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Protein engineering of a cold-active β-galactosidase from *Arthrobacter* sp. SB to increase lactose hydrolysis reveals new sites affecting low temperature activity

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Abstract We examined variants of an especially cold-active β-galactosidase (BgaS) to better understand features affecting enzyme activity at temperature extremes. We targeted locations corresponding to a region in the LacZ enzyme previously shown to increase activity and decrease thermostability. Changes in this region of BgaS consistently caused the elimination or reduction of activity. A gene (*bga*S3) encoding a loss of function variant was subjected to random mutagenesis to restore activity and discover potential interactions important in cold activity. Gene sequences from the resulting library indicated that only two amino acid alterations, E229D and V405A, were required to restore activity. Genes with combinations of these mutations were constructed and their enzymes purified. Enzymes with the E229D/V405A/G803D alterations (BgaS6), or E229D/V405A (BgaS7) had similar thermal optima and thermostabilities as BgaS. BgaS7, however, showed a 2.5-fold increase in catalytic activity at 15°C and hydrolyzed 80% of lactose in skim milk in less than half the time of BgaS at 2.5°C. Computer-generated models predicted that the substitutions at positions 229 and 405 yielded fewer contacts at the enzyme's activating interface. Results from regional saturation mutagenesis supported this hypothesis and suggested that not easily predicted, subtle, cooperative intramolecular interactions contributed to thermal adaptation.

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Introduction

One of the intriguing questions about extremophiles growing at high or low temperatures is how their enzymes have adapted to maintain activity. There has been considerable interest in determining the structural and mechanistic features responsible for the thermal properties of enzymes (Rossi et al. 2003; Sheridan et al. 2000; Zartler et al. 2001) and many suggestions for manually adjusting the 'thermostat' of individual enzymes have been made, such as changing specific amino acids (Oikawa et al. 2001; Russell 2000) and altering enzyme flexibility (Zavodszky et al. 1998). However, no universal rules have emerged. The focus of the early work in this field was with thermophilic proteins (Sato et al. 1977; Ulrich et al. 1972), but cold-active enzymes are also of considerable importance both for providing insight into thermal adaptation and for their biotechnological applications.

In an effort to increase the knowledge concerning cold-active enzymes, we characterized a β-galactosidase (BgaS) from the Antarctic *Arthrobacter* isolate SB (Coker et al. 2003). This enzyme, BgaS, is of interest because it has the lowest temperature optimum of any characterized BgaS (more than 35°C below the mesophilic LacZ enzyme from *Escherichia coli*) and retains about 50% of its optimal activity at 0°C, a temperature where LacZ has little to no activity. Similar to the LacZ enzyme from *E. coli*, BgaS is a family two glycosyl hydrolase, active as a tetramer, and disassociates into inactive monomers above its optimum temperature (Coker et al. 2003; Hennrissat and Davies 1997; Nichtl et al. 1998). The BgaS enzyme, however, is heat-labile above 25°C and loses all activity after less than 10 min at 37°C. At their respective optimum

temperatures, the activity of LacZ is about three times higher than BgaS; however, BgaS has five times higher specific activity at 10°C than the LacZ enzyme and is more active at 0°C than a previously reported β-galactosidase with a temperature optimum at 26°C (Coker et al. 2003; Fernandes et al. 2002). This high activity at near-freezing temperatures, an unusually low temperature optimum, and thermolability at moderate temperatures make the BgaS enzyme an intriguing model for studying the mechanisms responsible for cold-activity.

In addition, its special cold-active biochemical properties make BgaS a candidate for commercial use by the dairy industry to hydrolyze lactose in refrigerated milk for lactose-intolerant consumers (Dahlquist 1983) and in whey to eliminate pollution problems associated with its disposal (Hoyoux et al. 2001). Enzymes from mesophilic organisms (Replius 1983) are currently used to hydrolyze lactose in milk but the use of a cold-active, heat-labile enzyme could have economic advantages. Although the BgaS enzyme already has activity at low temperatures, its potential for industrial applications would be enhanced by increased specific activity, since rapid lactose hydrolysis with little enzyme would decrease costs.

Here we combined directed and random mutagenesis approaches in order to determine whether the specific activity of an enzyme (BgaS) already having a low temperature optimum could be further increased, and if so, what alterations were necessary. We used information from the *E. coli* LacZ enzyme to test the effects of specific mutations in the *bga*S gene and then created random mutations to explore additional regions affecting cold-activity. Random mutagenesis and the separation and analysis of the individual mutations showed that two unpredicted alterations (E229D, V405A) could affect enzyme activity. These results show the value of a random mutagenesis approach and the separation and testing of individual mutations for revealing amino acid interactions that might not be predicted. Furthermore, they are consistent with reports (Panasik Jr. et al. 2000; Wintrode et al. 2000; Zartler et al. 2001) suggesting that local alterations rather than global changes (amino acid composition, salt bridges, and so on) can increase cold activity.

Materials and methods

Mutagenesis

The nucleotide alterations in *bga*S required to change the codons to encode aspartic acid rather than glycine at the 802, 803, and 805 residues were created using the QuikChange kit with the following primers and their complements (underlined nucleotides are changes from wild-type sequence):

802—5′ GGCGCGCACCGACGGATAACGACG ACGGAGCGGGCCGCGGC 3′

803—5′ GGCGCGCACCGACGGATAACGAC GGCGACGCGGGCCGCGGC 3′

805—5′ GGCGCGCACCGACGGATAACGACGG CGGAGCGGACCGCGGC 3′.

Ethyl methanesulfonate (EMS) mutagenesis was performed by incubating 4 μg of the pΔα18N-G803D construct with varying concentrations of EMS (0, 100, 150, 200 mM) at 37°C for 2 h and stopped by adding sodium thiosulfate (Rhaese and Boetker 1973). Aliquots of the quenched mutagenesis reaction were then transformed into *E. coli* ER2585 F′ cells (F′ Tn10::*pro*AB 81, Δ*lac*Z, *lac*Y$^+$) and screened on Luria-Bertani (Miller 1972) plates containing 100 μg ampicillin ml^{-1} and 0.01% of the chromogen 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Transformants showing X-Gal hydrolysis were transferred to M-9 plates (Miller 1972) containing 0.2% lactose, vitamins (Gibco), and 100 μg ampicillin ml^{-1}.

Saturation mutagenesis was performed in the regions of the *bga*S gene encoding residues 229 and 405 using the Quick Change protocol with the following primers and their complements:

229 area—5′ CCGGCCGGCGGCNTCNACNAC NTCNGGCTGCGCACCTCA 3′

405 area—5′ ACCCACGGTNTTNCCNTTNAG NAGTGGGCCGGC 3′, where N equals all possible nucleotides.

Template for each mutagenesis contained the G803D and E229D or V405A alteration (i.e., the 229 area primer used a construct with the V405A/G803D alterations as a template). Amplified plasmid was then treated with *Dpn*I to remove template plasmid and transformed into *E. coli* ER2585F' cells. Transformants were then screened for X-Gal hydrolysis at 18°C. All mutated genes were sequenced at the Penn State Nucleic Acid Sequencing Facility with an ABI model 3100 sequencer.

Enzyme purification

The enzymes used for all biochemical characterizations were purified from *E. coli* MC1061 (DE3) cells containing a pET28a vector in which had been ligated the gene encoding the wild-type or mutant form of the *bga*S gene into *Nde*I and *Sal*I sites to create an *N*-terminal 6X-Histidine tag on the protein. An *E. coli* transformant containing one of the constructs was grown at 37°C in 500 ml of Terrific Broth (Miller 1972) supplemented with 90 μg kanamycin ml^{-1} until the optical density at 600 nm was 0.45. Cells were transferred to 18°C and incubated until the optical density at 600 nm was 0.6, then isopropyl-β-D-thiogalactoside (IPTG) was added (final concentration of 0.1 mM), and incubation continued at 18°C for 16 h. Following centrifugation, the cell pellet was resuspended in 3 ml of Z buffer without β-mercaptoethanol (designated as modified Z-buffer and containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ at pH 7.0) per gram. The cells were disrupted with one pass

through a French pressure cell (18,000 lb/in^2) and the supernatant of the spun lysate was mixed with an equal volume of cold column wash buffer (modified Z buffer, 300 mM NaCl, 5 mM imidazole, pH 7.0), and loaded onto a TALON SuperFlow column (ClonTech). The column was washed with eight column volumes of wash buffer while increasing the imidazole concentration from 5 to 30 mM. Elution buffer (modified Z-buffer, 300 mM NaCl, 150 mM imidazole) was added, and the eluted enzymes were then dialyzed against 3 l of modified Z-buffer using 10,000 molecular weight cut-off Slide-A-Lyzer casettes (Pierce) and stored at 5°C (no more than 4 ml of pooled fractions were used in dialysis). The dialyzed enzymes were subjected to denaturing electrophoresis on a 10% polyacrylamide gel (SDS-PAGE) and a single species was visible upon staining with Coomassie blue indicating greater than 95% purity.

Biochemical characterization

Protein concentrations were measured using the bicinchoninic acid method (Wiehlman et al. 1988) with bovine serum albumin as a standard. For all specific activities, one unit is defined as the amount of enzyme needed to release 1 μmol of *o*-nitrophenol/min. Hydrolysis of nitrophenol groups was detected spectroscopically at 420 nm using a Genesys2 spectrophotometer (Spectronic Instruments, Inc.). Control experiments demonstrated that all assays were within linear ranges for enzyme and product concentrations.

The thermodependency of activity was determined by incubating the enzyme in modified Z-buffer containing 2.2 mM *o*-nitrophenyl-*β*-D-galactoside (ONPG) for 5 min at temperatures ranging from 0 to 50°C. Reactions were stopped by the addition of 1 M Na_2CO_3. The standardization of this protocol yielded highly reproducible thermodependency of activity results that permitted reliable comparisons of different enzymes. The thermostabilities of the enzymes were determined by incubating at 15, 30, and 37°C and removing aliquots for up to 120 min. The enzymes were then immediately assayed at 15°C in the same manner as the thermodependency of activity assays.

The substrate specificities of the enzymes were determined by assaying at 15°C for 5 min in modified Z-buffer containing 2.2 mM (final concentration) of various nitrophenyl substrates. Substrates tested were ONPG, *p*-nitrophenyl-*β*-D-galactoside (PNPG), *o*-nitrophenyl-*β*-D-fucopyranoside (ONPF), *o*-nitrophenyl-*β*-D-glucoside, or *p*-nitrophenyl-α-D-galactoside.

The metal studies were performed on enzymes that had been dialyzed individually for 14 h in 100 mM MOPS (pH 7.0) with and without 1–10 mM of $MgCl_2$, $MnCl_2$, $CaCl_2$, $CuCl_2$, NaCl, and KCl. After dialysis, each enzyme was assayed at 15°C for 5 min in 100 mM MOPS (pH 7.0) buffer containing 2.2 mM ONPG.

The Michaelis–Menten kinetic parameters (V_{max} and K_m) were determined by plotting the substrate concentration versus initial velocity for each reaction and subjecting the data to nonlinear regression analysis using the analysis program EnzymeKinetics (version 1.5; Trinity Software, Fort Pierce, FL, USA) and verified using the Windows Non-Lin program (M. Johnson and D. Yphantis, University of Virginia, Charlottesville, USA). Kinetic assays with ONPG were done at concentrations from 0.1 to 5 mM and performed at 5, 10, 15, and 20°C in modified Z-buffer. Lactose kinetics were performed with substrate concentrations from 1 to 100 mM at 15°C in modified Z-buffer containing 2 U ml^{-1} hexokinase, 2 U ml^{-1} glucose-6-phosphate dehydrogenase, 1 mM ATP, and 0.5 mM NADP. The production of NADPH, created through the coupled assay of hexokinase and glucose-6-phosphate dehydrogenase, was detected at 340 nm using a Genesys2 spectrophotometer. Controls demonstrated that the hexokinase and glucose-6-phosphate were in excess and not rate limiting for the reactions. The K_i values were determined using varying concentrations of the inhibitors D-galactose and lactose with 0.1 to 5 mM concentrations of ONPG as the substrate. Plus or minus values listed for all kinetics experiments represent the standard deviation using at least six replicates.

Cysteine titration

Titrations were performed by incubating the active enzyme in titration buffer: 150 mM phosphate buffer containing 2 mM dithiobisnitrobenzoic acid (DTNB, ε_{412} = 14,100). Reactions were scanned in a Genesys2 spectrophotometer at 412 nm at 5-min intervals (Ishmael et al. 2001). The total number of cysteines was determined by unfolding the protein in titration buffer containing 8 M urea (final concentration). Reactions were then assayed in the same manner as the folded protein. Values reported for the folded and unfolded enzyme are the average of three separate experiments (nine replicates).

Skim milk assays

The hydrolysis of lactose in milk was monitored by incubating 98 μg of purified BgaS, BgaS6, and BgaS7 individually in 1 ml of commercial skim milk (Schneider Valley Farms, PA, USA) at 2.5°C for 45 min, 2, 4, 6, 12, and 24 h. The enzymes were inactivated by incubating the assay tubes at 60°C for 10 min and proteins were precipitated with trichloroacetic acid and filter sterilized for use in HPLC analysis. Lactose hydrolysis and the evolution of glucose and galactose were detected by refraction index on a Waters HPLC system equipped with a BioRad Aminex HPX-87H column using 5 mM H_2SO_4 as the mobile phase.

Computer-based models of BgaS

The three-dimensional structure of BgaS was modeled by importing the ClustalW alignment generated at the T-COFFEE site (Notredame et al. 2000) into the SWISS-MODEL (version 3.51) program at the ExPASy server (Peitsch 1996). Several structures of the LacZ enzyme (1bgl, 1dp0, 1jyn, 1jz7) were used as templates (Berman et al. 2000). Analysis and figures were done in the SWISS-PROT Pdb Viewer (version 3.51).

Materials

All reagents were of the highest purity available. Restriction endonucleases and bovine serum albumin were purchased from Promega. All DNA oligonucleotides were synthesized by Integrated DNA Technologies. QuikChange kit and pET28a were purchased from Stratagene. The purity of metals used were as follows: magnesium chloride hexahydrate (JT Baker) ACS reagent, 100.4%; manganese chloride (Fisher) certified ACS, 98.9%; calcium chloride dehydrate (Fisher) certified ACS, 100.6%; copper chloride dehydrate (Sigma) ACS reagent, 100.1%; sodium chloride (Fisher) certified ACS, 99.8%; potassium chloride (EM Science) meets ACS specifications, 99.0–100.5%. All other materials were from Sigma unless otherwise noted.

Results

Directed and EMS mutagenesis

Previous studies (Martinez-Bilbao et al. 1991) had shown that changes in the *E. coli* LacZ enzyme at position 794 increased the k_{cat} with lactose fivefold, decreased its activity with ONPG twofold, and also rendered the enzyme more heat-labile. In order to examine similar changes to the BgaS enzyme, we aligned the sequences for LacZ, BgaS, and other β-galactosidase genes from *Arthrobacter* species and evaluated the comparable regions (Coker et al. 2003; Fowler and Zabin 1978; Karasova-Lipovova et al. 2003; Trimbur et al. 1994). We found three glycines in BgaS (802, 803, 805) that might correspond to the one at position 794 in LacZ and used site-directed mutagenesis to change the codons from ones for glycine to aspartic acid. Each construct was characterized and the activity of purified enzymes measured (Table 1). Although the resulting properties differed from those found with a similar LacZ variant (G794D), the reduced activities demonstrated that this region was also critical for BgaS activity (Table 1). Furthermore, a loss of function BgaS variant (BgaS3) provided the basis for a direct selection of mutants with alterations reversing this loss and possibly highlighting residues important in cold activity.

Table 1 Specific activities at 15°C of family 2 β-galactosidase (BgaS) from *Escherichia coli* (LacZ) and *Arthrobacter* sp. SB and its mutant enzymes using *o*-nitrophenyl-β-D-galactoside (ONPG) as the substrate

Enzyme	Amino acid alteration(s)	Specific activity (U/mg)
BgaS	None	25.4 ± 0.8
BgaS2	G802D	0.5
BgaS3	G803D	< 0.5[a]
BgaS5	G805D	18.1
BgaS6	E229D/V405A/G803D	30.2 ± 0.9
BgaS6a	E229D/G803D	< 0.5[a]
BgaS6b	V405A/G803D	< 0.5[a]
BgaS7	E229D/V405A	46.4 ± 0.6
BgaS7a	E229D	27.2 ± 3.3
BgaS7b	V405A	27.5 ± 0.8
LacZ	None	14.5 ± 0.7

[a]Activity was below the detectable range

To obtain enzymes with secondary alterations, a plasmid construct containing the *bga*S3 gene was subjected to EMS mutagenesis, transformed into an *E. coli* host, and the resulting 49,200 transformants screened for X-Gal hydrolysis of which only 28 showed a return of function by hydrolyzing X-Gal at 18°C. To ensure that hydrolysis was due only to changes within the gene, each gene was excised from its vector (which had been subjected to the mutagenesis) and ligated into an unmutated vector. The new 28 constructs were then transformed into an *E. coli* host and tested for the ability to grow with lactose as the sole carbon source. Each grew at 15°C but not at 30 or 37°C, similar to transformants with *bga*S. The doubling times of the transformants were also measured at 15°C, and those exhibiting the three fastest (8–16 h compared with 10 h for transformants carrying the *bga*S gene) were selected for further study and the mutated genes sequenced.

Analyses of mutants

The sequences of the three mutated genes showed ten different missense mutations and ten silent mutations. No true revertants were observed, probably due to the selection of transformants with growth rates differing from that of wild type. Two alterations, E229D and V405A, were present in all three sequences suggesting they were important for restoring activity. To test this, we created constructs, which coded for the E229D and V405A alterations together and individually in a G803D (*bga*S6, *bga*S6a, *bga*S6b) and wild type background (*bga*S7, *bga*S7a, *bga*S7b) (Table 1). Transformants each containing one of these six constructs were then screened for X-Gal hydrolysis and growth on M9-lactose media. The results showed that the *bga*S6 construct and all three *bga*S7 constructs were able to support X-Gal hydrolysis and growth on lactose in an *E. coli* host. The *bga*S6a (E229D, G803D) and *bga*S6b (V405A, G803D) constructs were unable to hydrolyze either substrate

demonstrating that both the E229D and V405A mutations are needed to restore activity in the mutant background. Once purified, the specific activity of BgaS7a (E229D) and BgaS7b (V405A) enzymes was measured and found to be similar to BgaS (Table 1), showing that the individual mutations do not alter enzyme activity in a wild type background.

Thermodependence of activity and thermostability

To examine other effects of all three alterations (E229D, V405A, and G803D), the BgaS6 and BgaS7 enzymes were purified and further characterized (Fig. 1). The BgaS6 (E229D, V405A, G803D) and BgaS7 (E229D, V405A) enzymes showed a 19% and 83% increase, respectively, in specific activity compared to BgaS and had thermal optima similar to the wild-type BgaS (Table 1; Fig. 1a). Further, BgaS6 and BgaS7 retained between 65% and 60% of their activity at 0°C which corresponded to an increase in specific activity of 1.5 and 2.0 times compared to the wild-type enzyme. The activity of the LacZ enzyme was also tested as a reference point (Fig. 1a). Interestingly, at their respective optimum temperature, the activity of LacZ is about three times higher than BgaS. However, the activity of BgaS7 is 80% of LacZ at their respective optimum.

The thermostabilities of the BgaS, BgaS6 and BgaS7 enzymes were measured (Fig. 1b) and all three enzymes were stable at 15°C for at least two hours and lost activity in less than 10 min at 37°C. BgaS, BgaS7, and BgaS6 lost all detectable activity after 75, 90, and 120 minutes, respectively, at 30°C.

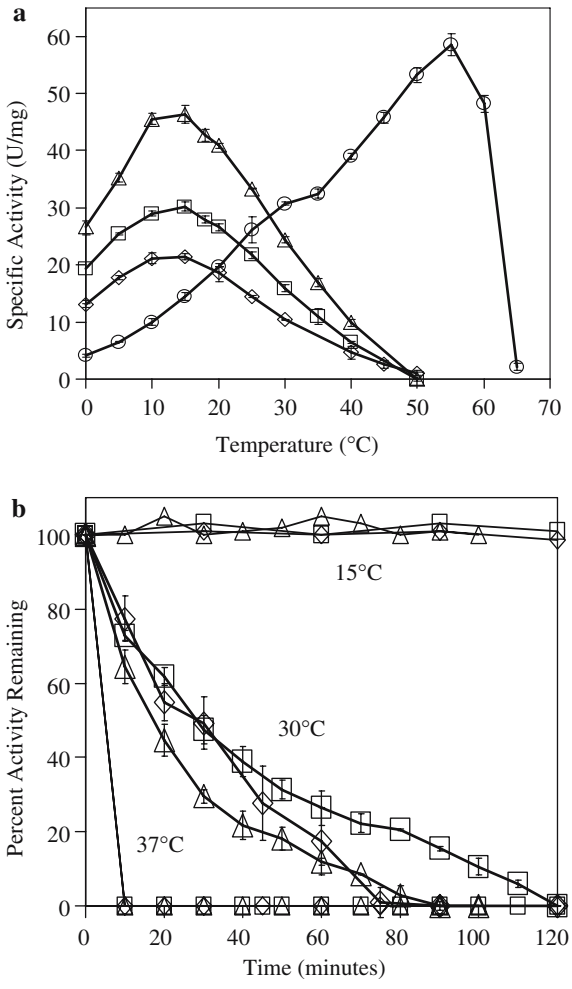

Fig. 1 Activity and stability of the purified β-galactosidase (*BgaS*) from *Arthrobacter* sp. SB and its mutant enzymes. **a** Thermodependency of activity for BgaS (*diamonds*), BgaS6 (E229D, V405A, G803D) (*squares*), BgaS7 (E229D, V405A) (*triangles*), and the *Escherichia coli* LacZ enzyme (*circles*) using *o*-nitrophenyl-β-D-galactoside (*ONPG*) as the substrate. **b** Thermostabilities of purified BgaS (*diamonds*), BgaS6 (*squares*), and BgaS7 (*triangles*) at 15, 30 and 37°C. Specific activity corresponding to 100% activity was 25.4, 30.2, 46.4 U/mg for BgaS, BgaS6, and BgaS7, respectively. *Error bars* in both panels represent the standard deviations using at least six replicates performed at a particular temperature or time point

Substrate specificity, metal requirements, and cysteine titrations

Because the substrate preferences of the LacZ G794D enzyme had been altered (Martinez-Bilbao and Huber 1994), we determined the substrate specificities of the BgaS, BgaS6 and BgaS7 enzymes by assaying with several chromogenic substrates (Table 2). The BgaS6 and BgaS7 enzymes had 94.6% of their ONPG activity with PNPG as the substrate, but showed less than 2% of their ONPG activity with the other substrates tested, similar to the BgaS enzyme. The exception was that BgaS7 had marginal activity (2%) with the substrate ONPF.

Our previous data showed that the BgaS enzyme lost activity when dialyzed in a buffer without metal ions and that the addition of magnesium ions restored partial activity (Coker et al. 2003). Therefore, metal studies were conducted with the purified BgaS6 and BgaS7 enzymes following dialysis to remove the existing metals. About 60% of the BgaS activity was restored by the addition of 5 mM Mg^{2+}; however, BgaS6 had only 0.1% of its original activity restored, while none of the other metals restored activity to BgaS6. The BgaS7 enzyme had about 7% activity restored with 10 mM Mg^{2+}, 6% with 10 mM Mn^{2+}, and 5% with 10 mM K^{+}. It regained no activity with 1–10 mM Cu^{2+} and had minimal activity with 1–10 mM Ca^{2+} and Na^{+} (1.5 and 3.0% respectively).

Titration of cysteine side chains was performed with DTNB on the BgaS, BgaS6 and BgaS7 enzymes. The BgaS sequence has four cysteines per monomer, and averaged values from three separate experiments showed that in the unfolded forms of all three enzymes, DTNB was able to titrate 3.5 cysteines per monomer. Average titrations of three separate experiments using the folded forms of BgaS and BgaS6 showed 0.8 and 0.6 cysteines titrated per monomer, respectively. However, average titrations with the native BgaS7 enzyme showed 1.6

Table 2 Percent activity of the purified β-galactosidase (BgaS) from *Arthrobacter* sp. SB and its mutant enzymes with different nitrophenol-derived chromogenic substrates

Substrate	Enzyme[a]			
	BgaS	BgaS3 (G803D)	BgaS6 (E229D/ V405A/G803D)	BgaS7 (E229D/V405A)
o-Nitrophenyl-β-D-galactoside	100	< 0.1[b]	100	100
p-Nitrophenyl-β-D-galactoside	84.1	< 0.1[b]	94.6	94.6
o-Nitrophenyl-β-D-fucopyranoside	< 0.1[b]	< 0.1[b]	< 0.1[b]	2.0
p-Nitrophenyl-β-D-cellobioside	< 0.1[b]	< 0.1[b]	< 0.1[b]	< 0.1[b]
o-Nitrophenyl-β-D-glucoside	< 0.1[b]	< 0.1[b]	< 0.1[b]	< 0.1[b]
p-Nitrophenyl-β-D-galactoside	< 0.1[b]	< 0.1[b]	< 0.1[b]	< 0.1[b]

[a]Activities corresponding to 100% specific activity are 26.9, 30.2 and 46.4 U/mg for BgaS, BgaS6 and BgaS7, respectively. Reported values are the average of at least six replicates.
[b]Activity was below the detectable range

cysteines per monomer were exposed (2–3 times the number observed for BgaS and BgaS6), suggesting that the E229D and V405A mutations create greater access for the DTNB molecule once the original G803D mutation is removed.

Kinetic and inhibition studies

In order to evaluate the activities of the mutant enzymes, kinetic values were determined (Table 3). The BgaS6 and BgaS7 enzymes had greater catalytic efficiency with ONPG as the substrate than BgaS at 10 and 15°C, primarily due to an increase in the k_{cat} values. Lactose inhibition assays (using ONPG as a substrate) showed that the BgaS6 (K_i = 10.4 ± 1.0 mM) and BgaS7 (K_i = 16.9 ± 1.6 mM) enzymes were less sensitive to this inhibitor compared to BgaS (K_i = 7.9 ± 0.6 mM) and LacZ (K_i = 1.0 ± 0.2 mM). However, D-galactose assays (using ONPG as a substrate) showed that the BgaS6 (K_i = 8.7 ± 0.6 mM) and BgaS7 (K_i = 7.3 ± 0.1 mM) enzymes were more inhibited by D-galactose compared with the BgaS (K_i = 12.5 ± 0.5 mM) and LacZ (K_i = 24.0 ± 0.2 mM) enzymes.

Hydrolysis of lactose

Because of the interest in improving lactose hydrolysis for potential commercial use, the kinetic values were also measured using lactose as the substrate. Although BgaS6 has roughly the same catalytic efficiency as BgaS, BgaS7 is 3.0 times more efficient than the BgaS enzyme with lactose at 15°C (Table 4).

Hydrolysis of lactose in commercial skim milk was also monitored using similar concentrations of BgaS, BgaS6, and BgaS7 at 2.5°C. After 45 min, 59% of the lactose was hydrolyzed by BgaS7 while BgaS6 hydrolyzed 47% and BgaS hydrolyzed only 24%. Eighty percent lactose hydrolysis was achieved in 3 h by BgaS7, 6 h by BgaS6, and 7.5 h by BgaS at 2.5°C.

Location of altered amino acids in a modeled structure

In an effort to examine the effects of the amino acid substitutions on catalytic activity at low temperatures, we produced homology-based models of the enzymes in this study using the SWISS-MODEL program (Fig. 2). Models were based on the LacZ enzyme from *E. coli*, as it is the only known structure of a family two

Table 3 Steady-state kinetic parameters for the purified β-galactosidase (BgaS) from *Arthrobacter* sp. SB and its mutant enzymes with *o*-nitrophenyl-β-D-galactoside (ONPG)

[a]Values are the average of at least six replicates and the standard deviation. Reactions were performed with ONPG concentrations from 0.1 to 5 mM at the various temperatures stated

	Temperature (°C)	K_m[a] (mM)	k_{cat}[a] (s^{-1})	k_{cat}/K_m[a] (s^{-1} mM^{-1})
BgaS	5	0.7 ± 0.03	43 ± 0.8	61 ± 4.0
	10	0.7 ± 0.05	57 ± 9	82 ± 21
	15	0.8 ± 0.1	100 ± 8	125 ± 19
	20	0.6 ± 0.1	110 ± 10	183 ± 40
BgaS6 ± E229D/ V405A/ G803D)	5	0.7 ± 0.08	48 ± 3	68 ± 10
	10	0.4 ± 0.04	53 ± 2	132 ± 4
	15	0.5 ± 0.05	110 ± 15	220 ± 30
	20	0.5 ± 0.08	92 ± 15	185 ± 29
BgaS7 ± E229D/ V405A)	5	0.8 ± 0.08	59 ± 7	74 ± 9
	10	0.5 ± 0.03	85 ± 8	170 ± 17
	15	0.9 ± 0.08	279 ± 26	310 ± 28
	20	0.6 ± 0.02	136 ± 3	227 ± 6

Table 4 Steady-state kinetic parameters for the purified β-galactosidase (BgaS) from *Arthrobacter* sp. SB and its mutant enzymes with lactose at 15°C

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
BgaS	11.5 ± 1.6	5.3 ± 0.3	0.5 ± 0.03
BgaS6 (E229D/V405A/G803D)	7.5 ± 0.8	4.1 ± 0.2	0.6 ± 0.1
BgaS7 (E229D/V405A	12.7 ± 1.8	18.7 ± 0.9	1.5 ± 0.3

β-galactosidase. Even though the BgaS enzyme has a 30% sequence similarity to LacZ, the model predicted that the native structure of BgaS is very similar to LacZ. Each enzyme appears to be composed of monomers containing five domains plus the α-fragment, which contains the first 51 amino acids and is important in formation of the tetramer. These combine to create a homotetramer that forms a diamond shape with two axes: a long interface and an activating interface (Fig. 2a).

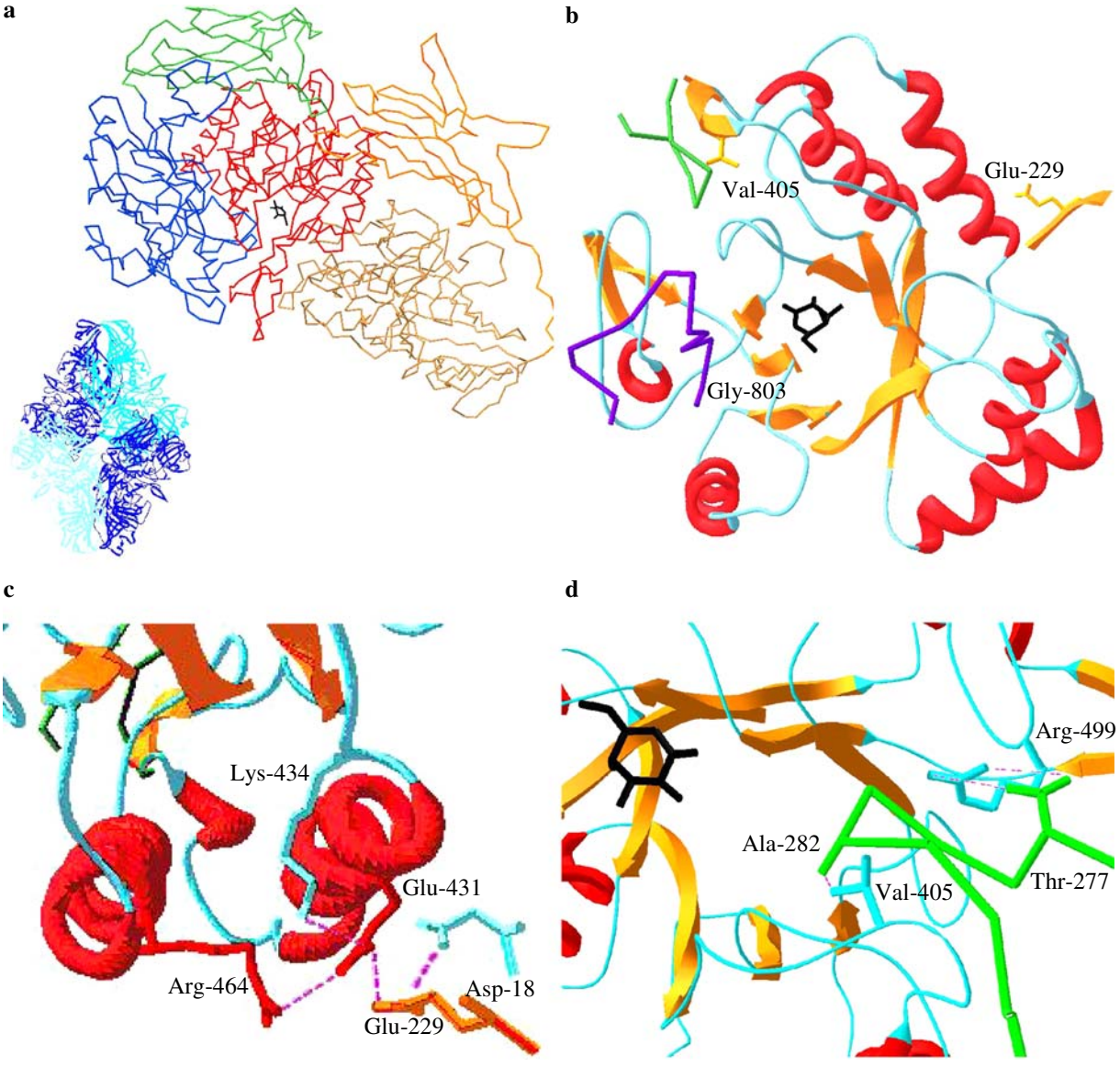

Fig. 2 SWISS-MODEL structures of the β-galactosidase (*BgaS*) from *Arthrobacter* sp. SB. **a** Tetramer (*lower image*) and one subunit of the BgaS enzyme highlighting Domain 1 (*blue*), Domain 2 (*green*), Domain 3 (*red*), Domain 4 (*orange*), and Domain 5 (*brown*). Galactose modeled into the active site is *black*. **b** Magnified view of the α/β barrel (active site) of Domain 3 (*red portion from part a*) showing the positions of three residues affecting activity in the variant enzymes. The loop from a neighboring monomer completing the active site is *green*. The loop containing Gly-803 is *purple* (803 is the first residue in the loop). Helices forming the four-helix bundle (part of the activating interface) are located on the top of the barrel. Galactose (*black*) is modeled into the active site. **c** Magnified view of the area around residue 229. Hydrogen bonds are shown in *pink* (*lower right*) and the loop donated from the neighboring monomer is *green*. **d** Magnified view of the area around residue 405. Hydrophobic and/or van der Waals interactions are shown in *pink* (*center and right center*). Galactose modeled into the active site is *black* and the loop from the neighboring monomer is *green*

According to the models, residue 803 lies within the enzyme's fifth domain at the base of a loop, corresponding well with our alignment with LacZ and similar enzymes from known *Arthrobacter* species (Gutshall et al. 1995; Trimbur et al. 1994) (Fig. 2b). Biochemical data from the *E. coli* LacZ enzyme has shown that this loop is important in catalysis and possibly substrate binding (Juers et al. 2003). Structural and mechanistic data have shown that this loop moves up to 10 Å and is a vital part of the "deep binding" mode of catalysis. Our models show that the G803D mutation is able to interact with an area that aligns with the LacZ Trp-999, a residue important in initial binding of the substrate (Huber et al. 2003) and Phe-601, a residue important for the switch from open to closed configuration of the active site (Juers et al. 2001). Although the physical make up of the BgaS loop differs from that of LacZ, it shares all three residues that stabilize the loop in both configurations. Residue Glu-229 lies in the second domain and is situated at the activating interface of the enzyme. It can make hydrogen bonds with residues 18 (α-peptide residue), 431 (member of a 4 helix cluster), and 464 (at the activating interface), while all other residues are over 5 Å away (Fig. 2c). The replacement of glutamate with aspartic acid eliminates these interactions due to the shorter side chain. Residue Val-405 is located in the third domain at the base of the potential active site and is a member of the activating interface (Fig. 2d). It can form hydrophobic and/or Van der Waals interactions with residues Arg-499 (directly across the active site) and residues 267–278 (members of a loop in a neighboring subunit that completes the active site). The mutation of residue 405 to alanine results in the loss of all these interactions.

The mutations at 229 and 405 are both able to interact with the activating interface of the molecule. Most of the interface is composed of the interactions between the active site portion of Domain 3 (where residue 405 is located) and the protruding loop from Domain 2 (Fig. 2b green loop) of the neighboring subunit. The remaining portion of the activating interface is composed of intrasubunit interactions between Domain 3 and the α-peptide, intersubunit interactions between Domain 3 (where residue 229 is located), and intersubunit interactions with a four-helix bundle.

Regional saturation mutagenesis

Based on the modeled structure of BgaS, we hypothesized that the E229D and V405A mutations were able to increase activity through an alteration of the activating interface. We used saturation mutagenesis to further explore these possible effects. Since both mutations are necessary to restore activity in the G803D background (Table 1), we used G803D constructs that also contained either E229D (*bga*S6a) or V405A (*bga*S6b) and primers to generate mutations at the other site. In the area around residue 229, we predicted that only mutations at 229 would lead to restored activity since, according to the models, it is the only residue predicted to make contact with the activating interface, via the four helix bundle. For the area surrounding residue 405, we expected alterations at adjoining amino acids to lead to restored activity since the models predicted contacts with the activating interface. Upon analysis of the sequences from active and inactive mutants created in this study, we found, as predicted, that for the area around residue 229, only the E229D mutation was able to restore activity. For the area around residue 405, we found that changes to alanine at 404 (S to A) and 405 (V to A) were able to restore activity, although the V405A alteration resulted in the highest activity (25.2 and 46.4 U/mg, respectively).

Discussion

In an effort to understand how enzymes with low temperature optima are able to increase activity at cold temperatures, we used a combined approach of site-directed and random mutagenesis. The directed mutagenesis examined a region in BgaS corresponding to one that is important for catalysis and thermostability in the *E. coli* LacZ enzyme. This approach was valuable and showed that the homologous region was critical for catalysis in BgaS. However, the alterations eliminated or reduced activity, rather than increasing hydrolysis of lactose as observed with LacZ, illustrating the complexity of amino acid interactions and the need to test predictions with additional enzymes.

The random mutagenesis of *bga*S3 showed that two alterations (E229D and V405A) were necessary to restore activity in the G803D background. Interestingly, in the absence of G803D (i.e., BgaS7), both changes were also required to increase the activity above wild type levels (Table 1; Fig. 1a). Thus, the E229D and V405A alterations act synergistically, not separately, in the BgaS6 (E229D, V405A, G803D) and BgaS7 (E229D, V405A) variants to increase the catalytic activity at low temperatures.

The thermal dependence of activity curves showed that this increase was present through the entire range of temperatures tested for BgaS6 and BgaS7 (Fig. 1a). Thermostability results at 15 and 37°C were similar for all three enzymes; however, BgaS6 and BgaS7 were active longer than BgaS at 30°C (Fig. 2b), suggesting that enzymes do not have to become more heat-labile in order to increase activity at lower temperatures, as we previously suggested (Coker et al. 2003; Sheridan et al. 2000).

Substrate specificity experiments showed that all three enzymes were similar with one exception, the ability of BgaS7 to weakly hydrolyze the substrate ONPF (Table 2). Kinetic studies showed that the BgaS7 enzyme was able to hydrolyze ONPG and lactose more efficiently than the BgaS or BgaS6 enzymes at low temperature. Skim milk studies showed that BgaS7 was

able to hydrolyze 80% of the lactose in skim milk in less than half the time of BgaS. The increase in inhibition by D-galactose for BgaS6 and BgaS7 suggests a reason for the quicker plateau in enzyme activity seen in the skim milk assays, compared to BgaS. Metal studies demonstrated that the addition of metals to dialyzed BgaS6 and BgaS7 failed to restore substantial activity indicating that the alterations prevented reinsertion of the depleted metals into the folded protein, which may be due to denaturation of the protein or aggregate formation upon removal of the metals. Cysteine titrations showed that the BgaS7 enzyme gave greater access to the DTNB molecule compared to BgaS and BgaS6 resulting in a two to three fold increase in the number of cysteines able to be titrated in the BgaS7 enzyme. The combination of these results establish that the substitution of only two amino acids (E229D and V405A) simultaneously affected enzyme activity, thermostability (at 30°C), metal binding, and the exposure of cysteine residues.

In an effort to explain the potential role of each alteration (G803D, E229D and V405A) in catalysis at low temperature, models of the enzymes were constructed (Fig. 2). According to our models, the 803 residue is a member of a loop, which has been shown to be mobile in the LacZ enzyme and important in substrate binding making it a vital part of the "deep binding" mode of catalysis (Juers et al. 2001, 2003). Gly-803 can interact with areas corresponding to those in LacZ important for stabilization of the mobile loop, substrate binding, metal coordination at the active site, and catalysis. Thus, one explanation for the loss of activity with the G803D alteration is that it changes the interaction with one or more of the stabilizing residues causing a continuously closed or rigid conformation of this loop, which is consistent with recent studies of the 794 area in LacZ (Juers et al. 2003). The E229D and V405A alterations are then able to either restore activity (BgaS6) or increase activity (BgaS7) through the loss of interactions along the activating interface of the enzyme.

Saturation mutagenesis data around the 229 and 405 area support the prediction of fewer interactions since only mutations with a shortened side-chain length, and thus, fewer interactions, restored activity in the G803D background. However, consistent with the model suggesting that the G803D alteration affects interactions with stabilizing residues, the BgaS7 enzyme, which lacks G803D, allowed greater access to cysteine residues and had a higher catalytic efficiency when compared to BgaS6, which contains the G803D alteration.

To date, most studies with cold-active enzymes (D'Amico et al. 2002; Kano et al. 1997Merz et al. 2000; Russell et al. 1998; Taguchi et al. 1998; Wintrode et al. 2000) have centered on increasing the activity of enzymes with marginal activity at low temperatures or comparing structures of enzymes to discern key differences. Counter to these studies, which suggest that global changes in an enzyme are required for low temperature activity, our data show that activity can be increased with only two changes. Because it is unlikely that the combination of the E229D and V405A alterations would have been predicted, these interactions would not have been discovered by traditional site directed mutagenesis of conserved regions proposed to be involved in activity. Furthermore, finding these unexpected regions in BgaS can be used to predict additional regions of the LacZ enzyme that can be explored for effects on substrate binding and thermostability. Thus, the random mutagenesis approach, combined with the separation and analysis of individual mutations, was valuable for discovering new residues and interactions that increased activity at low temperatures and suggests, as previously mentioned (Panasik Jr. et al. 2000; Wintrode et al. 2000; Zartler et al. 2001), that subtle, synergistic, and cooperative intramolecular interactions can contribute to thermal adaptation.

Similar to other cold-active enzymes, BgaS has less activity than its mesophilic homologue (LacZ) at their respective temperature optima. It has been suggested that this decreased activity represents an incomplete adaptation to the cold (Georlette et al. 2004). Two reasons cited for this are that cold-active enzymes usually have lower levels of specific activity at their optimum temperatures when compared to mesophilic homologues at their optima. Second, cold-active enzymes frequently have less activity at 0°C than their mesophilic counterparts have at 37°C. However, the data from BgaS7 suggests that "complete" adaptation is possible since its activity is similar to that of LacZ at their respective optima (47 vs 58 U/mg), and the activity of BgaS7 at 0°C approaches that of LacZ at 37°C (27 vs 35 U/mg) (Fig. 1a).

In addition to wanting to understand basic features of enzyme structure and activity, researchers would like to genetically engineer enzymes for specific industrial uses. In this report we altered a β-galactosidase to increase its activity at low temperature and demonstrated that the BgaS7 enzyme hydrolyzed 80% of the lactose in skim milk at 2.5°C in less than half the time of the wild-type enzyme. This increased lactose hydrolysis suggests that this cold-active enzyme could be a viable alternative for the dairy industry and we are continuing to test this possibility. Thus, our combined directed and random mutagenesis approach provided interesting novel mutations and a β-galactosidase with increased cold-activity and potential use. The observation, however, that two separate and unpredicted mutations yield this phenotype illustrates the limited understanding of the features responsible for enzyme activity at different temperatures and suggests that the ability to engineer enzymes for specific industrial use awaits further breakthroughs in knowledge.

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