Action of Designer Cellulosomes on Homogeneous Versus Complex Substrates

CONTROLLED INCORPORATION OF THREE DISTINCT ENZYMES INTO A DEFINED TRIFUNCTIONAL SCAFFOLDIN*

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In recent work (Fierobe, H.-P., Bayer, E. A., Tardif, C., Czjzek, M., Mechaly, A., Bélache, A., Lamed, R., Shoham, Y., and Bélache, J.-P. (2002) J. Biol. Chem. 277, 49621–49630), we reported the self-assembly of a comprehensive set of defined “bifunctional” chimeric cellulosomes. Each complex contained the following: (i) a chimeric scaffoldin possessing a cellulose-binding module and two cohesins of divergent specificity and (ii) two cellulases, each bearing a dockerin complementary to one of the divergent cohesins. This approach allowed the controlled integration of desired enzymes into a multiprotein complex of predetermined stoichiometry and topology. The observed enhanced synergy on recalcitrant substrates by the bifunctional designer cellulosomes was ascribed to two major factors: substrate targeting and proximity of the two catalytic components. In the present work, the capacity of the previously described chimeric cellulosomes was amplified by developing a third divergent cohesin-dockerin device. The resultant trifunctional designer cellulosomes were assayed on homogeneous and complex substrates (microcrystalline cellulose and straw, respectively) and found to be considerably more active than the corresponding free enzyme or bifunctional systems. The results indicate that the synergy between two prominent cellulosomal enzymes (from the family-48 and -9 glycoside hydrolases) plays a crucial role during the degradation of cellulose by cellulosomes and that one dominant family-48 processive endoglucanase per complex is sufficient to achieve optimal levels of synergistic activity. Furthermore, cooperation within a cellulosome chimeric between cellulases and a hemicellulose from different microorganisms was achieved, leading to a trifunctional complex with enhanced activity on a complex substrate.

Cellulosomes are extracellular macromolecular complexes produced by cellulolytic anaerobic bacteria that efficiently degrade cellulose (the most abundant biopolymer on earth) and related polysaccharides of the plant cell wall (1). Typical cellulosomes, such as those produced by Clostridium cellulolyticum and Clostridium thermocellum contain a scaffolding protein devoid of enzymatic activity that displays a powerful substrate-targeting family-3a carbohydrate-binding module (CBM)1 and numerous cohesin modules (eight and nine for the two respective species) (2, 3). The latter cohesins bind to the enzyme-dockerin dockerin modules in a species-dependent manner (4). The dockerin-dockerin interaction is Ca2+-dependent and of high affinity (Ka ≈ 109 M−1) (5–7). Within the given species, the dockerin component appears to bind to all of the cohesins with similar affinity, thus suggesting a random incorporation of the enzymes in the cellulosome (3, 8). Therefore, the relative abundance of the catalytic subunits in the cellulosomes is assumed to reflect the level of expression of the corresponding genes as demonstrated recently in the case of C. cellulolyticum using a genetic approach (9).

The modular nature of the cellulosomal subunits led to the proposal that the individual modules could be linked together genetically to form chimeric components whose combination would result in the construction of artificial “designer cellulosomes,” which could then be applied to improve the degradation of cellulolytic wastes (10). In previous studies (6, 11), the property of species specificity was indeed exploited to selectively incorporate desired enzymes into precise positions within chimeric minicellulosomes. In this approach, chimeric scaffolds were constructed that contained a cohesin from each species and an optional CBM. In addition, hybrid cellulosomes were constructed in which the native dockerins of three different C. cellulolyticum enzymes were replaced by a dockerin of divergent specificity derived from the other species. In total, a library of 75 different bifunctional designer cellulosomes was constructed by combining one of the five chimeric scaffolds with one of the

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1 The abbreviations used are: CBM, carbohydrate-binding module; SPR, surface plasmon resonance; RU, resonance unit; 5Ac, Cel5A appended with a C. cellulolyticum dockerin; 8Cc, Cel8C appended with a C. cellulolyticum dockerin; 9Gf, Cel9G appended with a C. cellulolyticum dockerin; 9Ec, Cel9E appended with a C. cellulolyticum dockerin; 9Fc, Cel9F appended with a C. cellulolyticum dockerin; 9Ac, Cel9A appended with a C. cellulolyticum dockerin; 9Mc, Cel9M appended with a C. cellulolyticum dockerin; 48Fc, Cel48F appended with a C. thermocellum dockerin; 48Fc, Cel48F appended with a C. thermocellum dockerin; 48Fc, Cel48F appended with a C. thermocellum dockerin; 90f, Cel9G appended with a R. flavefaciens dockerin; MOPS, 4-morpholinepropanesulfonic acid.
three dockerin-engineered hybrid cellulases (C. *lutilicus* Cel5A, Cel9E, and Cel48F with an appended *C. thermocellum* dockerin) in combination with one of the five available wild-type *C. lutilicus* cellulases (Cel5A, Cel8C, Cel9E, Cel48F, and Cel9G) (11). In most cases, controlled incorporation of the enzyme pairs into chimeric scaffolds enhanced the level of synergistic activity, especially toward recalcitrant celluloses such as microcrystalline cellulose. The stimulation of the activity was mainly a function of two factors: (i) enzyme proximity within the complex and (ii) the substrate targeting effect of the CBM. Among the 75 different designer cellulosomes tested, the most efficient contained two prominent cellulosomal enzymes, i.e. Cel48F and Cel9G (11). Nevertheless even the most potent bifunctional designer cellulose exhibited much less activity on cellulose compared with native cellulases purified from *C. lutilicus* (11).

To gain further insight into the function of natural cellulosomes, a new generation of trifunctional designer cellulosomes was developed in which three different enzymes could be integrated at specified positions within the complex. For this purpose, a third cohesin-dockerin device, derived from the cellulosomal system of the rumen bacterium *Ruminococcus flavefaciens*, was established (12). The affinity of the interaction was determined, and species specificity was verified. A new chimeric scaffoldin was then constructed that contained a CBM and three cohesins, one from each of the three species. A series of trifunctional designer cellulosomes was then self-assembled by combining the new chimeric scaffoldin with three different enzymes (selected from native or dockerin-engineered cellulases from *C. lutilicus* and a hemicellulase from *C. thermocellum*). The resultant designer cellulosomes were examined for their capacity to degrade microcrystalline cellulose and hatched straw substrates.

**Experimental Procedures**

**Plasmids and Strains**—The plasmids and encoded proteins are summarized in Fig. 1. The construction of pETscaf2 (6), pETFc (13), pETFcC (11, 14), pETEc (15), pETEc (16), pETEc (17), and pETMc (18) were described previously.

Plasmid pETscaf6 encoding the hybrid scaffoldin Scaf6 was derived from pETscaf2. The vector pETscaf6 was first modified to remove the stop codon at the 3′ end of the cohesin gene (underlined) introducing six His codons (italics) and a stop codon (underline). The resulting amplified fragment was cloned into NdeI-XhoI-linearized pET22b. Correct orientation of the cloned fragment was checked by restriction analysis of the resulting plasmid.

The plasmid pETCohrf encoding His-tagged cohesin 1 of *ScaB* was cloned into pETCohrf encoding His-tagged cohesin 1 from *ScaB* to remove an internal NdeI site using the mutagenic complementary primers 5′-GGATCC-GTAAGGATACCTTGTTTATCCGCTGTGCAG-3′ (NdeI site in boldface) introducing six His codons (italics) and a stop codon (underline). The resulting amplified fragment was cloned into NdeI-XhoI-linearized pET22b. Correct orientation of the cloned fragment was checked by restriction analysis of the resulting plasmid, pETCohrf.

The plasmid pETCohrf encoding His-tagged cohesin 1 of ScaB was subjected to nondenaturing PAGE as described previously (6). For the xylanase XynZt, the final purification was performed by gel filtration using an Akta system (Amersham Biosciences) and a HiLoad 26/60 Superdex preparatory grade column (Amersham Biosciences).

The concentration of purified proteins was estimated by absorbance (280 nm) in 6 M guanidine hydrochloride and 25 mM sodium phosphate, pH 7.0, using the program ProtParam tool (www.expasy.org/tools/protparam.html) and by quantitative amino acid analysis on a Beckman 6300 system using ninhydrin detection. The purified proteins were dialyzed by ultrafiltration against 10 mM Tris-HCl, pH 8.0, 1 mM CaCl₂, and stored at −20 °C.

**Purification of the Cellulosomes of *C. lutilicus***—The cellulosomes purified by *C. lutilicus* were purified as described previously (20) from a 5-day culture (1 liter) on a medium containing 7.5% (w/v) cellulose as the carbon source. The final protein concentration was determined by the method of Lowry et al. (21) using bovine serum albumin as the standard.

**Nondenaturing PAGE**—Samples (10 μl final concentration) were mixed at room temperature in 20 mM Tris maleate, pH 8.0, 1 mM CaCl₂, and 4 μl were subjected to PAGE (4–15% gradient) using a Phastsystem device (Amersham Biosciences).

**Stability of Chimeric Cellulosomes**—Proteins were mixed at 10 μM (final concentration) in 20 mM Tris maleate, pH 6.0, 1 mM CaCl₂, and 1 μl was subjected to PAGE (Fig. 4–15% gradient) using a Phastsystem device (Amersham Biosciences).

**Surface Plasmon Resonance (SPR)**—Experiments were performed using a BIACore system (BIACore, Umeå, Sweden) as described previously (5, 6) with 10 mM Tris maleate, pH 6.0, 1 mM CaCl₂, and 4 μl were subjected to PAGE (4–15% gradient) using a Phastsystem device (Amersham Biosciences).
model L + A → LA where L denotes the mobile ligand and A denotes the immobilized receptor. For all tested ligands, control experiments were performed by direct injection on the streptavidin-dextran layers.

**Enzyme Activity—**Hatched straw (0.05–0.8 mm) provided by Valagro (Poitiers, France) was incubated in distilled water under mild stirring for 3 h at room temperature, vacuum filtered on 2.7-μm glass filter, resuspended in water, and incubated for 16 h under mild stirring at 4°C. The suspension was filtered and washed three times with water, and the concentration of the residual material was estimated by dry weight.

The total acid-extractable reducing sugar content was determined according to Ref. 22 with some modifications: 300 mg (dry matter) were incubated for 1 h with 12 M H2SO4 at 35°C. The sample was diluted 12 times with distilled water and autoclaved at 120°C for 1 h. The pH was adjusted to 7 with NaOH, and the suspension was centrifuged for 15 min at 8000 × g. The reducing sugar content of the supernatant was determined using the Park and Johnson method (23), and the glucose content was examined using the glucose oxidase method (24). The released monosaccharides were also analyzed by high performance liquid chromatography on an NH2P50 column (Shodex, Tokyo, Japan) using 75% (v/v) CH3CN. The detection and quantification of the various monosaccharides were performed with a Varian (Palo Alto, CA) refractive index detector.

Kinetics were performed by incubating at 37°C 7.5 μl aliquots (40 μl) of the protein (10 μg) in 20 mM Tris maleate, pH 6.0, 1 mM CaCl2 with 4 ml of microcrystalline cellulose (Avicel PH101, Fluka, Buchs, Switzerland) or washed straw at 3.5 g/liter in 20 mM Tris maleate, pH 6.0, 1 mM CaCl2, 0.01% (w/v) NaN3. The final protein concentration was 0.1 μM. Aliquots (0.9 ml) were extracted at 0, 1, 6, and 24 h, centrifuged, and examined for soluble reducing sugars using glucose as the standard.

The xylanase activity of XynZt was determined on insoluble xylan (Sigma). Insoluble xylan was prepared according to Ref. 25 and resuspended at 10 g/liter in 20 mM Tris maleate, pH 6.0, 1 mM CaCl2. Four milliliters of substrate were incubated at 37°C or 60°C with an appropriate dilution of XynZt. Aliquots (0.9 ml) were extracted at specific intervals, centrifuged, and examined for reducing sugars using xylose as the standard. Activity on methyl ferulate was performed as described previously with some modifications: 1070 μl of an appropriate dilution of XynZt in 0.1 M MOPS, pH 6, was incubated at 37 and 60°C with 30 μl of 2.2 mM methyl ferulate (Aaspin, Oxon, UK) in the same buffer. The fluorescence release of ferulate was monitored by measuring the decrease in absorbance at 335 nm.

**RESULTS**

**Selection and Characterization of a Third Divergent Cohesin-Dockerin Device—**Previous studies have shown that complexation of cellulase pairs, appended with suitable dockerins, into hybrid minicellulases containing two divergent cohesins (derived from C. cellulolyticum and C. thermocellum) served to induce significant increases in cellulolytic activity, especially toward recalcitrant cellulose such as microcrystalline cellulose (6, 11). These reports also indicated that the most efficient bifunctional complexes remain approximately 10 times less active on microcrystalline cellulose (Avicel) compared to purified C. cellulolyticum cellulosomes, which may contain up to eight different enzymes (11). Furthermore, with hybrid scaffoldins bearing two divergent cohesins, only two enzymes with a 1:1 stoichiometry could be studied in the complexed state, although it is known that in natural cellulosomes some catalytic subunits are more abundant than others (20). For these reasons, a third specific cohesin-dockerin device was sought to increase the capacity of the hybrid cellulosomes. The first cohesin module of ScafB and the complementary C-terminal dockerin module of ScaA from R. flavefaciens were selected because affinity blotting experiments have shown that these modules interact strongly with each other (12). In addition, these modules are rather divergent from those found in Clostridium spp. and were classified, on the basis of sequence similarities, in a different phylogenetic group, termed type III (27). Cross-reactivity with type-I cohesins or dockerins from either C. cellulolyticum or C. thermocellum is therefore unlikely to occur.

To accurately determine the affinity constant of the cohesin-dockerin interaction in R. flavefaciens and verify the lack of cross-reactivity with its C. cellulolyticum and C. thermocellum counterparts, the His-tagged cohesin 1 from ScaB (termed Coh-rf) was overproduced in E. coli and purified. In parallel, Cel9G from C. cellulolyticum was engineered to bear the corresponding R. flavefaciens dockerin from ScaA thus generating 9GF (Fig. 1). The dockerin-borne cellulase hybrid was subsequently purified from the E. coli overproducing strain. Non-denaturing PAGE experiments (data not shown) of equimolar mixtures of Coh-rf with 9GF, 5Ac, or 48Ft (see scheme in Fig. 1) showed that Coh-rf can only interact with 9GF, thus demonstrating the species specificity of the cohesin-dockerin interaction of the R. flavefaciens system versus C. cellulolyticum and C. thermocellum. Similarly 9GF was unable to bind to the hybrid scaffoldin Scaf2 (Fig. 1), which contains one cohesin each from C. cellulolyticum and C. thermocellum but lacks Coh-rf. The interaction between 9GF (ligand) and Coh-rf (immobilized receptor) was analyzed by SPR, and kinetic constants of 4.1 × 10^5 s^-1 M^-1 and 1.1 × 10^-4 s^-1 were obtained for k_on and k_off, respectively. The value of the resulting K_app, 3.7 × 10^9 M^-1, was lower than that found for the C. thermocellum system (K_A > 10^11 M^-1) using the same technique (6, 28) but similar to the affinity constant of the C. cellulolyticum cohesin-dockerin interaction (K_A = 4.8 × 10^9 M^-1) (5). In the latter case, however, both rate constants of association and dissociation are faster. 5Ac and 48Ft at concentrations up to 1 μM failed to interact with the immobilized Coh-rf, whereas no binding of 9GF at 1 μM to immobilized Scaf2 was detected by SPR. Taken together, these data confirmed the high affinity of the R. flavefaciens cohesin-dockerin interaction and the lack of cross-reactivity with the corresponding modules originating from C. cellulolyticum and C. thermocellum. The selected cohesin and dockerin from the rumen bacterium thus constituted suitable building blocks for the construction of hybrid cellulosomes.

**Characteristics of the Trifunctional Chimeric Scaffoldin Scaf6**—A previous report has shown that the most efficient bifunctional complexes contain the endoglucanase Cel9G in combination with an endoprocessive cellulase (Cel48F or Cel9E) bound to scaffoldin Scaf1 or Scaf2 that contain two divergent cohesins and a single cellulose-binding CBM (11).

Some of the enzymes, notably Cel9G, also bear their own CBMs, but the latter do not bind to microcrystalline cellulose. Instead the enzyme-borne CBMs appear to function as part of the respective catalytic domain by “feeding” a single cellulose chain to the active site of the catalytic domain thus modifying the character of the enzyme. Based on our previous study (11), a new scaffoldin derived from Scaf2 was designed and overproduced in E. coli. This protein, named Scaf6 (Fig. 1), is identical to Scaf2 but contains cohesin 1 from R. flavefaciens Scab at its C terminus. The interaction of the scaffoldin Scaf6 with three different C. cellulolyticum enzymes bearing a dockerin derived from each of the three species (5Ac, 48Ft, and 9GF; Fig. 1) was analyzed by SPR. The hybrid scaffoldin was immobilized (110 ± 10 RU) onto the sensor chip, and the various cellulosases at a concentration of 15 nM were sequentially or simultaneously injected (Fig. 2). The resulting sensograms showed that each type of cohesin module binds to the corresponding dockerin-borne enzyme in an independent manner irrespective of the order of incorporation. As mentioned above, the sensograms also showed that although the R. flavefaciens and C. cellulolyticum docking systems display similar K_A values (3.7 and 4.8 × 10^9 M^-1, respectively), the association and dissociation rates are both visibly faster in the case of the C. cellulolyticum cohesin-dockerin interaction (Fig. 2). The salient component, slower dissociation of the R. flavefaciens cohesin-dockerin pair, implies its suitability in the construction of designer cellulosomes. Furthermore, under these saturating or near saturating conditions, the scaffoldin with cohesin 1 bound to its docking site on scaffoldin 2 mediates the binding of endoglucanase Cel9G to scaffoldin 2 in threefold excess.
designates the feruloic acid esterase catalytic module of XynZt. In the shorthand notation for the cellulases, numbers (Key to symbols)

R. flavefaciens *, note the CBMs associated with the enzymes (9Gf, 9Gc, 9Ec, and XynZt) are not cellulose-binding CBMs but serve as ancillary modules that

i.e. study to contain the same two catalytic modules (11), trifunctional complexes were designed in the present

one of the most efficient bifunctional chimeric cellulosomes

Cel9G (9Gc) bound to Scaf2 were previously found to generate

2000 IU/t toward carboxymethylcellulose (72,118 Da)

8Cc, and 9Mc (13, 14, 18) that display high specific activity

C. cellulolyticum was then supplemented with one of the available wild-type

9Gf) as two of the three enzyme components. This basic system

thus indicating a stoichiometry of 1:1:1:1 for the trifunctional

Complex Formation and Stability—Since Cel48F (48Ft) and

Cel9G (9Gc) bound to Scaf2 were previously found to generate

one of the most efficient bifunctional chimeric cellulosomes

(11), trifunctional complexes were designed in the present

study to contain the same two catalytic modules (i.e. 48Ft and

9Gf) as two of the three enzyme components. This basic system

was then supplemented with one of the available wild-type

C. cellulolyticum cellulases (Fig. 1): the endoglucanases 5Ac,

8Ce, and 9Mc (13, 14, 18) that display high specific activity

toward carboxymethylcellulose (>2000 IU/μmol) and amorphous

cellulose (57–336 IU/μmol) or the endo-processive cellulase

9Ec (15) for which the preferred substrate is amorphous

cellulose (72 IU/μmol). For each chimeric minicellulosome, the

complex formation was examined by nonnaturating PAGE.

Two representative examples are shown in Fig. 3. The stoichiometric mixtures of the three desired enzymes and Scaf6 resulted in a single band with altered mobility thus indicating that complete or near complete complexation was achieved in all cases. Earlier studies have shown that C. cellulolyticum cellulases in the free or complexed states (11) are stable at

37 °C since their carboxymethylcellulase activity remains un-

changed after 24 h of incubation at 37 °C. In the present study,

however, the stability at 37 °C of the tested minicellulosome

complexes were quite similar (550–588

conditions, the amounts of 5Ac (80–85 RU), 48Ft (110–120

RU), and 9Gf (110–115 RU) were in close agreement with the calculated

Rmax (maximum binding capacity of analyte in RU), thus indicating a stoichiometry of 1:1:1:1 for the trifunctional

complexes. The stoichiometry of the various complexes used in

the present work was also systematically verified by nonnaturating

PAGE as described below.

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Activity of the Trifunctional Cellulosomes on Microcrystalline

Cellulose—The activity of the trifunctional complexes containing

three different cellulases was investigated on 3.5 g/liter

microcrystalline cellulose and compared with that of the cor-

responding free enzyme mixtures. In addition, we examined the activity of the corresponding bifunctional complex, Scaf2:8Cc/48Ft, supplemented with free 9Gf (where 9Gf designates the wild-type C. cellulolyticum enzyme 5Ac, 8Cc, 9Ec, or 9Mc). The complexation of three enzymes into Scaf6 induced a strong increase in their combined activity as shown in Fig. 5. After 24 h, the enhancement in activity of the trifunctional complexes varied from 2.3-fold (Scaf6:8Cc/48Ft/9Gf, Fig. 5D) to 3.8-fold (Scaf6:8Cc/48Ft/9Gf, Fig. 5D) compared with the corresponding free enzyme systems. The trifunctional complexes were also found to be approximately twice as active on micro-

crystalline cellulose as the mixture containing the correspond-

ing Scaf2-based complex and free 9Gf. Interestingly the final

amounts of soluble sugars released in 24 h by the various

trifunctional complexes were quite similar (550–588 μm), whereas the amounts released by the corresponding free en-

zyme systems displayed significant variations (146–240 μm).

Impact of the Stoichiometry and the Location within the

Complexes—Cel48F has previously been shown to be one of the

three major components of the cellulosomes produced by C. cellulolyticum; the other two are the scaffoldin CipC and the

enzyme Cel9E (20). On the other hand, Cel9G is a relatively minor catalytic subunit (20). To explore the importance of the

Cel48F/Cel9G stoichiometry, trifunctional complexes were as-

bottom of the gel. Analysis of the same samples by SDS-PAGE

(Fig. 4, C and D) did not reveal any noticeable spontaneous

proteolysis of any of the four proteins participating in the

complex. The faint band appearing after 24 h in the nonnatur-

ating PAGE remained unidentified; nevertheless it was con-

cluded that the half-life at 37 °C of the tested minicellulosome

complexes is beyond 24 h.

Activity of the Trifunctional Cellulosomes on Microcrystalline

Cellulose—The activity of the trifunctional complexes containing

three different cellulases was investigated on 3.5 g/liter

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sponding free enzyme mixtures. In addition, we examined the activity of the corresponding bifunctional complex, Scaf2:8Cc/48Ft, supplemented with free 9Gf (where 9Gf designates the wild-type C. cellulolyticum enzyme 5Ac, 8Cc, 9Ec, or 9Mc). The complexation of three enzymes into Scaf6 induced a strong increase in their combined activity as shown in Fig. 5. After 24 h, the enhancement in activity of the trifunctional complexes varied from 2.3-fold (Scaf6:8Cc/48Ft/9Gf, Fig. 5D) to 3.8-fold (Scaf6:8Cc/48Ft/9Gf, Fig. 5D) compared with the corresponding free enzyme systems. The trifunctional complexes were also found to be approximately twice as active on micro-

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sembled that contained two molecular equivalents of either cellulase (i.e. Scaf6:9Gc/48Ft/9Gf and Scaf6:48Fc/48Ft/9Gf). The latter chimeric cellulosomes were assayed on microcrystalline cellulose, and the activity was compared with that of the corresponding free enzyme systems (Fig. 6). The complex containing two Cel9G subunits and one Cel48F was found to be more efficient than the complex displaying the reverse stoichiometry. Furthermore the bifunctional complex Scaf2:9Gc/48Ft exhibited similar activity compared with the trifunctional complex Scaf6:48Fc/48Ft/9Gf, thus suggesting that the second Cel48F introduced in the latter complex does not contribute to the overall activity.

The cohesin-dockerin interaction was shown to be nonspecific within the C. cellulolyticum system using biochemical and genetic approaches (3, 9). The various catalytic subunits would therefore be expected to be randomly incorporated into the scaffoldin CipC during assembly of the cellulosomes. To determine whether the maximum synergy between the basic enzyme pair (Cel48F and Cel9G) is only reached when the two complementary enzymes are bound at specific locations within the complex, the particular architecture of Scaf6 was exploited to anchor only one Cel48F and one Cel9G at different positions on the scaffoldin, whereas the dockerin components of the two enzymes were varied to enable their precise incorporation. Thus, three different bifunctional complexes were assembled on a trifunctional scaffoldin (i.e. Scaf6:48Fc/9Gf, Scaf6:48Ft/9Gf, and Scaf6:9Ec/48Ft). The resultant complexes were assayed on microcrystalline cellulose (Fig. 7), and the results were compared with those of the corresponding free enzyme pairs and the bifunctional scaffoldin-borne complex Scaf2:9Gc/48Ft. The three Scaf6-based complexes were found to display very similar activities, which, in turn, were similar to that exhibited by the Scaf2-based complex. Thus, the synergies triggered by complexation seem to be independent of enzyme location within the complex.

**Activity of the Trifunctional Cellulosomes on Hatched Straw**—In a former study (11), it was observed that the incor-
poration of enzyme pairs into bifunctional complexes increased their activity on recalcitrant celluloses such as microcrystalline cellulose, whereas on more tractable substrates, such as amorphous cellulose, the complexation of the enzyme pairs showed little or no advantage over free enzyme systems. In this earlier report, only pure celluloses of various crystallinity or degree of polymerization had been used (11). In the present study, in addition to the model cellulose substrate, Avicel, the bifunctional complexes were assayed on hatched straw, a complex, natural substrate that contains, in addition to cellulose, numerous other polysaccharides (e.g., xylan, etc., collectively referred to as hemicellulose). The washed straw was found to contain 3.3 mmol of acid-extractable reducing sugars/g of dry matter. Quantification of the glucose by high performance liquid chromatography analysis and the glucose oxidase method (see “Experimental Procedures”) indicated that the substrate contains approximately 40% cellulose (2.3 mmol of glucose/g of dry matter). The content of xylose was found to be 0.8 mmol/g of dry matter, whereas the amount of arabinose was around 0.1 mmol/g.

The activity of the various trifunctional complexes on straw is shown in Fig. 8. Control kinetics with the corresponding free enzyme systems and the corresponding bifunctional Scaf2-based complex supplemented with free 9Gf were performed. Compared with microcrystalline cellulose, the various samples were found to be less active on straw; nevertheless incorporation of the three different cellulases into Scaf6 also induced a drastic increase in the activity compared with free or partly complexed enzyme systems. After 24 h, the observed stimulations varied from 5.22-fold (Scaf6:5Ac/48Ft/9Gf, Fig. 8A) to 2.5-fold (Scaf6:8Cc/48Ft/9Gf, Fig. 8B) compared with free enzyme systems and bifunctional complexes. Contrary to microcrystalline cellulose degradation, the amount of released insoluble sugars varied considerably depending upon the third wild-type C. cellulolyticum enzyme incorporated into Scaf6 together with 48Ft and 9Gf. The following hierarchy was ob-

**Fig. 4.** Stability of the assembled trifunctional complexes. Samples containing 10 μM chimeric cellulosome Scaf6:8Cc/48Ft/9Gf (A and C) or 10 μM Scaf6:9Ec/48Ft/9Gf (B and D) were incubated at 37 °C. At 0, 1, 6, and 24 h (time of incubation indicated on top of the lanes), aliquots were subjected to nondenaturing PAGE (A and B) or SDS-PAGE (C and D). Note the mobilities of Scaf6, 48Ft, and 9Gf by SDS-PAGE are very similar, and the respective bands are indistinguishable in the gel.

**Fig. 5.** Kinetic studies of cellulose hydrolysis by the trifunctional complexes versus controls. The panels indicate the contribution of the third enzyme (5Ac (A), 8Cc (B), 9Ec (C), and 9Mc (D)) to the basic enzyme pair (48Ft and 9Gf). Curves are labeled as follows: trifunctional complexes (●) containing Scaf2:5Ac/48Ft/9Gf (A), Scaf2:8Cc/48Ft/9Gf (B), Scaf2:9Ec/48Ft/9Gf (C), and Scaf2:9Mc/48Ft/9Gf (D); free enzyme mixtures (○) containing 5Ac + 48Ft + 9Gf (A), 8Cc + 48Ft + 9Gf (B), 9Ec + 48Ft + 9Gf (C), and 9Mc + 48Ft + 9Gf (D); bifunctional complexes supplemented with free 9Gf (○): Scaf2:5Ac/48Ft + free 9Gf (A), Scaf2:8Cc/48Ft + free 9Gf (B), Scaf2:9Ec/48Ft + free 9Gf (C), and Scaf2:9Mc/48Ft + free 9Gf (D); bifunctional complex Scaf2:9Ec/48Ft (●). Unless otherwise stated, the final protein concentration in this and subsequent figures was 0.1 μM. Released soluble sugars were assayed by the ferricyanide method. The data show the mean of three independent experiments (variation within ±5%).
iewed: 5Ac > 9Mc > 8Cc > 9Ec. Nevertheless, as observed with the cellulose substrate, the complex containing two Cel9G subunits and one Cel48F was significantly more efficient on straw than the complex displaying the opposite stoichiometry (Fig. 9). Again, similar to the results observed for cellulose degradation, the binding of the basic enzyme pair at various positions on Scaf6 was essential for its activity on the activity of the resulting bifunctional complexes on straw (data not shown).

**Incorporation of Xylanase XynZt into Trifunctional Chimeric Cellulosomes—**Although C. cellulolyticum is able to grow on xylan (25), the genes encoding xylanases have not yet been described. On the other hand, several genes encoding hemicellulases have been identified and sequenced in C. thermocellum (29). Among the available C. thermocellum xylanase-encoding genes, the xynZ gene is of particular interest. The corresponding protein is itself a bifunctional enzyme (19). The enzyme contains a family-1 carbohydrate esterase catalytic module, a family-6 CBM, and a typical C. thermocellum type I dockerin module, and a family-10 xylanase catalytic module (afmb.cnrs-mrs.fr/CAZY). The protein (XynZt, Fig. 1) was appended with a C-terminal His tag, overproduced in E. coli, and purified. Since XynZt stems from a thermophyllic microorganism, the activity toward insoluble xylan and methyl ferulic acid was assayed at 37 °C and at the optimum temperature (60 °C). The residual activities at 37 °C were found to be 60.5% (5.47 IU/mol of reducing soluble sugars released in 24 h) compared with microcrystalline cellulose (2930 mol of reducing soluble sugars released in 24 h). The decrease in activity, however, was less dramatic (1.9-fold) than that of the trifunctional complexes containing exclusively C. cellulolyticum xylanoliticum cataytic subunits (up to 4.7-fold for Scaf6:9Ec/48Ft/9Gf, Figs. 5C and 8C). On microcrystalline cellulose, native cellulosomes at 0.1 μM were found to be 5.1-fold more active than the trifunctional complexes containing 5Ac, 8Cc, 9Ec, and 9Mc (Fig. 5B). On straw, the most efficient complex that only contained endoglucanase subunits derived from C. cellulolyticum (Scaf6:5Ac/48Ft/9Gf, Fig. 8A) was 6.5-fold less active than natural cellulosomes. This difference, however, was drastically improved (almost 2-fold) when the purified cellulosomes at 0.1 μM were compared with the XynZt-containing bifunctional complex Scaf6:48Ft/XynZt/9Gf (3.45-fold, Fig. 10).

**DISCUSSION**

Initial work on bifunctional designer cellulosomes that contain two enzymes (6, 11) has provided an effective model system for assessing many of the original assumptions regarding cellulose action. Using this approach, individual cellulosome modules could be mixed, matched, and expressed as viable hybrid proteins, essentially at will, such that the functionality of each module was retained. Production of stable, defined designer cellulosomes could thus be controlled by matching the cohesins of the chimeric scaffoldins with the complementary dockerins of the enzyme hybrids. The enzymes of the resultant designer cellulosomes exhibited enhanced synergy, and their fused species of divergent dockerin (derived from C. thermocellum or C. cellulolyticum) had little if any effect on the performance of the enzymes in the complex. Notably both enzyme proximity and substrate targeting were demonstrated experimentally to be important cellulosome parameters (11) in accord with previous speculation (30). This is the first time that these two features of the cellulosome could be assessed individually. The bifunctional designer cellulosomes indeed facilitated degradation of recalcitrant substrates (e.g. microcrystalline cellulose) but provided little or no advantage on tractable substrates (e.g. acid-treated cellulose) (11). Although revealing in terms of basic research, the overall cellulose degradation capacity of the designer cellulosomes was much lower than that of the native cellulosomes. Consequently further development of higher order complexes that bear combinations of additional enzymes is warranted.

The first step in extending the designer cellulosome concept
is to identify a new cohesin-dockerin pair that meets the following major criteria: high affinity and lack of cross-reactivity with the other cohesin-dockerin components of the system. The cohesin-dockerin pair selected from *R. flavefaciens* (12) indeed fulfilled these requirements and proved entirely suitable for use in trifunctional complexes.

It was shown previously in bifunctional complexes that inclusion of two cellulases, *i.e.* Cel48F and Cel9G, provided the most efficient cellulosome chimeras (11). Based on these data, these two enzymes were incorporated into all of the designer cellulosomes examined in this work. The resultant trifunctional complexes were severalfold more efficient on pure cellulose compared with the free enzyme systems (Figs. 5 and 11A). CBM and proximity effects did occur when the enzymes were incorporated into trifunctional complexes in accord with previous studies on bifunctional complexes (6, 11). The latter reports have shown that, for most tested enzyme pairs, both proximity and CBM effects contributed almost equally to the elevated activity of bifunctional complexes (11). In the case of Cel48F and Cel9G, each effect induced approximately a 2-fold increase in activity when this enzyme pair was bound to Scaf2 (11). Since all trifunctional complexes studied in this report contained this key enzyme pair, it was expected that both proximity and CBM effects remain prominent. The trifunctional complexes were also found to be significantly more active than the bifunctional complex either alone or in combination with the third enzyme in the free state. These results further attest to the contribution of enzyme proximity and to the superiority of the arrangement of numerous enzymes into a cellulosome complex. The importance of the efficiency afforded by these two cellulosomal enzymes was also borne out by the fact that they contributed about 75% of the overall enzymatic activity in the trifunctional cellulosomes irrespective of the third enzyme integrated into the complex (Figs. 5 and 11A).

On the other hand, when the more complex substrate (hatched straw) was used as the substrate, very different activity patterns were obtained (Figs. 8 and 11B). The enzyme mixtures in the free and/or complexed states were all signifi-
Bars given enzyme system and are summarized from Figs. 5, 6, 8, 9, and 10. The data represent the amount of released soluble sugars after 24-h reaction by the enzyme systems.

The enzyme Xylanase Z from C. thermocellum (Cel48F/Cel9G) supplemented with the additional enzyme as designated with (+) or without (−) the trifunctional chimeric scaffoldin (Scaf6). The data represent the amount of released soluble sugars after 24-h reaction by the given enzyme system and are summarized from Figs. 5, 6, 8, 9, and 10. Bars (in B) indicate the standard deviation for straw hydrolysis.

Fig. 11. Comparative solubilization of microcrystalline cellulose (A) and straw (B) by the various complexes and free enzyme systems. The composition of the complexes and free enzyme systems is indicated at the bottom of the graph; i.e., the basic enzyme pair (48F + 9G) supplemented with the additional enzyme as designated with (+) or without (−) the trifunctional chimeric scaffoldin (Scaf6). The data represent the amount of released soluble sugars after 24-h reaction by the given enzyme system and are summarized from Figs. 5, 6, 8, 9, and 10. Bars (in B) indicate the standard deviation for straw hydrolysis.

The impact of stoichiometry of the basic enzyme pair was similar for the degradation of both substrates. Clearly the trifunctional complex that included two copies of Cel9G and one of Cel48F was significantly more active than the bifunctional complex containing only the basic pair. In contrast, the trifunctional complex exhibiting the reverse stoichiometry (i.e., two copies of Cel48F and one of Cel9G) displayed essentially the same activity as the bifunctional complex (Fig. 11). This observation is intriguing because Cel48F is one of the major proteins in all of the known cellulosomes, including C. cellulolyticum, whereas Cel9G is but a minor component (20). The data imply that each individual cellulosomal complex would likely contain one processive family-48 cellulase rather than an averaged incremented distribution of this dominant cellulosomal enzyme (i.e., 0, 1, 2, etc.). This premise is consistent with the tandem position of the cipC and cel48F genes on the C. cellulolyticum genome; the two genes form part of an operon and lack an intergenic terminator (31, 32). With respect to the relatively low levels of Cel9G, the native cellulosome includes two other family-9 enzymes of identical modular architecture (Cel9F and Cel9J) (18), and these three enzymes together would presumably work in concert with Cel48F in a similar manner, thus enhancing the overall synergy displayed by the system.

The source of the appended dockerin (C. cellulolyticum, C. thermocellum, or R. flavefaciens) and relative position of the basic enzyme pair in the chimeric scaffoldin had essentially no effect on the specific activity and synergy of the designer cellulosomes (Fig. 7). Moreover the integration of the very large XynZt in a trifunctional configuration did not affect the observed cooperation between the basic pair of cellulases on cellulose (Fig. 11A). These results are consistent with the notion that long linkers connecting the various modules in the cellulosomal scaffoldins would provide conformational flexibility, thus enabling fine tuning in the interaction of individual catalytic modules in response to the local geometry of the substrate (33).

In the absence of a bona fide xylanase, the most efficient trifunctional complex exhibited activity levels on cellulose and straw that were only a fraction (5- and 6.5-fold less, respectively) of those achieved by the native cellulosomes. Such cellulosome chimeras containing only cellulases cannot compete with the native cellulosomes and their diverse content of enzymes. This is corroborated by the fact that incorporation of the genuine hemicellulase XynZt together with the basic pair of cellulases served to increase the activity on straw, and the resulting trifunctional complex is only 3.5-fold less active than the native cellulosomes. In contrast, the striking difference in activity between native cellulosomes and the trifunctional complexes, on pure cellulose as a substrate, remains intriguing. Of course, in the present study, we analyzed just a limited number of the known cellulosomal enzymes produced by C. cellulolyticum, and the action of a critical enzyme(s) may have been missed. In any case, the native cellulosomes have been shown to be heterogeneous with respect to enzyme content (32), and the synergistic activity among additional enzymes in higher order complexes or cooperation among different cellulosome complexes may be required for optimal degradation of the recalcitrant substrate. This study thus raises questions concerning the conformational flexibility of the cellulosomes, their enzymatic heterogeneity, their ability to cooperate, and their remaining uncharacterized components. These topics will be the subject of future investigation.

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Action of Designer Cellulosomes on Homogeneous Versus Complex Substrates: CONTROLLED INCORPORATION OF THREE DISTINCT ENZYMES INTO A DEFINED TRIFUNCTIONAL SCAFFOLDIN

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