0273-2289/91/3002—0129$02.00

Anomalous Dissociative Behavior of the Major Glycosylated Component of the Cellulosome of *Clostridium thermocellum*

ELY MORAG (MORGENSTERN),[1] EDWARD A. BAYER,*[,2] AND RAPHAEL LAMED[1]

[1]*Department of Biotechnology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel; and* [2]*Department of Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel*

Received June 15, 1990; Accepted July 3, 1990

ABSTRACT

The cellulosome of *Clostridium thermocellum* is a highly cohesive multienzyme complex that is capable of completely solubilizing insoluble cellulose. One of the major cellulosomal components, the glycosylated S1 subunit, is believed to play an important structural role and normally migrates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an M_r of 210,000. It is shown here that by simply altering the conditions (pH or ionic strength) of the environment prior to electrophoresis, a different migratory profile for S1 emerges, yielding a collection of bands, all of which migrate faster than the parent band. The original electrophoretic behavior of S1 can be reproduced on restoration of the original pH and ionic strength. These results may bear important significance for the physiological role of the S1 subunit in facilitating the observed synergistic action of the other (cellulolytic) components of the cellulosome.

Index Entries: Cellulosome; cellulase; SDS-PAGE, anamolous migration; *Clostridium thermocellum;* multienzyme complex; subunit dissociation.

*Author to whom all correspondence and reprint requests should be addressed.

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

Clostridium thermocellum, an anaerobic thermophilic cellulolytic bacterium, is known for its exceptional capacity to solubilize microcrystalline cellulose. This is accomplished mainly through the action of a high-molecular-weight multienzyme complex, termed the cellulosome (*1*). The cellulosome is composed of at least 14 major subunits (most of which express enzymatic activity), which are linked together by unusually tight noncovalent interactions. The molecular basis for the observed structural stability of the complex is unknown.

Recently, by supplementing a purified preparation of the cellulosome with a commercially available β-glucosidase, near-complete conversion of a 20% suspension of crystalline cellulose to glucose could be achieved (*2*). Thus, the cell-free production of cellulose breakdown products using a purified enzyme system may eventually graduate from a laboratory curiosity to a commercially feasible process. Consequently, there is growing incentive to analyze in detail the fine structure of the cellulosome, its subcomponents, and subunits. The information accumulated may be critical in designing a viable industrial process for the conversion of cellulose to useful products.

One of the major cellulosomal components, the S1 subunit, exhibited no detectable cellulase activity (*3*) and was shown to contain most of the covalently linked cellulosome-based carbohydrate (*4–6*). The apparent molecular mass of the S1 subunit was found to be about 210,000 by SDS-PAGE, and, in the absence of a defined enzymatic role for S1, we initially suspected a central structural and/or organizational role for this subunit.

The S1 was also found to be the major antigenic determinant of the cellulosome. Interestingly, immunochemical, lectin-mediated, and saccharide-specific labeling studies all suggested an apparent partial disintegration of the S1 subunit into smaller fragments. In addition, our attempts to isolate the S1 subunit to purity were often hampered by a frustrating disappearance of the characteristic high-mol-wt band in SDS-PAGE. We therefore decided to investigate further the possible causes for this phenomenon.

MATERIALS AND METHODS

Cellulosome Preparation

The cellulosome was prepared from the cell-free growth medium of cellulose-grown cells of *C. thermocellum* strain YS as described previously

(*7*). The samples were stored in aliquots at −20°C at a final concentration of 2.2 mg/mL in 50 m*M* Tris-HCl buffer (pH 7.5).

Preparation of Anticellulosomal (S1-Specific) Antibodies

Cellulosome-specific antibodies were prepared by raising antiserum in rabbits against wild-type cells of strain YS (*7*). The antiserum was then adsorbed onto cells of an adherence-defective mutant (strain AD2, which lacks exocellular cellulosome). The nonadsorbent fraction contained antibody species that bound selectively to the S1 band (*8*).

Procedures for Alteration of Preconditions

Ionic strength and pH of cellulosome samples (0.5 mg/mL) were altered by dialysis against 2 L of the appropriate solution (as indicated later in text). In "restoration" experiments, the preconditions were restored by successive dialyses against double-distilled water and 50 m*M* Tris-HCl (pH 7.5) prior to SDS-PAGE.

SDS-PAGE, Blotting, and Staining Procedures

SDS-PAGE was performed on a miniature electrophoresis assembly using 3% stacking gels and 6% separating gels. Samples were diluted with one-half vol of sample buffer (3% SDS, 10% glycerol, 5% mercaptoethanol, and 0.2% bromophenol blue in 62.5 m*M* Tris-HCl buffer, pH 6.8) and boiled for 5 min prior to electrophoresis. The running buffer consisted of 0.1% SDS in 25 m*M* Tris-glycine buffer, pH 8.9. Gels were stained for proteins with Coomassie Brilliant Blue R250, and for carbohydrates using the periodic acid Schiff procedure (*9*).

For blotting experiments, SDS-PAGE-separated cellulosome samples (10 μg) were transferred electrophoretically onto nitrocellulose sheets. Proteins were stained on the blots using amido black. The location of cellulosomal sugars was determined either by the enzyme hydrazide technique (*10*) or using a biotinylated form of isolectin GSI-B_4 from *Griffonia simplicifolia,* combined with avidin–biotin technology (*11*). Antigenic activity was similarly detected on blots using the biotinylated S1-specific antibody preparation.

RESULTS

Previous experiments had revealed that dialysis of the cellulosome against distilled water prior to SDS-PAGE led to a dramatic disappearance of the major 210-kDa band (unpublished results). In further investigating the effect of ionic strength on the mobility pattern of the S1 subunit, a

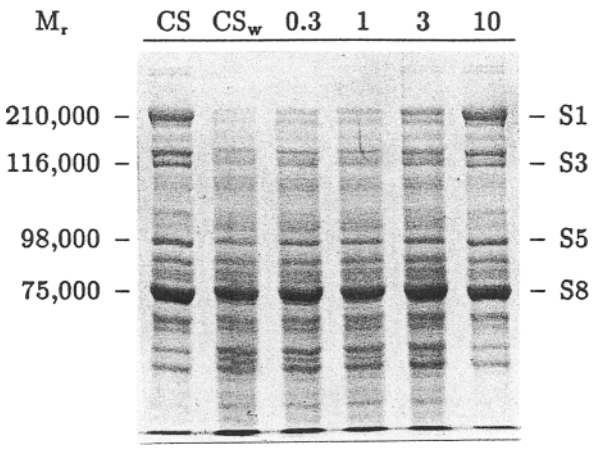

Fig. 1. Effect of ionic strength on disassociation of S1 during SDS-PAGE. Cellulsome samples (1 mg/mL) were dialyzed against Tris-HCl buffer, pH 7.5, at the indicated concentrations (m*M*) prior to introduction of sample buffer and SDS-PAGE. An undialyzed cellulosome sample (CS) and a sample dialyzed against double-distilled water (CS_w) are also shown in the figure. Note the disappearance at low salt concentration of the S1 subunit, which often left a weak doublet band at the same position.

striking dependence was observed (Fig. 1). Partial disintegration of S1 was observed following pretreatment of the cellulosome at intermediate levels of ionic strength (0.3–3 m*M* NaCl). In the presence of 10 m*M* salt, the SDS-PAGE mobility pattern of the cellulosomal subunits was retained. The acidity of the pretreatment medium was also clearly a factor (Fig. 2). At pH 4.5, the characteristic S1 band disappeared; above pH 5.5, the S1 band remained intact.

Close observation of the results in Figs. 1 and 2 revealed a series of new low-mol-wt bands that, in some cases, appeared to be masked by the native cellulosomal subunits. We therefore labeled the SDS-PAGE-separated products with S1-specific antibody (Fig. 3). Under standard conditions, the 210-kDa S1 band was the prominent band, but was also accompanied by antibody-induced labeling of a series of lower mol-wt bands. When the cellulosome was pretreated (either by dialysis or at low pH) prior to SDS-PAGE, the immunolabeling pattern showed a strengthening of the lower mol wt bands at the apparent expense of the S1 band. Similar results were achieved by employing sugar-specific labels (i.e., periodic acid Schiff's stain, GSI lectin, enzyme hydrazide); the intensity of the S1 band (stained by the latter techniques) was also reduced on pretreatment with acid or at low ionic strength (Fig. 3).

It was interesting to determine whether the alteration in the SDS-PAGE profile reflected a permanent alteration in the structure of the cellulosome, or whether the observed effects could be reversed simply by readjusting

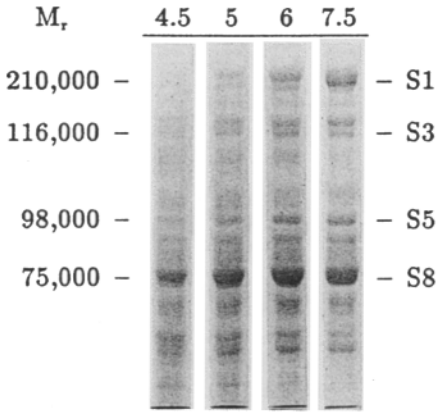

Fig. 2. Effect of pH on disassociation of S1 during SDS-PAGE. Cellulosome samples (1 mg/mL) were dialyzed against 10 m*M* sodium acetate buffer, pH 4.5 through 6, as indicated. Another sample was similarly dialzyed against 10 m*M* Tris-HCl, pH 7.5. The samples were then subjected to SDS-PAGE. Note again the disappearance of S1 at low pH; sometimes a slight reduction in the staining intensity of other bands (e.g., S2 an S3) could also be detected.

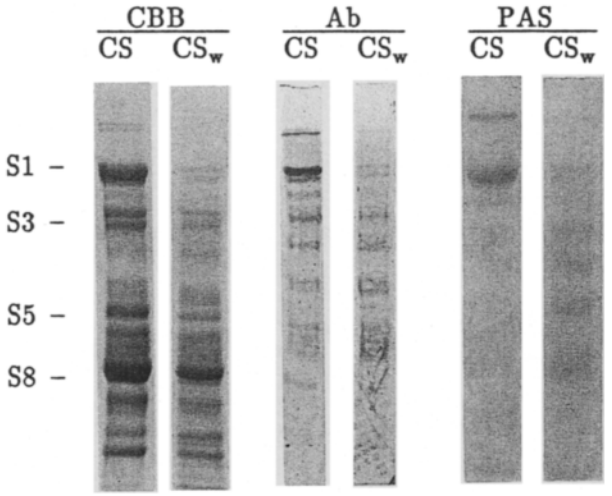

Fig. 3. Effect of ionic strength on immunolabeling and sugar-specific staining pattern of cellulosomal subunits in SDS-PAGE. Cellulosome samples (1 mg/mL) were either dialyzed against double-distilled water (CS_w) or not (CS) prior to SDS-PAGE. The gels were stained either with Coomassie Brilliant Blue (CBB) or periodic acid-Schiff's stain (PAS). A separate set of lanes were transferred electrophoretically to nitrocellulose membrane filters and labeled with an S1-specific antibody preparation (Ab).

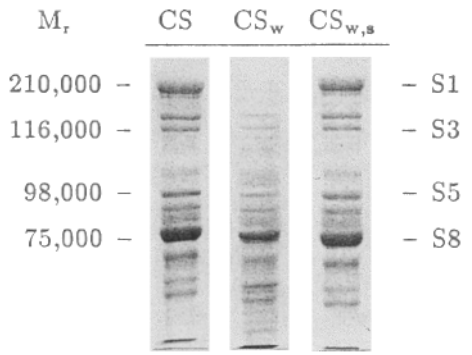

Fig. 4. Restoration of original mobility pattern of S1 in SDS-PAGE. A cellulosome sample (1 mg/mL) was dialyzed against double-distilled water (CS_w). Another sample was similarly treated, followed by a second dialysis step, against 50 m*M* Tris-HCl, pH 7.5 ($CS_{w,s}$). The respective SDS-PAGE mobility patterns were compared with an undialyzed sample dissolved in the same buffer (CS).

the ionic strength or pH *prior* to SDS-PAGE. Indeed, the original mobility pattern of the S1 subunit could be restored experimentally, as shown in Fig. 4. Likewise, the immunochemical and carbohydrate profiles could be rectified by appropriate preadjustment of either ionic strength or pH (data not shown).

DISCUSSION

Since the original description of the cellulosome in *C. thermocellum* (*1,3*), the largest mol wt component, the 210-kDa S1, has been considered to be a distinctive, integral, salient part of the cellulosome. Although no enzymatic role has been attributed to this subunit, the importance of the S1 to cellulosome structure has been intimated by its high antigenicity, high carbohydrate content, exposed position on the cellulosome, and exclusive in situ association with the cellulosome complex (*12*). In a recent article (*13*), an S1 analog derived from the cellulosome of a different strain of *C. thermocellum* was purified following SDS-PAGE and shown to promote degradation of crystalline cellulose when combined with a purified preparation of another major cellulosomal subunit.

SDS-PAGE is a routinely employed technique that has been used for the last two decades for efficient separation of polypeptides according to their relative molecular mass. By boiling samples in SDS, noncovalent inter- and intramolecular bonds are usually counteracted completely. Polypeptide structures are converted from globular or ordered forms to randomly extended chains that, in the presence of the detergent, migrate through the gel matrix according to their size. It is emphasized here that

the observed anomalous migratory behavior of the S1 band reflects the preconditions (i.e., acidity or ionic strength) existing prior to boiling the samples in SDS. It should also be noted that boiling in sample buffer serves to normalize the conditions for all samples on electrophoresis. The effect is reversible in that the original status of the precondition medium can be experimentally reproduced, resulting in a restoration of the initial SDS-PAGE profile.

Several possible explanations could account for the observed alteration in the S1 band. First, the data may indicate that the S1 subunit is actually composed of smaller component(s), which implies that its original migratory position (i.e., M_r 210,000) may be misleading, and represents a relatively uniform aggregation of these components. Alternatively, low pH or ionic strength may cause a conformational change (e.g., incomplete unfolding) yielding different species that exhibit different (faster) SDS-PAGE migration rates. Another possibility is that such conformational changes in vitro may render the S1 subunit unusually sensitive to selective covalent breakdown by subsequent steps in the SDS-PAGE process.

Several cases of anomalous SDS-PAGE behavior of proteins have been recorded in the literature. These include the incomplete dissociation of subunits, stable oligomerization of components, and conformation-induced discrepancies (*14–18*). In addition, compared to unglycosylated proteins the migratory rate of glycoproteins in SDS-PAGE is notoriously heterogeneous and deviant, and it should be remembered that the S1 subunit is a glycoconjugate. In any event, although SDS-PAGE is a routine and standard technique for analysis of polypeptides, it is clear that it is not uniformly appropriate in the strict sense for all systems.

The importance of initial salt concentration and pH to the integrity of the S1 band should help us to obtain better preparations of the purified component. Cloning and expression of (nonglycosylated) S1 may also contribute complementary information concerning the composition and properties of this subunit. Preliminary data using strains of *C. thermocellum* other than YS have indicated that the anomalous electrophoretic behavior of the major glycosylated cellulosomal subunit (the S1 analog) is a general property of this bacterium.

It is interesting to note that the observed pH effect may have physiological implications, since the pH optimum for cellulase activity of the cellulosome is about 5.5, similar to that which induces the anomalous SDS-PAGE pattern. In addition, previous reports (*19,20*) have indicated that the ionic strength of the solution has a remarkable effect both on the adsorption of the cellulosome to cellulose, and on the degradation of the insoluble substrate by the cellulosome. Moreover, the effective range of ionic strength coincides with that described here. It was suggested in our earlier report (*20*) that conformational changes in the cellulosome complex could account for these phenomena, and it is appealing to speculate

here that such conformational changes are effected by alterations in the state of the S1 subunit. It also follows that dramatic conformational changes may accompany adsorption of the cellulosome to cellulose. Indeed, preliminary evidence suggests that binding of the cellulosome to cellulose also serves to promote the effects described in this communication, thus linking the putative conformational alterations with the initial event in cellulose degradation.

REFERENCES

1. Lamed, R., Setter, E., Kenig, R., and Bayer, E. A. (1983), *Biotechnol. Bioeng. Symp.* **13,** 161–181.
2. Lamed, R., Kenig, R., Morag (Morgenstern), E., Calzada, J. F., de Micheo, F., and Bayer, E. A. (1991), *Appl. Biochem. Biotechnol.* **27,** 173–183.
3. Lamed, R., Setter, E., and Bayer, E. A. (1983), *J. Bacteriol.* **156,** 827–836.
4. Bayer, E. A. and Lamed, R. (1986), *J. Bacteriol.* **167,** 828–836.
5. Hon-nami, K., Coughlan, M. P., Hon-nami, H., and Ljungdahl, L. G. (1986), *Arch. Microbiol.* **145,** 13–19.
6. Gerwig, G. J., de Waard, P., Kamerling, J. P., Vliegenthart, J. F. G., Morgenstern, E., Lamed, R., and Bayer, E. A. (1989), *J. Biol. Chem.* **264,** 1027–1035.
7. Lamed, R. and Bayer, E. A. (1988), *Methods Enzymol.* **160,** 472–482.
8. Bayer, E. A., Kenig, R., and Lamed, R. (1983), *J. Bacteriol.* **156,** 818–827.
9. Fairbanks, G., Steck, T. L., and Wallach, D. H. F. (1971), *Biochemistry* **10,** 2606–2617.
10. Bayer, E. A., Ben-Hur, H., and Wilchek, M. (1987), *Anal. Biochem.* **161,** 123–131.
11. Wilchek, M. and Bayer, E. A., eds. (1990), *Methods in Enzymology,* vol. 184, Academic, Orlando, FL, 746 pp.
12. Lamed, R. and Bayer, E. A. (1988), *Adv. Appl. Microbiol.* **33,** 1–46.
13. Wu, J. H. D., Orme-Johnson, W. H., and Demain, A. L. (1988), *Biochemistry* **27,** 1703–1709.
14. Henderson, D., Eibl, H., and Weber, K. (1979), *J. Mol. Biol.* **132,** 193–218.
15. McMichael, J. C. and Ou, J. T. (1979), *J. Bacteriol.* **138,** 969–975.
16. Creamer, L. K. and Richardson, T. (1984), *Arch. Biochem. Biophys.* **234,** 476–486.
17. Bayer, E. A., Safars, M., and Wilchek, M. (1987), *Anal. Biochem.* **161,** 262–271.
18. Berenguer, J., Faraldo, M. L. M., and de Pedro, M. A. (1988), *J. Bacteriol.* **170,** 2441–2447.
19. Ljungdahl, L. G., Peterson, B., Eriksson, K.-E., and Wiegel, J. (1983), *Curr. Microbiol.* **9,** 195–200.
20. Lamed, R., Setter, E., and Bayer, E. A. (1985), *Enzyme Microb. Technol.* **7,** 37–41.