

Increasing the thermal stability of cellulase C using rules learned from thermophilic proteins: a pilot study[☆]

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Abstract

Some structural features underlying the increased thermostability of enzymes from thermophilic organisms relative to their homologues from mesophiles are known from earlier studies. We used cellulase C from *Clostridium thermocellum* to test whether thermostability can be increased by mutations designed using rules learned from thermophilic proteins. Cellulase C has a TIM barrel fold with an additional helical subdomain. We designed and produced a number of mutants with the aim to increase its thermostability. Five mutants were designed to create new electrostatic interactions. They all retained catalytic activity but exhibited decreased thermostability relative to the wild-type enzyme. Here, the stabilizing contributions are obviously smaller than the destabilization caused by the introduction of the new side chains. In another mutant, the small helical subdomain was deleted. This mutant lost activity but its melting point was only 3 °C lower than that of the wild-type enzyme, which suggests that the subdomain is an independent folding unit and is important for catalytic function. A double mutant was designed to introduce a new disulfide bridge into the enzyme. This mutant is active and has an increased stability ($\Delta T_m = 3$ °C, $\Delta(\Delta G_u) = 1.73$ kcal/mol) relative to the wild-type enzyme. Reduction of the disulfide bridge results in destabilization and an altered thermal denaturation behavior. We conclude that rules learned from thermophilic proteins cannot be used in a straightforward way to increase the thermostability of a protein. Creating a crosslink such as a disulfide bond is a relatively sure-fire method but the stabilization may be smaller than calculated due to coupled destabilizing effects. © 2002 Elsevier Science B.V. All rights reserved.

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[☆] This work is dedicated to Prof. Rainer Jaenicke, with esteem and appreciation.

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1. Introduction

The structures of homologous enzymes from thermophilic and mesophilic sources are seemingly identical in most cases. Comparisons of related enzymes with different thermal stabilities [1] can teach us how to engineer proteins with increased thermal stability. Since thermal stabilization is achieved by the cumulative effect of small stabilizing and destabilizing contributions, it may be hard to predict the effect of individual amino acid replacements in a polypeptide sequence. In this work, we used a cellulose degrading enzyme, cellulase C (CelC) from a moderately thermophilic organism, *Clostridium thermocellum* (*C. th.*) as a model protein for a ‘pilot study’ to test whether the thermal stability of the enzyme can be increased by simple mutations using rules learned from comparative studies of thermophilic and mesophilic enzymes.

Cellulases have been widely studied and are being used for various industrial purposes because of their potential in biomass conversion and in the paper, textile and detergent industries. Some of the applications require heat stable enzymes that can work at elevated temperatures. An increasing number of heat stable cellulases have been described, however, the major factors stabilizing the structure of these enzymes are not well understood.

Cellulases are members of the glycosyl hydrolase enzyme families. The recent classification of glycosyl hydrolases [2] comprises more than 80 families. The catalytic domain of the enzymes belonging to the same family probably evolved from a common, distant ancestor [3–5]. Crystal structures have been reported for members of several families.

Being an endoglucanase, cellulase C from *Clostridium thermocellum* rapidly fluidizes carboxymethylcellulose solutions. However, soluble β -glucans, cellodextrins and especially *p*-nitrophenyl-cellobioside (*p*NPC) are also good substrates. Cellulase C has no significant activity on cellobiose or *p*-nitrophenyl-glycoside [6]. It is a noncellulosomal enzyme [7,8].

The three-dimensional structure of cellulase C was determined by Dominguez et al. [9]. The enzyme, which belongs to family 5 of glycosyl hydrolases, consists of a catalytic domain having

an $(\alpha/\beta)_8$ barrel (or TIM barrel) fold and an additional 54-residue subdomain. This small subdomain contains 4 α -helices and a two-stranded β -sheet, inserted between strand 6 and helix 6 of the barrel, near the substrate binding cleft. Being a unique feature of cellulase C, this subdomain is only present in enzymes belonging to subfamily A3 of family 5 cellulases. Enzymatic hydrolysis of the glycosidic bond occurs via acid catalysis that requires a proton donor (Glu140) and a nucleophile (Glu280). The substrate binding cleft has been identified by binding studies in the crystal and is formed at the carboxy terminal end of β -strands 4 and 7 [10]. Cellulase enzymes of family 5 such as cellulase C hydrolyze the 1,4- β -glycosidic bond through a double displacement mechanism [11].

Although the relationship between stability and structure of a number of TIM barrel enzymes have been extensively studied [12], very little is known about the stability of family 5 cellulases. No hyperthermophilic structure so far has been resolved, and no data are available regarding their folding kinetics. On the other hand, their catalytic properties have been extensively studied [13,14]. Despite the importance of cellulases in industrial reactions at elevated temperatures, little attention has been paid to the conformational stability of these enzymes.

In this paper, we made an attempt to describe quantitatively the thermal and the GdmCl induced unfolding of *Clostridium thermocellum* cellulase C. Thereafter, we designed point mutations with the aim to increase the thermal stability of the enzyme, in a ‘pilot study’.

Because of the low sequence similarity between TIM barrel enzymes, no hyperthermophilic homologue of cellulase C has been identified. The design of the mutants was based on computer modeling studies using the 3D structure of cellulase C. The common fold and the moderate thermostability of this enzyme make it a very appropriate model enzyme for thermal stability studies on TIM barrel proteins. In the design of mutations, we used ‘rules’ of stabilization learned from comparative analyses of thermophilic proteins with their mesophilic and moderately thermophilic homologues [1]. We produced mutants in three categories. First, the most frequently

observed difference between thermophilic and mesophilic proteins is an increase in the number of electrostatic interactions [1]. Therefore, we created mutants potentially leading to the formation of new ion pairs or clusters. Second, thermophilic proteins also tend to contain shorter loop regions [15]. Therefore, we created a mutant where we deleted the small helical subdomain to make the chain shorter and to remove this possibly destabilizing subdomain. Third, crosslinks are known to increase conformational stability by decreasing the entropy of the unfolded state. Therefore, in a third type of mutant, we introduced two cysteine residues near the N- and C-termini of the polypeptide chain in order to create a disulfide bridge, which was expected to increase thermal stability.

2. Materials and methods

2.1. Chemicals

All biochemicals were obtained from Fluka and Sigma Co. Other organic and inorganic chemicals were purchased from Reanal and Sigma Co.

2.2. Strains and growth media

E. coli cultures were grown in Luria broth or on Luria agar [16]. When appropriate ampicillin or chloramphenicol were added at a final concentration of 100 mg/l and 30 mg/l, respectively. *E. coli* strains JM109, BL21(DE3)plysS were used as plasmid hosts.

2.3. Plasmids and site directed mutagenesis

The plasmid containing the gene of *Clostridium thermocellum* cellulase C was a generous gift of Pierre Beguin (Institut Pasteur, Paris, France). All molecular biology procedures were carried out using standard methods [16]. Restriction endonuclease enzymes were purchased from New England Biolabs Co., and used according to the recommendations of the supplier.

The gene of cellulase C was amplified by standard PCR method and inserted at the NdeI-BamHI site of plasmid pET21c, and transformed into *E. coli* strain BL21(DE3)plysS for increased yield of the enzyme. The in vitro mutagenesis

experiments were carried out by the Kunkel method using the pBluescriptII KS+ vector. The DNA sequences of the wild-type and mutant genes were confirmed by sequencing in both directions. The cells were grown to mid logarithmic phase at 37 °C in the presence of 100 mg/l ampicillin and induced by IPTG (1 mM) for overexpression of the wild-type or mutant proteins. After induction the expression was continued for an additional 3 h at 37 °C, then the cells were harvested by centrifugation.

2.4. Protein purification

Wild-type and disulfide+ mutant cellulase Cs were expressed in *E. coli* BL21(DE3)plysS cells containing the expression plasmids. The preparation of homogenous recombinant cellulase C was reported previously [17]. The same purification procedure was used with minor modifications.

2.5. Assay of enzyme activity

The catalytic activity of cellulase C was measured in 100-mM succinate buffer, pH 6.0, containing 0.2-mM pNPC. Initial rates were determined by monitoring the release of the nitrophenol group at 410 nm using Jasco V-500 spectrophotometer. Measurements were carried out at 60 °C as described previously [18].

2.6. Spectropolarimetry and DSC

Thermal unfolding curves and the apparent melting temperatures were determined by differential scanning calorimetry (Microcal VP-DSC) and UV circular dichroism (CD) measurements at 221 nm (Jasco J720). The heating rate was set to 1 °C/min. Protein samples were dialyzed overnight in 20 mM Tris pH 7.2 buffer and diluted to the same (0.4 mg/ml, 10^{-5} M) concentration unless stated otherwise. In the case of reducing conditions, the DTT concentration was set to 50 mM or 100 mM (Section 3).

2.7. Guanidine hydrochloride denaturation

All experiments were carried out at a protein concentration of 0.02 mg/ml and at 25 °C. Solu-

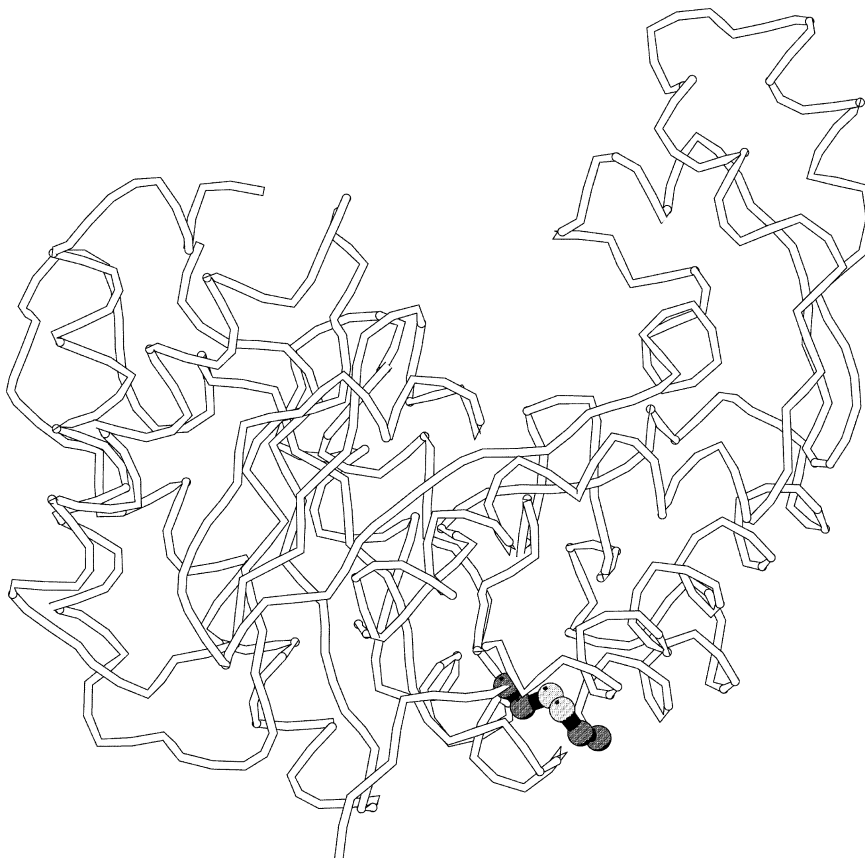


Fig. 1. Ribbon diagram of *Clostridium thermocellum* cellulase C indicating the position of the newly introduced disulfide bond between the N- and C-termini of the molecule.

tions contained 50 mM Tris buffer (pH 7.0), 0.01% Tween 20, 3 mM reduced glutathione (GSH), 0.3 mM oxidized glutathione (GSSG) and different concentrations of guanidine hydrochloride (ranging from 0 to 5 M). Samples were incubated at room temperature for at least 18 h. Denaturation curves were determined by circular dichroism (Jasco 720) at 221 nm. Guanidine hydrochloride stock solutions were prepared and their concentration determined as described previously [19]. Guanidine hydrochloride induced unfolding is almost completely reversible for both the wild-type and the disulfide+ mutant cellulase C. The yields of reconstitution were 90 and 91%, respectively.

2.8. Computer graphics and modeling

We used the InsightII graphical interface and the Discover molecular simulation program from Molecular Simulations, Inc. for molecular visualization and mutant design. All modeling studies were done using the known structure of cellulase C [9] (Protein Data Bank entry 1CEC). For mutations creating ion pairs, a rotamer library search [20] was performed to find suitable conformations for the mutated side chain. These conformations were further refined by energy minimization using the conjugate gradients algorithm. The deletion mutant was designed by selecting the sites of

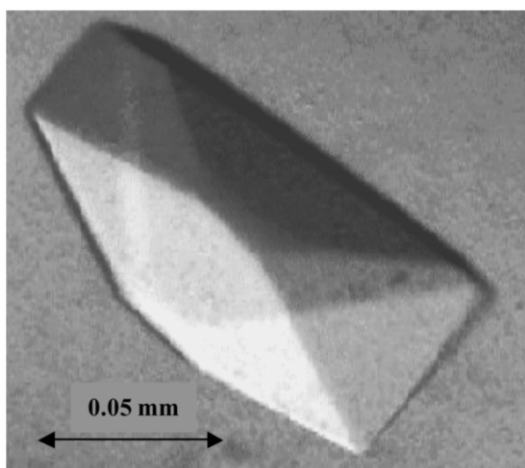


Fig. 2. Crystal form of disulfide+ mutant cellulase C. Bipyramid-shaped crystals belonging to the tetragonal space group were grown at a protein concentration of 3 mg/ml mixed with 2.45 mM ammonium sulfate in 20 mM Tris at pH 7.2, containing 2 mM *n*-octyl- β -D-glucopyranoside.

cleavage that are optimal for removing the small subdomain so that only a short loop remains instead. The double mutation creating a disulfide bond was designed using the SSSEARCH and SSBOND programs (Dr Andrew C.R. Martin, University College, London, personal communication). The former program generates a list of potential sites for introducing a disulfide bridge based on simple geometric criteria, and the latter program actually substitutes cysteines at the specified positions and performs a conformational search to find conformations with optimal separation of the sulfur atoms.

2.9. Crystallization

Crystallization was carried out in Limbro plates by the vapor diffusion hanging drop method at 4 °C as described for the wild-type enzyme [17]. All reservoir solutions contained 20 mM Tris solutions, pH 7.2. Drops contained reservoir and protein solutions at 1:1 ratio, the protein solution contained

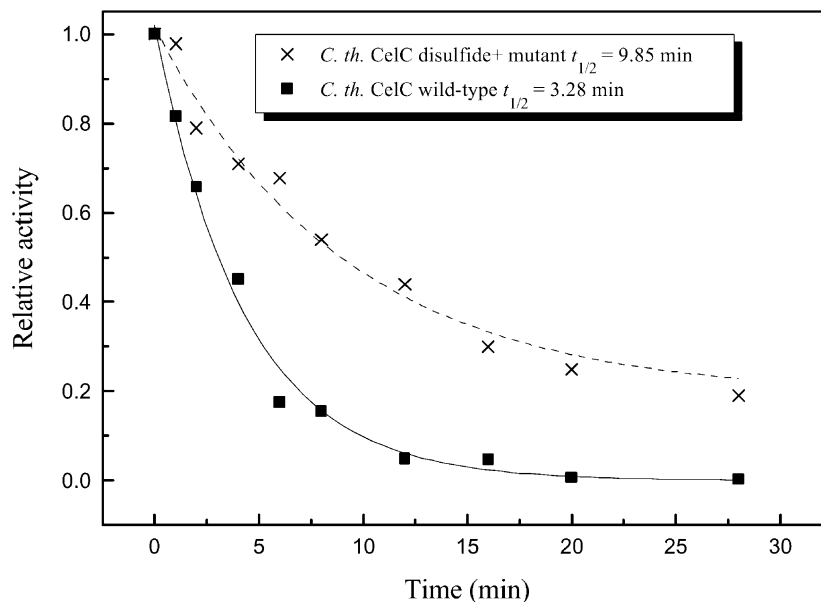


Fig. 3. Thermal inactivation curves of the wild-type *Clostridium thermocellum* cellulase C (■, $t_{1/2}$ = 3.28 min) and the disulfide+ mutant (×, $t_{1/2}$ = 9.85 min). $t_{1/2}$ is the time while the enzyme loses 50% of its activity during incubation at 70 °C. Incubations were performed in 20 mM Tris buffer, pH 7.2 at 70 °C. The measurements were carried out at 60 °C in pH 6.0 succinate buffer, containing 0.2 mM *p*NPC. The final protein concentration was 0.008 mg/ml.

Table 1
Possible ion pair creating mutations and the effect of the mutation on the thermal stability

	Partners forming potential ion pairs	T_m (°C) (DSC)	ΔT_m (°C)
Wild-type		70.4	–
Ile293Arg	Glu333, Glu290, Glu297	67.6	–2.8
Glu332Arg	Glu39	69.4	–1.0
Asn9Asp	Arg46	63.9	–6.5
{Asn220Asp, Thr222Asp}	{Lys209, Arg221 Lys209, Arg221, Lys224}	68.3	–2.1

3 mg/ml protein. Diffraction experiments were carried out on an in-house Rigaku R-AXIS II instrument.

2.10. Other methods

Free sulfhydryl groups were titrated with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in denaturing conditions (6 M guanidine hydrochloride) as described previously by Creighton [21]. The reaction was performed in cuvettes at room temperature. The amount of free thiol was calculated from the liberated 2-nitro-5-thiobenzoate anion using a molar extinction coefficient of 13700/M/cm. Protein concentration was determined by UV absorbance at 280 nm (the theoretical molar extinction coefficient was used: $1.776 \text{ (g/l*cm)}^{-1}$) as well as the BCA method using BSA as standard.

3. Results and discussion

The aim of the present study is to find out how effectively the 'rules' of stabilization learned from comparisons of homologous proteins with different thermostabilities can be used to design a mutant protein with increased thermal stability. In general, minor changes in the protein structure are responsible for the increased thermostability [22]. In the last decades, several structural features have been identified that could contribute to thermostability, including an increased number of surface ion pairs [1,23], a strengthened hydrophobic core [24,25], disulfide bonds [26,27], a decreased number of cavities [28], enhanced helix–dipole interactions and several other factors [29,30]. Using some of these 'rules' we designed mutations in the structure of cellulase C.

3.1. Design of the mutants

Mutations potentially contributing to the formation of new ion pairs were designed by the following procedure. Based on visual inspection of the positions of the charged residues in a 3D structural model, side chains were selected where the incorporation of a negative or a positive charge could create favorable interactions with the surrounding charged residues. These side chains were actually mutated in the program and tested by rotamer search and energy minimization (see Methods) to make sure that they can form favorable interactions with their surroundings. In the end, five appropriate mutants were selected for experimental study (Table 1). In the Ile293Arg mutant, Arg is surrounded by three nearby Glu residues. In the mutant Glu332Arg, Arg can potentially form an ion pair with Glu39, a residue near the N-terminus, thereby creating a link between the two terminal regions. In the double mutant Asn220Asp, Thr220Asp, a network of ion pairs was expected to form (see Table 1). A buried ion pair was also designed: in the Asn9Asp mutant, a buried ion pair can be expected between Asp9 and Arg46.

The subdomain deletion mutant was designed as described in the Methods section. The region 202–249 was selected for removal.

Disulfide bonds can be designed into proteins using simple geometric criteria [31]; the SSSEARCH and SSBOND programs we applied (see Methods) are based on such criteria. From the 46 residue pairs generated by SSSEARCH, the

Ala6-Arg340 pair was selected since this pair spans the longest peptide stretch and therefore a disulfide link introduced here was expected to have a high stabilizing effect. The SSBOND program and molecular modeling was used to make sure that cysteines introduced at these sites can assume a native-like conformation, without overlapping surrounding atoms, while creating a disulfide bond.

3.2. Ion pair mutants

In comparative studies, surface or partially buried ion pairs have been found to play an important role in thermostability. In a comprehensive study, we analyzed a number of thermophilic structures with their mesophilic and moderately thermophilic homologues and found that the most frequently observed characteristic of thermophilic proteins is an increased number of ion pairs [1].

In *Clostridium thermocellum* cellulase C, we have replaced selected amino acid residues to create an ion pair with one or more existing charged residues in their neighborhood. Table 1 lists the mutations.

As Table 1 shows, each mutation destabilized the structure, while the activity of the enzymes remained essentially the same at 60 °C. Clearly, the destabilizing effects, e.g. the new side chains interfering with existing stabilizing interactions, are stronger in all these cases than the possible stabilizing contribution of the introduced electrostatic interactions. Not surprisingly, the largest destabilization is observed with the Asn9Asp mutant where a buried residue was mutated.

Actually, in the literature, it is debated whether surface ion pairs have a stabilizing effect at all [32,33]. The high dielectric constant of water and the desolvation penalty coupled to ion pair formation are all factors that can decrease the possible stabilizing effect of ion pairs. However, at high temperatures, ion pairs are more likely to be stabilizing [34].

3.3. Subdomain deletion mutant

Loops are flexible regions in proteins that can serve as starting points for unfolding. In thermo-

philic proteins, loops are often shorter than in their mesophilic homologues [15]. We produced a deletion mutant of cellulase C to investigate the role of the 54 amino acid subdomain in thermal stability. Although this is not a loop (it has a well-defined internal structure), clearly it can destabilize the protein when it is more flexible than the TIM barrel domain it joins. In our mutant, the complete subdomain (from Pro202 to Asn249) was deleted. This mutant could be purified at nearly the same conditions as the wild-type enzyme, and could fold spontaneously as can be concluded from circular dichroism measurements (data not shown). The mutant, as could be expected, lost its activity on any substrates of cellulase C, but the melting temperature of the $(\alpha/\beta)_8$ barrel domain ($T_m = 67.0$ °C) was similar to that of the wild-type enzyme ($T_m = 70.4$ °C) as measured by circular dichroism and differential scanning calorimetry experiments. This suggests that the 54-residue subdomain may be an independent cooperative unit that is not tightly associated with the TIM barrel domain. Also, it should not be very flexible in solution since it does not destabilize the TIM barrel domain.

3.4. Introduction of a new disulfide bridge

Crosslinks linking two distant peptide segments obviously increase the conformational stability of proteins by decreasing the entropy of the unfolded state. Besides, the terminal regions of proteins are generally more flexible than other parts of the structure. Although the role of terminal regions in protein folding is still unclear, an increase in terminal flexibility may speed up the denaturation process, and promote irreversible aggregation. In single-domain proteins, there is a significant preference for the N and C terminal regions to be in close proximity [35]. This is also the case for cellulase C, which offers the possibility to link the two terminal regions with a crosslink.

A disulfide bridge has been introduced between residues 6 and 340 replacing Ala and Arg side-chains, respectively. Fig. 1. shows the ribbon model of the enzyme showing the location of the disulfide bond. The formation of the covalent

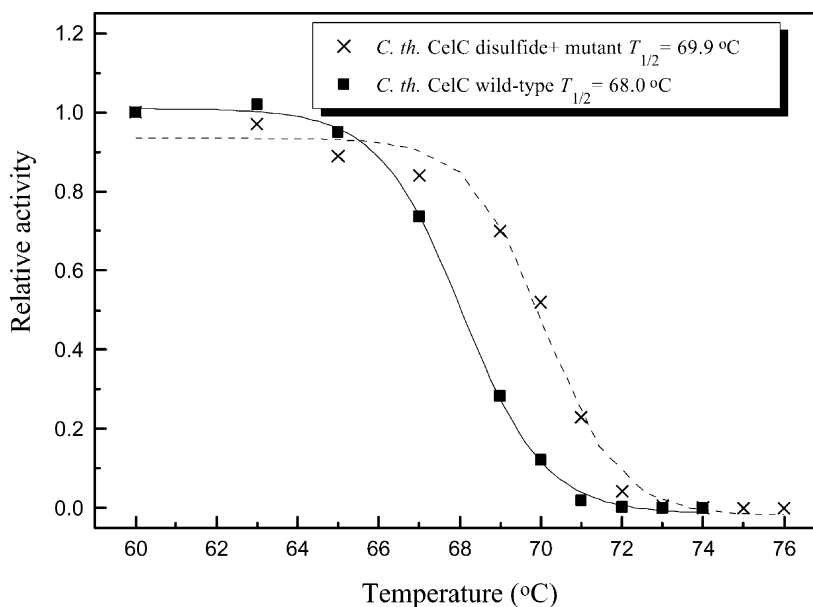


Fig. 4. Thermal inactivation curves of the wild-type *Clostridium thermocellum* cellulase C (■, $T_{1/2}=68.0$ °C) and the disulfide + mutant (×, $T_{1/2}=69.9$ °C). $T_{1/2}$ is the temperature at which the enzyme loses 50% of its activity during 10-min incubation. Incubations were performed in 20 mM Tris buffer, pH 7.2 for 10 min at increasing temperatures. The measurements were carried out at 60 °C in 100 mM succinate buffer at pH 6.0, containing 0.2 mM pNPC. The final protein concentration was 0.008 mg/ml.

disulfide bond was verified as described in *Materials and Methods*. Titration of the wild-type and mutant enzyme by DTNB under denaturing conditions showed that the disulfide bond has been established in the mutant enzyme.

3.5. Crystallization

The mutant enzyme with the extra disulfide bridge crystallizes into the same space group as the wild-type one, and the unit cell dimensions were the same within the limits of error ($a=b=130.6$ Å, $c=69.5$ Å, $\alpha=\beta=\gamma=90^\circ$), indicating highly similar 3D structures. Crystals (see Fig. 2), grown at 2.45 M ammonium sulfate conditions, were measured. Crystals were flat, their dimensions were approximately $0.2 \times 0.15 \times 0.1$ mm. The highest resolution achieved on the first frames was 3.6 Å, however, it was deteriorative on the later ones. Therefore we could not solve the structure at atomic resolution. We can assume that both the wild-type and mutant enzymes have the same conformation and the introduction of a disulfide

bridge between the two termini did not change the overall fold.

3.6. Stability

The enzyme with the extra disulfide bond showed an increased conformational stability against thermal denaturation in all heat denaturation experiments. A shift of 3 °C in T_m was observed. Unfortunately, a rigorous thermodynamic analysis of thermal unfolding curves from wild-type and mutant enzymes was not feasible due to the irreversibility of the thermal unfolding at the conditions used in this study. Figs. 3 and 4 show the thermal inactivation measurements of wild-type and disulfide bridge mutant cellulase C. Aggregation was observed in the last phase of thermal unfolding with both the mutant and the wild-type enzymes.

While the oxidized mutant showed a 3 °C increase in melting point, the reduced double mutant protein had a decrease in T_m of 4.2 °C (Figs. 5 and 6), relative to the wild-type enzyme. This is likely to be due to the removal of the

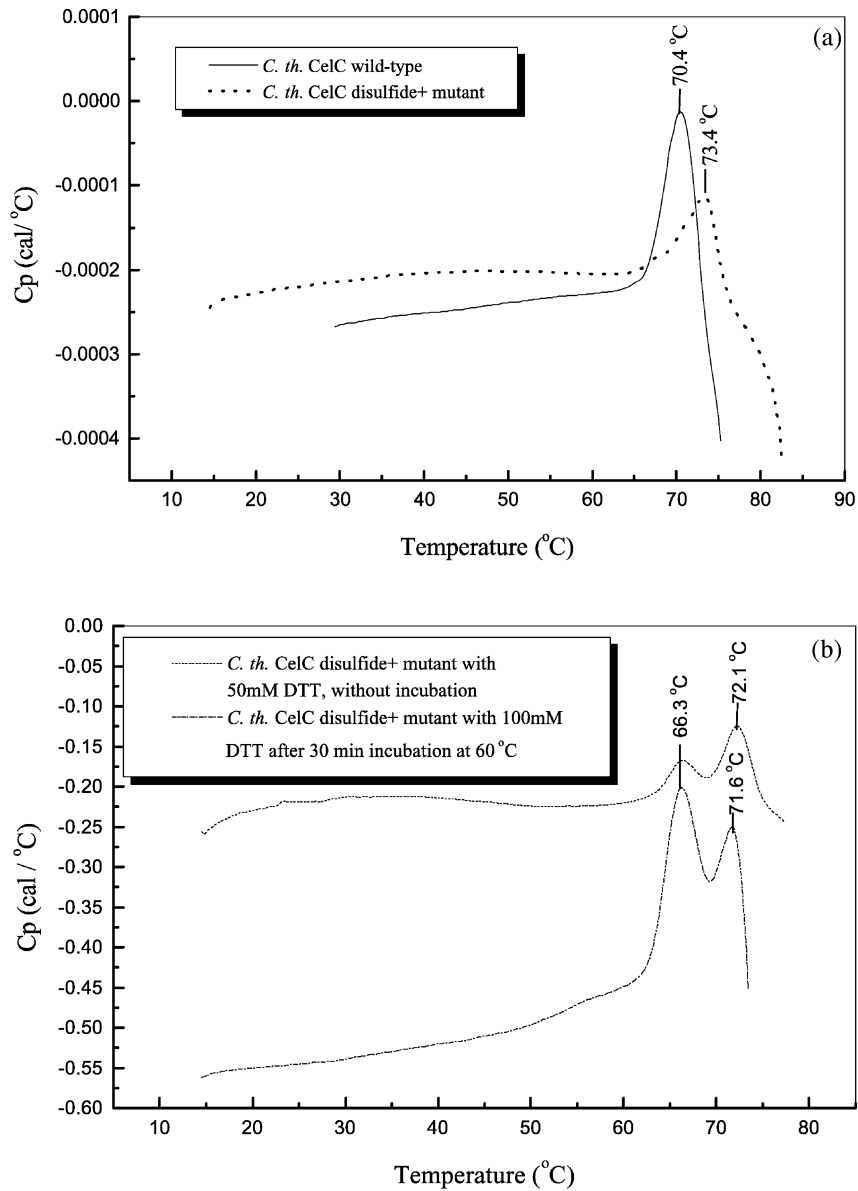


Fig. 5. Partial heat capacity curves of (a) the wild-type *Clostridium thermocellum* cellulase C (solid line, $T_m = 70.4$ °C) and the disulfide+ mutant (dashed line, $T_m = 73.4$ °C), and (b) the disulfide+ mutant cellulase C with 50 mM DTT, without incubation (dotted line, $T_{m1} = 66.3$ °C, $T_{m2} = 72.1$ °C) and with 100 mM DTT after 30 min incubation at 60 °C (dash-dotted line, $T_{m1} = 66.3$ °C, $T_{m2} = 71.6$ °C). The measurements were carried out with a heating rate of 60 °C/h in 20 mM Tris buffer pH 7.2, the protein concentration was 0.4 mg/ml. The unfolding was irreversible.

favorable interactions formed by the alanine and arginine residues that were replaced by cysteines in the mutant. For the reduced enzyme, the calorimetric scans always showed two peaks instead of one as in the oxidized or the wild-type enzyme.

Increasing concentrations of added DTT and prolonged incubation times shifted the T_m to lower temperatures, but even after 30 min incubation with 100 mM DTT, the double peak remained indicating that this pattern does not originate from

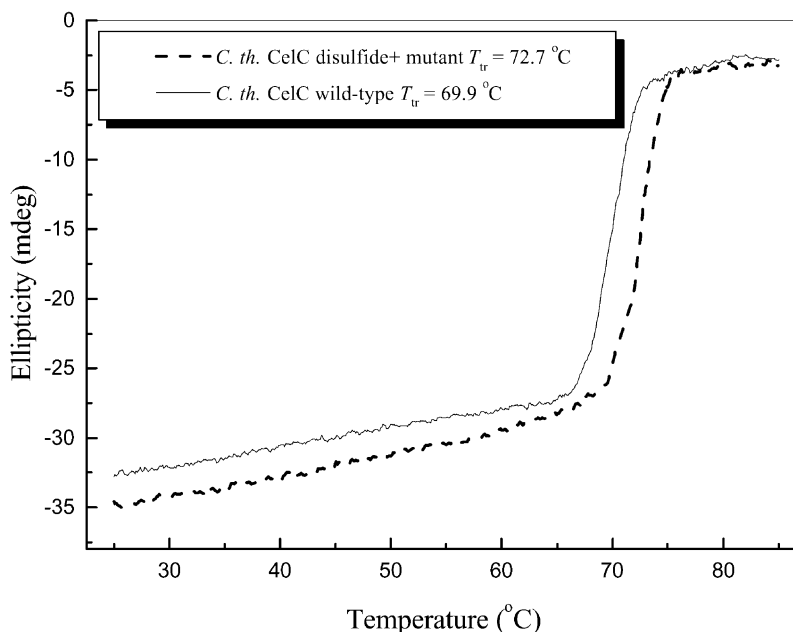


Fig. 6. Thermal unfolding of the wild-type *Clostridium thermocellum* cellulase C (solid line, $T_{tr}=69.9$ °C) and the disulfide+ mutant (dashed line, $T_{tr}=72.7$ °C) followed by CD spectroscopy at 221 nm. The transition temperatures obtained this way show the increased stability of the disulfide+ mutant cellulase C. The measurement was carried out in 100 mM Tris buffer pH 7.2, at a protein concentration of 0.4 mg/ml. The heating rate was 60 °C/h.

a mixture of oxidized and reduced enzymes. The disulfide bond of the mutant is certainly accessible to DTT, which is also indicated by a very slow oligomerization of the mutant during storage (data not shown). The explanation of the appearance of a second peak in the calorimetric curves remains uncertain. It can be argued that in the absence of the disulfide bond, the TIM barrel domain is destabilized and after thermal denaturation of this domain, a cooperative substructure, possibly the small helical subdomain, still remains.

Since heat denaturation was irreversible, we performed equilibrium denaturation measurements, using guanidine hydrochloride (GdmCl) as a denaturant, to obtain unfolding free energies [19]. The normalized ellipticity values as a function of guanidine hydrochloride concentration are shown in Fig. 7. Using the method suggested by Pace [19] we have calculated the midpoints of the GdmCl denaturation curves and the ΔG values for GdmCl induced unfolding. The data are summarized in Table 2.

Fig. 7 and the data in Table 2 clearly indicate that the ΔT_m values observed in the thermal unfolding experiments (Figs. 5 and 6) reflect a real thermodynamic stabilization for the disulfide+ mutant. The calculated change in ΔG is 1.73 kcal/mol.

The major effect of the crosslink on the stability results from a decrease in the conformational entropy of the unfolded molecule. The entropic contribution of a covalent crosslink to a protein stability may be estimated using the following equation [36]:

$$\Delta S(\text{conf}) = -2.1 - 3/2 R \ln n \quad (1)$$

where n is the number of residues between the crosslinked side chains. Using this equation we can predict the ΔG change associated with the introduction of the disulfide bond. The calculated value is 5.8 kcal/mol, larger than that obtained from the GdmCl unfolding experiments. This discrepancy is explained by an enthalpic contribution that may in part result from the unfavorable inter-

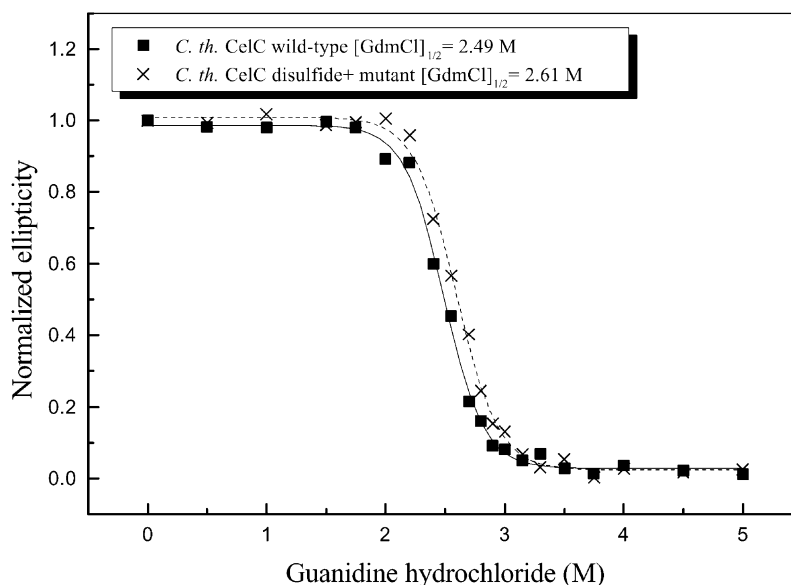


Fig. 7. Equilibrium guanidine hydrochloride denaturation of the wild type *Clostridium thermocellum* cellulase C (■) and the disulfide+ mutant (×). The data points represent the normalized changes in ellipticity of solutions in 50 mM Tris buffer at pH 7.0, containing 3 mM GSH, 0.3 mM GSSG and 0.01% Tween 20. The measurements were carried out at different guanidine hydrochloride concentrations (0–5 M) at 25 °C, the protein concentration was 0.02 mg/ml. The yields of reconstitution were 90% and 91% for the wild type and the disulfide+ mutant cellulase C samples, respectively.

actions of the introduced cysteine residues or a residual structure in the unfolded state.

4. Conclusions

Thermal adaptation is a good example how nature can alter the thermal stability of enzymes while preserving their specific catalytic function. In both research and industry it is of major interest to find ways to design enzymes with increased conformational stability. *Clostridium thermocellum* cellulase C is a good target for such an attempt, since this is a single domain (α/β)₈ TIM barrel type protein with industrial significance.

Our previous comparative studies revealed certain tendencies that relate increased thermal stability to evolutionary amino acid replacements. There were successful attempts to use rationally designed mutants to increase the thermal stability of enzymes.

In the literature, several successful mutations are presented e.g. thermolysin-like protease was made hyperstable by 8-fold mutations, which included an extra disulfide bond [37]. Another successful attempt was to introduce a new ion cluster into the subunit interface of *E. coli* IPMDH [23]. The proteins in these reports are usually small and the changes are at the surface or at

Table 2
Parameters characterizing the GdmCl induced denaturation at 25 °C

Protein	[GdmCl] _{1/2} ^a (M)	$\Delta G^{\text{H}_2\text{O}}$ (kcal/mol)	$\Delta(\Delta G)$ (kcal/mol)
Wild-type cellulase C	2.49	6.30	–
Disulfide+ cellulase C	2.61	8.03	1.73

^a Midpoint of the guanidine hydrochloride denaturation curve.

subunit interfaces. An attempt of Oshima's group [38] to replace a sequential motif at the surface of a mesophilic isopropylmalate dehydrogenase by that of the thermophilic variant led to destabilization.

Our experiments show that even careful design is not always sufficient to consider all effects of a side chain replacement, particularly if it is inside the complex polypeptide matrix. The rules learned from thermophilic proteins cannot be used in a straightforward way to produce proteins with increased thermostability. Creating a crosslink, such as a disulfide bond, is a relatively sure-fire way to increase stability due to the inevitable entropic effect, but even in this case, associated destabilizing effects may lead to a smaller stabilization than expected from a simple entropy calculation.

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