Biochemical and Biophysical Research Communications **265,** 57–61 (1999)
Article ID bbrc.1999.1629, available online at http://www.idealibrary.com on IDEAL®

# *In Vitro* Heat Effect on Functional and Conformational Changes of Cyclodextrin Glucanotransferase from Hyperthermophilic Archaea

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Received October 4, 1999

**The *in vitro* heat effect on protein characteristics of thermostable enzyme was examined using a cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) from the hyperthermophilic archaeon *Thermococcus* sp. B1001 as a model protein. The recombinant form of CGTase was obtained as an inclusion body from *Escherichia coli* cells harboring a plasmid which carried the B1001 CGTase gene (*cgtA*). CGTase was solubilized by 6 M urea, refolded, purified to homogeneity, and heat treated at 80°C for 20 min. Enzyme characteristics were examined compared with those of unheated CGTase. Cyclization activity was increased by *in vitro* heat treatment, while hydrolysis activity was decreased. The heated and unheated CGTases were analyzed for structures by circular dichroism (CD). The near- and far-UV CD spectra indicated that the structure of unheated CGTase with low cyclization activity was different from that of heated CGTase with high activity. Differential scanning calorimetry of unheated CGTase showed two absorption peaks at 87 and 106°C with increasing temperature. After heat treatment, the minor peak at 87°C disappeared, suggesting that heat-dependent structural conversion occurred in CGTase. These results indicate that the thermal environment plays an important role for the protein folding process of thermostable CGTase.** 

***Key Words:*** **archaea; CGTase; conformation; structural maturation; hyperthermophile.**

The amino acid sequence possesses the information for specifying the three-dimensional structure of a protein. In addition to genetic information, some accessory factors are considered to play an important role for proper protein folding. In hyperthermophilic microorganisms which can grow above 90°C, additional unique factors such as high concentration of potassium ion [1] and the novel sugar diinositol-1′-phosphate, 2,3-diphosphoglycerate [2] or molecular chaperons [3, 4] are considered to be involved in protein structure formation. Furthermore, recent studies have indicated that high temperature itself has significant effects on the proper folding of thermostable proteins. Effect of heat treatment on proper oligomerization was well studied for homohexameric thermostable glutamate dehydrogenase (GDH) from *Pyrococcus kodakaraensis* [5, 6]. Recombinant *Pk*-GDH heterologously expressed in *Escherichia coli* formed a different structure from that of natural *Pk*-GDH. Upon *in vitro* heat treatment, the structure of recombinant *Pk*-GDH was converted to a different form which was closer to the natural form. In addition, proper subunit assembly by heat treatment was observed in a heterooligomeric indolepyruvate ferredoxin oxidoreductase (IOR), which were composed of two kinds of subunits [7, 8]. However, heat dependent manner of thermostable protein which is composed of several domain units has been unclear. To obtain further information of heat induced structural conversion, cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) has been focused as a model protein.

CGTase catalyzes the degradation of starch into cyclodextrin (CD) which possesses closed-ring structure joined by $\alpha$-1,4-glucosidic bonds [9–15]. According to the number of glucose unit (G6, G7, and G8), CDs are named $\alpha$-, $\beta$-, and $\gamma$CDs, respectively [16]. Previously, we isolated the hyperthermophile *Thermococcus* sp. B1001 as a unique CGTase producing archaeon [17]. Subsequently, the CGTase gene (*cgtA*) was cloned and the nucleotide sequence was determined [18]. CGTase from B1001 produced mainly $\alpha$CD (more than 85% of total product) with quite small amount of $\beta$- and $\gamma$CDs

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0006-291X/99 $30.00

from starch at extremely high temperature (90°C) [17]. Interestingly, B1001 CGTase showed very low level of amylase activity when its activity was measured by liberated reducing sugar [17]. Tertiary structures of various CGTases were determined from *Bacillus circulans* 251, *Thermoanaerobacterium thermosulfurigenes* EM1, *Bacillus stearothermophilus*, and *Bacillus circulans* No. 8 [13, 15, 19, 20]. The structural studies of CGTase revealed that CGTase was composed of four or five domains. $NH_2$-terminal domains are similar to the structure of $\alpha$-amylase. COOH-terminal domains are unique to the CGTase, hence the COOH-terminal domains are considered to be involved in cyclization of linear maltooligosaccharide [19, 21, 22]. In the case of thermostable CGTase which is functional in extremely thermal environment, $NH_2$-terminal and COOH-terminal domains seem to be suitably arranged for an efficient CD production. In the present study, B1001 CGTase was obtained as a recombinant protein and *in vitro* heat effect on enzyme characteristics and protein structure were studied.

## MATERIALS AND METHODS

*Microorganisms, plasmids, and media.* *Thermococcus* sp. B1001 was isolated from the hot spring at Tottori prefecture, Japan [17]. *Escherichia coli* strains used in DNA manipulations were JM109 (*recA*1, *supE*44, *endA*1, *hsdR*17, *gyrA*96, *relA*1, *thi*, Δ (*lac-proAB*)/F′ [*traD*36, *proAB*, *lacI*$^q$, *lacZ* Δ*M15*]). *E. coli* BL21 (DE3) ($F^-$, *ompT*, *hsd*$S_B$ ($r_B^-$, $m_B^-$), *gal* (λ*cI* 857, *ind*1, *Sam*7, *nin*5, *lacUV*5-T7 gene 1), dcm (DE3)) was used to overexpress *cgtA*. *E. coli* strain JM109 was cultivated in Luria–Bertani medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter of deionized water, pH 7.0) at 37°C. NZCYM medium (10 g NZ amine, 5 g yeast extract, 1 g casamino acids, 5 g NaCl and 2 g $MgSO_4 \cdot 7H_2O$ per liter of deionized water, pH 7.0) was used for BL21 (DE3) cultivation with 50 μg/ml ampicillin. Plasmids pUC19 and pET-21a were used as cloning and expression vectors, respectively.

*Recombinant DNA manipulations.* The DNA sequence of B1001 CGTase gene was submitted to the EMBL/GenBank/DDBJ database and was assigned Accession No. AB025721. The expression vector, pCGTA, was constructed as described previously [18]. Small scale preparation of *E. coli* plasmid DNA was achieved by using Wizard Miniprep DNA purification kit (Promega Japan, Tokyo, Japan) and large scale plasmid DNA preparation was performed by Qiagen plasmid Maxi kit (Qiagen, Chatsworth, CA).

*Purification of recombinant CGTase.* Recombinant form of B1001 CGTase was produced in aerobically cultured *E. coli* BL21 (DE3) cells harboring pCGTA at 37°C. When optical density ($OD_{660}$) reached 0.45, gene expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyraoside) for 4 h. *E. coli* cells were harvested by centrifugation. Cells were sonicated in 50 mM Tris–HCl (pH 8.0) buffer and an insoluble fraction was recovered by centrifugation. The insoluble fraction was treated with 2% Triton X-100, 10 mM EDTA (pH 8.0) and sonicated again. Inclusion bodies were harvested by centrifugation and washed twice with a buffer (50 mM Tris–HCl, 30 mM NaCl, pH 8.0). The obtained suspension was solubilized in a refolding solution (6 M urea, 50 mM Tris–HCl, 1 mM DTT (dithiothreitol) pH 8.0) for 1 h according to the reported procedure [23]. The full unfolded proteins were refolded by dialysis against a refolding buffer (50 mM Tris–HCl, 1 mM DTT, pH 8.0) and then applied to anion-exchange column (Hi-TrapQ, Amersham Pharmacia Biotec., Uppsala Sweden). Eluted fractions were combined and the solution was dialyzed against 50 mM Na-phosphate buffer (pH 7.0). The obtained solution was concentrated and further purified by gel filtration (Superdex 200HR 10/30, Amersham Pharmacia Biotec., Uppsala Sweden)

*Enzyme assay.* The αCD synthesis activity was examined by measurement of liberated αCD by the methyl orange method [24]. The reaction mixtures (3 ml), containing 600 μl of 5% (w/v) soluble starch in 50 mM sodium acetate buffer (pH 5.5) and 105 μl of 1 mM methyl orange solution in 50 mM sodium acetate buffer (pH 5.5), 1,795 μl of 50 mM sodium acetate buffer (pH 5.5) and 500 μl of enzyme solution, was incubated at 40°C or 60°C for 5 min. The reaction was terminated by adding 150 μl of 6 N HCl at 16°C. The optical density at 505 nm was measured. One unit of CGTase activity was defined as the amount of enzyme which released 1 μmol of αCD per min.

The hydrolysis activity was examined by measurement of liberated reducing sugar by dinitrosalicylic acid method [25]. The 300 μl aliquot of enzyme solution was mixed with 300 μl of 1% soluble starch in 0.2 M sodium acetate buffer (pH 5.5) and incubated at 40°C for 10 min. One unit of hydrolysis activity was defined as the amount of enzyme which released 1 μg of reducing sugar per min.

*Circular dichroism (CD) measurement.* The circular dichroism (CD) spectra were measured with a J-720W automatic spectropolarometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan) based on published procedure [26]. The far-UV (205–250 nm) and near-UV (250–320 nm) spectra were obtained using solution containing the 200 μg/ml and 1 mg/ml protein, respectively, in 50 mM Na-phosphate buffer (pH 7.0). The 2 mm optical path cell was utilized for scanning. CD measurement was performed at 25°C. The experiment was repeated 40 times and average value was used for the analysis.

*Differential Scanning Calorimetry (DSC) measurement.* DSC scans for measuring the change in excess heat capacity for unheated and heated CGTases were performed using nano-DSCII Differential Scanning Calorimeter 6100 (Calorimetry Sciences Corporation, UT) with golden cells with a volume of 0.3 ml at scanning rates of 1°C/min. Degassing during the calorimetric experiments was prevented by maintaining an additional constant pressure of 3.0 bar over the liquid in the cells. The measurements were performed in 50 mM Na-phosphate buffer at pH 7.0. Each protein concentration was adjusted to 1.0 mg/ml. The calorimetric enthalpy of each sample was analyzed using the equipped software.

## RESULTS AND DISCUSSION

*Expression of CGTase and refolding.* The CGTase gene (*cgtA*) from *Thermococcus* sp. B1001 was cloned into the an expression plasmid pET-21a and resulting plasmid was named pCGTA [18]. Overexpression of CgtA was archived in *E. coli* BL21 (DE3) cells harboring pCGTA as described in materials and methods [18]. Most CGTase was obtained as an insoluble form. Inclusion form of CGTase was unfolded with 6 M urea *in vitro*. Refolded fraction was used for further purification. Purification was archived by anion-exchange and gel filtration chromatographies. The purified CGTase was analyzed by native PAGE and SDS–PAGE. The mobility of active band as determined by starch-degrading activity staining coincided with that stained with Coomassie brilliant blue (Fig. 1b), indicating that purification of CGTase was accomplished. The extract from *E. coli.* BL21 (DE3) harboring pET-21a did not show any active bands by starch-degrading activity staining (data not shown). The purified CGTase was not detected as a single band by SDS–PAGE probably

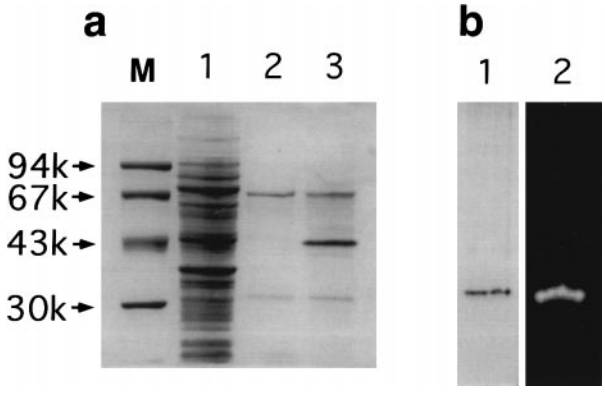


**FIG. 1.** Behavior of CGTase in SDS–PAGE (a) and native-PAGE (b). (a) The 0.1% SDS-10% polyacrylamide gel electrophoresis of *E. coli* extracts harboring pCGTA and purified CGTase. Samples were visualized by Coomassie brilliant blue staining. Lanes: 1, soluble fraction from *E. coli* cells (pCGTA); 2, insoluble fraction from *E. coli* cells (pCGTA); 3, purified CGTase by anion exchange and gel chromatographies; M, molecular weight markers (phosphorylase *b*, 94,000; albumin, 67,000; ovalbumin, 43,000; Carbonic anhydrase, 3000). (b) The native–7.5% polyacrylamide gel electrophoresis of the purified CGTase. Lanes: 1, CGTase by Coomassie brilliant blue staining; 2, CGTase by amylolytic activity staining.

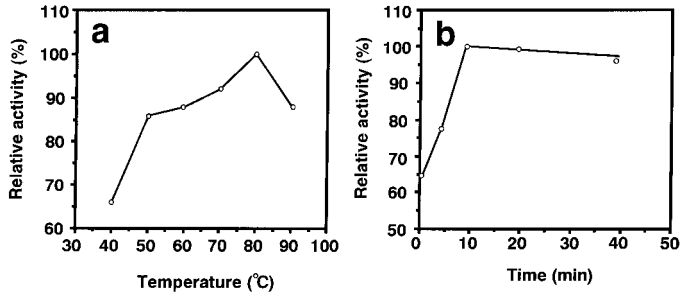


**FIG. 2.** Effect of heat treatment on CGTase activity. (a) Effect of various temperatures. Refolded CGTases were heated for 10 min at the indicated temperatures (30–90°C) and then cyclization activity was measured. (b) Effect of incubation time. Refolded CGTase was heat treated at 80°C for various periods and then cyclization activity was measured.

due to incomplete denaturation (Fig. 1a). Natural CGTase purified from B1001 required severe heat treatment (110°C for 5 min) for complete denaturation to detect a single band by SDS–PAGE [17].

*Heat effect on cyclization activity.* High temperature seems to have an effect on the proper folding of thermostable proteins [5–8]. Especially for secretion enzymes, thermal environment might be responsible for proper folding process because there are no accessories such as chaperonins in the extracellular environment. To examine the effect of heat treatment on enzyme characteristics, the purified CGTase was heat treated for 10 min at various temperatures. After heat treatment, samples were centrifuged and the cyclization activity of supernatant was measured at 60°C (Fig. 2a). Most enzymes from mesophilic microorganisms are generally thermolabile and enzyme loses its activity by heat treatment. However the cyclization activity of B1001 CGTase was increased as the temperature for the heat treatment increased, except for the activity of the enzyme treated at 90°C. Heat treatment at 80°C showed the highest enhancement of the activity which is 1.5 times higher than that of the enzyme without heat treatment. To examine the most effective condition for heat treatment, the CGTase was treated at 80°C for various periods (Fig. 2b). The cyclization activity was increased with incubation period and reached to the plateau level in 10 min.

*Kinetic studies on heated and unheated CGTases.* As mentioned above, CGTase activity was increased by *in vitro* heat treatment. To compare enzyme characteristics of heated CGTase (80°C, 20 min) with those of unheated one, kinetic parameters were obtained. The cyclization activity from soluble starch was measured at 40°C to avoid heat effect while enzyme assay was performed. The $K_m$ and $k_{cat}$ values of heated CGTase for cyclization activity were 0.87 mg · ml$^{-1}$ and 80 min$^{-1}$, respectively, which were 0.75 and 1.3 times higher than those of unheated CGTase ($K_m$, 1.1 mg · ml$^{-1}$; $k_{cat}$, 60 min$^{-1}$). Values of $k_{cat}/K_m$ for unheated and heated CGTases were 55 and 92 ml · mg$^{-1}$ · min$^{-1}$, respectively. The heated CGTase has 1.5 times higher cyclization power than unheated one. These results indicated that CGTase obtained higher affinity to substrate and catalytic power by heat treatment. CGTase catalyzes both cyclization and hydrolysis reactions at the same active site in $NH_2$-terminal domain. To obtain further information about heat effect, cyclization and hydrolysis activities for two distinct CGTases were compared. As shown in Table 1, cyclization activities of CGTase was increased by heat treatment, however hydrolysis activity was decreased. Natural CGTase purified from B1001 cells showed very low level of hydrolysis activity by monitoring reducing ends [17], which was a unique feature of B1001 CGTase. Both unheated and heated CGTases showed hydrolysis activity, indicating that enzyme characteristics of recombinant CGTase were different from those of natural one. However, heated CGTase showed higher cyclization activity and less hydrolysis activity than unheated one, suggesting that recombinant CGTase acquired native like enzyme characteristics by heat treatment.

**TABLE 1**

Effect of Heat Treatment on Cyclization and Hydrolysis Activity

| CGTase | Cyclization activity (U · mg$^{-1}$) | Hydrolysis activity (U · mg$^{-1}$) |
|---|---|---|
| Unheated | $4.8 \times 10^4$ (100%) | $5.1 \times 10^3$ (100%) |
| Heated | $6.7 \times 10^4$ (140%) | $4.1 \times 10^3$ (80%) |

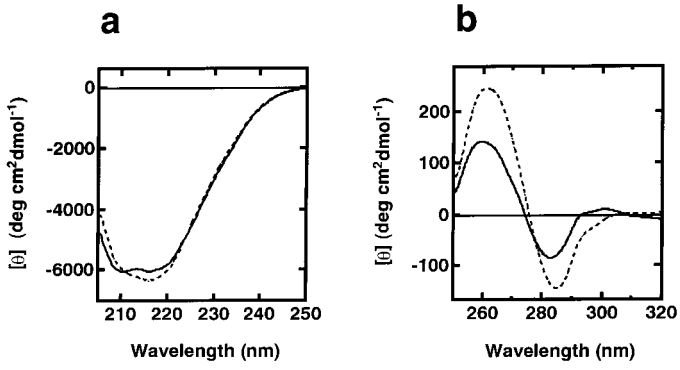


**FIG. 3.** CD spectra of heated and unheated CGTases. (a) Far-UV spectra. (b) Near-UV spectra. The heat treatment of CGTase was carried out at 80°C for 20 min. Each spectrum was obtained using solution containing 1 mg/ml protein in 50 mM Na-phosphate buffer (pH 7.0) in a 2-mm optical path cell. CD measurement was performed at 25°C. The spectra of heated and unheated CGTase were shown by dashed and solid lines, respectively.

On the basis of structural analysis, $NH_2$-terminal domains were similar to the structure of TAKA-amylase and COOH-terminal domains were unique to the CGTase [13, 15, 19, 20]. Hence, it was supposed that COOH- terminal domains of CGTase were related to CD production. *In vitro* heat treatment may induce structural change resulting in optimization of protein conformation for cyclization of maltooligosaccharide. Otherwise, hydrophobic circumstance near catalytic sites in $NH_2$-domain might be optimized for cyclization. To obtain further information on structural change, CGTase was examined for profiles of circular dichroism (CD).

*CD spectra of heated and unheated CGTases.* Heated and unheated CGTases showed different specific activities and kinetic parameters. These results prompt us to analyze the secondary and tertiary structures of these two CGTases. As shown in Fig. 2b, CGTase needed at least 10 min heat treatment at 80°C for activity enhancement. The result indicates enough treating time is necessary for the conversion. The sample of heated CGTase was prepared by treating protein solution at 80°C for 20 min. The far-UV CD spectrum of unheated CGTase showed a single minimum peak at 217 nm, whereas far-UV CD spectrum of heated CGTase showed two peaks at 222 nm and 208 nm (Fig. 3a). This implied that structural change occurred by heat treatment. The structural change was further confirmed by near-UV spectra (Fig. 3b). The spectrum of unheated CGTase showed broad minimum peak at 283 nm and maximum peak at 260 nm. With heat treatment, the spectrum of heated CGTase showed minimum and maximum peaks at 285 and 262 nm respectively, and higher peak values were observed than those of unheated CGTase (Fig. 3b). The results of the far-UV and near-UV CD spectra indicate the structural change with heat treatment.

*DSC measurement of heated and unheated CGTases.* To obtain further information about heat dependent structural conversion, differential scanning calorimetry (DSC) was performed. Based on the result of Fig. 2b, only 10 min incubation at 80°C enhanced enzymatic activity, suggesting that some structural conversion occurred in CGTase during incubation. To avoid the heat effect while DSC measuring, the scanning rate was adjusted to 1.0°C/min. DSC profiles of heated and unheated CGTases were shown in Fig. 4. DSC signals of the unheated CGTase appeared in two peaks. Minor symmetric and major asymmetric peaks were observed at 87 and 106°C, receptively, in the scan of unheated CGTase with increasing temperature. The enzyme was irreversibly unfolded at above 110°C, because repeating heating of the samples revealed almost complete irreproducibility of denaturation curves upon recurrent heating. The possible reason for the irreversibility of unheated CGTase is the protein propensity to aggregate in a denatured state. The signals of heated CGTase were different from those of unheated CGTase. The small adsorption peak at around 87°C disappeared. The major peak at around 106°C appeared and also was irreversible above the temperature. Heat dependent activation of CGTase may associate with heat absorption at around 87°C. The results of CD and DSC imply that the conformational change occurred by heat treatment. If the scanning rate was decreased, unheated CGTase might be converted to heated form after 80°C during DSC measurement. The second peak appeared at 106°C would become identical to that of heated CGTase (107°C). We think that our experimental condition was suitable to find the distinctive profiles between heated and unheated CGTases.

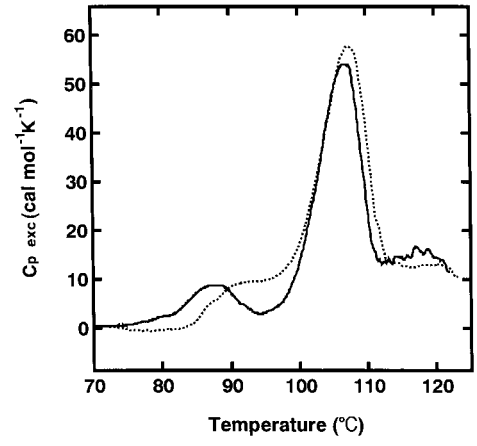


**FIG. 4.** DSC measurements of heated and unheated CGTases. Heated CGTase was prepared by treating protein solution at 80°C for 20 min. DSC scan for each enzymes was performed using solution containing 1 mg/ml protein in 50 mM Na-phosphate buffer (pH 7.0) at a scan rate of 1°C/min. The solid and dashed lines indicate the profiles of the unheated and heated CGTases, respectively.

*Heat-induced conformation relating with CGTase activity.* In the case of hyperthermophilic microorganisms, high temperature itself has significant effects on the proper folding of proteins. It has been reported that the thermostable proteins expressed in *E. coli* do not form a suitable structure without sufficient heat treatment. Glutamate dehydrogenase (GDH) from *P. kodakaraensis* [5, 6] or *Pyrococcus furiosus* [27], D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Thermotoga maritima* [28–30] and histones molecules from *Methanothermus fervidus* [31] needed *in vitro* heat treatment for proper protein folding. The recombinant form of thermostable protein purified in the absence of heat treatment may be in an intermediate stage for protein folding. In the present study, heat absorption at 87°C found in DSC analysis suggests the existence of an intermediate stage in CGTase folding. Intermediate form also seems functional as a enzyme because unheated CGTase possessed cyclization activity.

Domain dependent reversible or irreversible unfolding has been observed in glucoamylase 1 from *Aspergillus niger* (GA1). GA1 consists of three parts, catalytic domain, starch binding domain (SBD) and linker region [32, 33]. DSC analysis on GA1 and deletion derivative GA2 which lacks SBD has shown that unfolding of catalytic domain follows an irreversible one-step mechanism with no observable intermediates. In contrast, unfolding of SBD seemed to be reversible [32]. Reversible unfolding/refolding processes of SDB occurred independently from the catalytic domain of GA1 [32]. SBD of GA1 shows sequence similarity with COOH-terminal domain of CGTase [19]. The COOH-terminal domain of CGTase may show reversible unfolding/refolding processes if this is examined by DSC. Folding process of $NH_2$-terminal and COOH-terminal domains of CGTase seem to be different. The unheated CGTase might possess the fully folded and unfolded regions. In the absence of heat treatment, $NH_2$-terminal domain of CGTase might be folded, while COOH-terminal domain might remain unfolded. Heat probably induces full folding of COOH-terminal domain, resulting in complete folding of whole protein.

Further structural analysis is needed for more detailed speculation. CGTase from hyperthermophile B1001 would be a good model to investigate the temperature dependent folding process. For precise structural analysis, crystallization of unheated and heated CGTases are in progress.

## ACKNOWLEDGMENT

This work was supported by a grant from CREST (Core Research for Evolutional Science and Technology, Japan).

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