Volume 291, number 2, 185–188 FEBS 10255 October 1991

ADONIS 0014579391009658

Interaction of the duplicated segment carried by *Clostridium thermocellum* cellulases with cellulosome components

Kostas Tokatlidis[1], Sylvie Salamitou[1], Pierre Béguin[1], Prasad Dhurjati[2] and Jean-Paul Aubert[1]

[1]*Unité de Physiologie Cellulaire and URA 1300 C N R S, Departement des Biotechnologies, Institut Pasteur, 28, rue du Dr Roux, 75724 Paris Cedex 15, France* and [2]*Department of Chemical Engineering, University of Delaware, Newark, Delaware 19716 USA*

Received 15 July 1991

The function of the non-catalytic, duplicated segment found in *C thermocellum* cellulases was investigated Rabbit antibodies reacting with the duplicated segment of endoglucanase CelD cross-reacted with a variety of cellulosome components ranging between 50 and 100 kDa ^{125}I-labeled forms of CelD and of xylanase XynZ carrying the duplicated segment bound to a set of cellulosome proteins ranging between 66 and 250 kDa, particularly to the 250 kDa S_L (or S1) subunit ^{125}I-labeled forms of CelD and XynZ devoid of the duplicated segment failed to bind to any cellulosome protein The duplicated segment appears thus to serve to anchor the various cellulosome subunits to the complex by binding to S_L, which may be a scaffolding element of the cellulosome

Duplicated segment, Cellulosome, Endoglucanase CelD, Xylanase XynZ, *Clostridium thermocellum*

1. INTRODUCTION

Clostridium thermocellum is a thermophilic bacterium that degrades crystalline cellulose with a high efficiency [1]. The *C. thermocellum* cellulase system consists of a highly active, extracellular multienzyme complex with M_r=2–4 MDa termed cellulosome [2,3].

The amino acid sequences deduced from the nucleotide sequences of 8 endoglucanase and 1 xylanase genes established so far, revealed that although the various polypeptides, as a whole, are not closely related, all of them except endoglucanase CelC carry a highly conserved duplicated segment of 22 amino acids The two segments are linked by a variable spacer of 10–17 residues and are located near the C terminus, except in endoglucanase CelE and xylanase XynZ where they are found in the middle of the polypeptide [4–7]. These observations raise three main questions. (i) is the duplicated segment predicted from nucleotide sequences present in the subunits constituting the cellulosome, (ii) is it specific for cellulosome subunits, and (iii) does it play a role in cellulosome assembly?

To address these questions, experiments with endoglucanase CelD and xylanase XynZ were performed Antibodies recognizing the duplicated segment of CelD were used in Western blots to probe for cellulosome components bearing this region. In addition, forms of CelD [8,9] and XynZ [5] with or without the repeated segment (Fig. 1) were labeled with ^{125}I and used in blotting experiments to examine their interaction with cellulosome components [10,11]

The results reported here support the hypothesis that the duplicated segment plays a role in anchoring the various subunits to the cellulosome

2 MATERIALS AND METHODS

2 1 *Purification of proteins*

The 63 kDa form of CelD was purified from cytoplasmic extracts of *E coli* TG1(pCT608) [4] and the 65 kDa form from cytoplasmic inclusion bodies of *E coli* TG1(pCT603) [8] The 65 kDa polypeptide derives from a 68 kDa form containing the integrity of the duplicated segment, which is present in inclusion bodies but is subsequently proteolyzed once the material extracted from inclusion bodies is dialyzed in the presence of 5 M urea [9] Proteolysis could be largely prevented by purifying the urea-solubilized fraction by gel filtration with a Sephacryl S-300 (Pharmacia) column equilibrated with 0 1 M Tris-HCl, pH 8 5, containing 5 M urea followed by dialysis The obtained preparation consisted of 60–80% 68 kDa form and of 20–40% 65 kDa form (data not shown)

The 54 kDa form of XynZ was purified from inclusion bodies formed in *E coli* TG1(pCT1223) [12] as described for 65 kDa CelD [8] The truncated 42 kDa form of XynZ, devoid of the conserved domain, was purified from *E coli* TG1(pCT1214) [12] according to [11] until the DEAE column step

2 2 *Preparation of antiserum*

500 μg of 68 kDa CelD preparation were mixed with an equal volume of Freund's complete adjuvant and injected into New Zealand White rabbits. The second and third injections were done with Freund's incomplete adjuvant in two-week and one-month intervals using the same quantity of protein A final injection of 100 μg was

Correspondence address P Béguin, Unité de Physiologie Cellulaire and URA 1300 C N R S, Département des Biotechnologies, Institut Pasteur, 28, rue du Dr Roux, 75724 Paris Cedex 15, France Fax (33) (1) 45 68 87 90

done intravenously two months after the third injection and the serum was collected 10 days after the final injection

2.3 *Radiolabeling of CelD and XynZ*

The labeling reaction [13] was carried out at room temperature in 20 μl 0.1 M Tris-HCl pH 7.5, containing 20 μg of purified protein, 0.5 μg of lactoperoxidase and 100–200 μCi $Na^{125}I$ (carrier-free, Amersham). Four aliquots of 1 μl of 0.003% hydrogen peroxide were added at 1-min intervals and the reaction was stopped by the addition of 20 μl of a 5 mM solution of L-tyrosine. Excess iodide was removed by gel filtration on a Sephadex G-25 column equilibrated with 0.1 M Tris-HCl, pH 7.5, containing 0.6 mg/ml of BSA.

2.4 *Purification of the cellulosome*

The cellulosome was purified from *C. thermocellum* NCIB 10682 culture supernatant by cellulose affinity chromatography and gel filtration on Sepharose CL-4B (Pharmacia) according to [14].

2.5 *Western blotting*

Samples were heated for 5 min at 100°C in 2% SDS/5% β-mercaptoethanol sample buffer, followed by SDS-PAGE, transfer of the separated proteins onto nitrocellulose and detection of immunoreactive bands [15]. The anti-CelD antiserum was saturated with a crude extract of *E. coli* TG1(pUC19) [11].

2.6 *Binding of CelD and XynZ to cellulosome components*

Cellulosome components were separated by SDS-PAGE and transferred onto nitrocellulose [15]. Bands recognized by CelD and XynZ were revealed as described for the detection of immunoreactive bands, except that ^{125}I-labeled CelD and XynZ were used instead of antiserum plus ^{125}I-labeled protein A.

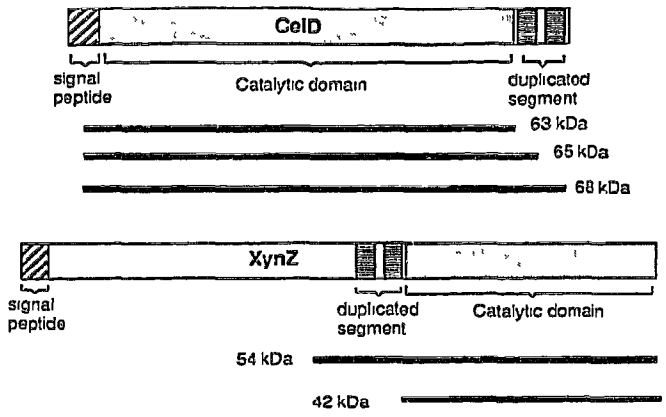

Fig. 1. Domain structure of CelD and XynZ. The extent of the catalytic domain was defined by deletion analysis of *celD* and *xynZ* (data not shown). The portion of CelD and XynZ present in the more or less truncated forms of the two proteins is indicated by horizontal bars. Each species resulted from the fusion of the indicated portion of the gene with the start of *lacZ* carried by pUC vectors [4,8,12].

3. RESULTS

3.1. *A number of proteins of the cellulosome bear epitopes immunologically similar to the conserved, duplicated segment of CelD*

Isolation of the intact, 68 kDa form of CelD provided a means of raising antibodies recognizing the conserved domain.

Among proteins of the crude *C. thermocellum* super-

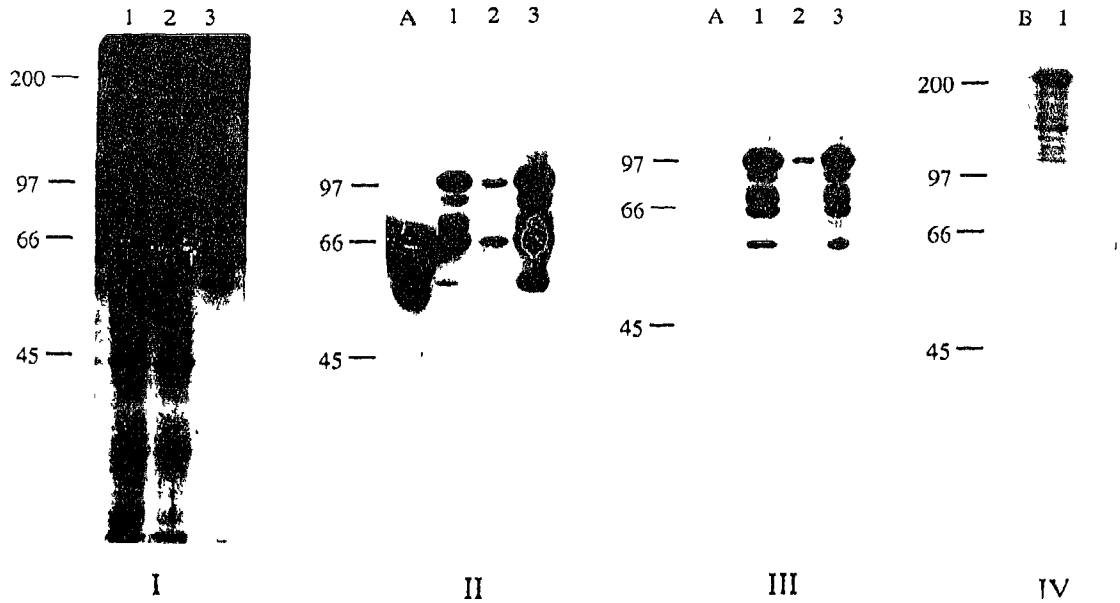

Fig. 2. *C. thermocellum* proteins recognized by antibodies directed against 68 kDa CelD. I, Coomassie blue-stained gel; II–IV, Western blots revealed with antiserum raised against 68 kDa CelD; II, antiserum preadsorbed with *E. coli* TG1(pUC19) extract; III, antiserum preadsorbed with *E. coli* TG1(pCT608) extract (containing 63 kDa CelD); IV, antiserum preadsorbed with *E. coli* TG1(pCT608) extract plus purified 68 kDa CelD. 1, crude *C. thermocellum* supernatant; 2, fraction non-adsorbed on a cellulose affinity column; 3, fraction ad- sorbed on a cellulose affinity column. In panels I–III, the amount of *C. thermocellum* protein loaded in each sample corresponded to the same percentage of each fraction. 2.8 μg 63 kDa CelD and 2 μg 68 kDa CelD were loaded in lanes A and B, respectively.

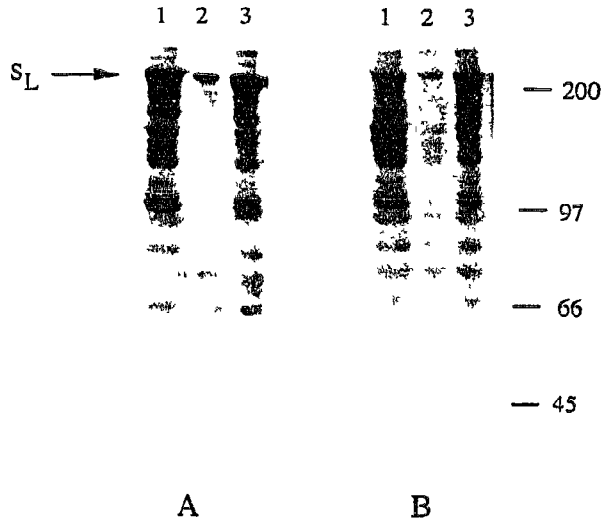

Fig 3 Components of the cellulosome interacting with CelD and XynZ carrying the duplicated segment The proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane The membrane was incubated in the presence of 5 4 10^4 cpm/ml radioactive CelD (panel A) or XynZ (panel B), with a specific activity of 4 10^5 cpm/μg, followed by washing and autoradiography Protein samples loaded in lanes 1, 2 and 3 are the same as in Fig 2

natant, the antiserum recognized a set of 6–8 bands ranging between 50 and 100 kDa (Fig 2, panel II, lane 1). Most of the recognized species were adsorbed on a cellulose affinity column (lanes 2 and 3) and were associated with a complex larger than 1 MDa (data not shown), indicating that they corresponded to cellulosome components Recognition was not prevented by preincubating the antiserum in the presence of excess 63 kDa protein (Fig. 2, panel III), except for a 63 kDa component which did not adsorb to cellulose and may correspond to partially truncated CelD (lanes 2 of panels II and III) Controls showed that the original antiserum recognized 63 kDa CelD (panel II, lane A) and that blocking of antibodies recognizing the 63 kDa epitopes was successful (Panel III, lane A) No band was recognized in a crude extract of *E. coli* TG1(pUC19) (data not shown) The results suggested that the recognition of multiple cellulosome components was due to the presence of epitopes structurally related to the COOH-terminal, duplicated segment of 68 kDa CelD

3 2 *Affinity of CelD and XynZ carrying the duplicated segment for cellulosome components*

In order to demonstrate that recognition of the cellulosome bands was due to the duplicated segment epitopes, the antiserum was preincubated in the presence of excess purified 68 kDa CelD before using in Western blotting experiments The result is shown in Fig. 2 panel IV Recognition of purified 68 kDa CelD and of cellulosome bands previously labeled by the antiserum preadsorbed with 63 kDa CelD was abolished Surprisingly, a 250 kDa component, which corresponded to the S_L/S1 component described by others [3,16], was strongly labeled, along with a set of less intensely labeled bands with a lower M_r This led us to suppose that the antiserum in this case did not bind directly to the bands detected but through the intermediary of 68 kDa CelD that presumably bound to the detected components first

To check this hypothesis, cellulosome components were separated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with ^{125}I-labeled 68 kDa CelD Autoradiography revealed a series of cellulosome-specific bands ranging mostly between 95 and 250 kDa, S_L being the most prominent (Fig. 3A) A very similar pattern was obtained with ^{125}I-labeled 54 kDa XynZ, the only similarity of which with CelD lies in the duplicated segment (Fig 3B) No band was revealed, even after 5-fold longer exposure, when the same experiment was repeated with the ^{125}I-labeled 63 kDa and 65 kDa forms of CelD, with the 42 kDa form of XynZ or upon incubation of ^{125}I-labeled 68 kDa CelD with proteins from a crude *E coli* extract (data not shown)

4 DISCUSSION

Experiments done with antibodies showed that the duplicated segment, which is highly conserved among *C thermocellum* cellulases [12], is present in several proteins that are part of the cellulosome The labeled polypeptides had M_r between 50 and 100 kDa, a range corresponding to subunits S13 to S5 listed by Lamed and co-workers [3]. The duplicated segment was not detected in non-cellulosomal polypeptides present in the culture supernatant. The results extend previous observations showing that CelD, CelE, and XynZ, which contain the duplicated segment, are part of the cellulosome [11,14,17], whereas CelC, the only endoglucanase known so far that does not contain the duplicated segment, is not associated with the multienzyme complex (R Lamed, personal communication).

Two hypotheses have been proposed for the function of the duplicated segment [12]. The first one suggests a cellulose binding function. This hypothesis appears less plausible since it was shown [18] that binding of endoglucanase CelE from *C thermocellum* to crystalline cellulose did not involve the duplicated segment The second hypothesis suggests that the duplicated segment serves to anchor the various components to the cellulosome

Incubation of cellulosome components transferrred to nitrocellulose with ^{125}I-labeled 68 kDa CelD provided direct evidence that the duplicated segment mediates anchoring of the catalytic subunits to the cellulosome A specific set of bands binding 68 kDa CelD was revealed, among which S_L was the most prominent. The same set of bands was revealed upon incubation with

^{125}I-labeled 54 kDa XynZ, a protein of which the only feature common with CelD is the presence of the duplicated segment. Conversely, no band was detected when forms of the same proteins devoid of the duplicated segment were used as probes Our results may also explain the observation of Morag et al [19] that mild proteolytic cleavage releases the S8 subunit from the cellulosome The size (7 kDa) of the excised portion putatively involved in cohesion of S8 to the cellulosome is consistent with the size of the conserved 65–70 residue region containing the duplicated segment.

The structural role of S_L (or its 210 kDa equivalent termed S1 found in *C thermocellum* YS) has long been surmised [10,14], and S_L was shown to promote the binding to the substrate of the catalytic subunit S_S [20]. The identity of the lower molecular weight bands is unclear. Most of them have M_r between 95 and 200 kDa, a range devoid of most major cellulosome components listed by Lamed and co-workers except S2 and S3 [3]. They may represent a class of scaffolding proteins different from S_L. Alternatively, they could match with some of the lower M_r bands into which $S1/S_L$ reportedly dissociates upon dialysis against distilled water or low pH buffers (R. Lamed, personal communication).

As S_L and other minor bands revealed by the ^{125}I-labeled probes do not react with antibodies directed against the duplicated segment, it should bind to some complementary determinants in the cellulosome Identification and characterization of these determinants, particularly those present on the S_L subunit, should prove a fascinating goal

Acknowledgements We thank A Meier for help in growing *C thermocellum* and harvesting culture supernatants K T was supported by Presidential Young Investigator Grant ECE-8552492 awarded to P D 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 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] Lamed, R , Setter, E , Kenig, R and Bayer, E A (1983) Biotechnol Bioeng Symp. 13, 163–181
[4] Chauvaux, S , Beguin, P , Aubert, J -P , Bhat, K M , Gow, L A , Wood, T M and Bairoch, A (1990) Biochem J 265, 261–265
[5] Grepinet, O and Beguin, P (1986) Nucleic Acids Res 14, 1791–1799
[6] Hall, J , Hazlewood, G P , Barker, P J and Gilbert, H J (1988) Gene 69, 29–38
[7] Yagüe, E , Beguin, P and Aubert, J -P (1990) Gene 89, 61–67
[8] Joliff, G , Beguin, P , Juy, M , Millet, J , Ryter, A , Poljak, R and Aubert, J -P (1986) Bio/Technology 4, 896–900
[9] Tokatlidis, K , Dhurjati, P , Millet, J , Béguin, P and Aubert, J -P (1991) FEBS Lett 282, 205–208
[10] Lamed, R and Bayer, E A (1988) in FEMS Symposium No 43 Biochemistry and Genetics of Cellulose Degradation (Aubert, J -P , Beguin, P and Millet, J , eds) pp 101–116 (Academic Press, London)
[11] Grépinet, O , Chebrou, M -C and Beguin, P (1988) J Bacteriol 170, 4576–4581
[12] Grepinet, O , Chebrou, M -C and Beguin, P (1988) J Bacteriol 170, 4582–4588
[13] Marchalonis, J J (1969) Biochem J 113, 299–305
[14] Lamed, R and Bayer, E A (1988) Adv Appl Microbiol 33, 1–46
[15] Towbin, H , Staehelin, T and Gordon, J (1979) Proc Natl Acad Sci USA 76, 4350–4354
[16] Wu, J H D , Orme-Johnson, W H and Demain, A L (1988) Biochemistry 27, 1703–1709
[17] Hazlewood, G P , Davidson, K , Clarke, J H , Durrant, A J , Hall, J and Gilbert, H J (1990) Enzyme Microb Technol 12, 656–662
[18] Durrant, A J , Hall, J , Hazlewood, G P and Gilbert, H J (1991) Biochem J 273, 289–293
[19] Morag, E , Halevy, I , Bayer, E A and Lamed, R (1991) J Bacteriol 173, 4155–4162
[20] Wu, J H D and Demain, A L (1988) in FEMS Symposium No 43 Biochemistry and Genetics of Cellulose Degradation (Aubert, J -P , Beguin, P and Millet, J , eds) pp 117–131 (Academic Press, London)