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ORIGINAL PAPER

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Analysis of the function of a hyperthermophilic endoglucanase from *Pyrococcus horikoshii* that hydrolyzes crystalline cellulose

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Abstract A hyperthermophilic β-1,4 endoglucanase was identified in *Pyrococcus horikoshii*, a hyperthermophilic archaeon. In order to clarify the function of the protein in detail, structural and catalytic site studies were performed using protein engineering. By removing some of the C-terminal sequence of the ORF of the endoglucanase (PH1171), two types of recombinant proteins were expressed from one ORF, using *Escherichia coli*. One exhibited endoglucanase activity, and the other did not. An SD-like sequence was identified in the ORF of the endoglucanase. By removing the SD-like sequence without changing the amino acid sequence of the endoglucanase, one recombinant endoglucanase was prepared effectively from *E. coli*. From the analysis of the N- and C-terminal regions of the ORF, this endoglucanase appears to be a secreted and membrane-binding enzyme of *P. horikoshii*. A mutation analysis of the endoglucanase, using the synthetic substrate, indicated that Glu342 is a candidate for the active center and plays a critical role in the activity of the enzyme. Additional catalytic amino acid residues were not found. These results indicate that the catalytic residue of the enzyme is different from that of typical family 5 endoglucanase, even though it has a high homology to the endoglucanase from *Acidothermus cellulolitticus*. The activity of the enzyme, using carboxy methylcellulose and crystalline cellulose as the substrates, was increased, but not for a synthetic low-molecular substrate when a carbohydrate-binding module of chitinase from *P. furiosus* was added to the C-terminal region.

Keywords Active site · Cellulase · Chitinase · Disulfide bond · Endoglucanase · Hyperthermophile · Site-directed mutagenesis

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Introduction

A hyperthermophilic endoglucanase, EGPh, with the capability of hydrolyzing crystalline cellulose from *Pyrococcus horikoshii* was reported previously (Ando et al. 2002). This was a highly promising finding, since enzymatic hydrolysis of crystalline cellulose, a major component of biomass, has long been awaited. Furthermore, cellulases are used in large quantities in the textile industry for the bio-polishing of cotton products. This process is essential for the removal of excess fibers and to create a soft texture. Although the genome sequences of several hyperthermophilic archaea have been elucidated, few cellulases have been identified. A cellulase recently obtained from *P. furiosus* showed no activity toward crystalline cellulose (Bauer et al. 1999).

However, a demonstration of activity toward crystalline cellulose is insufficient to understand the catalytic mechanism of such an enzyme, which is needed to improve its activity. We therefore undertook studies using synthetic substrates. Furthermore, we examined the function of the enzyme in detail, using five mutant enzymes produced by protein engineering. Calorimetric measurements were carried out to determine the thermostability of the enzyme using differential scanning calorimetry (DSC).

Materials and methods

Construction of plasmids

All primers used in experiments are listed in Table 1. Mutation experiments were performed by polymerase chain reaction (PCR)-mediated site-directed mutagenesis (Mullis et al. 1986). To obtain the wild-type recombinant enzyme (EGDsc) by truncation of its N-terminal signal sequence and C-terminal region of full-length EGPh, we constructed the expression vector pETEGDSC. DSF and DSCR primers were used for in the amplification of the *EGDSC* gene. The DNA fragment obtained was inserted into *Nde*I and *Bam*HI sites of a pET11a expression vector (Novagen, Madison, Wis., USA). All PCRs were performed by using KOD DNA polymerase (TOYOBO, Kyoto, Japan), and the sequences of the genes were verified by DNA sequencing using ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, Cal., USA).

Removal of an SD-like sequence

The mutant gene (*SD2 M*) that was constructed by removing an SD-like sequence from the *EGDSC* gene was regenerated by PCR, using DSF and DSCR as primers and DNA fragments as templates, obtained by two separate PCR runs using DSF and SD2MR, and DSCR and SD2MF. The resulting *SD2 M* gene was digested with *Nde*I and *Bam*HI, and was inserted into the pET11a vector to obtain the pETSD2 M plasmid.

Mutation of the candidates for the active site

Three mutant proteins, E201Q (Glu 201 → Gln), E342Q (Glu 342 → Gln), and D355 N (Asp 355 → Asn) were constructed. Primers 1 (E201QR for E201Q, E342QR for E342Q, and D355NR for D355 N) and 2 (E201QF for E201Q, E342QF for E342Q, and D355NF for D355 N) were designed. For each mutation, two separate PCRs were used to amplify the 5′ portion (DSF and primer 1) and 3′ portion (DSCR and primer 2) of the *EGDSC* gene. The mutant DNA fragments were regenerated by a second PCR, using DSF and DSCR as primers. The full-length mutant DNA fragments were digested with *Nde*I and *Bam*HI and inserted into the corresponding site of the pET11a expression vector.

Mutation of four Cys residues

The mutant ΔCYS (Cys106, 159, 372, and 412 were changed to Ala residues) was also constructed by site-directed mutagenesis. The DNA fragments were amplified by four separate PCRs, using DSF and CA106R, CA106F and CA159R, CA159F and CA372R, CA372F and CA412R, and then the full-length Δ*CYS* gene was regenerated by a second PCR using the four DNA fragments as the templates, and DSF and DSCR as the primers. The resulting mutated DNA fragment was digested with *Nde*I and *Bam*HI and inserted into the corresponding site of the pET11a vector.

Fusion of the EGDsc protein with the carbohydrate-binding module of chitinase

We constructed the fusion protein EG *Pf*ChiCBM by using EGDsc and the carbohydrate-binding module (CBM) of chitinase from *Pyrococcus furiosus* (Chhabra et al 2002). (The sequence of the CBM domain in *Pf*ChiA (PF1233) is available at http://gib.genes.nig.ac.jp/single/index.php?spid=Pfur_DSM3638.) PCR amplification of the *EGDSC* gene without stop codon

Table 1 Primers used in this study

Name	Nucleotide sequence (5′-3′)[a]
DSF	CCAGTA**CATATG**GAAAATACAACATATCAAACACCG
DSCR	TG**GGATCC**TCAAGAACTTTTGGAACAACTATC
SD2MF	CGCTTGGTGGGG TGG TAATCTAATG
SD2MR	CATTAGATT ACC ACCCCACCAAGCG
E201QF	ATCTAAAGAAT CAGCCTCATAGTGTT
E201QR	AACACTATGAGGCT GATTCTTTAGAT
E342QF	CAGTTGTAATAGGA CAGTTTGGAGGAAAATATG
E342QR	CATATTTTCCTCCAAACT GTCCTATTACAACTG
D355NF	CGATCCAAGGAATGTTATATGGC
D355NR	GCCATATAACATTCCTTGGATCG
CA106F	GCAATAAGACTTCCTTTC GCTACTGAGTCTGTAAAACCA
CA106R	TGGTTTTACAGACTCAGTA GCGAAAGGAAGTCTTATTGC
CA159F	GACTATCATAGGATAGGA GCCACTCACATAGAACCCCTC
CA159R	GAGGGGTTCTATGTGAGTG GCTCCTATCCTATGATAGTC
CA372F	GGATGATAGAGAATAAATTT GCTGATTTCTTTTACTGGAGCTGG
CA372R	CCAGCTCCAGTAAAAGAAATCA GCAAATTTATTCTCTATCATCC
CA412R	GTAGGGATCCGTACTTCAAGAACTTTTGGAA GCACTATCCATCAATCTCTTCAG
CBMF	CG**GGATCC**ACCACTACAACTACCCCTGTCC
CBMR	C**GAATTC**TCATGTCCATATGTCAATTACTTGTCG
DSCBR	CG**GGATCC**AGAACTTTTGGAACAACTATCCATC

[a]Restriction sites are indicated in *boldface*. The mutation interests are *underlined*

was carried out using the DSF and DSCBR primers, and EGPH as a template. PCR amplification of the CBM region was carried out using the CBMF and CBMR as primers, and genomic DNA from *P. furiosus* as the template. The resulting *EGDSC* gene without stop codon was digested with *Nde*I and *Bam*HI and inserted into the corresponding site of a pET21a vector (Novagen). The resulting plasmid was digested with *Bam*HI and *Eco*RI and then ligated with the PCR fragment of the CBM region digested with the same restriction enzymes.

Expression of the proteins

The constructed plasmids were introduced into *Escherichia coli* strain BL21(DE3)pLysS (for the expression of E201Q, E342Q, D355 N, and ΔCYS) or strain Rosetta (DE3) (for that of EG *Pf*ChiCBM). Each transformant was cultured in LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37°C until an OD_{600} of 0.2 was reached, and isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added (0.01 mM). After a 20-h culture at 37°C, the cells were harvested by centrifugation (8,000 *g*, 5 min, 4°C). The cells were homogenized by ultrasonication, and then the homogenates were heated at 85°C (for E201Q, E342Q, D355 N, and EG *Pf*ChiCBM) or 75°C (for ΔCYS) for 30 min. After removing the cell debris and the precipitated protein by centrifugation (28,000 *g*, 20 min), the mutant and fusion proteins were isolated and purified to homogeneity by ammonium sulfate precipitation, anion exchange chromatography using HiTrap Q, hydrophobic interaction (using a HiTrap Phenyl) column chromatography, and HiPrep S-200 gel-filtration chromatography.

Western blotting experiment

BALB/c mice were immunized with 50 μg EGDsc in complete Freund adjuvant (Wako Pure Chemical, Osaka, Japan) and then at weekly intervals with incomplete Freund adjuvant until anti-EGDsc antibodies were detected in the sera. The antisera were examined for specificity by an enzyme-linked immunosorbent assay, which confirmed that the anti-EGDsc detected EGDsc. Transformants harboring the pETEGDSC or pETSD2 M plasmid were cultured at 37°C in LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol until an absorbance at 600 nm of 0.6 was reached. After the addition of 0.5 mM IPTG, a further 5-h cultivation was performed. The cells were harvested by centrifugation and then homogenized by ultrasonication. The cell homogenates (1.2 μg protein) were loaded onto a 15–20% gradient gel. After electrophoresis, the proteins were electro-transferred onto a polyvinylidene difluoride membrane. The membrane was then incubated with the mouse–anti-EGDsc antisera in Tris-buffered saline, followed by incubation with horseradish peroxidase-conjugated goat–anti-mouse IgG (Sigma, St. Louis, Mo., USA). The antibody to EGDsc was detected using a Lumi-Light plus (Roche Diagnostics, Mannheim, Germany).

DSC

DSC measurements were carried out using a nanoDSCII instrument (Calorimetry Sciences, Spanish Fork, Utah, USA) with platinum tubing cells having a volume of 0.3 ml. Proteins were dialyzed against 50 mM phosphate buffer, pH 8.0 (for EGDsc and Δ CYS) or pH 7.0 (for 16 k protein). The dialysate buffer was used as a reference solution for the DSC scan. Samples containing 1.0 mg/ml protein were heated at 1°C/min from 0 to 125°C.

Activity measurement

The hydrolytic activities of the enzymes toward CMC (Wako Pure Chemical) and Avicel SF (Asahi Kasei, Tokyo, Japan) were measured by the modified Somogi–Nelson methods (Hiromi et al. 1963) at 85°C in 100 mM acetate buffer (pH 5.6). Activities against *p*-nitrophenyl cellobiose [(G2-PNP) Sigma] were measured spectrophotometrically from the absorbance of *p*-nitrophenol compared to that for G2-PNP at 420 nm, 50°C in 100 mM acetate buffer (pH 5.6).

Results and discussion

We constructed the recombinant hyperthermophilic endoglucanase (EGDsc) of *Pyrococcus horikoshii* by removing the C-terminal (411–459 amino acid residues) region from EGPh (PH1171) and the expression level was increased by 60-fold or more in *Escherichia coli* without any loss of activity (unpublished data). In the sequence of EGPh, an SD-like sequence of another protein was found (Fig. 1). Using *E. coli* and the pET system, this EGDsc was expressed. In a Western blotting experiment using the anti-EGDsc antisera, however, it was revealed that two types of recombinant proteins were expressed in *E. coli* (Fig. 2). The molecular weight of one was estimated to be 43,000 and the other, 16,000. The two recombinant proteins were isolated and purified using HiTrap Q and HiTrap Phenyl Sephacryl S-100 (Amersham, Piscataway, N.J., USA) chromatography. The activities of EGDsc against CMC, Avicel SF, and G2-PNP were then measured (Table 2). The protein corresponding to M_r 45,000 was determined to be EGDsc. However, the protein corresponding to the M_r 16,000 molecule (16 k protein) had no endoglucanase activity. A DSC experiment indicated that EGDsc was a hyperthermostable protein ($T_m = 96$°C) but 16 k protein

was not ($T_m = 71°C$) (Fig. 3; Table 3). By changing the SD-like sequence (AGGA) (Fig. 1) to TGGT without changing the amino acid sequence, it was possible to eliminate the expression of the 16 k protein in *E. coli* (Fig. 2). These results suggest that two different proteins are expressed in *P. horikoshii* cells from the ORF (PH1171). From the structural analysis of an endoglucanase from *Acidothermus cellulolyticus* (Sakon et al. 1996) exhibiting high sequence identities with EGPh, the structure of EGPh appears to be a $(\beta/\alpha)_8$ barrel, and the 16 k protein appears to consist of three α helices and three β strands at the C-terminus of the $(\beta/\alpha)_8$ barrel. However, the role of 16 k protein appears to be different from that of EGPh.

The C-terminal region of EGPh contains a unique amino acid sequence; CGPA-[hydrophobic amino acids]-[basic amino acids] (Fig. 4). We found many ORFs containing a similar sequence in the genome of *Pyrococcus* spp. The precursors of most surface proteins on Gram-positive bacteria have a C-terminal hydrophobic domain and a charged tail, preceded by a conserved LPXTG motif that signals the anchoring process. This motif is the substrate for an enzyme, referred to as sortase, which has transpeptidation activity and functions in the cleavage of the LPXTG sequence and attachment of the protein to the peptideglycan (Lee et al. 2002). The endoglucanase activity was observed in the membrane fraction of *P. horikoshii* cells (0.02 U/mg protein: one unit is defined as the amount of enzyme required to produce reducing power equivalent to 1 mg of glucose per hour at 85°C), which was prepared by precipitation from a homogenate of *P. horikoshii* cells

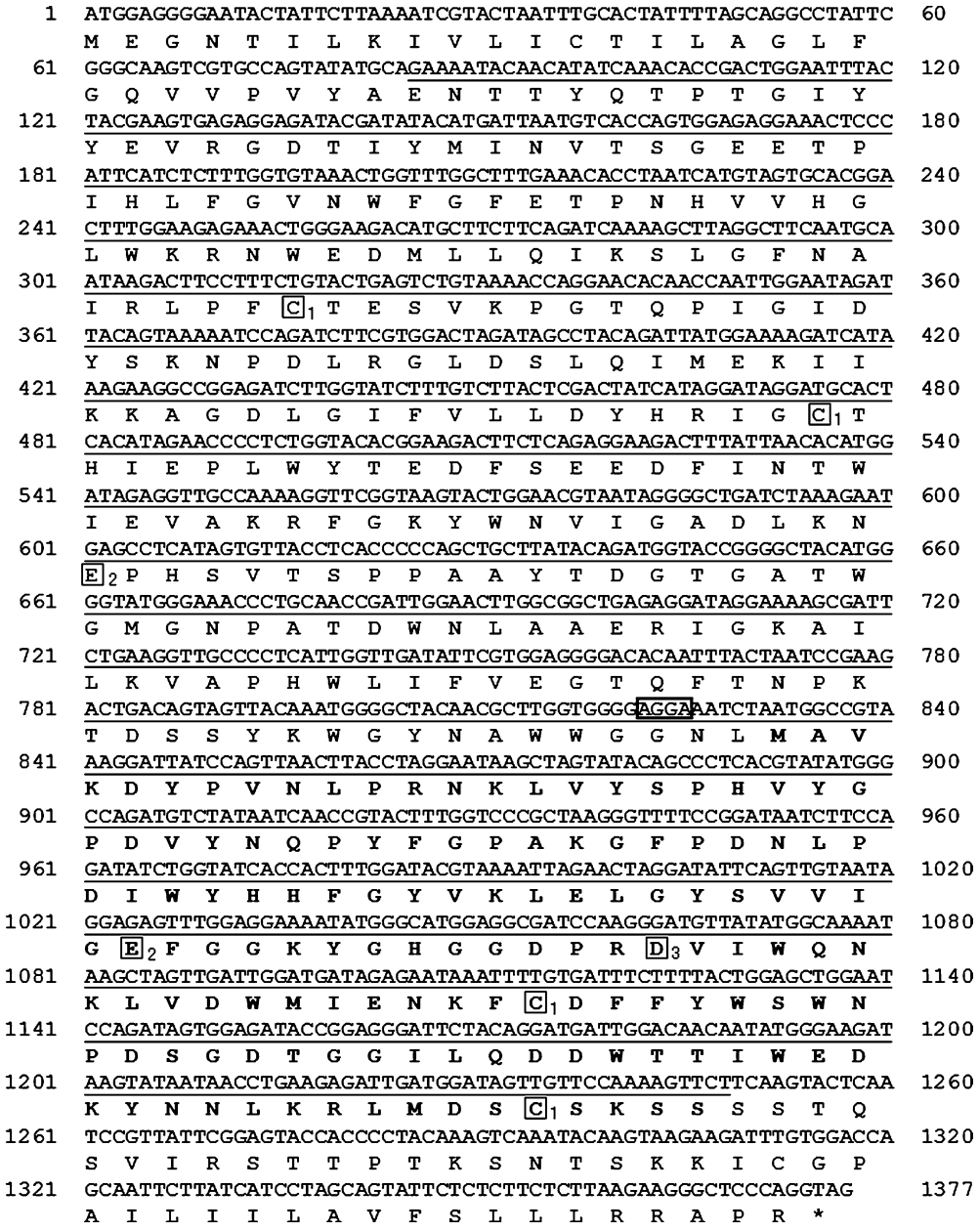

Fig. 1 Nucleotide and deduced amino acid sequence of EGPh. The nucleotide sequence of the *EGDSC* gene is *underlined*. The deduced amino acid sequence of EGPh is represented by *single notation under the nucleotide sequence*. The possible second SD-like sequence is *boxed*. The amino acid sequence of the 16 k protein is shown in *boldface*. The *boxes 1, 2*, and *3* indicate the amino acid residues changed to Ala (*box 1*), Gln (*box 2*), and Asn (*box 3*) in the mutation analysis

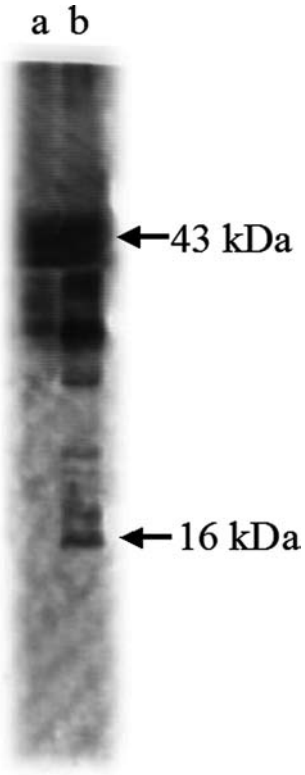

Fig. 2 Western blot analysis of homogenates of *Escherichia coli*, which express the two *EGDSC* genes. Homogenates (1.2 μg protein) of *E. coli* harboring the *EGDSC* gene without a second SD-like sequence (**a**) and native *EGDSC* gene (**b**) were loaded on polyacrylamide gel

Table 3 Calorimetric data for the recombinant enzymes

Enzymes	T_m (°C)	ΔH (J/g)
EGDsc	95.5	36.3
ΔCYS	89.5	35.2
16 k protein	70.9	16.8

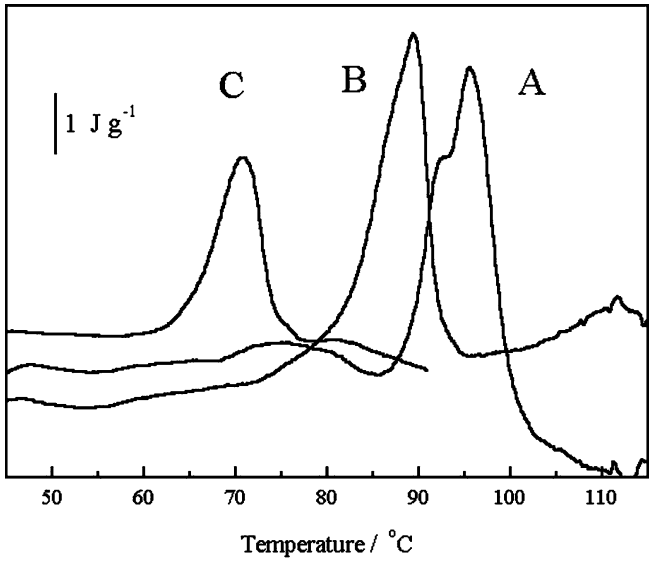

Fig. 3 Differential scanning calorimetry tracings of the recombinant enzymes. **A** EGDsc (1 mg/ml) in 50 mM sodium phosphate, pH 8.0; **B** ΔCYS (1 mg/ml) in 50 mM sodium phosphate, pH 8.0; and **C** 16 k (1 mg/ml) in 50 mM sodium phosphate pH 7.0

Table 2 Activities of the recombinant enzymes. Activities toward CMC and Avicel SF were measured at 85°C in 100 mM acetate buffer (pH 5.6), using 0.5% CMC or Avicel SF as substrates. The reducing ends were measured according to the modified Somogi–Nelson methods (Hiromi et al. 1963). Activity toward G2-PNP was measured at 50°C. The increase in absorbance at 420 nm due the production of *p*-nitrophenol was measured. The concentration of *p*-nitrophenol was calculated by comparing with the standard curve of *p*-nitrophenol solution in 100 mM acetate buffer (pH 5.6). The k_{cat} values were determined from the initial velocity measured in the presence of excess concentration of the substrates

Enzymes	Specific activity (k_{cat})(1/s)		
	CMC	Avicel SF	G2-PNP
EGDsc	34.8 ± 1.17	0.191 ± 0.095	1.432 ± 0.032
EG *Pf*ChiCBM	164 ± 10.3	0.347 ± 0.052	1.223 ± 0.025
E201Q	3.51 ± 0.33	ND[a]	0.575 ± 0.043
E342Q	< 0.1	ND	< 0.001
D355 N	5.87 ± 0.291	ND	0.566 ± 0.019
ΔCYS	22.9 ± 0.102	ND	0.634 ± 0.027

[a] *ND* Not determined

after ultracentrifugation (100,000 *g*, 60 min) (unpublished data). Therefore, the C-terminal region of the ORFs appears to play some role in the anchoring and binding process to the cell surface of *Pyrococcus* sp., since the proteins contain the signal peptide-like sequence and have high identities to the membrane-bound permease transporter (Fig. 4).

EGPh has a high sequence homology to an endoglucanase from the *A. cellulolyticus* EGAc (Sakon et al. 1996; Michael et al. 1994). Using a low-molecular synthetic substrate, we attempted to characterize the recombinant enzymes in detail. An alignment analysis of the two endoglucanases suggested that Glu residues (E201 and E342) were the active sites of EGPh (Ando et al. 2002; Sakon et al. 1996). Furthermore, an Asp residue (D355) was presumed to be one of the candidates for the active site, as evidenced by an analysis based on a structural analysis of EGAc (Sakon et al. 1996). In order to examine the active site of this enzyme, we prepared wild-type EGPh (EGDsc) by the truncation of its N-terminal signal sequence and C-terminal region and three mutant enzymes by site-directed mutagenesis. The mutant enzymes were prepared and purified using the same methods as were used for EGDsc. The circular dichroism spectra of these three proteins had the same profiles over the temperature range (25–95°C) examined. This indicates that the overall structure and thermostability of this enzyme were not influenced by these mutations. The characteristics of the mutant enzymes are shown in Table 1. The mutation of E342 to Q completely extinguished its activity for all substrates

Fig. 4 Alignment of the C-terminal region of some proteins of *Pyrococcus* spp. The C-terminal regions of proteins having a high sequence homology to that of EGPh are aligned. A *bold bar* indicates the region of the S, P, T-rich linker-like sequence. A *box* indicates the sequence of the CGPA region. A *double bar* indicates the hydrophobic region. The Arg and Lys residues at the C-terminus are shown in *boldface*. PH0502, PH0807, and PH1962 are the putative oligopeptide-binding proteins of *P. horikoshii*; PF0357 and PF1209 are the putative dipeptide-binding protein and the ABC-transporter of *P. furiosus*, respectively; PAB0627 and PAB1343 are the putative oligopeptide transport permease and dipeptide transport protein of *P. abyssi*

examined. However, the activity of the mutants E201Q and D355 N was retained. The k_{cat} values for E201Q and D355 N for G2-PNP were decreased to about 40% that of EGDsc, but the K_m values were similar to that of EGDsc. However, they had no apparent activity with respect to Avicel SF. These results indicate that the E342 residue is essential for enzyme activity. However, the other candidates, the E201 and D355 residues, are not required for activity and have no effect on substrate affinity. Thus, the active site and the catalytic residue of EGDsc appear to be different from other endoglucanases in family 5, despite their similar structure (Sakon et al. 1996). The other catalytic residue's counterpart could not be determined because the protein sequence contains a large number of candidates. This question may be clarified by a crystal structure analysis in future studies.

The cellulose-binding domain of EGPh has not been identified (Ando et al. 2002). It was reported that the activity of the endoglucanase is increased when a CBM is added (Chhabra et al. 2002). Therefore, we constructed the fusion protein EG*Pf*ChiCBM, using the EGDsc and CBM of chitinase from *P. furiosus*. The recombinant fusion protein EG*Pf*ChiCBM was prepared and purified using the same methods as were used for EGDsc. The optimum pH and temperature were the same as those for EGDsc. The kinetic parameters of EG-*Pf*ChiCBM toward G2-PNP were similar to that of EGDsc (Table 2). On the other hand, the activities toward CMC and Avicel SF were increased by about 4.7-fold and 2.2-fold, respectively (Table 2). Thus, the active site of EGDsc is not influenced by the fused CBM. However, the presence of CBM causes an increase in hydrolytic activity toward cellulose substrates. The construction of a fusion protein with CBMs from other thermophilic endoglucanase might represent a strategy for achieving an even higher activity toward cellulose.

EGDsc contains four Cys residues and these residues have been proposed to form two disulfide bonds from the similar structure of EGAc (Sakon et al. 1996). This disulfide bond is thought to contribute to the hyperthermostability of the enzyme by decreasing the conformational entropy of the backbone in the denatured state (Matsumra et al. 1989). We prepared a mutant enzyme (ΔCYS) without two disulfide bonds by changing four Cys residues to Ala residues, and examined the product with respect to activity and thermostability. By removing the disulfide bond, the activity was decreased (Table 2) and the T_m value was also decreased to 89.5°C (Fig. 3; Table 3). However, the small difference (6°C) in T_m values between EGDsc and ΔCYS indicates that these disulfide bonds do not contribute to the hyperthermostability of the enzyme (Matsumura et al. 1989). ΔCYS was still relatively thermostable and exhibited the same optimum temperature as EGDsc. Other factors, aside from disulfide bonds, appear to be important for the hyperthermostability of the enzyme. These results indicate that the disulfide bond of the enzyme is peripherally related to the active site but is not essential for hyperthermostability. The relationship between the thermostability and disulfide bond is not still clear (Kanaya et al. 1991, Wakarchuk et al. 1994). A detailed study of the disulfide bond in hyperthermophilic protein should be informative.

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