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Unorthodox Intrasubunit Interactions in the Cellulosome of *Clostridium thermocellum*

Identification of Structural Transitions Induced in the S1 Subunit

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ABSTRACT

Clostridium thermocellum, an anaerobic thermophilic cellulolytic bacterium, produces an extremely cohesive, very high-molecular-mass, multicellulase-containing complex termed the *cellulosome*. One of its components, the S1 subunit, is a nonenzymatic, 210 kDa glycopolypeptide. Upon preconditioning of the intact cellulosome with low-ionic-strength or low-pH solutions, the S1 subunit separates in hot sodium dodecyl sulfate (SDS) solutions into a series of defined lower-molecular-mass subcomponents. Under the same conditions, the purified S1 subunit demonstrated the same behavior. Higher levels of glycosylation associated with the larger S1 subcomponents. The data support alterations in the conformational state of the S1 structure that lead to its disintegration induced by combined treatments with SDS and heating. Evidence is provided that this phenomenon may reflect a physiological response of the cellulosome, since similar alterations in S1 appear to accompany its binding to cellulose.

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Index Entries: Subunit dissociation; conformational transitions; SDS-PAGE; anomalous migration; cellulosome; multienzyme complex; cellulose degradation; *Clostridium thermocellum*.

INTRODUCTION

The degradation of cellulose by a variety of different cellulolytic bacteria appears to be mediated by remarkably similar types of cellulose-binding, multienzyme complexes termed *cellulosomes* (*1–6*). Characteristically, cellulosomes contain different enzymes (e.g., endoglucanases, exoglucanases, and xylanases) as well as other apparently nonenzymatic components, the most notable of which are glycopolypeptide components, which may have one of several functions, including proposed roles in cellulosomal organization, cellulose-binding, and association of the enzymatic components into the cohesive complex.

The first cellulosome to have been described belongs to the anaerobic, thermophilic, cellulolytic bacterium *C. thermocellum* (*1,7,8*). Its properties have been well documented since its initial description, and its in-depth functional and structural characterization continues to be subject to extensive study (*4,9,10*).

One of the most intriguing components of the cellulosome from *C. thermocellulm* is the nonenzymatic 210 kDa glycopolypeptide, the S1 subunit (*8,11,12*). The oligosaccharide moiety(ies) of this very large cellulosomal subunit has recently been elucidated (*13*). However, study of the S1 subunit has been hampered throughout the years for two reasons: First, the structure of the cellulosome is remarkably stable to denaturation, and its separation into its component parts is experimentally difficult; only treatment with SDS at elevated temperatures has been shown to consistently dissociate the cellulosome complex into its collection of subunits (*4,5*). Secondly, an unusual pH- and ionic strength-related anomaly in the SDS-PAGE mobility pattern of the S1 subunit has recently been described (*14*); this has been the source of a great deal of confusion regarding the precise identification of cellulosomal components.

In this work, we present additional insight into the anomalous mobility properties of the S1 glycopolypeptide subunit from *C. thermocellum*. For the purpose of this study, the S1 subunit was isolated from the intact cellulosome, and its response to pH and ionic strength was assessed. The phenomenon is shown to reflect an inherent consequence of S1 structure that appears to simulate its natural conformational transition upon binding to cellulose.

MATERIALS AND METHODS

Materials

The cellulosome of *C. thermocellum* strain YS was prepared by the affinity digestion method (*15*). Phosphoric acid-swollen amorphous cellulose was prepared as described previously (*16*). Anticellulosomal (S1-specific) antibodies were prepared by subtractive adsorption of rabbit antiwhole cell antibodies onto adhesion-defective mutant cells as reported earlier (*6*). Microcrystalline cellulose (Avicel) was a product of Merck (Darmstadt, Germany). All other chemicals and biochemicals were from Sigma Chemical Co. (St. Louis, MO).

Preconditioning Procedures: Low-Ionic Strength, Low-pH Treatments

Cellulosome samples (0.5 mg/mL in Tris-HCl buffer, pH 7.5) were either dialyzed overnight against double-distilled water for establishing low-ionic-strength conditions or adjusted to pH 3.5 with acetic acid for low-pH preconditioning. The samples were then mixed with 1 vol of sample buffer, which consisted of 3% SDS, 5% mercaptoethanol, and 10% glycerol in 62.5 m*M* Tris-HCl buffer, pH 6.8. Regardless of the preconditioning protocol used for the individual samples, after addition of the sample buffer, the final ionic strength and pH of all samples were essentially identical. That is, all samples were at essentially the same pH and ionic strength at the onset of the heating step (10 min at the temperature indicated later in the text) and during the subsequent electrophoresis.

Effect of Temperature on SDS-PAGE Profile of Cellulosome

In order to assess the effect of temperature on the SDS-PAGE pattern, samples of cellulosome and purified S1 were incubated for 10 min at different temperatures between 37 and 100°C following the preconditioning procedure (at low ionic strength or low pH) but before electrophoresis.

Experiments with Protease Inhibitors

In some experiments, protease inhibitors (including TLCK, TPCK, pepstatin, and EDTA at a final concentration of 1 m*M* and PMSF at 10 μg/mL) were added to the sample buffer in order to examine the potential action of endogenous proteolytic enzymes on the disintegration of S1.

Isolation of S1 Subunit

Cellulosome samples were separated by preparative SDS-PAGE, and the position of the bands was determined by coomasie brilliant blue staining of a representative lane. The S1 band was excised and subjected to electroelution at a constant voltage (200 V) for 20 h in a Biotrap electroeluter cell (Schleicher & Schuell, Dassel, Germany). The electroelution buffer consisted of 10 m*M* ammonium bicarbonate, pH 8.3.

Adsorption Experiments

Aliquots (0.1 mL) of either 1 mg amorphous cellulose or 10 mg Avicel (depending on the experiment) in 50 m*M* Tris-HCl buffer, pH 7.4, were mixed with incremented amounts of 5–50 μg S1. After 15 min, the suspensions were centrifuged, and the supernatant fluids were analyzed by enzyme immunoassay on dot blots employing rabbit anti-S1 antibodies.

SDS-PAGE

The SDS-PAGE was performed in 6% gels as described (*6*), modified for a BioRad minigel apparatus. Blotting onto nitrocellulose paper and subsequent immunochemical staining or αGal-specific labeling was conducted using anticellulosomal (S1-specific) antibody or GSI-B4 isolectin from *Griffonia simplicifolia*, respectively, according to published procedures (*14*). For SDS-PAGE of the isolated, amorphous cellulose-adsorbed S1 subunit, cellulose pellets were washed once with Tris buffer. The resultant pellet was resuspended in 0.1 mL sample buffer, boiled for 10 min, and centrifuged; the supernatant fluids were subjected to electrophoresis as described above.

RESULTS

Effect of Preconditions on SDS-PAGE Profile of Intact Cellulosome

Figure 1 shows the results of a series of experiments in which cellulosome was maintained either under standard conditions (50 m*M* Tris buffer, pH 7.5) or at low ionic strength or low pH. The respective cellulosome sample was then mixed with sample buffer and heated at various temperatures prior to SDS-PAGE. The results indicated that the specific set of preconditions dictates the electrophoretic behavior of the cellulosomal components during SDS-PAGE, described as follows.

As reported in earlier work (*4,5*), for unconditioned cellulosome samples (left-hand panel of Fig. 1, designated CS), high-mol-wt subcellulosomal complexes are evident at low sample-heating temperatures (*see* bands labeled F0 and F1 in samples heated below 70°C). In addition,

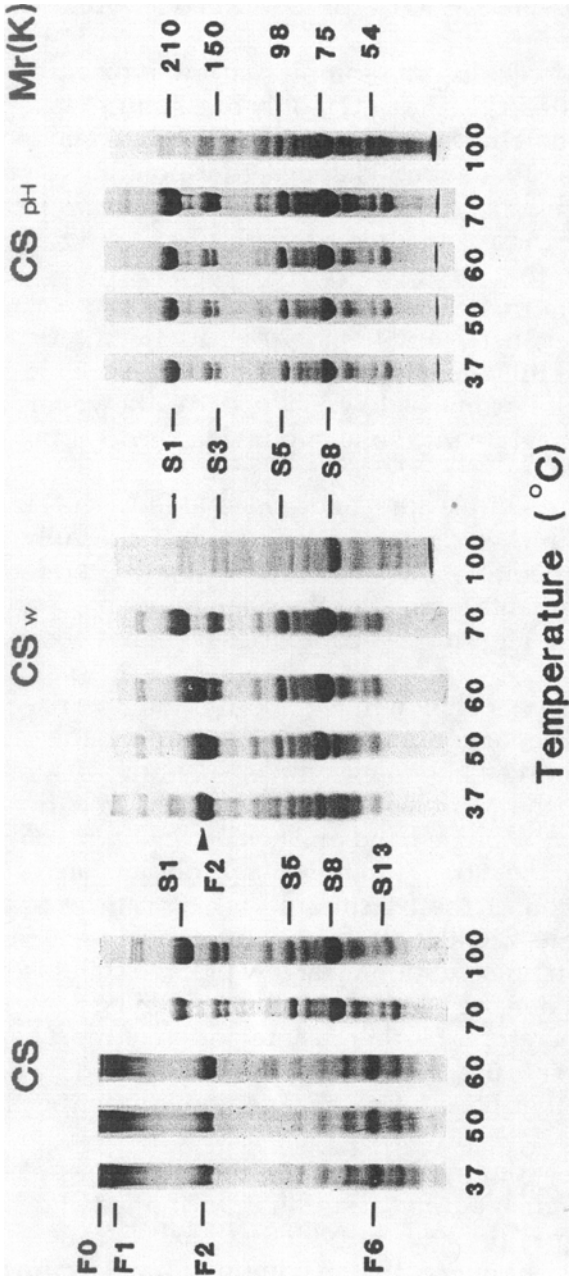

Fig. 1. Effect of temperature on SDS-PAGE profiles of native and preconditioned cellulosome samples. Samples of unconditioned cellulosome in 50 m*M* Tris buffer (CS) or preconditioned cellulosome samples [either subjected to dialysis against water (CS_W) or brought to pH 3.5 with acetic acid (CS_{pH})] were mixed with standard SDS-containing sample buffer and incubated for 10 min at the indicated temperature. The resultant samples were electrophoresed on 6% polyacrylamide gels, as described in the text. The markers indicate the previously determined positions of either partially dissociated cellulosomal subcomplexes or partially denatured subunits (i.e., fractions F0, F1, and so on). The previously characterized position of known fully denatured cellulosomal subunits (S1, S3, and so on) are also given along with their corresponding values for relative molecular mass (M_r).

other bands appear to represent only partially denatured subunits. For example, the cellulosomal component termed band F2 (which is formed at low temperatures) migrates slower at high temperatures (band S1 at 100°C), and band F6 migrates as S8 at 100°C (unpublished immunochemical and electroelution data).

Upon preconditioning of cellulosome samples at low ionic strength (*see* panel of 5 lanes, designated CS_w in Fig. 1), only one subunit, the S1, appeared to exhibit marked temperature-dependent alterations in its migratory pattern. All of the other subunits appeared to migrate as fully denatured subunits, and high-mol-wt complexes were not observed even at low temperatures. Under these low-ionic-strength preconditions, the behavior of S1 was similar to that described above for the unconditioned samples, i.e., at low temperature, the subunit migrated as the partially denatured F2 band; however, between 50 and 70°C, its migratory rate changed to match that of the fully denatured subunit (compare with pattern of the unconditioned cellulosome at 100°C). At 100°C, however, the S1 subunit appeared to disintegrate into subcomponents, as documented previously.

In the case of cellulosome samples preconditioned at low pH (Fig. 1, panel CS_{pH}), all of the subunits, *including S1,* migrated as the fully denatured material at low temperatures; again, at 100°C, the S1 tended to disintegrate into smaller fragments (similar to the phenomenon observed for low ionic strength). Again, high-mol-wt complexes were not observed at any temperature (*14*).

Thus, the electrophoretic data shown in Fig. 1 can be summarized as follows: Under the standard set of conditions (i.e., Tris buffer), the cellulosome appears to be in its most stable state; complete denaturation takes place only upon boiling of the samples in sample buffer, and the S1 subunit appears to migrate as a single band at 210 kDa. Upon preconditioning at low ionic strength, most of the cellulosomal subunits are easily denatured, with the exception of the S1 subunit, which can be totally denatured (i.e., unfolded to its 210-kDa state) only upon heating at 70°C in the presence of SDS. Upon preconditioning at low pH, the cellulosome is least stable, and complete denaturation of *all* subunits can be achieved in sample buffer at low temperature. Further disintegration of the S1 subunit to lower M_r subcomponents (i.e., <210 kDa) can be obtained by boiling of cellulosome samples that have been preconditioned in either low ionic strength or low pH.

As reported in the earlier communication (*14*), the effects of preconditioning are completely reversible upon restoration of the initial conditions of ionic strength and pH prior to introduction of SDS-containing sample buffer. In some cases, it appears that in addition to the disappearance of the S1 subunit, other high-molecular-mass subunits may also disintegrate to lower-molecular-mass subcomponents.

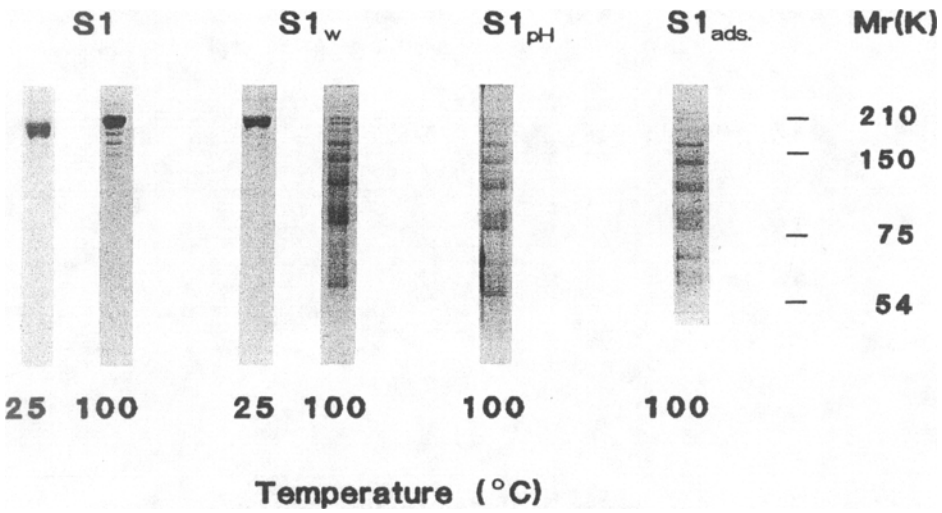

Fig. 2. The purified S1 subunit: electrophoretic mobility of the unconditioned, preconditioned and cellulose-adsorbed forms. The unconditioned, purified subunit (labeled S1 in the figure) was mixed with sample buffer and incubated for 10 min at either 25 or 100°C, as indicated, prior to SDS-PAGE. Purified samples of S1, preconditioned at either low ionic strength ($S1_w$) or low pH ($S1_{pH}$), were similarly treated with sample buffer and electrophoresed. An additional sample of purified S1 was adsorbed onto amorphous cellulose ($S1_{ads}$) before SDS-PAGE. Note that the mobility of the unconditioned S1 (incubated with SDS at 25°C) was measurably faster than that of the boiled subunit but slightly retarded compared to its cellulosome-based *in situ* form (*see* Figure 1, F2 band in the 37°C lane of the CS panel). Under the same conditions, the preconditioned, purified $S1_w$ migrated to the M_r 210-kDa position (*see* Fig. 1, compare with the position of F2 in CS_w).

Isolation and Electrophoretic Mobility of Purified S1 Subunit

In order to further clarify the character of the above-described changes in the SDS-PAGE profile of the S1 cellulosomal subunit, we proceeded to isolate the 210 kDa band by the electroelution procedure described in the Methods section. The isolated band was subjected again to SDS-PAGE after appropriate preconditioning, and the results are shown in Fig. 2.

When the isolated S1 subunit (dissolved in Tris buffer) was incubated in sample buffer at room temperaure and subjected to SDS-PAGE, a single band was observed that migrated slightly faster than the fully denatured 210 kDa subunit (Fig. 2). The rate was not quite as fast, however, as that noted for the F2 band (Fig. 1) derived from the intact cellulosome similarly treated. Thus, successive SDS treatments at room temperature

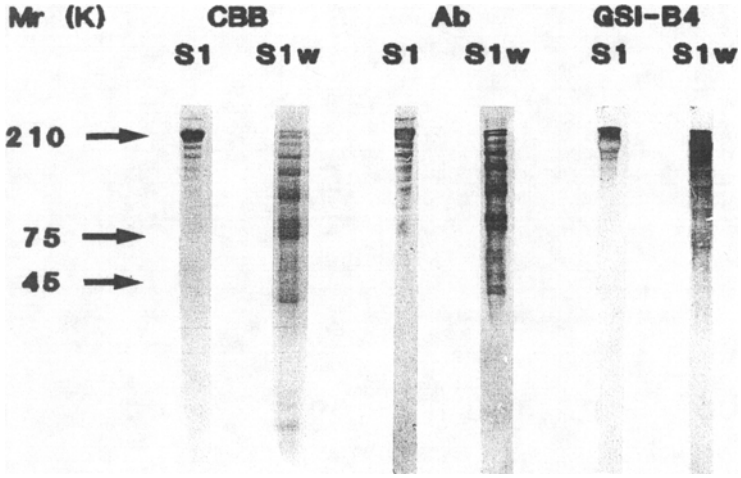

Fig. 3. Immunolabeling and αGal-specific staining profile of the purified S1 subunit. The isolated S1 subunit was either preconditioned at low ionic strength ($S1_w$) or not (S1). Gels were stained with coomassie brilliant blue (CBB) or blotted onto nitrocellulose membrane filters. The blots were labeled either with S1-specific antibody (Ab) or αGal-specific isolectin from *G. simplicifolia* (GSI-B4).

and rechromatography apparently cause alterations in the S1 structure that account for the faster mobility rate.

Boiling the isolated S1 yielded a major band at the usual position (i.e., ca. 210 kDa) plus a series of faint but persistent lower-mol-wt bands (Fig. 2). Upon dialyzing the purified S1 against either distilled water or low-pH solution prior to electrophoresis, the amplitude and number of lower-mol-wt bands clearly increased. Indeed, immunolabeling the purified S1, preconditioned by dialysis under low ionic strength, further emphasized this phenomenon (Fig. 3). Under these conditions, therefore, the isolated S1 subunit clearly behaves in a manner similar to the results reported earlier (*14*), which were based on immunolabeling experiments of SDS-PAGE–separated subunits derived from the intact cellulosome. The possibility of proteolysis during the preconditioning process and subsequent SDS treatment as the cause of the observed phenomenon was unlikely, since the inclusion of proteolytic inhibitors in the sample buffer failed to prevent the anomalous electrophoretic behavior of the purified S1 subunit.

Additional information regarding the structure of the S1 subunit was revealed from lectin-based staining of the purified S1. The resulant staining profile (Fig. 3) was very different from that observed for either the coomassie blue-stained S1 or the immunolabeling of the same material under the same conditions. In the case of the saccharide-specific label, lectin tended to stain the higher-mol-wt bands, suggesting that the bulk of the oligosaccharides were mainly associated with these components of

the S1. The lower-mol-wt components appeared to contain nominal amounts of oligosaccharide.

Adsorption of S1 to Cellulose

The adsorption of the isolated S1 subunit to cellulose was examined using either microcrystalline cellulose (Avicel) or amorphous cellulose as substrates. The capacity to adsorb S1 was determined to be 0.4 μg/mg in the case of Avicel and 15 μg/mg for amorphous cellulose. Upon binding to cellulosics, the mobility pattern of the S1 subunit in SDS-PAGE was altered. In the case of the purified subunit, the resultant profile resembled very closely that of the S1 dialyzed against low ionic strength or low pH (Fig. 2). Alterations in mobility of S1 were also observed for the subunit derived from the intact cellulosome, adsorbed onto cellulosic substrates (data not shown).

DISCUSSION

The importance of the S1 subunit in the cellulosome was recognized early in its study because of its relatively high levels within the complex, dominant antigenicity, unique glycosylation pattern, and appearance only as a component in large complexes (*1,4,8*). The absence of any demonstrable enzymatic activity in S1 created a mystery as to its function in the complex, and a structural or organizational role was tentatively assigned to this subunit. A possible role as a cellulose binding factor has also been suggested. More recently, the S1 was found to exhibit anomalous migratory behavior in electrophoresis, and what had appeared to be a nonspecific reaction of S1-specific antiserum (including monoclonal preparations) with other cellulosomal components is currently regarded as indirect evidence for its decomposition upon boiling in the presence of SDS.

The findings presented in this communication indicate that by subjecting the intact cellulosome to low-pH or low-ionic-strength conditions, the state of the S1 subunit is altered in two separate ways: First, the transition temperature of unfolding in the presence of SDS is reduced dramatically; and second, at higher temperatures, the S1 subunit appears to undergo further disintegration, which results in the accumulation of a remarkably reproducible series of smaller subcomponents. A similar effect was obtained by adsorption of the isolated S1 subunit to cellulose.

Current observations appear to be consistent with conformational changes in the S1 structure as illustrated schematically in Fig. 4. In the native state (Fig. 4A), the S1 subunit is in a folded conformation and serves as an attachment matrix for assembly of the other (catalytic) subunits. For the unconditioned cellulosome (Fig. 4B), contact with SDS at room temperature causes a release of many of the catalytic subunits from

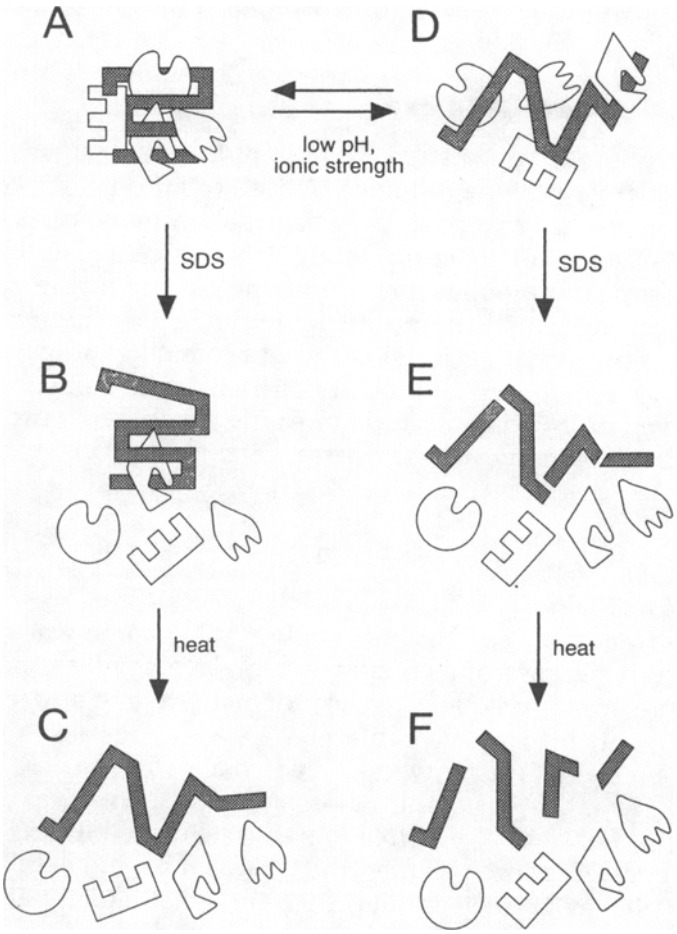

Fig. 4. Simplified schematic representation of proposed conformational changes in the S1 structure during dissociation of the cellulosome and denaturation of its components. The S1 subunit is represented by the shaded structure; other cellulosomal subunits are drawn as unshaded forms. The different conformational states of the S1 subunit **(A–E)** and its disintegration products **(F)** are based on electrophoretic data presented in Figs. 1–3. For the sake of simplicity, structural alterations of the other subunits are not shown.

the matrix, thus yielding subcomplexes such as the F0 and F1 cellulosomal fractions. In some cases, all of the catalytic subunits may be released from the S1, but the S1 remains in a partially folded state, reflected in the F2 band (Fig. 1). Many of the catalytic subunits are also in a partially folded state (e.g., F6), whereas others may be fully denatured even without heating. Nevertheless, for the unconditioned cellulosome, extensive denaturation of the S1 and other subunits occurs only upon its incubation at high temperatures (e.g., boiling) in the presence of SDS (Fig. 4C).

Changing the solution conditions from neutral, high-ionic-strength buffers to low ionic strength or low pH, results in a reversible structural

alteration in S1 to a sensitive, extended conformation (Fig. 4D). Subsequent incubation of this preconditioned cellulosome with SDS at moderate temperatures causes complete release of the catalytic subunits from the conglomerate (Fig. 4E). Moreover, the S1 subunit reverts to a presumably fragile conformation; upon boiling, the integrity of the 210-kDa structure is irreversibly lost (Fig. 4F), resulting in the series of the observed S1 subcomponents (Fig. 1).

The proposed conformational changes do not appear to be dependent on the presence of catalytic subunits, since all of the phenomena recorded for the S1, which resides in the intact cellulosome, could be demonstrated with the isolated subunit (Figs. 2,3). Moreover, the involvement of proteases in the production of S1 subcomponents is unlikely, since the effects of the preconditioning are in fact reversible or more accurately, can be aborted immediately following the dialysis step (Fig. 4A–D). In addition, the presence of protease inhibitors failed to prevent disintegration of the S1.

The results of this study suggest that disintegration of the S1 into defined subcomponents can occur only if it exists in a sensitive conformation. Such a conformational state is induced either by low ionic strength or low pH. The pH data (*14*) appear to reflect a titration of resident carboxylic groups, and may suggest the presence of very stable salt bridges. On the other hand, the resistance of the cellulosome complex to dissociation by SDS and the susceptibility of the S1 structure to low ionic strength may indicate the presence of strong hydrophobic bonding.

The apparent molecular masses of the various defined S1 subcomponents do not provide evidence for a minimal component size or a stoichiometric assessment of the component parts. Rather, the resultant SDS-PAGE profile appears to indicate a successive disassociation or fragmentation of the S1 subunit. Moreover, the subcomponents of the S1 subunit do not all share the same high degree of glycosylation. Out of at least 10 bands, only about half (mainly the larger components) interacted strongly with the αGal-specific lectin.

At this point, the molecular consequences of decomposition via successive pretreatment, SDS, and heating are not yet known; i.e., it is not at all clear whether the S1 subunit itself represents an unusually stable, relatively defined, noncovalent cohesion of component parts or the 210-kDa band comprises a single glycosylated polypeptide chain that is decomposed into subcomponents via breakage of unusually sensitive peptide bonds. Precedents for both possibilities, i.e., exceptionally strong noncovalent protein–protein interactions (*17*) and spontaneous peptide-bond cleavage (*18*) have been described in the literature.

Direct binding of the isolated S1 to cellulose was demonstrated here for the first time, giving credence to previous suggestions that this glycopolypeptide component plays a role as a cellulose-binding factor (*1,7,8,12*). The binding of S1 to cellulose also leads to its complete disintegration into

smaller components upon heating in the presence of SDS, again suggesting consequent conformational changes in its structure that are apparently similar to those generated by low salt or low pH. The question thus remains as to whether S1 contains the major cellulose-binding domain of the cellulosomal complex, which serves both to target the associated cellulolytic subunits as well as perturb the physical state of the insoluble substrate. The conformational changes in S1 that are proposed to accompany the binding of the cellulosome in fact may effect a redistribution of the various enzymes in an organized fashion on the cellulose surface, thus contributing to their synergistic action.

The significance of these findings is even greater in view of the recent evidence (*19*) that directly demonstrates the binding of cellulolytic components of the cellulosome to S1, a process that appears to be dependent on reiterative sequences common to these enzymatic components. It remains to be seen which of the S1 subcomponents is indeed involved in the binding of the other components of the cellulosome and which are involved in other processes (e.g., in cellulose binding or interconnection of the S1 subcomponents to each other). The cloning and expression of the different subcomponents as well as continued biochemical studies appear to be required for furthering our understanding of this unique set of organizing components.

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