunits, cellulases Cel9B (B), Cel9K (K), Cel9R (R), Cel48S (S), and double rings which assemble in the presence of ATP–Mg^{2+}. The electrophoretic mobility of rosettazymes (Rz), rosettasome–cohesin double rings (Rc), rosettasome–cohesin subunits and free enzymes (s/e) are indicated at the left. The addition of rosettasome–cohesin subunits (r-c) and/or ATP–Mg^{2+} is indicated along the bottom. In the absence of ATP–Mg^{2+} rosettasome–cohesin subunits do not assemble into double rings (lane 1), while in the presence of ATP–Mg^{2+} the double rings are evident (lane 2). The mobilities of the cellulases in the absence of rosettasome–cohesin are shown in lanes 3, 5, 7, and 9. The mobilities of the enzymes bound to rosettasome–cohesin subunits or double rings are shown in lanes 4, 6, 8, and 10.

gle dockerin-containing cellulase from *Thermobifida fusca* (Cel5A) and the resulting structure with a high local concentration of the cellulase showed an enhanced specific activity on carboxymethyl cellulose compared to the free enzyme (Heyman et al., 2007). Structures like those assembled on SP1 and the rosettasome described here have the advantage that they can accommodate a larger number of enzymes on a single particle. This may be a significant advantage in creating complexes to break down more chemically complex substrates like the hemicellulose present in cellulosic biomass. The complex we have created, with one cohesin module fused to each rosettasome subunit, we can bind up to 18 enzymes at interchangeable sites. Our work has confirmed and extended the concept that self-assembling protein complexes can act as scaffolds for synergistic enzymes with distinct activities.

The rosettasome from the hyperthermo-acidophilic archaeon *Sulfolobus shibatae*, is a group II chaperonin that self-assembles in the presence of ATP and magnesium ions into double rings (Yaoi et al., 1998). Unlike other group II chaperonins that have eight subunits per ring, rosettasomes have nine subunits per ring (Trent et al., 1991). In vivo, the native rosettasome consists of mixtures of three closely related subunits (alpha, beta, and gamma) in different ratios, depending on the growth conditions of *Sulfolobus* (Kagawa et al., 2003) but we have demonstrated that in vitro double rings can form from the beta subunit alone (Koeck et al., 1998). Furthermore, this ring-forming ability is retained even after significant mutations of the beta subunit (Paavola et al., 2006). Using a mutant beta subunit in which the termini were relocated by circular permutation to an exposed position on the apical domain, we fused the cohesin-2 module from the *C. thermocellum* CipA protein to the carboxyl terminus of the permuted beta subunit and transformed the beta double rings into a scaffold for dockerin-containing cellulases, as shown in Fig. 1. The illustration shows the mutant beta subunit with cohesin attached to the apical domain (Fig. 1a) and the subunit positioned in the assembled double rings (Fig. 1b) indicates how cohesins cluster on opposite ends of the rings. The structure we refer to as a rosettazyme is composed of dockerin-containing enzymes bound to the cohesins as illustrated (Fig. 1c, side view; Fig. 1d, end view).

1. Mixtures of cellulases form rosettazymes

Using native polyacrylamide gel electrophoresis (PAGE), we confirmed that cohesin-containing beta subunits assembled into double rings and that both the subunits and the double rings bind dockerin-containing cellulases from *C. thermocellum* (Fig. 2). In the presence of ATP–Mg^{2+}, the subunit–cohesin fusions formed double rings as indicated by a significant shift in electrophoretic mobility (Fig. 2, lanes 1 and 2). While the persistence of the subunit band (Fig. 2, lane 2) suggested that not all subunits assembled into rings, previous research with rosettasomes indicates that structures can be influenced by electrophoresis (Yaoi et al., 1998) and we determined that in these experiments the structures were influenced by the pH of the electrophoretic buffers (data not shown). The ratio of subunit to double ring bands in native PAGE therefore represents a qualitative indication of rosettasome assembly and sets a quantitative lower limit for the efficiency of assembly.

The interaction between purified dockerin-containing cellulases from *C. thermocellum* – Cel9B (also known as CelF (Mishra et al., 1991)), Cel9K (Kataeva et al., 1999), Cel9R (Zverlov et al., 2005a,b), Cel48S (Kruus et al., 1995) and cohesin-containing subunits or double rings – was clearly demonstrated by changes in electrophoretic mobilities (Fig. 2, lanes 3–10). The high-affinity binding between cohesin and dockerin was indicated by the disappearance of the bands in polyacrylamide gels corresponding to the cellulases in the presence of cohesin-containing subunits and double rings. The presence of enzymes bound to subunits in samples of double rings and enzymes is presumed to be due to the disassembly of double rings caused by electrophoresis (as noted above). Combining the four cellulases with beta subunits lacking cohesin or assembled rosettasomes lacking cohesin produced no change in electrophoretic mobilities of the enzymes or the rosettasomes,