Volume 304, number 1, 89–92 FEBS 11137 June 1992

Involvement of separate domains of the cellulosomal protein S1 of *Clostridium thermocellum* in binding to cellulose and in anchoring of catalytic subunits to the cellulosome

Sylvie Salamitou, Kostas Tokatlidis, Pierre Béguin and Jean-Paul Aubert

Unité de Physiologie Cellulaire and URA 1300 CNRS, Département des Biotechnologies, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France

Recieved 30 March 1992

Fragments of the 250 kDa S1 subunit of the *Clostridium thermocellum* cellulosome were obtained by protease-induced or spontaneous degradation. All detectable fragments, down to a mass of about 30 kDa, retained the ability to bind to ^{125}I-labelled endoglucanase CelD, one of the catalytic subunits of the cellulosome. Several fragments were able to bind both to cellulose and to CelD. However, some fragments that could still bind to CelD did not have the ability to bind to cellulose. Therefore, S1, a putative scaffolding protein of the cellulosome, is likely to carry two separate types of domains, one of which binds to cellulose, while the other type binds to the various catalytic subunits of the complex.

Cellulosome; Scaffolding protein; S1 subunit; *Clostridium thermocellum*

1. INTRODUCTION

Clostridium thermocellum, an anaerobic and thermophilic bacterium, possesses a highly efficient cellulose-degrading system [1]. This cellulase system consists mainly of an extracellular high molecular weight complex, termed the cellulosome, which binds strongly to cellulose [2].

The cellulosome is composed of at least 14 different polypeptides (listed as S1 to S14 on the basis of SDS-PAGE separation by Lamed et al. [2]), most of which exhibit cellulolytic [2] or xylanolytic activity [3–5]. However, no definite catalytic activity has been ascribed to S1, the largest subunit. S1, a 210–250 kDa protein, also termed S_L [6], was proposed to act as a cellulose-binding factor and/or as a scaffolding protein of the complex [7,8]. The latter hypothesis was recently supported by the observation that catalytic subunits of the complex, such as endoglucanase CelD or xylanase XynZ, were able to bind to S1. Binding is mediated by a non-catalytic domain, containing a duplicated segment of 22 residues, which is present in several of the catalytic subunits of the complex [9].

The large size of S1 suggests that the protein may consist of several domains, some of which could be involved in anchoring catalytic subunits to the complex, while other(s) would be responsible for binding to cellulose. To check this hypothesis, subfragments of S1 generated by *Staphylococcus aureus* V8 protease were tested for the capacity to interact with intact, ^{125}I-labelled endoglucanase CelD and for their affinity for cellulose. The properties of subfragments of S1 spontaneously generated in the absence of protease were also analyzed.

Correspondence address: P. Béguin, Unité de Physiologie Cellulaire, Département des Biotechnologies, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15 France. Fax: (33) (1) 45 68 87 90.

2. MATERIALS AND METHODS

2.1. *Electroelution of S1*

The cellulosome was purified from *C. thermocellum* NCIB 10682 culture supernatant by cellulose affinity chromatography [2]. Samples of cellulosome were boiled for 5 min in Laemmli sample buffer [10] and loaded onto 10% SDS-polyacrylamide gels. After electrophoresis the S1 band was identified by staining a strip of the gel with Coomassie brilliant blue, and the corresponding region was cut from the unstained part of the gel. Gel pieces were inserted into a dialysis bag and the protein was electroeluted in Tris-glycine buffer (25 mM Tris, 193 mM glycine) containing 0.1% SDS at 10 V/cm for 6 h. The preparation was dialyzed against 20 mM Tris-HCl buffer, pH 7.7, to remove SDS. Buffers and dialysis bags were autoclaved and gloves were worn to minimize contamination by proteases.

2.2. *Partial digestion with* Staphylococcus aureus V8 protease

6 μg S1 protein was treated for 1 h at 37°C with various concentrations of *S. aureus* V8 protease (Sigma) in 60 μl Tris-glycine buffer in the presence or absence of 0.1% SDS. The incubation was terminated by adding an equal volume of a twofold concentration of sample buffer [10] and heating for 5 min at 100°C.

2.3. *Cellulose binding test*

10 mg Avicel (Macherey-Nagel) was incubated for 90 min at room temperature with 60 μl 20 mM Tris-HCl, pH 7.7, containing 8 μg of dialyzed S1 protein and centrifuged. 30 μl of supernatant, i.e. the unbound fraction, were collected. The pellet was washed 4 times with 30 μl 20 mM Tris-HCl, pH 7.7. Cellulose-bound material was eluted by adding 30 μl of a twofold concentration of sample buffer followed by heating for 5 min at 100°C. The various fractions were analyzed

by SDS-PAGE [10]. *S. aureus* V8 protease-generated subfragments were obtained by treating 8 μg S1 for 60 min with 10 μg/ml protease in the presence of 0.1% SDS. After heating at 100°C for 5 min the digested protein was dialyzed against 20 mM Tris-HCl, pH 7.7. The dialyzed material was precipitated by adding 2 vols. of acetone (pre-chilled to −70°C), taken up in 60 μl 20 mM Tris-HCl, pH 7.7, and tested for cellulose binding.

2.4. *Detection of protein fragments binding to ^{125}I-labelled CelD*

Samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes [11]. Bands showing affinity for the duplicated segment of CelD were detected by incubating the membranes with ^{125}I-labelled CelD [9].

3. RESULTS

3.1. *Proteolytic digestion of S1 by* Staphylococcus aureus *V8 protease*

Fig. 1A shows that in the presence of 0.1% SDS, treatment of S1 with *S. aureus* V8 protease generated a set of discrete subfragments ranging from 200 kDa to about 30 kDa. All of the subfragments detected by Coomassie blue staining retained the capacity to bind CelD (Fig. 1B). Protease treatment in the absence of SDS was less efficient (Fig. 1C). This indicates that protease-sensitive regions became more exposed in the presence of SDS. A set of minor bands showing a similar pattern was also observed in the sample that was not treated with protease (lane 1 of each panel), suggesting spontaneous degradation of S1.

3.2. *Binding to cellulose of S1 subfragments*

The S_L protein (= S1) is able to promote binding to cellulose of the catalytic cellulosome subunit S_S [8]. Recently, Morag et al. [12] reported that purified S1 could bind to cellulose. Upon binding, the protein became susceptible to conversion by hot SDS into a set of components of lower M_r values [12]. Therefore, the cellulose-binding properties of S1 and its subfragments were investigated. Fig. 2A confirms that S1 and its spontaneously generated subfragments could bind to cellulose and be eluted in the presence of SDS. However, adsorption to cellulose did not, in our hands, lead to an increased sensitivity to SDS. Fig. 2B shows that among S1 fragments generated by *S. aureus* V8 protease some polypeptides retained the capacity to adsorb to cellulose and to bind ^{125}I-labelled CelD, while others, which could still bind CelD, could no longer adsorb to cellulose.

3.3. *Conditions affecting the spontaneous degradation of purified S1*

Morag et al. reported the conversion of S1 to lower M_r species upon dialysis against low ionic strength or low pH buffers followed by SDS treatment [12,13]. In the absence of added protease a somewhat similar, but much less extensive, disintegration of S1 was also observed in this work. Therefore, we investigated the conditions that influenced this phenomenon. Fig. 3 shows that when the sample was heated at 37°C in sample

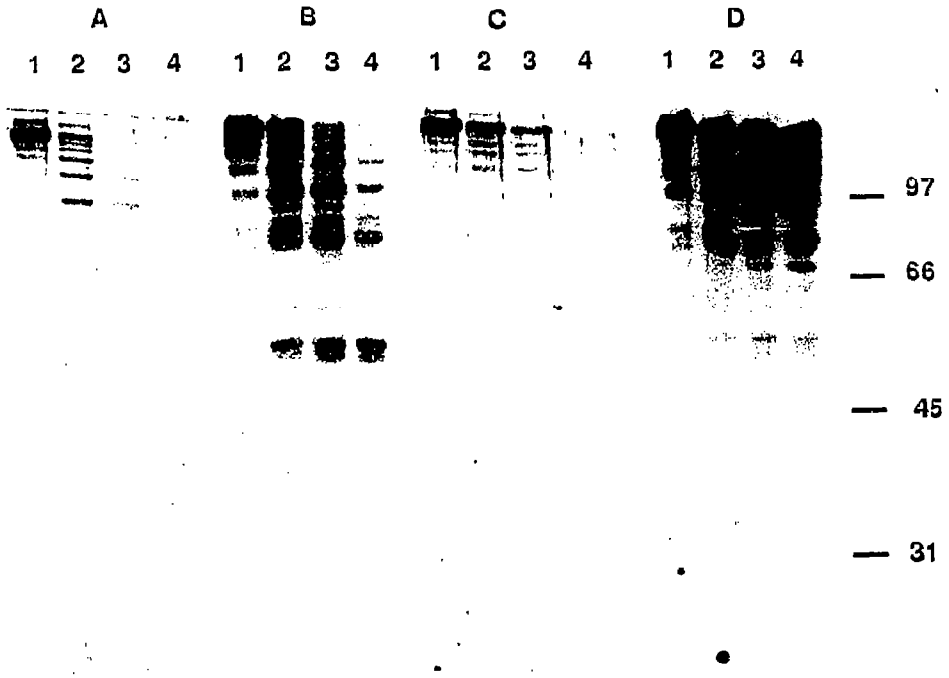

Fig. 1. Partial digestion of S1 with *Staphylococcus aureus* V8 protease. In panels A and B, digestion was performed in Tris-glycine buffer containing 0.1% SDS. In panels C and D digestion was performed in Tris-glycine. A and C, Coomassie blue-stained gels; B and D, autoradiograms of polypeptides interacting with ^{125}I-CelD. 2 μg of treated S1 were loaded in each lane. Concentrations of *S. aureus* V8 protease during the incubation were: lane 1, 0 μg/ml; lane 2, 10 μg/ml; lane 3, 20 μg/ml; lane 4, 50 μg/ml. No band was revealed upon incubation with the ^{125}I-labelled 63 kDa form of CelD lacking the duplicated segment (data not shown).

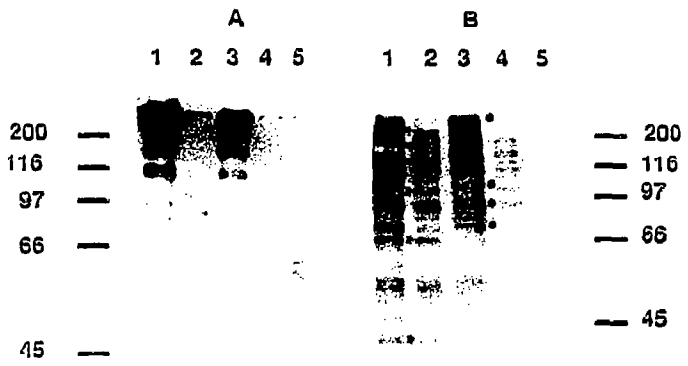

Fig. 2. Binding to cellulose of S1 subfragments that interact with ^{125}I-labelled CelD. (A) Undigested S1. (B) Subfragments of S1 generated by *S. aureus* V8 protease. Lane 1, sample prior to adsorption to cellulose; lane 2, non-bound fraction; lane 3, fraction eluted from cellulose with sample buffer; lanes 4 and 5, first two washes of unadsorbed material with 20 mM Tris-HCl pH 7.7. Stars indicate subfragments unable to bind to cellulose but retaining the ability to interact with the duplicated segment of CelD. Filled circles indicate subfragments retaining the two binding properties.

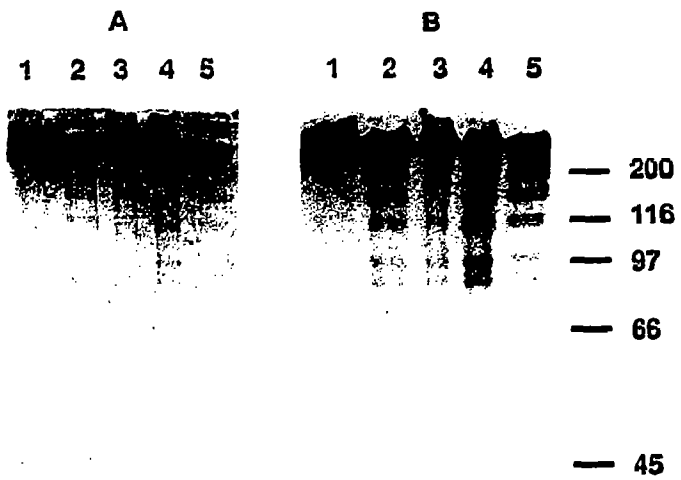

Fig. 3. Migration pattern of S1 and S1-derived polypeptides in SDS-PAGE following various treatments. (A) Coomassie blue-stained gel. (B) Autoradiogram of polypeptides interacting with ^{125}I-CelD. 4 μg S1 was loaded in each lane. Lane 1, S1 was incubated for 60 min at 37°C in sample buffer [10]; lane 2, S1 was incubated for 5 min at 100°C in sample buffer; lane 3, S1 was incubated for 5 min at room temperature in 80% HCOOH-0.1% SDS and vacuum-dried. The pellet was washed 3 times with distilled water, resuspended in sample buffer and incubated for 60 min at 37°C; lane 4, S1 underwent the same treatment as in lane 3 but was incubated for 5 min at 100°C in sample buffer; lane 5, S1 was dialyzed against distilled water prior to heating for 5 min at 100 °C in sample buffer.

buffer a single band was observed (lane 1). Heating for 5 min at 100°C led to a slightly slower rate of migration of S1 and generated a set of minor polypeptides ranging from 90 to 220 kDa (lane 2). A similar observation was made by Morag et al. [12]. An identical set of bands was observed when the sample was treated with 80% HCOOH containing 0.1 % SDS prior to incubation at 37°C in sample buffer (lane 3). The strongest intensity of the lower M_r bands was obtained by treating the sample with 80% HCOOH containing 0.1% SDS followed by heating at 100°C in sample buffer (lane 4). This particularly harsh treatment is known to result in the dissociation of the highly stable protein complexes found in *Anabaena* gas vesicles (G. Guglielmi, personal communication). Prior dialysis of electroeluted S1 against distilled water did not alter the pattern observed for the sample that was heated at 100°C (lane 5). In our hands, prior dialysis of the purified cellulosome against distilled water or 10 mM sodium acetate buffer, pH 4.5, also failed to change, detectably, the migration of any cellulosomal component, including S1, in SDS-PAGE gels (data not shown).

The same samples were probed for binding of ^{125}I-labelled CelD. Fig. 3B shows that all polypeptides detected by Coomassie blue staining (Fig. 3A) were able to bind CelD.

4. DISCUSSION

Down to a mass of 30 kDa all fragments of S1 generated by *S. aureus* V8 protease retained the ability to bind CelD carrying the duplicated segment common to cellulosomal cellulases and xylanases of *C. thermocellum*. This result puts an upper limit to the size of the domain(s) of S1 that bind catalytic subunits of the cellulosome. Indeed, the complete sequence of a gene encoding a polypeptide cross-reacting immunologically with S1 was recently determined. The sequence of the polypeptide comprises eight homologous domains of about 165 residues, which might correspond to the anchoring sites for the various catalytic subunits (U.T. Gerngross and A.L. Demain, personal communication).

Purified S1 binds to cellulose. Since it was possible to generate subfragments that still bound CelD, but no longer bound to cellulose, S1 must carry at least one cellulose-binding domain that is distinct from the domain(s) responsible for anchoring cellulolytic and xylanolytic enzymes. The size of fragments that did not bind to cellulose (up to about 200 kDa) suggests that the cellulose-binding domain is located one or the other terminus of the protein, as is often the case with cellulolytic and xylanolytic enzymes [14–16].

Therefore, the results reported here are consistent with the hypothesis that S1 is a multi-domain protein with a dual function. Adsorption of the complex to the substrate would be performed by the cellulose-binding domain, possibly in cooperation with other cellulose-binding domains carried by some of the catalytic subunits [17]. Organization of the various catalytic subunits along S1 would be mediated by several anchoring do-

mains. Indeed, electron micrographs have shown that the cellulosome consists of globular particles (the catalytic subunits ?) which are linked at regular intervals to a central, fibrous material (the S1 subunit ?) [18]. An example of scaffolding protein carrying repeated anchoring domains is the aminoacyl-tRNA synthetase complex of *Drosophila* [19]. The complex contains a 190 kDa subunit comprising two catalytic regions separated by a central domain. The central domain, which has been proposed to serve as a template for the association of other subunits of the complex, contains six repeated units of 75 amino acids each.

The lower M_r bands generated spontaneously from purified S1 in SDS-PAGE analysis are reminiscent of the lower M_r bands derived from S1 described by Morag et al. upon dialysis under low pH or low ionic strength conditions followed by SDS-PAGE [12, 13]. Accurate comparison of the two patterns was not possible due to our failure, for unknown reasons, to reproduce these observations. At any rate, our results indicate that if S1 consists of a multimeric complex, this complex must be extremely stable since dissociation after HCOOH + SDS treatment is, at best, very incomplete. Alternatively, the observed pattern could be due to the cleavage, either spontaneous or induced by some protease present in the sample, of a large polypeptide containing labile peptide bonds. The cloning of a gene encoding a single 197 kDa polypeptide cross-reacting immunologically with the 250 kDa S_L (= S1) glycoprotein (U.T. Gerngross and A.L.Demain, personal communication) argues in favour of the latter hypothesis. Dissociation of a multisubunit complex would require S1 to be post-translationally processed into fragments that cling together tightly. Such fragments would have to have highly similar N-terminal sequences, since residues 3–7 from the amino teminus of the S1 band could be unambiguously determined (J. d'Alayer, personal communication). The fact that degradation of S1 by *S. aureus* V8 protease mimicked, in part, the spontaneous degradation pattern also supports the hypothesis that the latter may result from peptide bond cleavage.

Acknowledgements: We thank E.A. Bayer, R. Lamed, U.T. Gerngross and A.L. Demain for providing information prior to publication, J. d'Alayer for performing amino-terminal sequence analysis of S1, A. Meier for help in growing *C. thermocellum* and harvesting culture supernatants, and G. Guglielmi for helpful discussions. S.S. was the recipient of a fellowship from Agence Française pour la Maîtrise de l'Energie under contract with the Lyonnaise des Eaux Company. K.T. was supported by Presidential Young Investigator Grant ECE-8552492 to P. Dhurjati, University of Delaware. This work was supported by Grants CPL C 462 from the Commission of the European Communities and by research funds from the University of Paris 7.

REFERENCES

[1] Johnson, E.A., Sakajoh, M., Halliwell, G., Madia, A. and Demain, A.L. (1982) Appl. Environ. Microbiol. 43, 1125–1132.
[2] Lamed, R., Setter, E. and Bayer, E.A. (1983) J. Bacteriol. 156, 828–836.
[3] Grépinet, O., Chebrou, M.-C. and Béguin, P. (1988) J. Bacteriol. 170, 4576–4581.
[4] Kohring, S., Wiegel, J. and Mayer, F. (1990) Appl. Environ. Microbiol. 56, 3798–3804.
[5] Morag, E., Bayer, E.A. and Lamed, R. (1990) J. Bacteriol. 172, 6098–6105.
[6] Wu, J.H.D., Orme-Johnson, W.H. and Demain, A.L. (1988) Biochemistry 27, 1703–1709.
[7] Lamed, R. and Bayer, E.A. (1988) Adv. Appl. Microbiol. 33, 1–46.
[8] Wu, J.H.D. and Demain, A.L. (1988) in: Biochemistry and Genetics of Cellulose Degradation, FEMS Symposium No. 43 (Aubert, J.-P., Béguin, P. and Millet, J., eds.) pp. 117–131, Academic Press, London.
[9] Tokatlidis, K., Salamitou, S., Béguin, P., Dhurjati, P. and Aubert, J.-P. (1991) FEBS Lett. 291, 185–188.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[12] Morag, E., Bayer, E.A. and Lamed, R. (1992) Appl. Biochem. Biotechnol. (in press).
[13] Morag, E., Bayer, E.A. and Lamed, R. (1991) Appl. Biochem. Biotechnol. 30, 129–136.
[14] Tomme, P., van Tilbeurgh, H., Pettersson, G., van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T. and Claeyssens, M. (1988) Eur. J. Biochem. 170, 575–581.
[15] Gilkes, N.R., Warren, R.A.J., Miller Jr., R.C. and Kilburn, D.G. (1988) J. Biol. Chem. 263, 10401–10407.
[16] Hazlewood, G.P., Laurie, J.I., Ferreira, L.M.A. and Gilbert, H.J. (1992) J. Appl. Bacteriol. 72, 244–251.
[17] Durrant, A.J., Hall, J., Hazlewood, G.P. and Gilbert, H.J. (1991) Biochem. J. 273, 289–293.
[18] Mayer, F., Coughlan, M.P., Mori, Y. and Ljungdahl, L.G. (1987) Appl. Environ. Microbiol. 53, 2785–2792.
[19] Cerini, C., Kerjan, P., Astier, M., Gratecos, D., Mirande, M. and Sémériva, M. (1991) EMBO J 10, 4267–4277.