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N-terminal fusion of a hyperthermophilic chitin-binding domain to xylose isomerase from *Thermotoga neapolitana* enhances kinetics and thermostability of both free and immobilized enzymes

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Abstract

Immobilization of a thermostable D-xylose isomerase (EC 5.3.1.5) from *Thermotoga neapolitana* 5068 (TNXI) on chitin beads was accomplished via a N-terminal fusion with a chitin-binding domain (CBD) from a hyperthermophilic chitinase produced by *Pyrococcus furiosus* (PF1233) to create a fusion protein (CBD-TNXI). The turnover numbers for glucose to fructose conversion for both unbound and immobilized CBD-TNXI were greater than the wild-type enzyme: k_{cat} (min^{-1}) was approximately 1000, 3800, and 5800 at 80°C compared to 1140, 10350, and 7000 at 90°C, for the wild-type, unbound, and immobilized enzymes, respectively. These k_{cat} values for the glucose to fructose isomerization measured are the highest reported to date for any XI at any temperature. Enzyme kinetic inactivation at 100°C, as determined from a bi-phasic inactivation model, showed that the CBD-TNXI bound to chitin had a half-life approximately three times longer than the soluble wild-type TNXI (19.9 hours vs. 6.8 hours, respectively). Surprisingly, the unbound soluble CBD-TNXI had a significantly longer half-life (56.5 hours) than the immobilized enzyme. Molecular modeling results suggest that the N-terminal fusion impacted subunit interactions, thereby contributing to the enhanced thermostability of both the unbound and immobilized CBD-TNXI. These interactions likely also played a role in modifying active site structure, thereby diminishing substrate-binding affinities and generating higher turnover rates in the unbound fusion protein.

Keywords

xylose isomerase; hyperthermophile; *Thermotoga neapolitana*; immobilization; chitin-binding domain

Introduction

Due to their intrinsic thermostability, enzymes from hyperthermophilic microorganisms have been considered for improving existing biocatalytic processes and generating novel routes for biotechnological products.^{1–4} In either case, immobilization of the enzyme is often desirable to avoid contaminating the product stream, as well as to recover and recycle the biocatalyst.^{5,6} High temperature bioprocessing conditions present certain challenges for enzyme immobilization. Enzyme entrapment in a porous solid phase, such as cross-linked

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agarose beads, necessitates that the matrix be impervious to thermal conditions, but the overall process efficiency can be diminished at any temperature due to mass transfer limitations.^{7–9} Non-covalent and covalent attachment strategies have previously been employed for immobilizing hyperthermophilic enzymes.^{10–14} However, based on electrostatic and hydrophobic multipoint interactions between the protein and immobilization surface, physical adsorption of hyperthermophilic enzymes may not be sufficiently strong at elevated temperatures to prevent displacement. Immobilization by covalent attachment often involves using toxic chemicals, such as carbodiimides and succinic anhydride,^{15,16} to modify the support surface and bind the enzyme. These treatments can result in diminished enzyme activity due to the immobilization process, thus impacting the biocatalytic capacity and accelerating enzyme inactivation.

One attractive method for enzyme immobilization involves engineering a fusion protein containing an affinity tag that binds the enzyme to a support matrix. Many examples of this strategy have been reported,¹⁷ including metal affinity tags based on poly-His residues inserted at the N- or C-terminus that are commonly used for small scale recombinant protein purification. Carbohydrate-binding domains (CBDs), discrete protein folding domains responsible for binding insoluble carbohydrate substrates such as chitin and cellulose, have also been used as affinity tags for protein purification and enzyme immobilization.¹⁸ Several CBDs have been identified through the characterization of cellulases and chitinases and the analysis of genome sequence data (for a complete listing, see the CAZY database at <http://afmb.cnrs-mrs.fr/CAZY/>). The thermostability of the CBD is an important consideration for enzyme immobilization at elevated temperatures. Unfortunately, CBDs are rare in the sequenced genomes of hyperthermophilic microorganisms, and CBDs from less thermophilic sources may not be sufficiently thermostable for high temperature bioprocessing applications. For instance, the CBD from the mesophilic soil bacterium *Cellulomonas fimi* denatured at 78°C when bound to crystalline, bacterial cellulose.¹⁹ Although rare in hyperthermophiles, chitin-binding domains (CBDs) have been identified in chitinases produced by hyperthermophilic archaea: ChiA in *Thermococcus kodakarensis*^{20–23} and ChiA-ChiB (PF1233-PF1234) in *Pyrococcus furiosus*.^{24,25} The three-dimensional structure of a CBD from *P. furiosus* has been recently reported²⁶, providing some insights into how these polypeptides function at elevated temperatures. CBDs from *P. furiosus* have been used to create cellulose-degrading fusion proteins,^{27–29} but have not been used to immobilize enzymes on chitin. Chitin, a β -1,4-linked N-acetyl-D-glucosamine homopolymer is one of the most abundant natural polysaccharides and is the main structural component of fungal cell walls and arthropod exoskeletons.³⁰ Chitin is inexpensive, chemically inert, and has low non-specific binding affinity for most proteins,¹⁹ properties which make it attractive for affinity-based enzyme immobilization support.^{31–33}

Xylose isomerases (XI), which find wide industrial use as “glucose isomerases” in the production of high fructose corn syrup³⁴ represent the largest commercial application of an immobilized enzyme. XIs are classified into two groups (class I and class II), based on primary amino acid sequence homology. The class II enzymes contain an additional 30–40 amino acid insert at the N-terminus that is not present in the class I enzymes.³⁵ The functional role of this N-terminal configuration in class II XIs is not known. The crystal structures for class I and class II XIs reveal that large parts of their assemblies are virtually superimposable,³⁶ however, structural differences do exist, primarily localized at the C-terminal loop and N-terminus. The C-terminal loop in both XI classes makes many inter-subunit contacts in the assembled tetrameric enzyme, although none of these contacts appear to be involved with the interface that contains the active site.^{36,37} Current commercial processes for HFCS production utilize class I XIs, but highly thermostable and thermoactive class II XIs have been identified in hyperthermophilic bacteria of the genus *Thermotoga*.^{38–41} These enzymes offer the prospect of operation at elevated temperatures

for extended periods of time, taking advantage of favorable equilibrium characteristics of ketose over the aldose.³⁸ The inactivation behavior of the *Thermotoga neapolitana* XI (TNXI) was previously examined when immobilized by covalent linkage to glass beads.¹⁴ However, this approach would not be suitable for large-scale bioprocess applications. Although chitin has previously been used as an immobilization support for XIs from mesophilic bacteria, this required cross-linking with glutaraldehyde.^{42,43} There has been no reports of XIs immobilized through the addition of CBD tags. Here, we explore the prospect of affinity immobilization of the class II XI from *T. neapolitana* to chitin via a fusion to a hyperthermophilic CBD from *P. furiosus*.

Materials and Methods

Bacterial Strains and Plasmids

E. coli strains XL1-Blue (Novagen, Madison, WI) and BL21(DE3) (Novagen) were used for cloning and expression of the *T. neapolitana* 5068 *xyIA* gene (TNXI), respectively. The wild-type and N-terminal deletion mutants were cloned as an *NdeI*-*Hind III* insert into pET22b(+) (pTNXI).⁴¹ The CBD-TNXI plasmid (pTNXICBD) was created from the pTNXI, as described below.

Production of *xyIA* N-terminus Deletion Mutants

N-terminal truncations of *xyIA* were introduced via PCR. Forward primers were designed to introduce an *NdeI* restriction site before the desired truncation. The plasmid pTNXI was used a template and each forward primer was designed to begin at various intervals along the N-terminus of *xyIA*. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). PCR products were treated with *DpnI* to degrade the pTNXI template prior to cloning into pET22b(+). Plasmids with N-terminal truncations are listed in Supplementary Table 1.

Assembly of the CBD-TNXI Fusion Construct into pCBD-TNXI

The *xyIA* gene was cloned as *NdeI*-*HindIII* inserts into plasmid pET22b(+), thereby creating pTNXI and its derivatives.⁴¹ Based on sequence analysis of *chiB* (PF1233) in *P. furiosus* (DSM3638),²⁴ primers were designed to clone the chitin-binding domain for insertion into pTNXI. The primer set 5'-CAA CGC TAC ATA TGA CAA CTA CCC CTG-3' (forward primer) and 5'-GAG TTG TCA TAT GAG TTG GTG TCC AAA TG-3' (reverse primer) was used to clone a ~350 base pair fragment of the *chiB* gene containing the CBD from *P. furiosus* (DSM 3638) genomic DNA. Both primers included an *NdeI* restriction site (underlined) to insert the CBD at the N-terminus of pTNXI. The pTNXI was digested with *NdeI* followed by treatment with shrimp alkaline phosphatase (SAP), and the de-phosphorylated plasmid was ligated with the *NdeI* treated PCR product and transformed into *E. coli*. The resulting colonies were screened to check for correct orientation of the insert as follows. Plasmid mini-preps were prepared from 3–5 ml overnight cultures (Qiagen, Valencia, CA). Positive results from digests were also confirmed via PCR, using the *chiB* forward primer and the pTNXI C-terminus primer.

Purification of Recombinant TNXI and CBD-TNXI

Both recombinant wild-type TNXI and CBD-TNXI were purified from 1-L cultures grown on LB medium. After centrifugation for 10 min at $4,000 \times g$, the cells were re-suspended in 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS) (pH 7.0), containing 5 mM MnSO_4 and 0.5 mM CoCl_2 (i.e., buffer A). Cells were disrupted by two consecutive passes through a French Pressure cell (ThermoSpectronic, Waltham, MA), using a pressure drop of 11,000 psi. After centrifugation at $5,000 \times g$, the supernatant was heat-treated for 15 min at 65°C.

The precipitated material was separated by centrifugation at $5,000 \times g$ for 30 min. The soluble fraction was filtered by a $0.45 \mu\text{m}$ filter and loaded on a DEAE-Sepharose Fast-Flow column equilibrated with buffer A. The protein was eluted with a linear 0–0.5 M NaCl gradient in buffer A, and the active fractions were analyzed by SDS-PAGE. Partially purified enzymes were loaded onto a Q-Sepharose column and eluted with a linear 0–0.5 M NaCl gradient in buffer A. Active fractions were combined and concentrated in a stirred ultrafiltration cell (Amicon, Beverly, MA), dialyzed against buffer A, and stored at 4°C . Protein concentrations were assayed by the method of Bradford using bovine serum albumin as the standard.

Kinetics for TNXI, and Unbound and Immobilized CBD-TNXI

Michaelis-Menten kinetics were determined based on time-course conversion of D-glucose D-fructose at 80 and 90°C . Briefly, $50 \mu\text{L}$ of substrate containing 10–500 mM D-glucose, 5 mM MnCl_2 and 0.5 mM CoCl_2 was pre-heated for 15 minutes at 80 or 90°C and then added to $50 \mu\text{L}$ of enzyme solution ($\sim 1 \text{ mg/ml}$). After 0.5 minute, the solution was placed on ice to stop the reaction. Abiotic isomerization was accounted for with a negative control, which contained only the substrate and buffer solution without the enzyme. Samples were processed by micro-centrifugation on 3 kDa membrane filters (Microcon) prior to analysis. The reaction products were measured using a resorcinol assay for ketose determination.⁴⁴ To test the amount of fructose produced, $50 \mu\text{l}$ of the mixed reaction solution was aliquoted into 96 well microplate and $150 \mu\text{l}$ of a 1:1 mixture of 0.05% resorcinol in ethanol and $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12 \cdot \text{H}_2\text{O}$ in concentrated HCl was added. For color development, the plate was incubated at 80°C for 40 min. The absorption was measured using a Perkin Elmer HTS 7000 Plus microplate reader at 490 nm for D-fructose. One unit of D-xylose isomerase catalyzed the formation of $1 \mu\text{mol}$ ketose (fructose) min^{-1} at 80°C in this assay system.

Temperature Optimum

The effect of temperature on CBD-TNXI activity was determined in 100 mM Mops buffer, containing 1 mM CoCl_2 . The $\Delta\text{pK}_a/\Delta T$ of Mops (-0.011) was taken into account to ensure a pH of 7.0 at all temperatures assayed. The enzyme (0.10 mg/mL) and substrate (300 mM fructose) were pre-incubated separately for one minute at the temperature of interest in either a Perkin-Elmer Cetus PCR system (Perkin-Elmer) (temperatures $\leq 100^\circ\text{C}$) or a heat block (temperatures $> 100^\circ\text{C}$). Then the enzyme and substrate were combined in equal volumes ($50 \mu\text{l}$ each) and incubated for 5 minutes. The reaction was stopped by submersion in an ice bath. The amount of glucose produced was quantified as previously described. One unit of isomerase activity is defined as the amount of enzyme that produces $1 \mu\text{mol}$ of product per minute under the assay conditions.

Differential scanning calorimetry (DSC)

Melting temperatures for free and immobilized enzymes were determined with a Nano-Cal differential scanning calorimeter (Calorimetry Sciences Corp., Provo, UT), using a scan rate of 1°C/min . All TNXI variants (TNXI conc. 1.0 mg/mL) were dialyzed overnight at 4°C against 2 L of 50 mM Mops, pH 7.0, containing 5 mM MgSO_4 and 0.5 mM CoCl_2 . The CBD-TNXI (1.1 mg/mL) fusion protein was immobilized onto chitin following dialysis. CBD-TNXI was dialyzed overnight at 4°C against 2 L of 50 mM Mops, pH 7.0, with 5 mM MnCl_2 and 0.5 mM CoCl_2 for metal-bound enzyme or 50 mM Mops, pH 7.0, with 10 mM EDTA for apo enzymes. Dialysis buffer was used as a baseline for the free enzymes and dialysis buffer containing chitin beads was used as the baseline for the immobilized enzyme. Each sample was scanned from 25°C to 125°C and the data was analyzed by vendor-provided software.

TNXI immobilization on chitin and thermoactivity/thermostability assays

CBD-TNXI (1.5 mg/mL) was mixed with chitin beads (New England Biolabs, Beverly, MA) and incubated overnight at 4°C. The weight of the chitin beads was measured before and after immobilization, and at least 1 mg of protein was loaded onto the beads for each experiment. The enzyme-chitin mixture was then heated to 55°C for 15 minutes and poured into jacketed, fritted glass columns (0.7 × 15 cm) (Sigma Chemical Co., St. Louis, MO) to drain. The chitin beads were re-suspended in 40 mL of buffer A and centrifuged at 8,000 × g for 10 minutes; the supernatant was discarded to remove any unbound enzyme. The beads were re-suspended for a final wash in 5 mL of buffer A and spun for 5 minutes at maximum speed in a Denville 260D centrifuge at 14,000 × g. The supernatant was discarded and the beads were re-suspended in 5 mL of a 600 mM fructose solution in buffer A, which was incubated in a water bath at 75°C for 15 minutes and then placed on ice. A 5 mL sample of the fructose solution was incubated along with the samples to correct for non-enzymatic isomerization. A 2 mL sample was taken from each of the chitin bead solutions and spun for 5 minutes at maximum speed in a Denville 260D centrifuge (14,000 × g) to remove the beads from the suspension. The supernatant was analyzed for glucose concentration, as described previously.

To determine the effects of temperature on the immobilized enzyme, the beads were suspended in buffer A to achieve a concentration of 0.5 mg/mL. The substrate (600 mM fructose) was prepared in 50 mM Mops buffer with the pH adjusted to 7.0 at the incubation temperature. The reaction was initiated by combining 100 µL of each solution and then incubated for 15 minutes in a water bath at the temperature of interest. A control with buffer and no enzyme was also incubated to account for non-enzymatic isomerization. The reaction was stopped by immersion in an ice bath; the amount of glucose formed was quantified, as previously described.

The thermostability of CBD-TNXI immobilized onto chitin beads was also investigated at 90 and 100°C. The fusion protein was immobilized as described previously in buffer A, with the resulting wet bead weight of ~ 1.0 mg. The substrate (100 mM glucose) was prepared in 50 mM Mops buffer (pH 7.0), containing 5 mM MnCl₂ and 0.5 mM CoCl₂ at pH 7.0, and heated to the desired temperature. The reaction was initiated by combining 100 µL of substrate with 100 µL of the wet bead solution, and then tracked at either 90 or 100°C for 17 hours. Time course samples were taken and analyzed using the previously described resorcinol assay.

Structural Modeling

The *P. furiosus* CBD amino acid sequence was added *in silico* to the N-terminus of TNXI at the *NdeI* site so that the fusion protein could be examined using the protein modeling program 3D-jigsaw (<http://bmm.cancerresearchuk.org/~3djigsaw/>).⁴⁵ This program was used to predict a three-dimensional structure based on homology to the existing structures of TNXI (1A0E) and *P. furiosus* CBD (2crw) in the Protein Data Bank (PDB). The predicted 3D structure of CBD-TNXI was visualized and compared to the native TNXI structure using the UCSF Chimera molecular graphics program (<http://www.cgl.ucsf.edu/chimera/>).⁴⁶

Results and Discussion

CBD fusion site and role of N-terminus in the structure and function of TNXI

CBDs can be engineered into fusion proteins to serve as N-terminal, internal, or C-terminal affinity tags.^{47–49} The β/α₈ barrel motif predominates in the TNXI structure and includes the catalytic site, which precludes engineering an internal CBD. Previously, the C-terminus of a class I XI from an *Arthrobacter* species was removed post-translationally without

affecting the stability or activity of the enzyme.³⁷ However, the proximity of the C-terminus to the other subunits in class II XIs makes it a less than ideal location than the N-terminus, which extends away from the rest of the enzyme. In fact, analysis of the available TNXI structural data suggest possible complications with formation of subunit interactions if a C-terminal CBD fusion was used for TNXI immobilization.

While it appears that the N-terminus of TNXI and of other class II XIs does not play a critical structural role, no experimental information is available on this issue. Comparison of amino acid sequence information from class I and class II XIs suggests that at least a portion of the class II N-terminus plays a role in protein structure (see Figure 1). This question was investigated here through a series of N-terminal deletion mutants, created by systematically eliminating sets of residues after the initial methionine. A 41-amino acid (aa) deletion mutant (TNXI_41) completely eliminated the class II N-terminal insert from the enzyme (based on comparison to the class I XI from *Streptococcus murinus*³⁸), truncating the enzyme at the start of a $\beta\alpha_8$ barrel fold (Figure 2). A 34-aa deletion mutant (TNXI_34), on the other hand, creates an N-terminus that corresponded in length to class I XIs. However, neither mutation produced a soluble or active enzyme, indicating that some portion of the class II TNXI N-terminus is required for proper conformation. Mutants TNXI_12 and TNXI_24 were then created to help determine the residual length of N-terminus needed to produce an active enzyme. TNXI_24 was inactive, while the TNXI_12 mutant isomerized fructose to glucose at normal assay temperatures. The TNXI_12 mutant behaved exactly as wild-type TNXI during purification, and had a comparable temperature optimum (90–95°C). However, the TNXI_12 mutant did not exhibit the characteristic two melting transitions (99.5°C and 110°C) observed for the native enzyme in the presence of two activating metals.^{14,40,41} Instead, the TNXI_12 had only a single melting transition at 105.4°C (data not shown). To further examine the extent that the N-terminus was needed for active enzyme, mutants covering the range from 13–23 aa were created. The mutants up to TNXI_18, with the exception of TNXI_16, were able to isomerize fructose, while deletions TNXI_19 and higher produced inactive, insoluble enzyme. This indicates that some portion of the N-terminal insert is critical for a properly folded and active enzyme. Analysis of the crystal structure revealed that residues 1–12 are situated outside the core structure and not associated with subunit interactions. In the region around residues 16–20, there are more hydrophobic interactions with the surface of the $\beta\alpha_8$ fold and the surface of other subunits, which corresponds to the point where the enzyme will no longer properly fold.

CBD-TNXI immobilization

CBDs are classified into 14 different families based on amino acid sequence, binding specificity, and structure.¹⁸ While the binding of some CBDs to cellulose is reversible (such as the Family 1), the Family 2 CBDs bind irreversibly and exhibit little or no enzyme leakage.⁵⁰ In fact, several Family 2 CBDs have been used commercially as affinity tags for purification.⁵¹ In view of this, the Family 2 CBD from *P. furiosus* chitinase ChiB (PF1233)²⁴ was selected to create a fusion protein at the N-terminus of TNXI. This CBD was previously used to create a fusion protein with a *Thermotoga maritima* endoglucanase.²⁷

To test for immobilization efficacy and non-specific binding, both recombinant *E. coli* crude extract containing TNXI-CBD and purified recombinant TNXI without the CBD, were incubated in a solution containing chitin beads at 4°C overnight. Initially, both enzyme samples decreased in protein concentration by about 0.7 mg/mL. However, after washing the chitin with buffer, the activity of the TNXI-CBD beads was over 20 times greater than the beads with TNXI (430 U/mg vs. 15 U/mg specific activity, respectively). When the beads were treated with a Coomassie blue protein dye (Bio-Rad protein assay), the TNXI-CBD

bound beads turned blue indicative of protein, while the wild-type TNXI beads remained white.

Thermostability of TNXI, Unbound CBD-TNXI and Immobilized CBD-TNXI

Differential scanning microcalorimetry was used to examine the melting behavior of the wild-type TNXI and the unbound and immobilized CBD-TNXI. Previous results with TNXI had shown that wild-type enzyme exhibited two thermal transitions that were associated with the binding of divalent cations Co^{2+} and Mn^{2+} ; while the apo-enzyme was characterized by a single melting transition.⁴⁰ Similar results were observed in this study, although the temperatures of the two transitions varied for the halo-enzyme. TNXI-apo melted at 97.5°C, while transitions at 100°C and 112°C were observed in the presence of divalent cations Co^{2+} (0.5 mM) and Mn^{2+} (5.0 mM) (see Table 1). The apo version of the immobilized enzyme melted at 104°C, with transitions at 87°C and 110°C for the halo enzyme. The unbound CBD-TNXI showed a lower melting transition temperature for both the halo and apo version of the soluble enzyme (60°C/110°C and 71°C, respectively).

Enzyme inactivation was determined for the three versions of TNXI. Inactivation data was analyzed using the bi-phasic inactivation model described previously.⁴⁰ The wild-type enzyme exhibited a half-life of 6.8 ± 0.6 min at 100°C, compared to 19.9 ± 1.5 min for the immobilized enzyme (see Table 2). Surprisingly, the unbound CBD-TNXI had a significantly longer half-life (56.5 ± 9.0 minutes) at 100°C than either the bound CBD-TNXI or the wild-type.

Kinetics of TNXI, Unbound CBD-TNXI and Immobilized CBD-TNXI

The kinetics of the wild-type TNXI was compared to the unbound and immobilized CBD-TNXI. The conversion of glucose to fructose was monitored at 80°C, the growth temperature optimum for *T. neapolitana*, and 90°C, closer to the temperature optimum for the enzyme. Data were fit to a Michaelis-Menten model that accounts for substrate inhibition and reversible product formation; assuming in all cases that TNXI was dimeric and contained one active site per monomer.⁴¹ Substrate inhibition was found to be minimal for all three versions of TNXI. Turnover numbers (k_{cat}) for both TNXI and the immobilized CBD-TNXI increased approximately 25% from 80 to 90°C, although the immobilized enzyme was 6-fold more active at both temperatures (see Table 3). K_M 's for both immobilized and wild-type TNXI also increased between 80 and 90°C, such that the effect of temperature on the catalytic efficiencies in both cases was minimal. In contrast, k_{cat} for the unbound CBD-TNXI increased from 3,798 min^{-1} at 80°C (4 times that for TNXI) to 10,353 min^{-1} at 90°C (9 times that for TNXI at 90°C and 50% higher than the immobilized CBD-TNXI at 90°C). Furthermore, the K_M for the unbound CBD-TNXI (158 mM) was comparable to the immobilized CBD-TNXI (122 mM) at 80°C but nearly 4-fold higher (536 mM vs. 149 mM) at 90°C. Thus, the addition of the CBD at the N-terminus significantly increased conversion rates of glucose to fructose, with the immobilized version being more active at 80°C and the unbound version most active of all at 90°C. Note that the k_{cat} values observed here for the unbound and immobilized versions of TNXI represent the highest ever reported for the biocatalytic conversion of glucose to fructose for any enzyme at any temperature.⁵²

Molecular basis for the stabilization and enhanced activity of CBD-TNXI

Molecular modeling tools were used to gain insights into the reasons for the improvements observed in stabilization and activation of TNXI with a CBD fusion partner. The structure of CBD-TNXI was predicted using 3D-jigsaw,⁴⁵ based on the solved structures of TNXI (PDB 1a0e) and the *P. furiosus* CBD from PF1233 (PDB 2cwr). The modeling program Chimera⁴⁶ was used to create the threaded dimeric structure. Initial comparison of the predicted dimeric

forms of unbound CBD-TNXI and soluble TNXI showed a more flexible and open active site in the fusion protein. Upon closer examination and determination of residue displacements (Table S2), the CBD-TNXI and TNXI active site residues were, indeed, not superimposable. A previous report showed that the VAL185THR mutation in the *Thermus thermophilus* xylose isomerase (TTXI), and the same mutation in TNXI, improved the catalytic efficiency of these enzymes on glucose.⁵³ In the TTXI mutant case, the VAL185THR mutation contributed to additional hydrogen bonding of Thr to the glucose's C6-OH resulting in a lower K_m for glucose and increased catalytic efficiency. The TRP138PHE mutation in TTXI also improved thermal stability, doubling the half-life of the enzyme at 85°C.⁵⁴ Here, the CBD fusion could have enabled hydrogen bonding to produce the same effect (Figure 3). The proximity of CBD residues GLU47 (15.68 Å), GLY48 (13.95 Å), TYR51 (16.01 Å), GLY50 (14.13 Å), and ASN49 (10.34 Å) to VAL185/253 creates the possibility of favorable interactions (strong and weak hydrogen bonds, electrostatic interactions, and van der Waals interactions) that stabilized glucose in the active site.⁵⁵ In any case, this local shift in active site residue orientation likely affected the enzyme-substrate binding properties and/or the reactivity of the residues during catalysis leading to the increased catalytic efficiency and increased K_M . Molecular modeling results also suggest that the N-terminal fusion could impact subunit interactions, thereby contributing to the enhanced thermostability of both the unbound and immobilized CBD-TNXI. Indeed, the inactivation data (Table 2) show that the soluble and immobilized states of CBD-TNXI (56.5 min and 19.9 min, respectively) are more thermostable than the native TNXI (6.8 min).

Conclusions

Immobilization strategies for thermostable enzymes are more limited because of the constraints arising from elevated temperatures. One option is the use of thermostable CBDs fused to the enzyme of interest, which is demonstrated here with the pairing of a hyperthermophilic xylose isomerase from *T. neapolitana* (TNXI) to a CBD associated with a chitinase from the hyperthermophilic archaeon *P. furiosus*. The increased conformational flexibility in the active site in the unbound and immobilized CBD-TNXI not only improved substrate to product turnover but also enhanced thermostability for this hyperthermophilic enzyme. This fortuitous result led to the most thermostable and thermoactive xylose isomerase yet reported. Detailed structural analysis is needed to more definitively identify the active site structure in the fusion protein and possible implications away from the catalytic center. Also, the possibility that, upon immobilization, enzyme-support interactions adversely affected biocatalyst function cannot be ruled out. In any case, this work demonstrates that hyperthermophilic enzymes can be paired with hyperthermophilic CBDs to create fusion partners that are functional at elevated temperatures, and in this instance, have improved biocatalytic and thermostability properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

N-terminal sequences of representative Class I (*Arthrobacter missouriensis* – AMXI and *Streptomyces murinus* – SMXI) and Class II (*Thermotoga neapolitana* - TNXI and *Thermus thermophilus* - TTXI) xylose isomerases showing additional amino acid residues for the Class II enzymes conserved tryptophan (W) and phenylalanine (F) residues shown in bold. α -helices represented as , β -sheets as .

#Amino acids Deleted	12	13	14	15	16	17	18	19	20	21	22	23	24	34	41
Active?	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	No	No	No	No

Figure 2. Consequence of TNXI N-terminal mutations

Bolded portion represents the maximum number of amino acid residue deletion that still retained activity Class I *Streptomyces murinus* (SMXI) N-terminus shown for comparison.

Figure 3. Influence of CBD on non-covalent bonding with active site residues (eg, VAL185)
The displacement distances from VAL185 to GLY48 (13.95Å), GLU47 (15.68Å), to ASN49 (10.34Å), to GLY50 (14.13Å), and to TYR 51 (16.01Å) suggest the possibility of interaction of active site residues to the N-terminal CBD.

Table 1

Differential Scanning Calorimetry of TNXI and CBD-TNXI.

Enzyme	Format	Melting Transition(s)	Added to Reaction Buffer *
TNXI	Soluble	100°C, 112°C	5 mM Mn ²⁺ , 0.5 mM Co ²⁺
TNXI (apo)		97.5°C	10 mM EDTA
CBD-TNXI	Unbound	60°C, 110°C	5 mM Mn ²⁺ , 0.5 mM Co ²⁺
CBD-TNXI (apo)		71°C	10 mM EDTA
CBD-TNXI	Immobilized	87°C, 110°C	5 mM Mn ²⁺ , 0.5 mM Co ²⁺
CBD-TNXI (apo)		104°C	10 mM EDTA

*
50 mM MOPS, pH 7.0

Table 2

Thermostability of Soluble and Immobilized TNXI.

Enzyme	Format	Half-life (min) at 100°C
TNXI	Soluble	6.8 ± 0.6
CBD-TNXI	Soluble	56.5 ± 9.0
CBD-TNXI	Immobilized	19.9 ± 1.5

Table 3

Kinetic Parameters for Wild-type, Unbound and Immobilized TNXI.

Enzyme	Format	T (°C)	V _{max} (U/mg)	K _M (mM)	K _i (mM)	k _{cat} (min ⁻¹)	k _{cat} /K _M (mM ⁻¹ min ⁻¹)
TNXI	Soluble	80	19.1 ± 3.4	65.2 ± 9.0	--	971	14.9
		90	22.4 ± 1.3 *	88.5 ± 16.5 *	--	1139	12.9
CBD-TNXI	Unbound	80	74.7 ± 17.0	157.8 ± 0.4	27.5 ± 1.7	3798	24.1
		90	203.6 ± 15.9	536.1 ± 17.3	--	10353	19.3
CBD-TNXI	Immobilized	80	113.6 ± 11.9	121.5 ± 153	16.9 ± 16	5776	47.5
		90	136.9 ± 11.0	148.9 ± 4.4	--	6961	46.7

* Data from Vielle et al (1995)