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GENETIC ENGINEERING OF XYLOSE ISOMERASE
THERMOZYMES FOR ENHANCED ACTIVITY, STABILITY, AND UTILITY

presented by

DINLAKA SRIPRAPUNDH

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of the requirements for

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John E. Kinsella
Major professor

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GENETIC ENGINEERING OF XYLOSE ISOMERASE THERMOZYMES FOR ENHANCED ACTIVITY, STABILITY, AND UTILITY

By

Dinlaka Sriprapundh

A DISSERTATION

Submitted to
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in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

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ABSTRACT

GENETIC ENGINEERING OF XYLOSE ISOMERASE THERMOZYMES FOR ENHANCED ACTIVITY, STABILITY, AND UTILITY

By

Dinlaka Sriprapundh

Molecular determinants responsible for high thermostability of the xylose isomerase from hyperthermophilic eubacterium *Thermotoga neapolitana* (TNXI) were identified by comparative thermostability and site-directed mutagenesis studies with the less thermostable counterpart enzyme from thermophilic eubacterium *Thermoanaerobacterium thermosulfurigenes* (TTXI). Despite their highly similar structures and amino acid sequences (70.4 % identity), no obvious differences in the enzyme structures can explain the differences in TNXI's stability compared to that of TTXI except for a few additional prolines and fewer Asn+Gln in TNXI. TNXI has 2 additional prolines in the Phe59 loop (Pro58 and Pro62). This loop helps forming another enzyme subunit's active site. When the 2 prolines in TNXI were substituted with the corresponding amino acids present in its less thermostable counterpart, TTXI, all mutant enzymes showed significant loss in thermostability compared to the wild-type TNXI. These data confirmed the hypothesis that prolines indeed play important roles in TNXI thermostability by reducing its entropy of unfolding.

TNXI's active site was engineered to improve its catalytic efficiency toward glucose. The TNXI V185T mutant derivative was three times more efficient in glucose isomerization than the wild-type TNXI. Although this mutant derivative was highly thermostable and highly active at 97°C, it was less than 10% as active at 60°C and

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required neutral pH to work. To customize this TNXI mutant derivative, a directed evolution approach was applied to the TNXI V185T to improve its activity on glucose at low temperature and low pH. After two successive rounds of random mutagenesis and low temperature/low pH activity screening, a new mutant, TNXI 1F1, was obtained that exhibited dramatic improvement of glucose isomerase activity at low temperature and low pH as compared to TNXI V185T. TNXI 1F1 (V185T/L282P/F186S) with one mutation relatively distant (L282P) from the active site and the other (F186S) within the active site of the enzyme, was more active than TNXI V185T over all temperatures and pHs. TNXI 1F1 was also more stable than TNXI and TNXI V185T and this may have resulted from additional H-bond formation between Ser186's sidechain and the neighboring L229 residue's mainchain structure. This H-bond would strengthen local conformation and the affinity of E231 co-ordination with the structural metal.

Biochemical properties and fructose productivities of TNXI 1F1 and Gensweet™, a commercially available glucose isomerase were also compared. TNXI 1F1 displayed higher catalytic efficiencies on glucose at low or high temperature and pH ranges and had greater thermal stability than Gensweet™ despite having similar temperature optima of activity. This greater thermal stability together with the superior kinetic parameters on glucose render TNXI 1F1 an excellent candidate for the industrial glucose isomerization process based on the lifetime fructose productivity estimation.

Dedicated to my family, Mr. Udom and Mrs. Achara Sriprapundh

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I would like to express my sincere appreciation and deepest gratitude to Professor J. Gregory Zeikus, who is my scientific advisor and philosophical mentor, for his guidance, encouragement, and patience through my graduate school years. I cannot thank him enough for all the support he has provided me for the successful completion of this research project. I would also like to thank all of the members of my guidance committee, Drs. John Linz, James Pestka, and Gale Strasburg, for their advice and invaluable time.

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I owe my success to my former advisor Dr. Sirirat Rengpipat for giving me the opportunities, her enormous support and encouragement through tough times. Without her assistance, this work could not have been completed.

I am most grateful to my best friend Paweena Limjaroen for her dedication and encouragement. I could not have undertaken this endeavor without her help and

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understanding. My deepest appreciation goes to my family and friends who supported me while accomplishing this work.

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LITERATURE REVIEW

XYLOSE ISOMERASE AND HIGH FRUCTOSE CORN SYRUP

Xylose isomerase (XI) (D-xylose ketol isomerase; EC 5.3.1.5), generally known as glucose isomerase (GI), catalyzes the interconversion of D-xylose to D-xylulose *in vivo* and D-glucose to D-fructose *in vitro* (Figure 1). Interconversion of xylose to xylulose provides a nutritional requirement in bacteria that thrive on decaying plant materials and also aids in the bioconversion of hemicelluloses to ethanol. Bioconversion of renewable biomass to fermentable sugars and ethanol is important in view of the rapid depletion of fossil fuels. Isomerization of glucose to fructose is of commercial importance in the production of high fructose corn syrup (HFCS) (Bhosale *et al.*, 1996). Xylose isomerase is one of the most important industrial enzymes in the food industry and also one of the three highest tonnage value enzymes, amylase and protease being the other two (Bhosale *et al.*, 1996).

The production of HFCS (Figure 2) from cornstarch comprises three major processes: (1) liquefaction of starch by α-amylase, (2) saccharification of starch slurry by the combined action of amyloglucosidase and debranching enzymes, and (3) isomerization of glucose by XI (or GI). The final product is a corn syrup containing a mixture of glucose and fructose with a greater sweetening capacity than that of sucrose. An equilibrium mixture of glucose and fructose (1:1) is 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose. The sweetening capacity of glucose is 70 to 75 % that of sucrose, whereas fructose is twice as sweet as sucrose (Barker, 1976). The price of HFCS is 10 to 20 % lower than that of sucrose on the basis of its sweetening power.

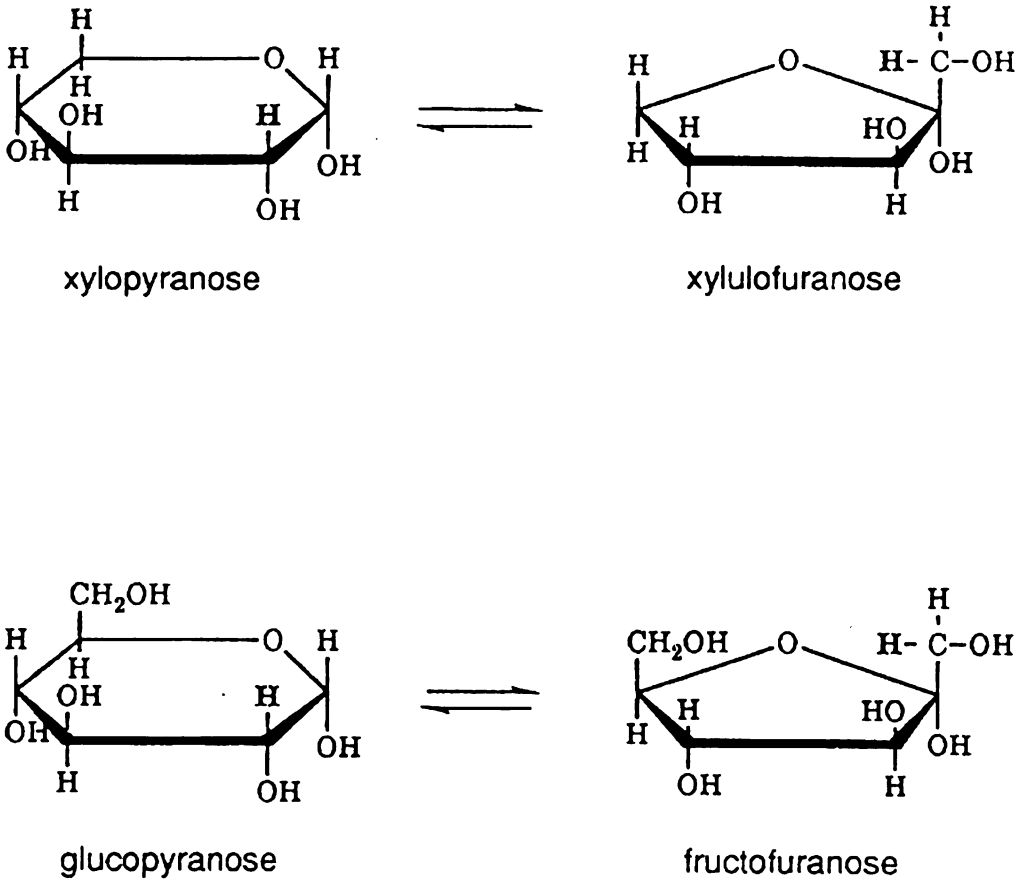

Figure 1: Illustration of the reaction carried out by xylose isomerases (XIs)

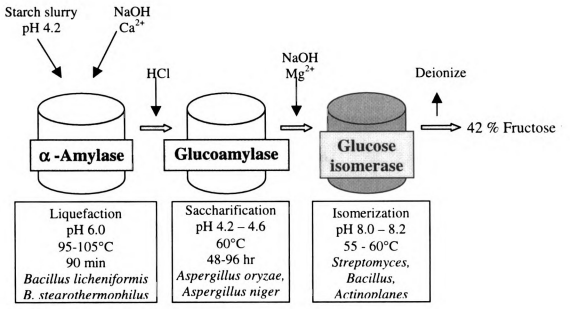

Figure 2: Starch processing into high fructose corn syrup (HFCS)

HFCS is preferred by the food industry since it does not pose the problem of crystallization, as is the case with sucrose. Moreover, fructose plays an important role as a diabetic sweetener because it is only slowly reabsorbed by the stomach and does not influence the glucose level in blood.

The major uses of HFCS are in the beverage, baking, canning, and confectionery industries. The annual world consumption of HFCS was estimated to have reached 10 million tons (dry weight) in 1995 (de Raadt *et al.*, 1994). In 1996, HFCS has almost completely replaced sucrose in beverage market in the United States. Beside its industrial importance, xylose isomerase also serves as an interesting model for studying structure-function relationships by advanced biochemical and genetic engineering techniques such as site-directed mutagenesis and directed evolution.

XI STRUCTURE, SEQUENCE HOMOLOGY, AND REACTION MECHANISMS

To elucidate structure-function relationships of xylose isomerases, the genes encoding XIs (*xylA*) sequences from various organisms have been compared. XIs can be classified into two groups based on their amino acid sequences and a 40-50 residue N-terminal extension (Vangrysperre *et al.*, 1988). Class I XIs contain about 390 amino acids and consist of those from *Streptomyces* spp., *Actinoplanes* spp., *Ampullariella* spp., *Arthrobacter* spp., and *Thermus thermophilus.* The enzymes from *Escherichia coli*, *Bacillus* spp., *Lactobacillus* spp., *Lactococcus* spp., *Thermoanaerobacterium thermosulfurigenes,* and *Thermotoga spp.* contain approximately 440 amino acids and are grouped as class II XIs. The enzymes being studied extensively in this work

(*Thermoanaerobacterium thermosulfurigenes* XI: TTXI and *Thermotoga neapolitana* XI: TNXI) belong to the class II XIs. The XI monomer consists of an eight-stranded parallel β-barrel surrounded by eight helices with an extended C-terminal tail that provides extensive contacts with a neighboring monomer (Figure 3). The active site pocket is defined by an opening in the barrel of which the entrance is lined with hydrophobic residues while the bottom of the pocket consists mainly of glutamate, aspartate, and histidine residues coordinated to two bivalent cations (Mg^{2+}, Mn^{2+}, or Co^{2+})(Whitlow *et al.*, 1991). Three type I and five type II XI's *xylA* sequences were aligned based on their sequence homology (Fig 4). In spite of the low homology between classes I and II enzymes, the amino acids involved in the substrate (H100, T140, E231, K233, D338) and metal ions binding (E231, E267, H270, D295, D306, D308, D338), as well as in catalysis (H100, D103, D338), are completely conserved (Fig. 5). Thus, the essential structure at the catalytic center of XIs appears to be analogous in class II XIs so far compared.

The subunit structure and amino acid composition of XI reveal that it is a tetramer or a dimer of similar or identical subunits associated with non-covalent bonds and is devoid of interchain disulfide bonds. Most known XIs are homotetramers with molecular masses of approximately 45 to 50 kDa per subunit, although some XIs have been found to be dimeric. The dissociation and unfolding of the tetrameric XI from *Streptomyces* sp. Strain NCIM 2730 revealed that the tetramer and the dimer are the active species whereas the monomer is inactive (Ghatge *et al.*, 1994).

The native *Thermotoga neapolitana* homotetrameric xylose isomerase is also expressed as a catalytically active and thermostable dimer in *E. coli* (Hess *et al.*, 1998).

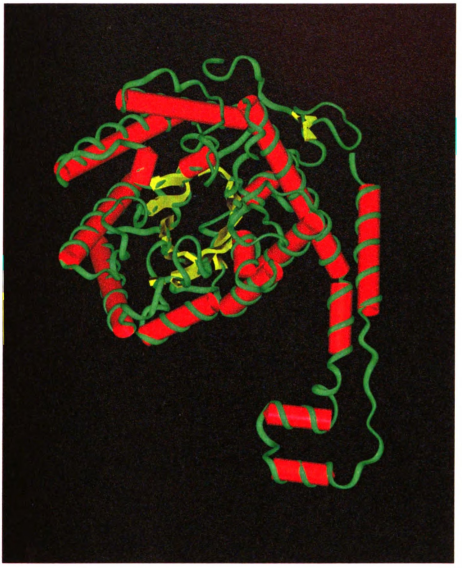

Figure 3: Three-dimensional structure of a subunit of *Thermotoga neapolitana* xylose isomerase (type II XI).

Figure 4: Homology alignment of xylose isomerase amino acid sequences from different organisms by the ClustalW analysis (http://www.ebi.ac.uk/clustalw). The sequences are T.m., *Thermotoga maritima*; T.n., *Thermotoga neapolitana*; T.t., *Thermoanaerobacterium thermosulfurigenes*; B.s., *Bacillus subtilis*; B.l., *Bacillus licheniformis*; E.c., *Escherichia coli*; A.m., *Actinoplanes missouriensis*; S.r., *Streptomyces rubiginosus*; Ar, *Arthrobactor* sp. (*) means amino acids are identical, (:) means conserved substitutions, and (.) means semi-conserved substitutions.

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T.m.          -------MAEFFPEIPKIQFEGKESTNPLAFRFYDPNEVIDGKPLKDHLKFSVAFWHTFV 53
T.n.          -------MAEFFPEIPKVQFEGKESTNPLAFKFYDPEEIIDGKPLKDHLKFSVAFWHTFV 53
T.t.          -------MNKYFENVSKIKYEGPKSNNPYSFKFYNPEEVIDGKTMEEHLRFSIAYWHTFT 53
B.s.          MAQSHSSSINYFGSANKVVYEGKDSTNPLAFKYYNPQEVIGGKTLKEHLRFSIAYWHTFT 60
B.l.          ---------MFFRNIGMIEYEGADSENPYAFKYYNPDEFVGGKTMKEHLRFAVAYWHTFD 51
E.c.          -------MQAYFDQLDRVRYEGSKSSNPLAFRHYNPDELVLGKRMEEHLRFAACYWHTFC 53
A.m.          ----------------------------MSVQATREDKFSFG------------LWTVG 19
S.r.          ----------------------------MNYQPTPEDRFTFG------------LWTVG 19
Ar            ----------------------------MSVQPTPADHFTFG------------LWTVG 19
                :.: ::      .      .. .: ..   :    :.. *.  ..    : .   *.

T.m.          NEGRDPFGDPTAERPWNRFSDPMDKAFARVDALFEFCEKLNIEYFCFHDRDIAPEGKTLR 113
T.n.          NEGRDPFGDPTADRPWNRYTDPMDKAFARVDALFEFCEKLNIEYFCFHDRDIAPEGKTLR 113
T.t.          ADGTDQFGKATMQRPWNHYTDPMDIAKARVEAAFEFFDKINAPYFCFHDRDIAPEGDTLR 113
B.s.          ADGTDVFGAATMQRPWDHYKG-MDLAKMRVEAAFEMFEKLDAPFFAFHDRDIAPEGSTLK 119
B.l.          ADGKDPFGDGTMFRAWNRLTHPLDKAKARAEAAFEFFEKLGVPYFCFHDVDIVDEGATLR 111
E.c.          WNGADMFGVGAFNRPWQQPGEALALAKRKADVAFEFFHKLHVPFYCFHDVDVSPEGASLK 113
A.m.          WQARDAFGDATRT------------ALDPVEAVHKLAEIGAYGITFHDDDLVPFGSDAQ 66
S.r.          WQGRDPFGDATRR------------ALDPVESVRRLAELGAHGVTFHDDDLIPFGSSDS 66
Ar            WTGADPFGVATRK------------NLDPVEAVHKLAELGAYGITFHDNDLIPFDATEA 66
                . * **  :   . .        :       ..   ::      *** *:   .

T.m.          ETNKILDKVVERIKERMKDSNVKLLWGTANLFSHPRYMHGAATTCSADVFAYAAAQVKKA 173
T.n.          ETNKILDKVVERIKERMKDSNVKLLWGTANLFSHPRYMHGAATTCSADVFAYAAAQVKKA 173
T.t.          ETNKNLDTIVAMIKDYLKTSKTKVLWGTANLFSNPRFVHGASTSCNADVFAYSAAQVKKA 173
B.s.          ETNQNLDMIMGMIKDYMRNSGVKLLWNTANMFTNPRFVHGAATSCNADVFAYAAAQVKKG 179
B.l.          ETFTYLDQMSSFLKEMMETSHVQLLWNTANMFTHPRYVHGAATSCNADVYAYAAAKVKKG 171
E.c.          EYINNFAQMVDVLAGKQEESGVKLLWGTANCFTNPRYGAGAATNPDPEVFSWAATQVVTA 173
A.m.          TR----DGIIAGFKKALDETGLIVPMVTTNLFTHPVFKDGGFTSNDRSVRRYAIRKVLRQ 122
S.r.          ER----EEHVKRFRQALDDTGMKVPMATTNLFTHPVFKDGGFTANDRDVRRYALRKTIRN 122
Ar            ER----EKILGDFNQALKDTGLKVPMVTTNLFSHPVFKDGGFTSNDRSIRRFALAKVLHN 122
                        :     :   :   *:* *::* :  *. *  . .:  ::  :.

T.m.          LEITKELGGEGYVFWGGREGYETLLNTDLGLELENLARFLRMAVEYAKKIGFTGQFLIEP 233
T.n.          LEITKELGGEGYVFWGGREGYETLLNTDLGFELENLARFLRMAVDYAKRIGFTGQFLIEP 233
T.t.          LEITKELGGENYVFWGGREGYETLLNTDMEFELDNFARFLHMAVDYAKEIGFEGQFLIEP 233
B.s.          LETAKELGAENYVFWGGREGYETLLNTDLKFELDDLARFMHMAVDYAKEIGYTGQFLIEP 239
B.l.          LDIAKELGAENYVFWGGREGYETLLNTDMKLELENLSSFYRMAVEYAREIGFDGQFLIEP 231
E.c.          MEATHKLGGENYVLWGGREGYETLLNTDLRQEREQLGRFMQMVVEHKHKIGFQGTLLIEP 233
A.m.          MDLGAELGAKTLVLWGGREGAEYDSAKDVSAALDRYREALNLLAQYSEDRGYGLRFAIEP 182
S.r.          IDLAVELGAETYVAWGGREGAESGGAKDVRDALDRMKEAFDLLGEYVTSQGYDIRFAIEP 182
Ar            IDLAAEMGAETFVMWGGREGSEYDGSKDLAAALDRMREGVDTAAGYIKDKGYNLRIALEP 182
              ::   ::*.:  * ****** *    .*:    :           :    *:   : :**

T.m.          KPKEPTKHQYDFDVATAYAFLKNHGLDEYFKFNIEANHATLAGHTFQHELRMARILGKLG 293
T.n.          KPKEPTKHQYDFDVATAYAFLKSHGLDEYFKFNIEANHATLAGHTFQHELRMARILGKLG 293
T.t.          KPKEPTKHQYDFDVANVLAFLRKYDLDKYFKVNIEANHATLAFHDFQHELRYARINGVLG 293
B.s.          KPKEPTAHQYDTDAATTIAFLKQYGLDNHFKLNLEANHATLAGHTFEHELRMARVHGLLG 299
B.l.          KPKEPTKHQYDFDAATTIAFLETYGLKDHFKLNLEANHATLAGHTFEHELRVAALHDMLG 291
E.c.          KPQEPTKHQYDYDAATVYGFLKQFGLEKEIKLNIEANHATLAGHSFHHEIATAIALGLFG 293
A.m.          KPNEPRGDILLPTAGHAIAFVQELERPELFGINPETGHEQMSNLNFTQGIAQALWHKKLF 242
S.r.          KPNEPRGDILLPTVGHALAFIERLERPELYGVNPEVGHEQMAGLNFPHGIAQALWAGKLF 242
Ar            KPNEPRGDIFLPTVGHGLAFIEQLEHGDIVGLNPETGHEQMAGLNFTHGIAQALWAEKLF 242
              **:**  .     ..   .*:.     .   .* *..*  ::   * : :  *     :

T.m.          SIDANQGDLLLGWDTDQFPTNIYDTTLAMYEVIKAGG-----FTKGGLNFDAKVRRASYK 348
T.n.          SIDANQGDLLLGWDTDQFPTNVYDTTLAMYEVIKAGG-----FTKGGLNFDAKVRRASYK 348
T.t.          SIDANTGDMLLGWDTDQFPTDIRMTTLAMYEVIKMGG-----FDKGGLNFDAKVRRASFE 348
B.s.          SVDANQGHPLLGWDTDEFPTDLYSTTLAMYEILQNGG-----LGSGGLNFDAKVRRSSFE 354
B.l.          SIDANQGDLLLGWDTDEFPTDLYSAVLAMYEILKAGG-----FKTGGINFDAKVRRPSFA 346
E.c.          SVDANRGDAQLGWDTDQFPNSVEENALVMYEILKAGG-----FTTGGLNFDAKVRRQSTD 348
A.m.          HIDLNGQHGPKFDQDLVFGHGDLLNAFSLVDLLENGP-DGAPAYDGPRHFDYKPSR-TED 300
S.r.          HIDLNGQNGIKYDQDLRFGAGDLRAAFWLVDLLES------AGYSGPRHFDFKPPR-TED 295
Ar            HIDLNGQRGIKYDQDLVFGHGDLTSAFFTVDLLENGFPNGGPKYTGPRHFDYKPSR-TDG 301
               :* *        :   *  .    .:   :::: .         *  :** *  * :
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T.m.            VEDLFIGHIAGMDTFALGFKIAYKLAKDGVFDKFIEEKYRSFKEGIGKEIVEGKTDFEKL 408
T.n.            VEDLFIGHIAGMDTFALGFKVAYKLVKDGVLDKFIEEKYRSFREGIGRDIVEGKVDFEKL 408
T.t.            PEDLFLGHIAGMDAFAKGFKVAYKLVKDRVFDKFIEERYASYKDGIGADIVSGKADFRSL 408
B.s.            PDDLIYAHIAGMDAFARGLKVAHKLIEDRVFEDVIQHRYRSFTEGIGLEIIEGRANFHTL 414
B.l.            DEDLFHAHIAGMDTYAVGLKVASRLLEDKALDQVIEERYESYTKGIGLEIKEGRTDLKKL 406
E.c.            KYDLFYGHIGAMDTMALALKIAARMIEDGELDKRIAQRYSGWNSELGQQILKGQMSLADL 408
A.m.            YDGVWESAKANIRMYLLLKERAKAFRADPEVQEALAASKVAELKTPTLNPGEGYAELLAD 360
S.r.            FDGVWASAAGCMRNYLILKERAAAFRADPEVQEALRASRLDELARPTA--ADGLQALLDD 353
Ar              YDGVWDSAKANMSMYLLLKERALAFRADPEVQEAMKTSGVFELGETTLNAGESAADLMND 361
                  .:  .  . :       : *  :  *  .:. :             .  ..   :

T.m.            EEYIIDKED-IELPSGK-QEYLESLLNSYIVKTIAELR------ 444
T.n.            EEYIIDKET-IELPSGK-QEYLESLINSYIVKTILELR------ 444
T.t.            EKYALERSQ-IVNKSGR-QELLESILNQYLFAE----------- 439
B.s.            EQYALNHKS-IKNESGR-QEKLKAILNQYILEV----------- 445
B.l.            AAYALENDH-IENQSGR-QERLKATVNRYLLNALREAPAGKETH 448
E.c.            AKYAQEHHLSPVHQSGR-QEQLENLVNHYLFDK----------- 440
A.m.            RSAFEDYDADAVGAKGFGFVKLNQLAIEHLLGAR---------- 394
S.r.            RSAFEEFDVDAAAARGMAFERLDQLAMDHLLGARG--------- 388
Ar              SASFAGFDAEAAAERNFAFIRLNQLAIEHLLGSR---------- 395
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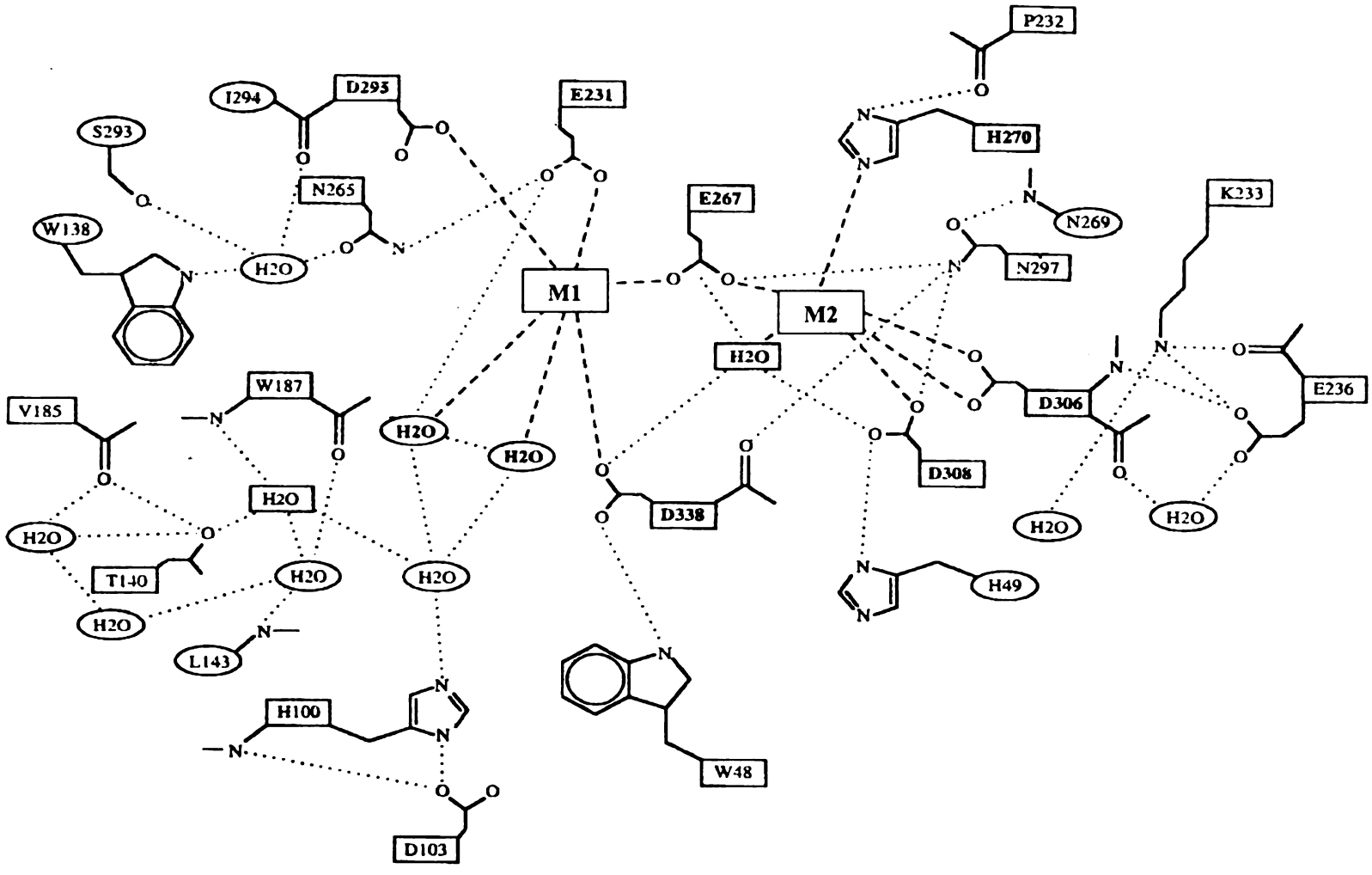

Figure 5: Schematic representation of the active site of TNXI. Residues interacting with metal ions and with the substrate are shown. Hydrogen bonds (light dashes) and metal bonds (heavy dashes) are indicated.

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The recombinant TNXI existed as a dimer as well as a tetramer with the ratio of dimer to tetramer of approximately 20:1. Although the structural features of the recombinant and native forms of TNXI differ in the degree of subunit assembly, their functional properties do not differ. The dimer is a catalytically viable and stable form of the enzyme.

Typically, two divalent cations (Mg^{2+}, Mn^{2+}, or Co^{2+}) per monomer are required for catalytic activity and stability of XIs. Two distinct metal binding sites, M1 and M2, have been identified in XIs: (i) the metal in site M1 is coordinated to four carboxylate groups; (ii) the metal in site M2 is coordinated to one imidazole and three carboxylate groups. These were initially referred to as the structural and catalytic metals, respectively (Whitlow *et al.*, 1991, and Marg and Clark, 1990). But these assumptions are no longer valid, since later studies showed that both metals are directly involved in catalysis (Jenkins *et al.*, 1992 and Allen *et al.*, 1994). Metal specificity depends on both the nature of substrate and on the enzyme type. *Thermus aquaticus* XI, a type I enzyme isomerizes glucose most efficiently in the presence of Mn^{2+}, but its activity toward xylose is highest with Co^{2+} (Lehmacher and Bisswanger, 1990). The type II *Bacillus coagulans* XI, on the other hand, isomerizes xylose most effectively in the presence of Mn^{2+}, while its activity toward fructose is highest with Co^{2+} (Marg and Clark, 1990). In our case, the three metals activate the TNXI. At any concentration, Co^{2+} is the best activating metal. Glucose isomerase activity in the presence of Mg^{2+} is approximately 40 % of the activity observed with the Co^{2+} enzyme. Poorly active, the Mn^{2+} enzyme show only 16 % of the activities observed with the Co^{2+} enzyme (Vieille *et al.*, 2001). The thermal stabilization of TNXI is also metal-specific: the Mn^{2+} enzyme is significantly more stable than the Co^{2+} and Mg^{2+} enzymes at 101°C.

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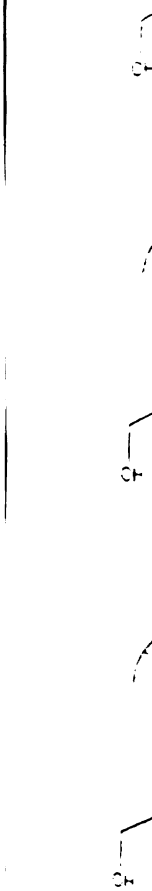
A mechanism of xylose isomerase catalysis was identified based on results of x-ray crystallographic studies on *Arthrobacter* or *Streptomyces* enzymes (Farber *et al.*, 1989; Collyer *et al.*, 1990) and biochemical properties exhibited by thermophilic enzymes obtained by site-directed mutagenesis of the *xylA* gene from *Thermoanaerobacterium thermosulfurigenes* (Lee *et al.*, 1990). The enzymatic interconversion of aldose to ketose by xylose isomerases involves binding of the substrate in the ring form, substrate ring opening, isomerization of the linear intermediate, intermediate ring closure, and release of the product. The isomerization step proceeds by a metal ion-assisted hydride-shift mechanism (Figure 6) (Farber *et al.*, 1989; Collyer *et al.*, 1990; Lee *et al.*, 1990), and this step, rather than ring opening, is rate determining (Lee *et al.*, 1990). D-xylose and D-glucose have identical atomic configuration, except for the presence of an additional $-CH_2OH$ group at the C-6 position in the glucose molecule. This extra hydroxymethyl group must therefore be responsible for the differences in the catalytic efficiency exhibited by xylose isomerase toward glucose versus xylose.

THERMOZYMES AND THEIR THERMAL STABILITY

Thermozymes are enzymes that evolved in thermophiles (organisms thriving at 50-80°C) and hyperthermophiles (organisms thriving at 80°C or above). Most archea and some bacteria are thermophiles and hyperthermophiles. These organisms have been isolated from all types of terrestrial and marine environments. Thermozymes developed unique structure-function properties above 70°C (Vieille and Zeikus, 1996; Vieille and



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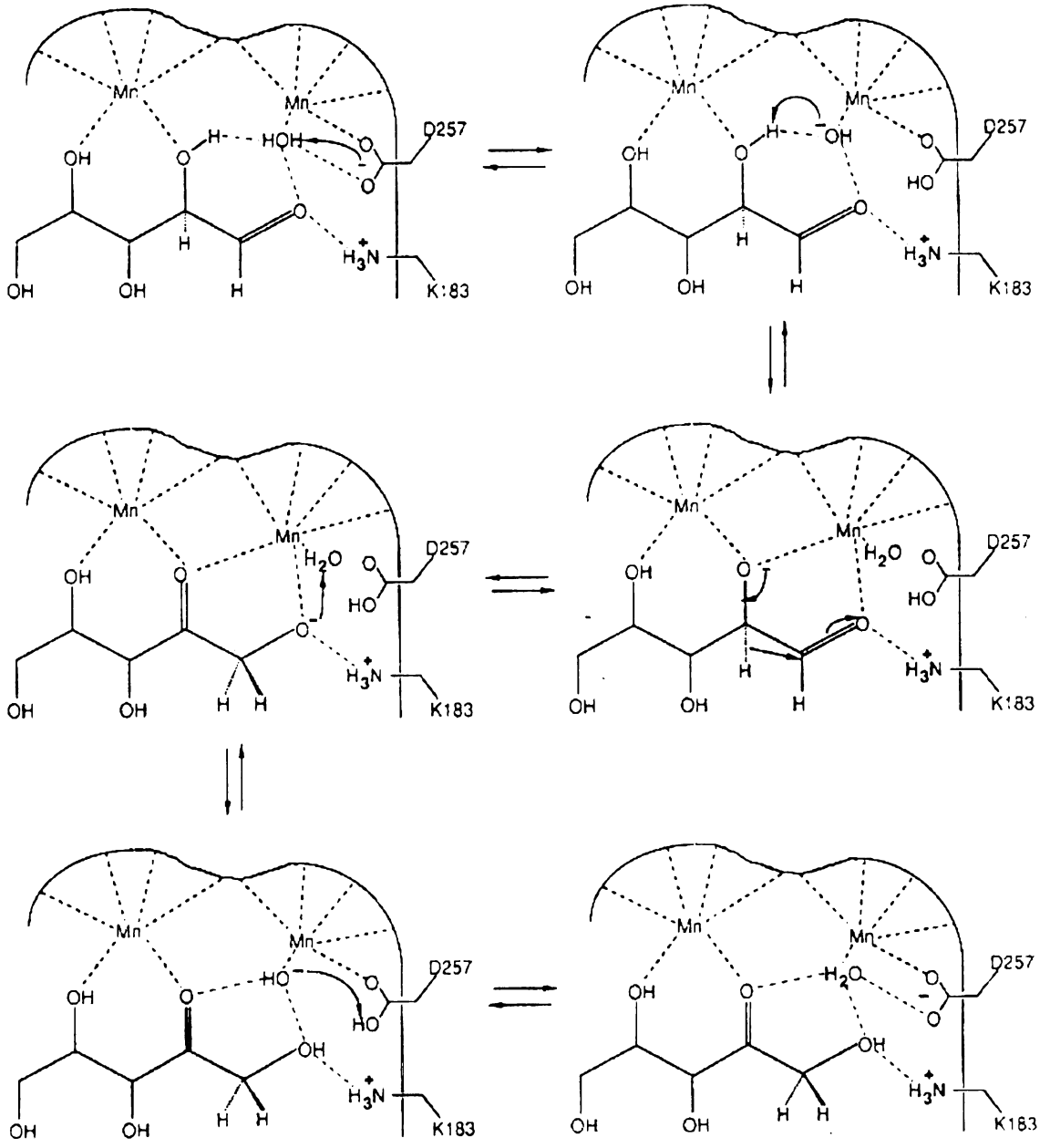

Figure 6: A metal-assisted hydride-shift mechanism of xylose in the *Streptomyces rubiginosus* xylose isomerase (type I XI). In type II XIs, Both Mn^{2+} are replaced by Co^{2+}. Corresponding residues of D257 and K183 in the *Thermotoga neapolitana* XI (TNXI) are D308 and K233, respectively.

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Zeikus, 2001). They have already been used in molecular biology (e.g. *Taq* DNA polymerase), starch-processing (e.g. α-amylases, glucose isomerases) industry, and are excellent catalytic candidates for several additional applications that require high stability including organic syntheses, diagnostics, waste treatment, pulp and paper manufacture, and animal feed (Vieille *et al.,* 1996). Intrinsically stable and active at high temperatures, thermozymes offer major biotechnological advantages over mesophilic enzymes (optimally active at 25-50°C) or psychrophilic enzymes (optimally active at 5-25°C): (i) once expressed in mesophilic hosts, thermozymes are easier to purify by heat treatment, (ii) their thermostability is associated with a higher resistance to chemical denaturants, and (iii) performing enzymatic reactions at high temperatures allow higher substrate concentrations, lower viscosity, fewer risks of microbial contamination, and higher reaction rates.

Protein stability has been actively studied for several decades starting with small, soluble, and monomeric enzymes (e.g. lysozyme and ribonuclease)(Dill, 1990). Having access to thermozymes allows us to determine what protein stabilization mechanisms are used in nature to gain extreme stability. Thermozymes are relatively similar to mesophilic enzymes: (i) their amino acid sequences are 40-85 % similar to those of their mesophilic counterparts (Vieille *et al.*, 1995 and Burdette *et al.*, 1996), (ii) their three-dimensional structures are superimposable (Davies *et al.*, 1993 and Fujinaga *et al.*, 1993), and (iii) they share the same catalytic mechanisms (Vieille *et al.*, 1995 and Voorhorst *et al.*, 1995). Therefore, their increased stability (as compared with their mesophilic counterparts) must be a result of differences in specific amino acid sequences. Since thermozymes are optimally active under more severely denaturing conditions than their

mesophilic counterparts, they need to be more rigid. This increased rigidity is essential for preserving their active center structures and protect them from unfolding. Such rigidity is demonstrated by lower hydrogen exchange rates and by lower susceptibility to proteolytic degradation and chemical denaturant or thermal unfolding (Veronese *et al.*, 1984, Wrba *et al.*, 1990, Kanaya and Itaya, 1992). Observations from large groups of enzymes indicate that stabilizing substitutions tend to improve the enzymes' packing efficiency (through cavity filling and increase in core hydrophobicity), and to increase overall enzyme rigidity through helix stabilization, electrostatic interaction optimization and conformational strain reduction.

Well-known and studied mechanisms of protein thermostabilization (Vieille and Zeikus, 1996; Vieille and Zeikus, 2001) are (i) hydrophobic interactions, (ii) packing efficiency and reduction in solvent-accessible hydrophobic surface, (iii) aromatic interactions, (iv) salt bridges, (v) disulfide bridges, (vi) hydrogen bonds, (vii) metal binding, (viii) reduction of conformational strain, (ix) reduction of the entropy of unfolding by proline substitution, (x) helix stabilization, (xi) stabilization of loops (xii) intersubunit interactions and oligomerization, (xiii) resistance to covalent destruction, and (xiv) post-translational modifications.

GENETIC AND PROTEIN ENGINEERING OF XIs

Thermostability mutations

A cluster of aromatic amino acid residues is present in the active site pocket of xylose isomerases isolated from different sources and the hydrophobic interactions

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among these aromatic amino acids were postulated to be one of the important forces that maintains the monomers associated into active dimers (Collyer *et al.*, 1990 and Whitlow *et al.*, 1991). Meng et al. (1993) explored the thermostability of mutants of aromatic residues (Trp48, Phe59, Trp138, Phe144, and Trp187) in *Thermoanaerobacterium thermosulfurigenes* XI) with Co^{2+} as a co-factor.

The Trp187His and Phe144Lys mutant enzymes were no longer resistant to the heat treatment at 75°C, which was one of the purification steps of the wild type and mutant enzymes. This indicates that the hydrophobic character of Trp187 and Phe144 is important in maintaining the enzyme structure at high temperature. The Trp138 substitutions with smaller hydrophobic residues had increased the thermostability at 85°C by 43-91 % without affecting the enzymes' activities. Since the indole group of Trp138 protrudes into the active site cavity, replacement of Trp138 with Phe, Met, or Ala reduced the area of active site hydrophobic surface and therefore enhanced thermostability. Trp48Arg mutation did not change the activity of the enzyme but it increased the stability of the enzyme by 60 %. The enhancement of thermostability in the Trp48Arg mutant enzyme was brought about because Arg48 could presumably fulfill the function of hydrogen bonding to Asp338 and thus leave the active site structure unchanged. The Phe59His mutant was relatively less stable with shorter half-live than the wild-type enzyme.

A different approach to improving thermostability was adopted by Quax et al. (1991) by converting lysine residues in *Actinoplanes missouriensis* XI (AMXI) to Arg residues. Special attention was given to preventing deleterious effects that could result from chemical modification of xylose isomerase by sugar components of high fructose

corn syrup, and in particular, from non-enzymatic glycation of key amino groups. Each AMXI subunit contains 20 lysine residues and two of them are located at the dimer/dimer interface, Lys252 and Lys294 (Gly304 and Lys340 in TNXI, respectively).

Lys252 is buried in the XI tetramer and is also involved in electrostatic interactions across the interdimer interface. The mutation Lys252Arg resulted in a 30 % increased in thermostabilty of the Mg-enzyme in solution with no loss in activity. More importantly, the half-life of the immobilized mutant at 70°C was 3-fold longer than that of the wild type. Lys252 would be accessible for glycation when the dimer dissociates, which would inactivate the enzyme by preventing reassociation of the dimer. This would explain why the immobilized mutant enzyme is more resistant to glucose inactivation.

Lys294 forms a salt bridge with the M1 ligand Asp256 (Asp308 in TNXI) and the M2 ligand Asp292 (Asp338 in TNXI). Lys294Arg had decreased thermostability in solution but very little after immobilization.

Chang *et al.* (1999) studied the structures of highly thermostable type I xylose isomerases from *Thermus thermophilus* (TthXI) and *Thermus caldophilus* (TcaXI) compared to those of less thermostable XIs from *Arthrobacter* B3728 (AXI) and *Actinoplanes missouriensis* (AMXI). Analyses of various factors that may affect protein thermostability indicate that the possible structural determinants of the enhanced thermostability of TcaXI/TthXI over AXI/AMXI are

(i) An increase in ion pair networks: the total ion pairs per tetramer of TcaXI/TthXI/AXI/AMXI are 150/163/93/107, respectively.

(ii) A decrease in the large intersubunit cavities: there is a clear correlation between the decreased number of large internal cavities and thermostability. The total

volume and surface area of cavities are 389/373/896/591 $Å^3$ and 938/887/1702/1136 $Å^2$ for TcaXI/TthXI/AXI/AMXI, respectively. All cavities in TcaXI/TthXI tetramers are small, whereas some of the cavities in AXI/AMXI tetramers are very large. The volume and surface area of the largest cavities in TcaXI/TthXI/AXI/AMXI are 38/30/193/122 $Å^3$ and 74/64/304/207 $Å^2$.

(iii) A removal of potential deamidation and isoaspartate formation sites: Deamidation and isoaspartate formation have been found to be one of the important processes leading to irreversible heat denaturation of protein at neutral pH (Ahern and Klibanov, 1985 and Aswad, 1990). They occur frequently when the sequences Asn-Gly, Asn-Ser, and Asp-Gly lie in highly flexible regions of the polypeptide chain. The analysis indicated that they are much fewer in TcaXI/TthXI (2/1) compared to AXI/AMXI (7/7).

(iv) Shortened loops and Proline residues: TcaXI and TthXI have a significantly shortened loop due to deletions, compared with AXI and AMXI. Compared with AXI and AMXI, TcaXI and TthXI have approximately five more proline residues.

A site-directed mutagenesis study of His219 residue of *Streptomyces rubiginosus* XI (His270 in TNXI) and its effect on activity and thermostability was done by Cha *et al.* (1994). This residue is conserved in all xylose isomerases. The three dimensional structure of the enzyme revealed that His219 is part of the octahedral coordination sphere of M2, One of the two metal ions (Mn^{2+}) in the active site. Substitutions of His219 with Ser, Glu, and Asn resulted in enzymes with the k_{cat} values of only 0.3-0.5 % of that of the wild-type enzyme. The K_m values of these mutant enzymes increased by 30-40 fold over

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the wild-type value. The His219Lys mutant enzyme did not exhibit any measurable activity.

Thermal denaturation studies indicated that the His219Ser and His 219Asn mutant enzymes are 5-8°C less stable, whereas His219Glu and His219Lys are 13-24°C less stable than the wild-type enzyme. In the His219Ser structure, a water molecule effectively replaced the Nε-2 atom of the imidazole ring of His219 and mediated the interaction between Mn^{2+} at the M2 site and Ser219. A similar water-mediated interaction between the metal ion and Asn219 was observed in the His219Asn mutant enzyme structure. On the other hand, no direct or water mediated interactions between the carboxyl group of Glu219 and the metal were observed. Whereas octahedral coordination was maintained for the metal at the M2 site in His219Ser and His219Asn, a pentahedral coordination with the metal at the M2 site was observed in His219Glu. Metal activation measurements supported the observation that metal binding is perturbed and is responsible for thermal lability of His219 mutant enzymes.

Active site mutations

Most active site mutations reduce or destroy activity but some are also relevant to enzyme thermostability (Hartley *et al.*, 2000). However, Meng *et al.* (1990) successfully used site-directed mutagenesis to switch the substrate preference of *Thermoanaerobacterium thermosulfurigenes* XI (TTXI) from xylose to glucose by redesigning its substrate binding pocket. In the *Arthrobacter* enzyme, the structure of the enzyme complex with the six carbon competitive inhibitor, sorbitol, indicated that the C6-hydroxymethyl group of the substrate (glucose) is oriented toward the bottom of the

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substrate-binding pocket and is adjacent (3.4-3.7 Å) to the residues Met87, Thr89, and Val134. These residues are highly conserved among XIs and correspond to Trp138, Thr140, and Val185, respectively, in the TTXI. Each of these residues was replaced with smaller amino acids to prove that they are part of the substrate-binding pocket in TTXI. Replacement of Trp138 with Phe, a smaller residue, produced an enzyme that had a higher catalytic efficiency (k_{cat}/K_m) for glucose than the wild-type enzyme due to both a decrease in K_m and an increase in k_{cat}. On the other hand, this mutation increased the K_m for xylose and decreased the catalytic efficiency for this substrate. This was suggested to be due to a more spacious pocket and this increased the freedom of movement of the xylose molecule, decreasing its binding efficiency. The enlargement of the binding pocket also decreased binding energy between the enzyme and the transition state resulting in the decrease of k_{cat}.

The Val185Thr mutant enzyme had a slightly lower K_m and a higher k_{cat} for glucose. Substitution with a Ser residue, which has a smaller sidechain but otherwise is equivalent to Thr, did not improve the glucose catalytic efficiency. Likewise, replacement with Ala did not significantly change either K_m or k_{cat}. Thus these data suggested that Val185 does not hinder glucose binding, but the Thr substitution may provide an additional hydrogen bonding, presumably to the C6-OH group of glucose.

Replacement of Thr140 with Ser increased the K_M for both xylose and glucose and resulted in lower catalytic efficiency for glucose. It can be concluded that Thr140 hydrogen bonds to the substrate but does not strictly hinder the binding of glucose. The double mutant enzymes Trp138Phe/Val185Thr and Trp138Phe/Val185Ser had a higher catalytic efficiency for glucose than the wild-type enzyme of 5- and 2-fold, respectively.

They also exhibited 1.5-and 3-fold higher catalytic efficiency for glucose than for xylose, respectively, thus they can be called the real "glucose isomerase" rather than xylose isomerase.

DIRECTED EVOLUTION OF ENZYMES

Directed evolution, also termed evolutionary engineering, has recently emerged as a key technology for biomolecular engineering and generating impressive results in the functional adaptation of enzymes to artificial, non-natural environments (Reetz and Jaeger, 1999; Pluckthun *et al.*, 2000; and Wintrode and Arnold, 2000). The approach is highly attractive because its principles are simple and do not require detailed knowledge of structure, function, or mechanism. Essentially like natural (Darwinian) evolution, directed evolution comprises the iterative implementation of (1) the generation of a "library" of mutated genes, (2) its functional expression, and (3) a sensitive assay to identify individuals showing the desired properties, either by selection or by screening. After each round, the genes of improved variants are deciphered and subsequently serve as parents for another round of optimization (Brakmann, 2001).

A series of experimental strategies have been developed for generating mutant libraries in the laboratory which differ in diversity. They can be divided into two main approaches, random mutagenesis and recombination. Random mutagenesis is a widely used strategy, which target whole genes. This may be achieved by passing cloned genes through mutator strains (Cox, 1976; and Greener *et al.*, 1996), by treating DNA with various chemical mutagens (Shortle and Nathans, 1978; Kadonaja and Knowles, 1985;

and Deshler, 1992), or by error-prone PCR (Leung *et al.*, 1989; and Cadwell and Joyce, 1992). Due to its simplicity and versatility, error-prone PCR (random PCR mutagenesis) emerged as the most common technique, which can result in mutation rate as high as 2 % per nucleotide position. The mutation rate may also be adjusted to lower values with alterations of PCR conditions. However, only a limited number of amino acid substitutions is accessible by this method since this reaction biases the distribution of mutation type in favor of transitions (A↔G and T↔C) over transversions (A/G↔C/T), and because multiple substitutions within a single codon are extremely rare. Complete permutation of a single amino acid position (saturation mutagenesis) may enable the finding of non-conservative replacements that are inaccessible by random PCR mutagenesis (Miyazaki and Arnold, 1999).

Recombination of DNA represents an alternative or additional approach for generating genetic diversity that is based on mixing and concatenation of genetic material from a number of parent sequences. Recombination may be advantageous in concentrating beneficial mutations which have arisen independently and may be additive, and likewise, in concentrating deleterious mutations which subsequently might be more efficiently purged from the population by selection (Zeyl and Bell, 1997; and Moore and Maranas, 2000). DNA shuffling was the first technique introduced for random in vitro recombination of gene variants created by random mutagenesis (Stemmer, 1994). It employs the PCR reassembly of whole genes from a pool of short overlapping DNA sequences (50-300 bp) generated by random enzymatic fragmentation of different parental genes. Alternative protocols include staggered extension process (StEP) (Zhao *et al.*, 1998) and random-priming recombination (Shao *et al.*, 1998). StEP recombination is

a PCR reaction with very short annealing and extension steps that promote the formation of premature extension products. The truncated strands may anneal randomly to a parent strand, thus combining the different sequences from different parental strands. Random-priming recombination, as an alternative to DNA shuffling, produces random fragments for reassembly by annealing of short, random primers to a certain template gene and extension by a polymerase.

In directed enzyme evolution, diversity is created on the DNA level, but selection or screening acts on the level of the encoded protein. Therefore, functional expression of the DNA libraries is a necessary prerequisite for the detection of improved enzyme variants. The most common approaches for recombinant protein expression employ the cellular transcription/translation machineries of well-established organisms such as *E. coli*, *S. cerevisiae*, or *B. subtilis*. Alternatively, a physical link between genotype and phenotype may be established by generating fusions between the protein of interest and a bacteriophage coat protein. Following intracellular assembly, recombinant phages express the protein variants on their surface while enclosing the appertaining genetic information within their genome (Johnson and Ge, 1999; and Smith and Petrenko, 1997).

The most challenging step in directed evolution experiments is to develop a screening or selection scheme that is sensitive to the desired properties. Selection can be used either *in vivo* or *in vitro*. *In vivo* selection is most often achieved by genetic complementation of hosts that are deficient in a certain pathway or capacity. *In vitro* enrichment procedures that are detached from cell survival may also be termed selection. These techniques have been developed for the biopanning of phage-displayed peptide libraries by binding to a ligand that is immobilized on an appropriate column matrix

(Brakmann, 2001). Recently, the approach has also been applied to the selective enrichment of phage-displayed functional enzyme libraries. Screening is an important alternative to selection. It enables a better control of the applied constraints, and also is more versatile, predominantly in unnatural environments, or with unnatural substrates. It usually requires that the mutant libraries are diluted and well-distributed which can be achieved by conventional plating of transformants on agar plates or filter membranes. This time-consuming step is sometimes accelerated using robotic systems. Common assays are based on visual or spectroscopic detection, for example formation, alteration, or destruction of colors or fluorescence characteristics. The determination of the optical parameters can also be accomplished by using automatic plate-readers, which enable a normalization of measured values to respective cell densities and may also be used to monitor the reaction kinetics. It should be noted that it is important to choose selective constraints that precisely reflect the desired property since the first law of directed enzyme evolution is "you get what you screen for" (Schmidt-Dannert and Arnold, 1999).

During the past few years, many enzymes have been successfully improved by directed evolution (Table 1). The narrow range of substrates accepted by natural enzymes often prevents their use in new synthetic and commercial applications. Thus, by far most results were efficient tuning of catalytic efficiency toward non-natural substrates. Thermostability of enzymes and enantioselectivity of specific bioconversions have also been improved by these approaches. These examples showed that directed evolution is a powerful and reliable tool for improving biocatalysts in reasonably short periods of time.

The only directed evolution approach (or random mutagenesis) performed on xylose isomerase reported to date was by Lonn *et al.* (2002). The thermophilic *Thermus*

thermophilus XI was subjected to one round of random PCR mutagenesis. It was screened for xylose isomerase activity at lower temperature than optimal by expression of the mutated genes in *E. coli* and replica-plated on McConkey agar plates, complemented with 1 % xylose followed by incubation at 30°C for 2 days. Three transformant colonies that were deeper red (suggesting higher XI activity) than the wild-type were selected and further characterized. Three amino acid substitutions were identified as Phe163Leu in domain I (C-terminal tail), and Glu372Gly/Val379Ala in domain II ($[\alpha/\beta]_8$ barrel). These mutant enzymes showed improved catalytic rate constants (k_{cat}) by up to nine times on both xylose and glucose with up to 26 times higher K_m values on xylose but relatively unchanged for glucose. All enzyme variants' relative activities on xylose are higher than the wild-type at low temperatures. These results suggested that amino acid substitutions distant from the catalytic center could lead to cold adaptation. There is a close relationship between molecular flexibility and function. Thermophilic enzymes are rigid and require high temperatures in order to gain sufficient molecular flexibility for activity. Their molecular structure must therefore be balanced between the requirements for stability and dynamics. They suggested that the sequence changes underlying the adaptation of *T. thermophilus* XI variants to temperatures lower than their optimal temperature, allow a higher degree of flexibility in areas that move during catalysis. Kinetic analysis demonstrated that the increase in the relative activity in the enzyme variants for xylose at low temperatures was indeed caused by an increase in k_{cat} and not by a decrease in the K_m value. This suggests that the mutant enzymes did not acquire higher affinity for the substrate than the wild-type enzyme at lower temperatures.

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In this study, the molecular determinants that are responsible for thermostability and activity toward glucose of a xylose isomerase from a hyperthermophilic eubacterium *Thermotoga neapolitana* (TNXI) will be identified. The method used in such work is site-directed mutagenesis based on the amino acid sequence and three dimensional structure comparisons between TNXI and a xylose isomerase from a thermophilic eubacterium *Thermoanaerobacterium thermosulfurigenes* (TTXI). Furthermore, the highly thermostable TNXI will be engineered by directed evolution to improve its activity on glucose at low temperature and pH. Finally, the resulting laboratory-evolved enzyme's biochemical properties and fructose productivity will be compared to those of the commercially available glucose isomerase, Gensweet™, to further ascertain its utility in industrial applications.

Table 1: Examples of enzymes that were successfully optimized using directed evolution.

Target enzyme	Target property	Change evolved	Approach	Reference
subtilisin E	activity in organic solvents	~ 170-fold increase in 60% dimethylformamide	error-prone PCR + screening	Chen & Arnold (1993); Arnold & Chen (1994)
β-lactamase	activity towards new substrate	32,000-fold greater resistance to cefotaxime	DNA shuffling + selection	Stemmer (1994)
para-nitrobenzyl esterase	activity towards pNB esters; activity in organic solvent	60-150 fold increase	error-prone PCR and DNA shuffling + screening	Moore & Arnold (1996); Moore *et al.* (1997); Arnold & Moore (1998)
β-galactosidase	activity towards new substrate; substrate specificity	66-fold increased activity; 1000-fold increase in substrate specificity	DNA shuffling + screening	Zhang *et al.* (1997)
aminoacyl-tRNA synthetase	aminoacylation of a modified tRNA	55-fold increase in activity	DNA shuffling + selection	Liu *et al.* (1997)
aspartate aminotransferase	activity towards β-branched amino and 2-oxo acids	10^5 increase	DNA shuffling + selection	Yano *et al.* (1997)
lipase	enantioselectivity in hydrolysis of p-nitrophenyl 2-methyldecanoate	increase in enantiomeric excess from 2% to 81%	error-prone PCR + screening	Reetz *et al.* (1997)
pNB esterase	thermostability	14 °C increase in Tm + increased activity at all temperatures	error-prone PCR, DNA shuffling + screening	Giver *et al.* (1998)
subtilisin E	thermostability	17 °C increase in Tm + increased activity at all temperatures	error-prone PCR, DNA shuffling + screening	Zhao & Arnold (1999)
subtilisin BPN'	activity at 10°C	2-fold increase	chemical mutagenesis + screening	Taguchi *et al.* (1998)
cephalosporinases	activity towards moxalactam	270-540-fold increased resistance	DNA shuffling of homologous genes + selection	Crameri *et al.* (1998)
kanamycin nucleotidyl	thermostability	increase 20°C	DNA shuffling + screening/selection	Hoseki *et al.* (1999)

transferase				
B. stearothermophilus LDH	remove fructose 1,6 bisphosphate requirement (FBP)	active without FBP	random mutagenesis + screening	Allen & Holbrook (2000)
phospholipase A_1	thermostability	increase Tm by 11°C without compromising activity	error prone PCR + screening	Song & Rhee (2000)
TEM-1 β-lactamase	activity towards cefotaxime	20,000 fold increase	high frequency random mutagenesis	Zaccolo *et al.* (1999)
myoglobin	peroxidase activity	25-fold increase	error-prone PCR + screening	Wan *et al.* (1998)
hydantoinase	enantioselectivity + total activity	inverted enantioselectivity, 3 x increase in total activity	error-prone PCR + screening	May *et al.* (2000)
xylose isomerase	activity at low temperature	improved k_{cat} at low temperatures	error prone PCR + screening	Lonn *et al.*(2002)

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CHAPTER II

ROLE OF PROLINE IN XYLOSE ISOMERASE THERMAL STABILITY: COMPARATIVE THERMOSTABILITY OF A THERMOPHILIC *Thermoanaerobacterium thermosulfurigenes* XYLOSE ISOMERASE (TTXI) AND A HYPERTHERMOPHILIC *Thermotoga neapolitana* XYLOSE ISOMERASE (TNXI)

ABSTRACT

Xylose isomerases (XIs) from *Thermoanaerobacterium thermosulfurigenes* (TTXI) and *Thermotoga neapolitana* (TNXI) are 70.4% identical in their amino acid sequences and have a nearly superimposable crystal structure. Nonetheless, TNXI is much more thermostable than TTXI. Except for a few additional prolines and fewer Asn+Gln in TNXI, no other obvious differences in the enzyme structures can explain the differences in their stability. TNXI has 2 additional prolines in the Phe59 loop (Pro58 and Pro62). Mutations Gln58Pro, Ala62Pro, and Gln58Pro/Ala62Pro in TTXI and their reverse counterpart mutations in TNXI were constructed by site-directed mutagenesis. Surprisingly, only the Gln58Pro mutation enhanced thermostability of TTXI (43 % longer half-life at 85°C), whereas, the Ala62Pro and Gln58Pro/Ala62Pro mutations both dramatically lowered the TTXI's stability. Analysis of the three-dimensional (3D) structure of TTXI and the predicted structure of its Ala62Pro mutant derivative indicated that a steric hindrance between Pro62-Cδ and Lys61-Cβ (2.92 Å) is responsible for the reduced thermostability of the mutant enzyme compared to the native TTXI. All the reverse counterpart mutations destabilized TNXI thus confirming that these 2 prolines play important roles in TNXI's thermostability.

INTRODUCTION

Xylose isomerase (D-xylose ketol isomerase; EC 5.3.1.5) (XI) is an intracellular enzyme found in a number of bacteria that utilize xylose as carbon substrate for growth (Chen, 1980). XI converts D-xylose to D-xylulose *in vivo* and also catalyzes the conversion of D-glucose to D-fructose *in vitro* (Takasaki *et al.*, 1969). This latter activity is used in industry for the production of high fructose corn syrup (HFCS) and xylose (i.e., glucose) isomerase is one of the largest volume commercial enzymes used today (Lee and Zeikus, 1991). Thermostable XIs with neutral or slightly acidic pH optima have a potential for industrial applications with the advantages of faster reaction rates, higher fructose concentrations at equilibrium, decreased viscosity of substrate and product streams, and less problems of by-products formation (Lee and Zeikus, 1991). Two XI groups have been identified; type I enzymes are shorter than type II enzymes by about 50 amino acids at their N-terminus (Vangrysperre *et al.*, 1990).

Two type II XIs have been studied extensively in our laboratory: one from a thermophile, *Thermoanaerobacterium thermosulfurigenes* (TTXI) and the other from a hyperthermophile, *Thermotoga neapolitana* (TNXI). The genes encoding these enzymes (*xylA* genes) were cloned, sequenced, and expressed in *Escherichia coli* (Lee *et al.*, 1990a, 1990b, Meng *et al.*, 1991, and Vieille *et al.*, 1995). TNXI has a higher turnover number, a lower K_m for glucose, and is more thermostable than any other known type II xylose isomerases (Vieille *et al.*, 1995). XIs are significantly stabilized and activated in the presence of divalent cations, especially by Mg^{2+}, Co^{2+}, and Mn^{2+}. Although TNXI and TTXI are highly similar (70.4% amino acid sequence identity), TNXI is significantly

more thermostable than TTXI with an optimum temperature for activity of 95°C (85°C for TTXI) and melting temperature in the presence of 0.5 mM Co^{2+} of 126°C (82°C for TTXI) (Vieille *et al.*, 1995). No obvious differences in the enzyme structures can explain the differences in their stabilities except for a few additional prolines and fewer Asn+Gln in TNXI (as seen from its alignment with TTXI).

Proteins can be stabilized by decreasing their entropy of unfolding (Matthews *et al.*, 1987). Prolines, with their pyrrolidine ring, can only adopt a few configurations. They restrict the configurations allowed for the preceding residue, and they can decrease the entropy of a protein's unfolded state. Substituting Pro for another carefully chosen residue can thus increase protein stability, provided that the newly introduced proline does not create volume interferences, and does not destroy stabilizing non-covalent interactions. The effect of prolines on protein stabilization has been studied by site-directed mutagenesis. The two T4 lysozyme mutants Ala82Pro (Matthews *et al.*, 1987) and Ile3Pro (Dixon *et al.*, 1992) illustrate the importance of carefully selecting the mutation location. With mutation Ala82Pro, the two conditions listed above were addressed, and the mutation stabilized the protein mainly by decreasing its entropy of unfolding. In mutant Ile3Pro, the substitution eliminated a hydrogen bond and the hydrophobic interactions created by Ile. The degree of enthalpic destabilization was greater than the entropy gained resulting in destabilization of the mutant enzyme. In Allen *et al.*'s study of *Aspergillus awamori* glucoamylase, three proline mutants: Ser30Pro, Asp345Pro, and Glu408Pro were constructed. The Ser30Pro mutation stabilized the protein because the mutation site allowed a residue conformation compatible with a proline, and because residue 29 (a valine) could adopt one of the

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conformations allowed for the residue preceding a proline (Matthews *et al*, 1987). The Glu408Pro mutation was destabilizing because the mutation site did not allow any conformation required for proline, whereas the Asp345Pro mutation did not affect stability due to the fact that the substitution destroyed the α-helix dipole in that region. Prolines 58 and 62 in TNXI are present in a large loop in which 14 hydrophilic residues surround Phe59. The corresponding TTXI residues are Gln58 and Ala62. The Phe59 loop participates in building the neighboring subunit's active site (Farber *et al.*, 1989; Whitlow *et al.*, 1991).

In the present report we test the hypothesis that substituting Gln58 and Ala62 with prolines will stabilize TTXI, and that the reverse mutations will destabilize TNXI.

MATERIALS AND METHODS

Bacterial strains and chemicals: *E. coli* strain BL21 (DE3) (Novagen, Madison, WI.) was used to overexpress the recombinant *T. thermosulfurigenes* and *T. neapolitana xylA* genes cloned in the pET23a and pET22b$^+$ vectors, respectively (Novagen). Media and growth conditions were the same as described by Vieille *et al.* (1995). Medium components and all other chemicals were reagent grade.

Site-directed mutagenesis and other DNA techniques: All DNA manipulations were performed using established protocols (Sambrook *et al.*, 1989; Ausubel *et al.*, 1993). Point mutations were introduced into the *T. neapolitana* and *T. thermosulfurigenes xylA* genes using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenic oligonucleotides (Table 1) were synthesized by the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University. Mutations were verified by DNA sequencing using the Thermosequenase sequencing kit (United States Biochemical, Cleveland, OH).

Protein purification: Recombinant enzymes were purified using the procedure of Vieille *et al.* (1995) followed by two additional steps. Partially purified enzymes were applied to a DEAE-Sepharose column (2.5x15 cm) equilibrated with 50 mM MOPS (pH 7.0) containing 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$ (buffer A), and enzymes were eluted using 500 ml of 0–250 mM NaCl gradient in buffer A. The pooled fractions from the DEAE-Sepharose

Table 1: Oligonucleotides and DNA templates used for site-directed mutagenesis. Bold and underlined nucleotides are the mutation sites.

Mutations	Oligonucleotides	DNA templates
TTXI:		
Gln58Pro	5'-GGAACAGAT**CCA**TTTGGCAAAGC-3' 3'-CCTTGTCTA**GGT**AAACCGTTTCG-5'	TTXI gene
Ala62Pro	5'-GATCAATTTGGCAAA**CCT**ACCATGC-3' 3'-CTAGTTAAACCGTTT**GGA**TGGTACG-5'	TTXI gene
Gln58Pro/Ala62Pro	5'-GGAACAGAT**CCA**TTTGGCAAACC-3' 3'-CCTTGTCTA**GGT**AAACCGTTTGG-5'	TTXI (Ala62Pro) gene
TNXI:		
Pro58Gln	5'-GGGAAGGGAT**CAG**TTCGGAGACCC-3' 3'-CCCTTCCCTA**GTC**AAGCCTCTGGG-5'	TNXI gene
Pro62Ala	5'-CCTTCGGAGAC**GCA**ACGGCCGATC-3' 3'-GGAAGCCTCTG**CGT**TGCCGGCTAG-5'	TNXI gene
Pro58Gln/Pro62Ala	5'-AGTTCGGAGAC**GCA**ACGGCCGATC-3' 3'-TCAAGCCTCTG**CGT**TGCCGGCTAG-5'	TNXI (Pro58Gln) gene

column were concentrated in a stirred ultrafiltration cell (MW cut-off 30 kDa)(Amicon, Beverly, MA), dialyzed twice against buffer A, applied to a Polybuffer column (Pharmacia, Uppsala, Sweden) equilibrated with 25mM histidine-HCl (pH 6.2), and eluted using a pH (6.0 to 4.0) gradient according to the manufacturer's instructions. The active fractions were pooled, concentrated in a stirred ultrafiltration cell (Amicon), and dialyzed twice against buffer A. Concentrated and homogenous enzymes were dispensed and stored frozen at -70°C.

Xylose isomerase assays: XI activity was routinely assayed with glucose as the substrate. The enzyme (0.06 mg/ml) was incubated in 50 mM MOPS (pH 7.0 at room temperature) containing 1 mM $CoCl_2$ and 1 M glucose at 60°C for 20 min. The reaction was stopped by cooling the tubes in ice. The amount of fructose produced was determined by the cysteine-carbazole-sulfuric acid method (Dische and Borenfreund, 1951). To determine the effect of temperature on XI activity, the assay mixtures were incubated at the temperatures of interest in a Perkin-Elmer Cetus GeneAmp PCR system 9600 (Perkin Elmer, Norwalk, CT) for 20 min. To determine the kinetic parameters, assays were performed in the presence of either 80 to 1,400 mM glucose or 20 to 900 mM xylose. The amounts of fructose and xylulose produced were determined as above. Absorbances were measured at 537 nm and 560 nm for xylulose and fructose, respectively. One unit of isomerase activity is defined as the amount of enzyme that produced 1 μmole of product per min under the assay conditions.

Thermostability assays: The time course of irreversible thermoinactivation was measured by incubating the enzyme (0.5-5 mg/ml) in 10 mM MOPS buffer (pH 7.0) containing 50 μM $CoCl_2$ (buffer B) at 85°C (TTXI derivatives) or 95°C (TNXI derivatives) in a Perkin-Elmer Cetus GeneAmp PCR system 9600 for various amounts of time, and by determining the residual glucose isomerase activity at 65°C (TTXI derivatives) or 80°C (TNXI derivatives). The first order rate constant, k, of irreversible thermoinactivation, was obtained by linear regression in semi-log coordinates of residual activity. Enzyme half-life was calculated from the equation: $t_{(1/2)} = \ln 2/k$.

Heat-induced enzyme precipitation: Heat-induced enzyme precipitation was monitored from 25°C to 100°C by light scattering (λ = 580 nm) using protein solutions (0.2 mg/ml) in buffer B. Absorbance measurements were conducted in 0.3 ml quartz cuvettes (path length = 1.0 cm), using a Gilford Response spectrophotometer (Corning, Oberlin, OH) equipped with a Peltier cuvette heating system. The increasing thermal gradient was 1.0°C min^{-1}. The temperature of 50% precipitation was the temperature at which the OD at 580 nm equals half of the difference between the baseline and the maximum ODs.

Analysis of TTXI and TNXI three-dimensional (3D) structures: Enzymes were visualized on an IRIS-4D25 computer (Silicon Graphics Computer System, Mountain View, CA) using the INSIGHT II graphic program (Biosym Technologies, San Diego, CA). Proteins Data Bank (PDB) files (#1A0C for TTXI and #1A0E for TNXI) were obtained from the Protein Data Bank website (www.rcsb.org/pdb).

RESULTS

TNXI Pro58 and Pro62 are present in a large loop in which fourteen hydrophilic residues surround Phe59 (Figure 1). According to crystallographic data, Phe59 (Phe26 in *Actinoplanes* XI) participates in the architecture of the neighboring subunit's active site (Farber *et al.*, 1989; Whitlow *et al.*, 1991). The corresponding residues in TTXI, Gln58 and Ala62, have backbone dihedral angles ([-66.57, -14.49] and [-57.91, 138.87], respectively) allowed for prolines (Nicholson *et al.*, 1988). With backbone dihedral angles of (-139.73, -175.10) and (-93.32, 176.20), respectively, TTXI residues Asp57 and Lys61 are in extended conformations, the most common conformation for residues preceding prolines (Nicholson *et al.*, 1988). This structural information suggests that mutations Gln58Pro and Ala62Pro would not create unfavorable backbone conformations and that they could stabilize TTXI.

Mutation Gln58Pro did not affect the optimum temperature for TTXI activity (i.e., 85°C), whereas mutation Ala62Pro and double mutation Gln58Pro/Ala62Pro decreased it by 7°C and 12°C, respectively (Figure 2A). Mutation Gln58Pro stabilized TTXI at 85°C: the enzyme's half-life was extended from 69 to 99 min (a 43% increase). With half-lives of 6.2 and 21 min at 85°C, respectively, Ala62Pro and Gln58Pro/Ala62Pro mutant TTXIs were significantly less thermostable than the wild-type enzyme (Figure 3A). These surprising results indicated that Pro in position 62 destabilized TTXI. Similar results were obtained in precipitation experiments. Mutation Gln58Pro increased TTXI's temperature of 50% precipitation by 6°C, whereas mutations Ala62Pro and Gln58Pro/Ala62Pro decreased it by approximately 4°C and 3°C, respectively (Table 2).

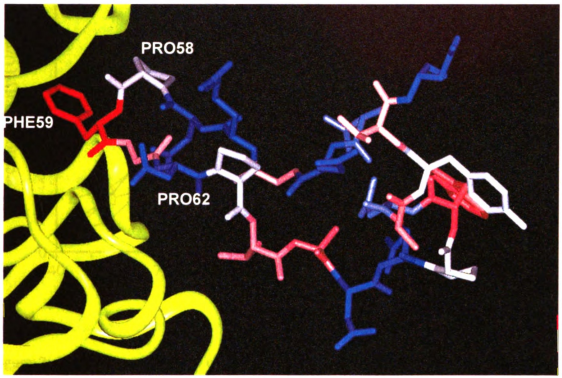

Figure 1: Three-dimensional structure of TNXI's Phe59 loop region. Only parts of the tetramer's subunits A and C are shown. Subunit C, shown in yellow ribbon, is interacting with subunit A's Phe59 loop. Residues in subunit A's Phe59 loop are colored based on their hydrophilicity (Blue-hydrophilic, Red-hydrophobic).

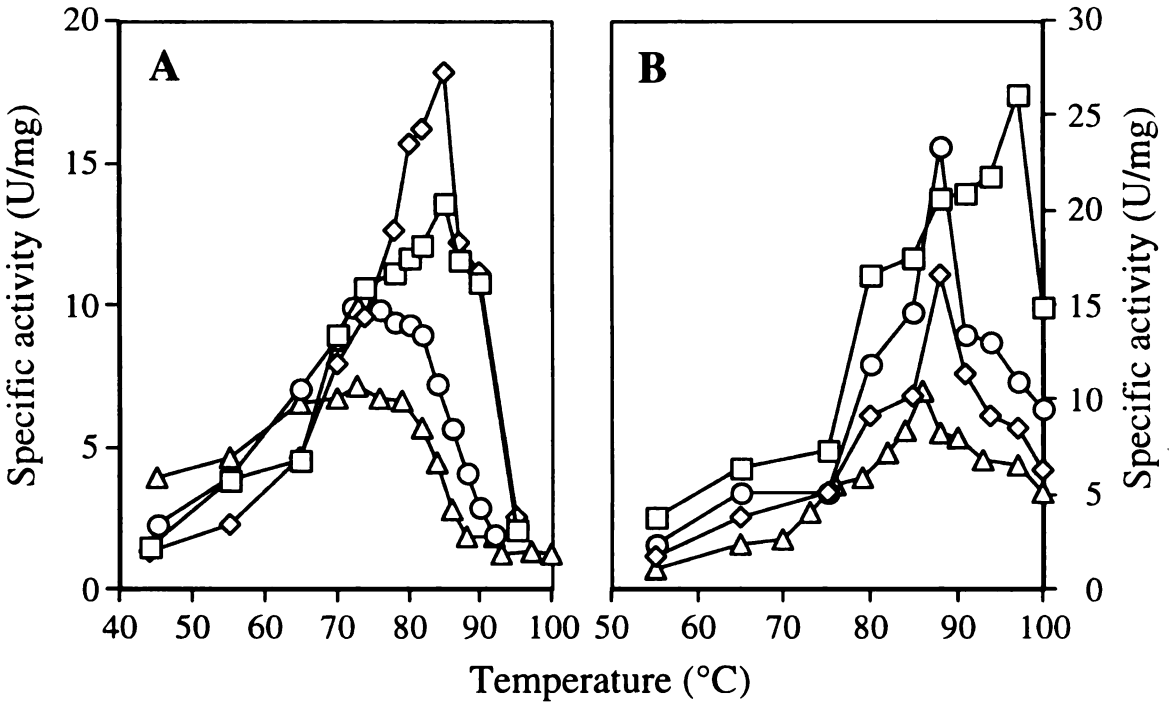

Figure 2: Effect of temperature on the specific activities of TTXI and TNXI and their Phe59 loop mutant derivatives. The substrate used was glucose. (A) TTXI and its mutant derivatives. Symbols: —□—: TTXI; ···◇···: Gln58Pro; ----○----: Ala62Pro; ----△----: Gln58Pro/Ala62Pro. (B) TNXI and its mutant derivatives. Symbols: —□—: TNXI; ···◇···: Pro58Gln; ----○----: Pro62Ala; ----△----: Pro58Gln/Pro62Ala.

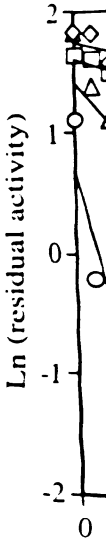

Figure 3: I

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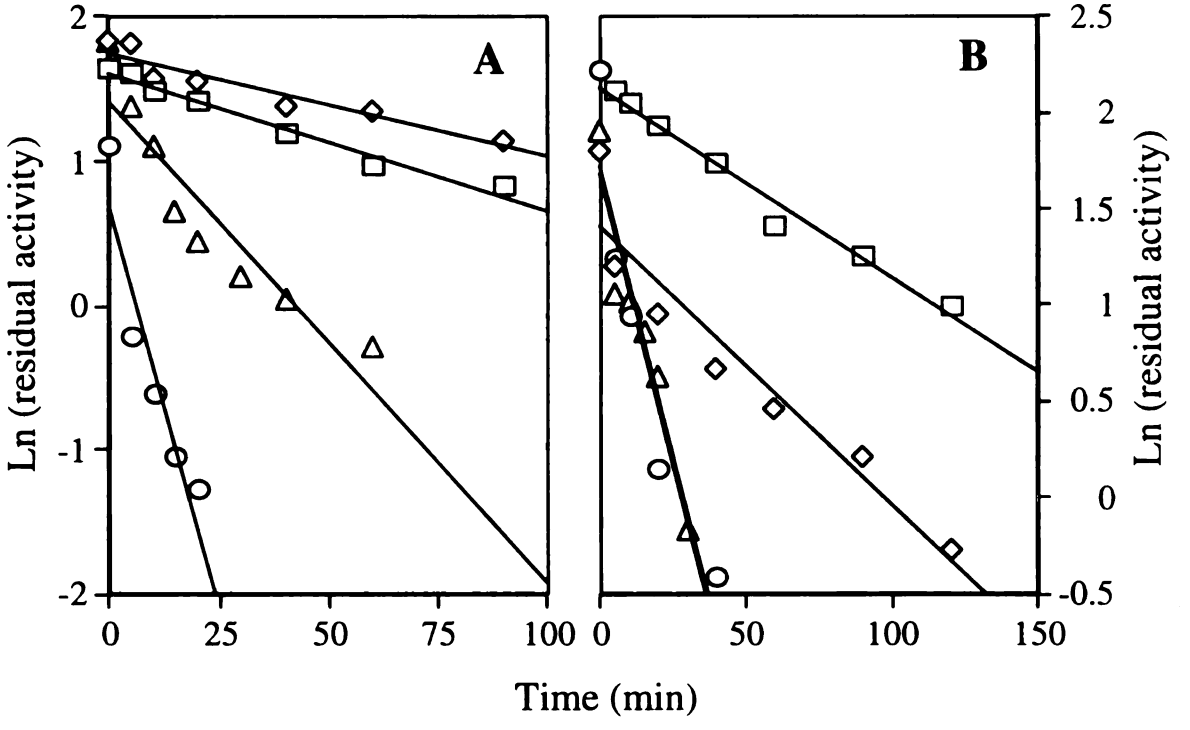

Figure 3: Inactivation curves of TTXI and TNXI and their Phe59 loop mutant derivatives (A) TTXI and its mutant derivatives at 85°C. Symbols: —□—: TTXI; ···◇···: Gln58Pro; ----○----: Ala62Pro; ----△----: Gln58Pro/Ala62Pro. Half-lives of TTXI, Gln58Pro, Ala62Pro, and Gln58Pro/Ala62Pro are 69.3, 99.0, 6.2, and 21.0 min, respectively. (B) TNXI and its mutant derivatives at 95°C. Symbols: —□—: TNXI; ···◇···: Pro58Gln; ----○----: Pro62Ala; ----△----: Pro58Gln/Pro62Ala. Half-lives of TNXI, Pro58Gln, Pro62Ala, and Pro58Gln/Pro62Ala are 69.3, 49.5, 7.1, and 7.1 min, respectively.

The reverse counterpart mutations in TNXI (Pro58Gln and Pro62Ala, and double mutation Pro58Gln/Pro62Ala) decreased the optimum temperature for TNXI activity (Figure 2B). Not surprisingly, thermoinactivation curves revealed that mutation Pro58Gln decreased TNXI's half-life from 69.3 min to 49.5 min (29% decrease). Both mutations Pro62Ala and Pro58Gln/Pro62Ala decreased TNXI's half-life to 11.6 min (83% decrease), which confirmed that these mutations were all destabilizing as we expected (Figure 3B). Again, these results were confirmed by precipitation experiments. The temperature of 50% precipitation of both Pro62Ala and Pro58Gln were 95.7°C, 1.1°C lower than that of the wild-type TNXI. Double mutant Pro62Ala/Pro58Gln precipitated at an even lower temperature than each single mutant enzyme, suggesting that these destabilizing effects are additive.

The kinetic features of the wild-type and mutant xylose isomerases with glucose and xylose as substrates were determined at 65°C for the TTXI series and at 80°C for the TNXI series (Table 3). With the exceptions of mutations Ala62Pro and Gln58Pro/Ala62Pro in TTXI, the mutations in TTXI and TNXI Phe59 loops did not significantly alter the enzymes' catalytic properties. The Ala62Pro mutation increased both TTXI's V_{max} and K_m on glucose, leaving its catalytic efficiency on glucose almost unchanged. This mutation had a much stronger effect on TTXI activity on xylose. It increased its affinity for xylose and its catalytic efficiency approximately 2.9 times. Mutation Gln58Pro/Ala62Pro had a more pronounced effect on TTXI activity on glucose. A 2.6-fold increase in its K_m for glucose decreased its catalytic efficiency almost 3-fold. These results indicate that mutations Ala62Pro and Gln58Pro/Ala62Pro altered TTXI's catalytic features and at the same time destabilized the enzyme.

Table 2: E

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Table 2: Effect of mutations on enzyme precipitation temperatures. Results are the means of three independent experiments.

Enzymes	Denaturation temperature for 50% precipitation (°C)
Wild-type TTXI	83.7 ± 0.2
Gln58Pro	89.9 ± 0.6
Ala62Pro	79.9 ± 1.0
Gln58Pro/Ala62Pro	80.8 ± 0.7
Wild-type TNXI	96.8 ± 0.4
Pro58Gln	95.7 ± 0.4
Pro62Ala	95.7 ± 0.6
Pro58Gln/Pro62Ala	94.0 ± 0.3

Table 3: Catalytic parameters of TTXI (at 65°C) and TNXI (at 80°C) and of their mutant derivatives. Results are the means of three independent experiments. K_m *and* V_{max} *values were obtained through direct fitting of the initial velocity data to the Michaelis-Menten*

Table 3: Catalytic parameters of TTXI (at 65°C) and TNXI (at 80°C) and of their mutant derivatives. Results are the means of three independent experiments. K_m and V_{max} values were obtained through direct fitting of the initial velocity data to the Michaelis-Menten equation. Results are the means of three independent experiments.

Enzymes	Glucose				Xylose			
	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Wild-type TTXI	114.1 ± 15.1	9.3 ± 0.5	465.0	4.1	41.9 ± 5.1	14.1 ± 0.8	705.0	16.8
Gln58Pro	120.0 ± 15.4	7.9 ± 0.4	395.0	3.3	43.3 ± 8.3	15.1 ± 1.5	755.0	17.4
Ala62Pro	153.1 ± 15.2	13.4 ± 0.6	670.0	4.4	13.7 ± 1.0	13.4 ± 0.4	670.0	48.9
Gln58Pro/Ala62Pro	298.4 ± 35.9	8.2 ± 0.6	410.0	1.4	67.9 ± 10.8	32.5 ± 3.6	1625.0	23.9
Wild-type TNXI	143.4 ± 20.3	15.1 ± 1.2	768.5	5.4	11.9 ± 1.7	14.6 ± 1.2	743.0	62.4
Pro58Gln	104.2 ± 13.7	8.7 ± 0.6	442.8	4.2	7.1 ± 0.8	9.5 ± 0.5	483.5	68.1
Pro62Ala	127.1 ± 11.1	12.2 ± 0.6	620.9	4.9	9.2 ± 0.8	14.6 ± 0.6	743.0	80.8
Pro58Gln/Pro62Ala	100.9 ± 18.7	9.3 ± 0.9	473.3	4.7	8.6 ± 1.7	9.1 ± 0.9	463.1	53.8

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DISCUSSION

The high degree of similarity between TTXI and TNXI and their significantly different thermostabilities and thermophilicities make comparative studies of these enzymes attractive for understanding the key molecular features responsible for their stability and activity differences (Vieille *et al.*, 1995). Multiple factors have been identified (Vieille and Zeikus, 1996) that can be responsible for a protein's high thermostability. They include packing efficiency, hydrophobic interactions, loop stabilization, reduction of entropy of unfolding, electrostatic interactions, etc. In our case, no obvious differences in TTXI and TNXI structures could explain their different thermostabilities except for a few additional prolines and fewer Asn+Gln in TNXI compared with TTXI.

Two additional prolines are found in a large "Phe59 loop" region in TNXI (Figure 1). These prolines are substituted with Gln and Ala in TTXI. Proteins can be stabilized by engineering proline into selected sites thereby decreasing the proteins' conformational entropy of unfolding (Allen *et al.*, 1998). In order for a proline substitution to stabilize a protein, at least four criteria have to be considered: (i) the mutation site should allow one of the conformations allowed for proline; (ii) the preceding residue should be able to adopt one of the conformations allowed for the residue preceding a proline; (iii) proline should not create volume interferences; and (iv) proline should not destroy stabilizing non-covalent interactions (Nicholson *et al.*, 1988). TTXI Gln58 and Ala62 have backbone dihedral angles allowed for prolines, and Asp57 and Lys61 have backbone dihedral angles allowed for residues preceding prolines. Despite these first two

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conformational requirements being satisfied, only mutation Gln58Pro stabilized TTXI. As seen in Figure 4A, the conformation of Gln58's sidechain is very close to that of the proline pyrrolidine ring. No volume interference is created by the Gln58Pro mutation. In the wild-type TTXI structure, Gln58 is not involved in any potentially stabilizing, non-covalent interactions (not shown). No enthalpic destabilization is expected with mutation Gln58Pro, and the stabilization it provides to TTXI probably entirely results from a reduction of the entropy of unfolding. On the other hand, the Ala62Pro mutation had a destabilizing effect on TTXI. In TTXI, Ala62's sidechain points toward a large cavity, and it is not in close vicinity of any other residues. So the Ala62Pro mutation does not eliminate any stabilizing non-covalent interactions. Detailed analysis of the Ala62Pro mutation modeled into the TTXI structure (Figure 5) suggests that Pro62's pyrrolidine ring (Cδ atom) is in close contact (within 2.92 Å) with Lys61's sidechain (Cβ atom). Carbon atoms have Van der Waals radii of 1.70-1.78 Å in protein. Optimal Van der Waals interactions between 2 carbon atoms would take place at approximately 3.4-3.5 Å. The unfavorable Van der Waals contact between Pro62-Cδ and Lys61-Cβ probably leads to local conformational changes. Not only are these changes destabilizing, they also affect the active site structure and the enzyme's interaction with the substrate. The overall destabilizing nature of mutation Ala62Pro indicates that the conformational destabilization of the native enzyme more than cancels the benefits of a potential decrease in unfolding entropy.

In conclusion, our data show that genetic engineering approaches can be utilized to identify amino acid residues responsible for extreme thermal stability of xylose (glucose) isomerase thermozymes. Both proline58 and 62 appear to stabilize TNXI's

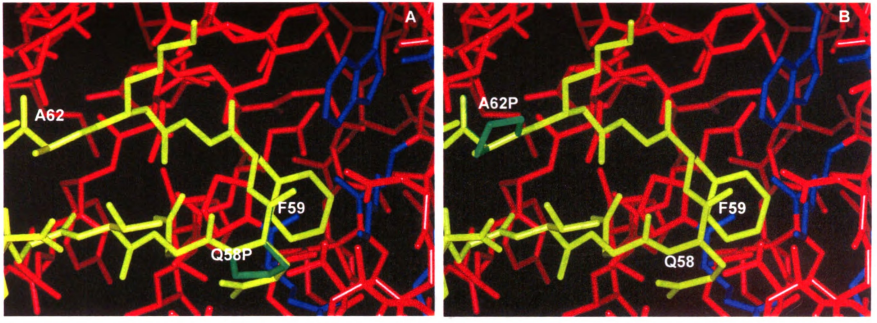

Figure 4: Three-dimensional structure of the Phe59 loop mutations in TTXI. (A) mutation Gln58Pro; (B) mutation Ala62Pro. Subunit C is in red. Subunit C active site residues are in blue. Subunit A's Phe59 loop is in yellow. Mutations are in green.

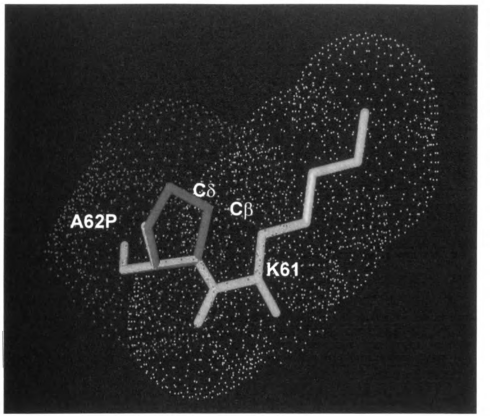

Figure 5: Van der Waals contacts between Lys61 and Pro62 in TTXI Ala62Pro mutant derivative. Carbon atom Van der Waals radii were arbitrarily fixed at 1.7 Å. Lys61 and Ala62 are in yellow. Pro62 is in green.

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Phe59 loop; whereas TTXI's thermal stability can be enhanced by the Gln58Pro mutation.

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CHAPTER III

ROLE OF SUBSTRATE BINDING POCKET IN *Thermotoga neapolitana* XYLOSE ISOMERASE (TNXI) ON GLUCOSE ISOMERASE ACTIVITY

ABSTRACT

The active site of xylose isomerase from the thermophilic *Thermoanaerobacterium thermosulfurigenes* (TTXI) has been previously engineered to improve its catalytic efficiency toward glucose and increase its thermostability. The same mutations were introduced into xylose isomerase from a hyperthermophilic *Thermotoga neapolitana* (TNXI), which shares 70.4 % amino acid sequence identity. Similar trends were observed, but to different extent. The TNXI Val185Thr was the most efficient mutant derivative with a 3.1 fold increase in its catalytic efficiency toward glucose whereas the double mutation Trp138Phe/Val185Thr was the best at increasing glucose catalytic efficiency in TTXI. With a maximal activity at 97°C of 45.4 U/mg on glucose, this TNXI mutant derivative is the most active type II XI ever reported. This "true" glucose isomerase engineered from a native xylose isomerase has now comparable kinetic properties on glucose and xylose.

INTRODUCTION

Xylose isomerase (D-xylose ketol-isomerase; EC 5.3.1.5) converts xylose to xylulose during xylose metabolism in various microorganisms (Chen, 1980). This enzyme also catalyzes the interconversion of glucose and fructose *in vitro* and has been used as an industrial biocatalyst for the production of high fructose corn syrup. It displays lower k_{cat} and higher K_m values for glucose than those for xylose. Specificity of enzymes toward their substrates is determined in part by molecular residues that provide for binding of the substrate and that maintain substrates steric configuration in the active site. The enzymatic interconversion of xylose/glucose (aldoses) to xylulose/fructose (ketoses) by xylose isomerases involves binding of the substrate in the cyclic form, linearization of substate, isomerization of the linear intermediate, product ring closure, and release of the cyclic product. The isomerization step proceeds by a metal ion mediated hydride shift mechanism, and this step, rather than substrate ring opening, is rate-determining (Lee *et al.*, 1990). Xylose and glucose have identical atomic configuration, except for the presence of an additional hydroxy-methyl (CH_2OH) group at the C-6 position in the glucose molecule. This extra hydroxymethyl group must therefore be responsible for the differences in the catalytic efficiency exhibited by xylose isomerase toward glucose versus xylose. The orientation of glucose as a substrate in the active site of the thermophilic enzyme from *Thermoanaerobacterium thermosulfurigenes* (TTXI) is such to position the C-6 end of hexose toward the His100 residue in the substrate-binding pocket. The residues in the thermophilic enzyme that are in contact with the C_6-OH group

of the substrate equivalent to those in the sorbitol-bound xylose isomerase from *Arthobacter* (Collyer *et al.*, 1990) are Trp138, Thr140, Val185, and Glu231.

In a previous study, genetic engineering was used to improve TTXI activity on glucose (not the natural substrate): the enzyme's substrate-binding pocket was enlarged, and the water-accessible surface area in the active site was altered (Meng *et al.*, 1991 and 1993). Substituting Trp138 with Phe, a smaller residue, decreased TTXI's K_m for glucose and increased its catalytic efficiency (k_{cat}/K_m) on glucose 2.6 times. This mutant enzyme was less active than the wild-type TTXI on xylose. Interestingly, this mutation doubled TTXI's half-life at 85°C (Meng *et al.*, 1993). Substituting Val185 with Thr, a polar residue, improved TTXI's catalytic efficiency on glucose. This improvement was thought to result from the creation of an additional hydrogen bond to glucose's C_6-OH group. The double mutation Trp138Phe/Val185Thr vastly improved TTXI's catalytic efficiency on glucose (5.7 times).

Since TTXI and the xylose isomerase from the hyperthermophilic *Thermotoga neapolitana* (TNXI) are more than 70 % identical in their amino acid sequences and have a nearly superimposable three-dimensional structure, the enhancement of glucose's catalytic efficiency would be expected from those substitutions in the TNXI. In this study, we introduced these mutations in TNXI to determine if TNXI activity and stability could be further enhanced by genetic engineering. All mutations were characterized by their kinetic parameters, optimum temperatures, half-lives at 85°C or 95°C, in order to compare their thermal stability and activity.

MATERIALS AND METHODS

Bacterial strains and chemicals: *E. coli* strain BL21 (DE3) (Novagen, Madison, WI.) was used to overexpress the recombinant *T. thermosulfurigenes* and *T. neapolitana xylA* genes cloned in the pET23a and pET22b$^+$ vectors, respectively (Novagen). Media and growth conditions were the same as described by Vieille *et al.* (1995). Medium components and all other chemicals were reagent grade.

Site-directed mutagenesis and other DNA techniques: All DNA manipulations were performed using established protocols (Sambrook *et al.*, 1989; Ausubel *et al.*, 1993). Point mutations were introduced into the *T. neapolitana* and *T. thermosulfurigenes xylA* genes using the QuikChange™ site-directed mutagenesis Kit (Stratagene, La Jolla, CA). Mutagenic oligonucleotides (Table 1) were synthesized by the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University. Mutations were verified by DNA sequencing using the Thermosequenase sequencing kit (United States Biochemical, Cleveland, OH).

Protein purification: Recombinant enzymes were purified using the procedure of Vieille *et al.* (1995) followed by two additional steps. Partially purified enzymes were applied to a DEAE-Sepharose column (2.5x15 cm) equilibrated with 50 mM MOPS (pH 7.0) containing 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$ (buffer A), and enzymes were eluted using 500 ml of 0–250 mM NaCl gradient in buffer A. The pooled fractions from the DEAE-Sepharose column were concentrated in a stirred ultrafiltration cell (MW cut-off 30 kDa)

Table 1: Oligonucleotides and DNA templates used for site-directed mutagenesis. Bold and underlined nucleotides are the mutation sites.

Mutations	Oligonucleotides	DNA templates
Trp138Phe	5'-GAAGCTCCTC**TTT**GGTACTGC-3' 3'-CTTCGAGGAG**AAA**CCATGACG-5'	TNXI gene
Val185Thr	5'-GAAGGGTAC**ACC**TTCTGGGGTG-3' 3'-CTTCCCATG**TGG**AAGACCCCAC-5'	TNXI gene
Trp138Phe/Val185Thr	5'-GAAGCTCCTC**TTT**GGTACTGC-3' 3'-CTTCGAGGAG**AAA**CCATGACG-5'	TNXI (Val185Thr) gene

(Amicon, Beverly, MA), dialyzed twice against buffer A, applied to a Polybuffer column (Pharmacia, Uppsala, Sweden) equilibrated with 25mM histidine-HCl (pH 6.2) ,and eluted using a pH (6.0 to 4.0) gradient according to the manufacturer's instructions. The active fractions were pooled, concentrated in a stirred ultrafiltration cell (Amicon), and dialyzed twice against buffer A. Concentrated; homogenous enzymes were dispensed and stored frozen at -70°C.

Xylose isomerase assays: XI activity was routinely assayed with glucose as the substrate. The enzyme (0.06 mg/ml) was incubated in 50 mM MOPS (pH 7.0 at room temperature) containing 1 mM $CoCl_2$ and 1 M glucose at 60°C for 20 min. The reaction was stopped by cooling the tubes in ice. The amount of fructose produced was determined by the cysteine-carbazole-sulfuric acid method (Dische and Borenfreund, 1951). To determine the effect of temperature on XI activity, the assay mixtures were incubated at the temperatures of interest in a Perkin-Elmer Cetus GeneAmp PCR system 9600 (Perkin Elmer, Norwalk, CT) for 20 min. To determine the kinetic parameters, assays were performed in the presence of either 80 to 1,400 mM glucose or 20 to 900 mM xylose. The amounts of fructose and xylulose produced were determined as above. Absorbances were measured at 537 nm and 560 nm for xylulose and fructose, respectively. One unit of isomerase activity is defined as the amount of enzyme that produced 1 μmole of product per min under the assay conditions.

Thermostability assays: The time course of irreversible thermoinactivation was measured by incubating the enzyme (0.5-5 mg/ml) in 10 mM MOPS buffer (pH 7.0)

containing 50 μM $CoCl_2$ (buffer B) 95°C in a Perkin-Elmer Cetus GeneAmp PCR system 9600 for various amounts of time, and by determining the residual glucose isomerase activity at 80°C. The first order rate constant, k, of irreversible thermoinactivation, was obtained by linear regression in semi-log coordinates of residual activity. Enzyme half-life was calculated from the equation: $t_{(1/2)} = \ln 2/k$.

Heat-induced enzyme precipitation: Heat-induced enzyme precipitation was monitored from 25°C to 100°C by light scattering (λ = 580 nm) using protein solutions (0.2 mg/ml) in buffer B. Absorbance measurements were conducted in 0.3 ml quartz cuvettes (path length = 1.0 cm), using a Gilford Response spectrophotometer (Corning, Oberlin, OH) equipped with a Peltier cuvette heating system. The increasing thermal gradient was 1.0°C min^{-1}. The temperature of 50% precipitation was the temperature at which the OD at 580 nm equals half of the difference between the baseline and the maximum ODs.

Analysis of TNXI three-dimensional (3D) structures: Enzymes were visualized on an IRIS-4D25 computer (Silicon Graphics Computer System, Mountain View, CA) using the INSIGHT II graphic program (Biosym Technologies, San Diego, CA). Proteins Data Bank (PDB) file (#1A0E for TNXI) was obtained from the Protein Data Bank website (www.rcsb.org/pdb).

RESULTS

The Val185Thr mutant and the double mutant Trp138Phe/Val185Thr TNXI derivatives had the same optimum temperature of activity (97°C) as the wild-type TNXI (Figure 1A). Interestingly, mutation Val185Thr almost doubled TNXI specific activity on glucose at 97°C (from 26.0 U/mg to 45.6 U/mg). Although the Trp138Phe mutation decreased TNXI optimum temperature to 87°C, the mutant enzyme still retained almost 80% of its activity at 97°C. The stability of these mutant enzymes was studied at 95°C (Figure 1B). With a half-life of 69.3 min, the Val185Thr mutation did not affect TNXI stability at 95°C. The Trp138Phe mutant (half-life of 87 min) and the Trp138Phe/Val185Thr double mutant (half-life of 99 min) enzymes were 25% and 43% more stable, respectively, than the wild-type TNXI.

When kinetic parameters were concerned, these three TNXI mutant enzymes showed the same trends as their equivalents in TTXI (Table 2). They all showed improved catalysis on glucose and poorer catalysis on xylose. Most of the catalytic efficiency increases were due to lower K_m's for glucose. Major differences existed though, between TTXI and TNXI derivatives. Whereas Trp138Phe is the best mutation in term of increasing TTXI catalytic efficiency on glucose, its effect on TNXI catalytic efficiency on glucose is only marginal. On the other hand, the mutation Val185Thr is better at increasing TNXI catalytic efficiency on glucose, than at increasing TTXI's. These differences are surprising, knowing that TTXI and TNXI active site structures are almost completely superimposable.

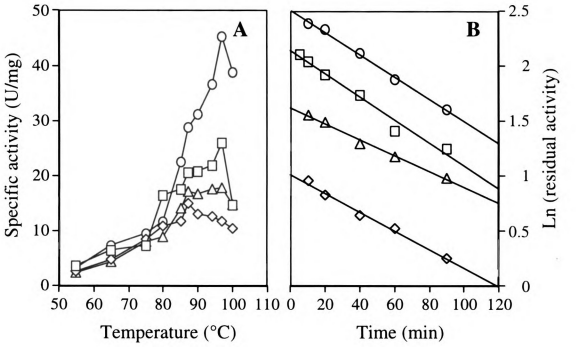

Figure 1: Activity and stability of TNXI and its active site mutant derivatives. (A) Effect of temperature on the specific activities of TNXI and its active site mutant derivatives. The substrate used was glucose. Symbols: —□—: TNXI; ···◇···: Trp138Phe; ····○····: Val185Thr; ····△····: Trp138Phe/Val185Thr. (B) Inactivation curves of TNXI and its active site mutant derivatives at 95°C. Symbols: same as in (A). Half-lives of TNXI, Trp138Phe, Val185Thr, and Trp138Phe/Val185Thr are 69.3, 87.0, 69.3, and 99.0 min, respectively.

Table 2: Catalytic parameters of TTXI (at 65°C) and TNXI (at 80°C) and of their mutant derivatives. Results are the means of three independent experiments. K_m and V_{max} values were obtained through direct fitting of the initial velocity data to the Michaelis-Menten equation. Results are the means of three independent experiments.

Enzymes	Glucose				Xylose			
	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Wild-type TTXI*	110 ± 7.6	12.8	640	5.8	12 ± 2.2	21.6	1100	97.2
Trp138Phe*	65 ± 7.4	19.4	970	15.0	46 ± 1.1	12.2	620	13.6
Val185Thr*	91 ± 7.0	17.6	880	9.7	13 ± 1.7	14.5	740	55.4
Trp138Phe/Val185Thr*	29 ± 3.7	19.0	950	32.9	36 ± 2.4	15.3	780	32.9
Wild-type TNXI	143.4 ± 20.3	15.1 ± 1.2	768.5	5.4	11.9 ± 1.7	14.6 ± 1.2	743.0	62.4
Trp138Phe	131.4 ± 39.4	15.7 ± 2.4	799.0	6.1	15.0 ± 1.7	8.7 ± 0.6	442.8	29.5
Val185Thr	63.5 ± 10.5	20.2 ± 1.6	1028.0	16.2	13.9 ± 1.3	15.0 ± 0.9	763.4	54.9
Trp138Phe/Val185Thr	46.3 ± 3.1	12.0 ± 0.3	610.7	13.2	145.0 ± 1.9	10.9 ± 0.8	554.7	37.0

*Data from Meng et al (1991)

DISCUSSION

The key molecular structure-function feature previously studied in *Thermoanaerobacterium thermosulfurigenes* xylose isomerase (TTXI) is residues in the active sites. Meng *et al.*, (1991) have shown that mutation Trp138Phe significantly increased TTXI catalytic efficiency on glucose (2.7 fold increase). This increase was suggested to result from a better accommodation of glucose in the substrate-binding site because Trp was substituted by a smaller residue, Phe. This substitution was also shown to double TTXI's half-life at 85°C (Meng *et al.*, 1993). The higher thermostability may be explained as the consequence of a reduction of the water-accessible hydrophobic surface area. Another mutation that has been shown to increase TTXI's catalytic efficiency on glucose was Val185Thr. This increase was attributed to additional hydrogen bonding of Thr to glucose's C6-OH.

When these mutations were introduced into TNXI, we observed the same trends in terms of catalytic features. All the mutant enzymes, including the double mutant Trp138Phe/Val185Thr, had a higher catalytic efficiency for glucose than the wild-type TNXI. These increases mainly resulted from a much lower K_m for glucose than that of TNXI. On the other hand, all these mutant TNXIs showed a lower catalytic efficiency for xylose like they also did in TTXI. W138F was the mutation that had the most significant effect on TTXI's catalytic efficiency on glucose. Here, TNXI V185T mutant derivative had the highest catalytic efficiency on glucose. This result came as a surprise since the two XIs' active sites were almost completely superimposable. As seen in figure 2, Trp138 and Val185 are almost completely superposed in TTXI and TNXI. The

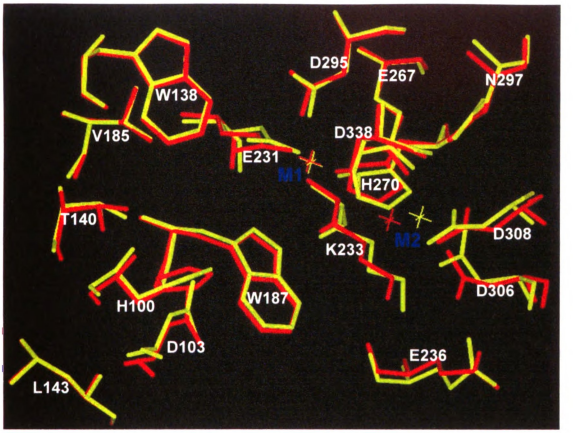

Figure 2: Superposition of TTXI and TNXI active sites. Single letter code is used for amino acid residues. TTXI residues are in yellow. TNXI residues are in red. Metal site I (i.e., structural metal, M1) and metal site II (i.e., catalytic metal, M2) are represented by crosses. Both metal sites are occupied by Co^{2+}in both enzymes. Co^{2+} in M2 of TTXI and TNXI are 1.23 Å apart.

conformations of the neighboring residues are also extremely conserved. The most significant difference between TTXI and TNXI active sites is the position of metal II (i.e., the catalytic metal, a Co^{2+} in both enzymes): the two cations are 1.23 Å apart. This shift of the catalytic metal might affect the enzyme-substrate binding properties and/or the catalytic metal's reactivity during catalysis, thus explaining why there was a difference in mutation effects on TTXI and TNXI catalytic activities on glucose. All TNXI mutant derivatives were as stable as (Val185Thr) or more stable than the wild-type TNXI (Trp138Phe and Trp138Phe/Val185Thr). These mutations did not increase TNXI stability to the same extent as they did in TTXI (Meng *et al.*, 1993). Mutation Trp138Phe might have the same stabilizing potential in TNXI as in TTXI, but another TNXI molecular feature probably becomes limiting for stability before the full stabilization potentially provided by the mutation can be reached. These findings prove that it is possible to further stabilize hyperthermophilic proteins. To our best knowledge, with a maximal activity on glucose at 97°C of 45.4 Units/mg, TNXI Val185Thr mutant derivative is the most active type II xylose isomerase ever reported.

In conclusion, our data show that genetic engineering approaches can be utilized to identify amino acid residues responsible for high catalytic activity of xylose (glucose) isomerase thermozymes. TNXI's thermal stability was further enhanced by the Trp138Phe substitution. The Val185Thr mutation significantly enhanced TTXI's and TNXI's V_{max} and k_{cat}/K_m for glucose isomerization to fructose. This significant catalytic enhancement of glucose isomerase activity was made possible in large part by the template enzyme's naturally evolved function as xylose isomerase. Genetic engineering altered the active site only to better accommodate glucose as the substrate.

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CHAPTER IV

DIRECTED EVOLUTION OF *Thermotoga neapolitana* XYLOSE ISOMERASE: HIGH ACTIVITY ON GLUCOSE AT LOW TEMPERATURE AND LOW pH

ABSTRACT

The *Thermotoga neapolitana* xylose isomerase (TNXI) is extremely thermostable and highly active at 95°C and above. Its mutant derivative, TNXI V185T, was the most active type II XI previously reported, with a catalytic efficiency (k_{cat}/K_m) of 25.1 s^{-1} mM^{-1} toward glucose at 80°C (pH 7.0). To further optimize this enzyme's potential industrial utility, two rounds of random mutagenesis and low temperature/low pH activity screening were performed using the TNXI V185T-encoding gene as the template. Mutants TNXI 3A2 (V185T/L282P) and 1F1 (V185T/L282P/F186S) were obtained after rounds one and two of random mutagenesis, respectively. TNXI 1F1 was more active than 3A2, which in turn was more active than TNXI V185T at all temperatures and pHs tested. TNXI 3A2 and 1F1 high activities at low temperatures were due to significantly lower activation energies (57 and 44 kJ/mole, respectively) than that of TNXI and V185T (87 kJ/mole). This observation suggested that TNXI 3A2 and 1F1's increased activity at low temperature is a consequence of their increased flexibility in the active sites. Although 3A2 was more active than TNXI and V185T, its kinetic stability (based on the enzymes' half life in different incubation conditions) was inferior to those of TNXI V185T possibly due to unfavorable van der Waal contacts of Pro282's pyrrolidine ring with neighboring mainchain atoms. This would, in turn, lead to conformational changes and eventually destabilize the enzyme. Unlike TNXI 3A2, 1F1 is more kinetically stable than TNXI and TNXI V185T. 1F1's enhanced stability is thought to be a result of additional H-bond formation between Ser186's sidechain and the neighboring L229 residue's mainchain structure. This, in turn, strengthens local conformation and the affinity of E231 co-

ordination with the structural metal, hence restoring the thermostability lost in 3A2. We showed here that low temperature/low pH activity of a hyperthermostable enzyme could be enhanced without costs to extreme thermal stability by directed enzyme evolution.

INTRODUCTION

Xylose isomerase (XI) (EC.5.3.1.5) is an intracellular enzyme found in bacteria that can utilize xylose as a carbon substrate for growth (Chen, 1980). Due to its ability to use glucose as substrate and convert it to fructose, XI is often referred to as glucose isomerase, and it is widely used in the industrial production of high fructose corn syrup (HFCS) (Meng *et al.*, 1993; Bhosale *et al.*, 1996). XI is one of the three highest tonnage value enzymes, amylase and protease being the other two (Bhosale *et al.*, 1996). Most industrially used XIs are isolated from mesophilic organisms (e.g., *Streptomyces spp.* and *Actinoplanes spp.*). The reaction temperature used in the current industrial glucose isomerization process is limited to 60°C because of by-product and color formations that occur at high temperature and alkaline pH, and because the isomerases themselves are not highly thermostable (Lee and Zeikus, 1991; Vieille and Zeikus, 2000). Thermostable XIs with neutral or slightly acidic pH optima have a potential for industrial applications. Performing isomerization at higher temperature than 60°C and neutral/slightly acidic pH with thermostable XI would allow faster reaction rates, higher fructose concentrations at equilibrium, higher process stability, decreased viscosity of substrate and product streams, and reduced by-products formation (Lee and Zeikus, 1991; Vieille and Zeikus, 2000).

The XI from the hyperthermophile *Thermotoga neapolitana* (TNXI) has been studied extensively in our laboratory. The gene encoding TNXI (*xylA*) was cloned, sequenced, and overexpressed in *Escherichia coli* (Vieille *et al.*, 1995). TNXI's active site was engineered by site-directed mutagenesis to increase its activity on glucose

(Sriprapundh *et al.*, 2000). The TNXI Val185Thr (V185T) mutant derivative is more active, more glucose-efficient, and as stable as the wild-type TNXI. It was also the most active type II XI ever reported. Although TNXI V185T is highly thermostable and highly active at 97°C, it is very poorly active (10 % of maximal activity) at the current industrial isomerization temperature (60°C) and it requires a neutral pH for optimal activity.

Rules for engineering protein activity and stability by rational design are likely to be protein-specific, and any such design effort would require prior detailed structural information. Numerous and intensive site-directed mutagenesis studies have probed this issue. Despite these efforts, considerable disagreement remains over which forces dominate stabilization mechanisms, and no generally applicable rules have been established (Giver *et al.*, 1998; Vieille and Zeikus, 2001). Although protein chemists continue to elucidate the relationships between the sequence, structure, and function of proteins, the extensive knowledge that is necessary for the application of rational engineering approaches is available for only a tiny fraction of known enzymes. Directed evolution, on the other hand, has proved to be useful for modifying enzymes in the absence of such knowledge (Kuchner and Arnold, 1997). In directed evolution, the process of natural evolution is accelerated in a test tube for selecting proteins with the desired properties (Moore and Maranas, 2000). A typical experimental cycle of directed evolution begins with the creation of a library of mutated genes. Among the methods that introduce mutations randomly along the entire length of a gene (Leung *et al.*, 1989, Stemmer, 1994, Zhao and Arnold, 1997, Shao *et al.*, 1998, Zhao *et al.*, 1998, and Ostermeier *et al.*, 1999), error-prone PCR has been used the most extensively. The mutated genes are then ligated into an expression vector and transformed into suitable

bacterial cells. A screening procedure is next employed to identify the few transformants expressing proteins/enzymes with improved properties. Random mutagenesis and screening are repeated several times depending on the extent to which the properties of the protein should be altered and on the effects of mutations observed in each generation. Interest in engineering enzymes using directed evolution has grown significantly in the past few years. It has been used to increase enzyme thermostability, activity on novel substrates, substrate specificity, and enantioselectivity. For example, six generations of random mutagenesis, recombination, and screening stabilized *Bacillus subtilis p*-nitrobenzyl esterase significantly (>14°C increase in T_m) without compromising its catalytic activity at lower temperature (Giver *et al.*, 1998).

Here we use the TNXI V185T-encoding gene as the template for directed evolution to develop an enzyme active at 60°C and acidic pH. We show that activity can be increased significantly at low temperature and acidic pH without cost to the enzyme thermal stability.

MATERIALS AND METHODS

Random mutagenesis: Random mutations were introduced into the TNXI V185T-encoding gene cloned between the *Nde*I and *Hind*III restriction sites of *pET*23a(+). PCR was performed with primers 5'-CGACTCACTATAGGGAGAC-3' and 5'-GGTGGTGCTCGAGTGCG-3' encoding sequences upstream of the *Nde*I site and downstream of the *Hind*III, respectively, in *pET*23a(+). The reaction mixture contained 100 ng plasmid DNA, 50 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.4 mM dCTP, 0.4 mM dTTP, 0.08 mM dATP, 0.08 mM dGTP, and 2.5 Units *Taq* DNA polymerase (Roche, Nutley, NJ) in a 50 µl reaction volume. Cycling parameters were 36 cycles of 95°C for 45 sec, 50°C for 45 sec, and 72°C for 3 min. Amplification of the 1.4-kb product was checked by running a small aliquot of the reaction on a 1 % agarose gel. The PCR product was purified using the Geneclean III kit (Bio101, Carlsbad, CA) and cloned back into the *Nde*I and *Hind*III sites of *pET*23a(+) using standard molecular biological techniques (Ausubel *et al,* 1993). For the second round of random mutagenesis, the gene encoding TNXI 3A2 was used as the template.

Construction of a mutant library: The plasmids resulting from random mutagenesis were transformed into electrocompetent *E. coli* HB101(DE3) cells (XI-deficient) created using the λDE3 lysogenation kit (Novagen, Madison, WI). Transformants were plated on Luria-Bertani (LB) agar containing 100 µg/ml ampicillin. After 16 hr of growth, single colonies were picked with sterile toothpicks and transferred into 24-well plates, each well containing 2 ml of LB plus 100 µg/ml ampicillin. Plates were then incubated overnight at 37°C on a shaker at 175 rpm to allow for cell growth. One hundred fifty microliters of

each culture were transferred to sterile 96-well plates. These plates were used to quantify bacterial growth by reading absorbance of the bacterial suspensions (OD_{595}) in a microplate reader (Dynatech, McLean, VA), before being stored at 4°C to save the original cultures. The rest of the cultures were pelleted by centrifugation at 1,000 g for 10 min and resuspended in 200 µl of 50 mM MOPS (pH 7.0) containing 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$ (i.e., buffer A). Bacterial suspensions were incubated with 50 µl of a 1 % (w/v) lysozyme solution at 37°C for 1 hr before being subjected to 3 freeze/thaw cycles (5 min in a dry ice-ethanol bath and 5 min in a 50°C water bath) to break the cells and release the enzymes into the supernatant. Cell-free crude extracts were then obtained by centrifugation at 1,000 g for 10 min and stored at 4°C for further use.

Screening the mutant library for increased activity on glucose at 60°C and low pH: The crude extracts were assayed for glucose isomerization in two conditions: 60°C (pH 7.0) and 80°C (pH 5.2). Assays were performed in microtiter plates with 150 µl of 100 mM MOPS (pH 7.0) or 100 mM sodium acetate (pH 5.2) containing 1 mM $CoCl_2$, 0.4 M glucose, and 10 µl of crude extract. The plates were incubated at 60°C (pH 7.0) or 80°C (pH 5.2) for 10 min and placed on ice to stop the reactions. The fructose produced was assayed using the resorcinol-ferric ammonium sulfate-hydrochloric acid method (Schenk and Bisswanger, 1998). Ten microliters of each reaction were transferred to a new set of microtiter plates and mixed with 40 µl of distilled water and 150 µl of a freshly prepared 1:1 mixture (v/v) of solution A (0.05 % resorcinol in ethanol) and solution B (0.216 g of $FeNH_4(SO_4)_2.12H_2O$ in 1L HCl). The plates were incubated in an 80°C water bath for 30 min to develop the color. The OD_{490} was measured with a microplate reader (Dynatech)

with 0-2.5 mM fructose as standards. A crude extract of HB101(DE3)*pET*23a(+) was used as the negative control on each plate. Crude extracts of HB101(DE3) expressing TNXI V185T and TNXI 3A2 were the positive controls in mutagenesis rounds one and two, respectively. Mutants with potentially higher activity on glucose than the positive control were selected on the basis of increases in both OD_{490} and OD_{490}/OD_{595} relative to the positive controls in the two rounds of mutagenesis. Mutants showing increased activity were screened a second time using crude extracts prepared from 5 ml cultures. These crude extracts were prepared as described above, before being heat-treated at 80°C for 15 min and centrifuged.

Oligonucleotide synthesis and DNA sequencing: PCR primers were synthesized by the Macromolecular Structure Facility, Department of Biochemistry and Molecular Biology at MSU. DNA sequences were determined either manually using the Thermosequenase kit (USB, Cleveland, OH) or automatically at the MSU Genomics Technology Support Facility.

Protein Purification: Recombinant enzymes were purified as described (Vieille *et al.*, 1995), followed by an additional ion-exchange chromatography step. Partially purified enzymes were applied to a DEAE-Sepharose column (2.5 x 15 cm) equilibrated with buffer A, and enzymes were eluted using a 500 ml linear 0–300 mM NaCl gradient in buffer A. The pooled fractions from the DEAE-Sepharose column were concentrated in a stirred ultrafiltration cell (30 kDa MW cut-off) (Amicon, Beverly, MA) and dialyzed

twice against buffer A. Concentrated, homogenous enzymes were dispensed and stored frozen at -70°C.

Glucose isomerase assays: TNXI and its mutants were assayed routinely with glucose as the substrate. The enzyme (1-1.5 mg/ml) was incubated in 100 mM MOPS (pH 7.0) [or 100 mM sodium acetate (pH 5.5)] containing 1 mM $CoCl_2$ and 0.4 M glucose at 80°C for 10 min. The reaction was stopped by transferring the tube to an ice bath. The amount of fructose produced was determined by the resorcinol-ferric ammonium sulfate-hydrochloric acid method (Schenk and Bisswanger, 1998). To determine the effect of temperature on activity, the enzymes were incubated in the reaction mixture at the temperatures of interest in a heated water (45-95°C) or oil bath (95-110°C) for 10 min. The effect of pH on activity was determined using the routine assay described above except that the MOPS buffer was substituted with 100 mM sodium acetate (pH 4.3-5.8), 100 mM PIPES (pH 6.1-7.0), or 100 mM EPPS (pH 7.2-8.1). All pHs were adjusted at room temperature, and the $\Delta pKa/\Delta t$'s for acetate, PIPES, and EPPS (0, -0.0085, and -0.011, respectively) (USB, Cleveland, OH) were taken into account for the results. To determine the kinetic parameters, assays were performed in 50 mM MOPS (pH 7.0) containing 10-1,500 mM glucose and 1 mM $CoCl_2$. One unit of glucose isomerase activity is defined as the amount of enzyme that produces 1 μmol of fructose per minute under the assay conditions.

Thermal inactivation assays: To obtain the apo-enzymes (metal-free enzymes), the purified enzymes were incubated overnight at 4°C in 50 mM MOPS (pH 7.0) containing

10 mM EDTA. They were then dialyzed twice against 50 mM MOPS (pH 7.0) containing 2 mM EDTA, and they were finally dialyzed twice against 50 mM MOPS (pH 7.0) without EDTA. $CoCl_2$ (0.5 mM) was added to the apo-enzymes and equilibrated at 4°C overnight before thermoinactivation assays. The time course of irreversible thermoinactivation was measured by incubating the enzymes (0.1-0.2 mg/ml) in either 10 mM MOPS (pH 7.0) or 10 mM sodium acetate (pH 5.5) at various temperatures for different periods of time in a heated water bath. Residual glucose isomerase activity was measured at 80°C as described above. The first order rate constant, k, of irreversible thermoinactivation was obtained by linear regression in semi-log coordinates. Enzyme half-lives were calculated from the equation: $t_{(1/2)} = \ln 2/k$.

Analysis of three-dimensional (3D) structures of TNXI and its variants: Enzymes were visualized on an IRIS-4D25 computer (Silicon Graphics Computer System, Mountain View, CA) using the INSIGHT II graphic program (Biosym Technologies, San Diego, CA). The TNXI pdb file (#1A0E) was obtained from the Protein Data Bank (www.rcsb.org/pdb).

RESULTS

Construction of mutant TNXI libraries and screening for activity on glucose at low temperature and low pH: TNXI V185T is optimally active at 95°C - 97°C, but its activity at 60°C does not exceed 10% of its optimal activity (Sriprapundh *et al.*, 2000). It retains only 20% of its optimal activity at pH 5.2. To increase this enzyme's activity at 60°C and at acidic pH, and to gain insight into the factors determining the effects of temperature and pH on activity, we subjected the TNXI V185T-encoding gene to sequential random mutagenesis and to low temperature/low pH activity screening. Random mutations were introduced into the gene by error-prone PCR. The PCR conditions used were suggested to yield an average of 1-2 mutations per gene, conditions deemed optimal for the improvement of specific properties by mutagenesis and screening (Arnold and Moore, 1997). After the first round of random mutagenesis, 1,000 transformants were screened for their activity on glucose at low temperature (60°C, pH 7.0) and at low pH (pH 5.2, 80°C). Thirty mutants were identified that showed significantly higher activity (> 30% increase) than TNXI V185T in both screening conditions. The phenotype of these mutants was tested again with heat-treated crude extracts prepared from 5 ml cultures. Higher activity on glucose was confirmed in only eleven out of the thirty crude extracts. XI expression level in these eleven crude extracts was checked by SDS-PAGE. Ten crude extracts showed higher XI content than the TNXI V185T control (data not shown). These ten mutants were discarded. The remaining mutant, TNXI 3A2, was purified to homogeneity. Once it was verified that TNXI 3A2 was significantly more active than TNXI V185T at 60°C and at pH 5.2, the gene

encoding TNXI 3A2 was used as the template in a second round of error-prone PCR and activity screening at low temperature and low pH. A library of ~1,500 transformants was screened using TNXI 3A2 as the positive control. A single mutant, TNXI 1F1, showed 80% and 40% increases in activity on glucose at 80°C (pH 5.2) and 60°C (pH 7.0), respectively, based on assays with heat-treated crude extracts. TNXI 3A2 and 1F1 were then purified to homogeneity. Their catalytic properties were studied in function of temperature and pH, and their thermostability was determined.

Effects of temperature and pH on TNXI 3A2 and 1F1 activities: The effect of temperature on 3A2 and 1F1 glucose isomerase activities is shown in Figure 1A in comparison to the activities of TNXI and TNXI V185T. Both 3A2 and 1F1 show significantly higher specific activity on glucose than TNXI and TNXI V185T at all temperatures. At their optimal temperatures of activity (i.e., 90°C for 1F1 and 95°C for 3A2), both mutants are ~ 3-fold more active than TNXI V185T. Activation energies (E_a's) for activity on glucose were calculated from the linear regressions shown in Figure 1B, using the equation $A = A_0e^{-Ea/RT}$. Whereas TNXI V185T shows the same activation energy as TNXI (i.e., 87 kJ/mole), 3A2 and 1F1 show significantly decreased E_a's (57 and 44 kJ/mole, respectively). These lower E_a's explain why 3A2 and 1F1 are as much as 7.3 and 12.3 times more active, respectively, than TNXI at 60°C, but only 4.2 and 4.8 times more active, respectively, than TNXI at 90°C.

The effect of pH on the activities of TNXI and its mutant derivatives is shown in Figure 2. 3A2 and 1F1 show significantly increased specific activity on glucose compared to TNXI and TNXI V185T over the entire active pH range. The activity

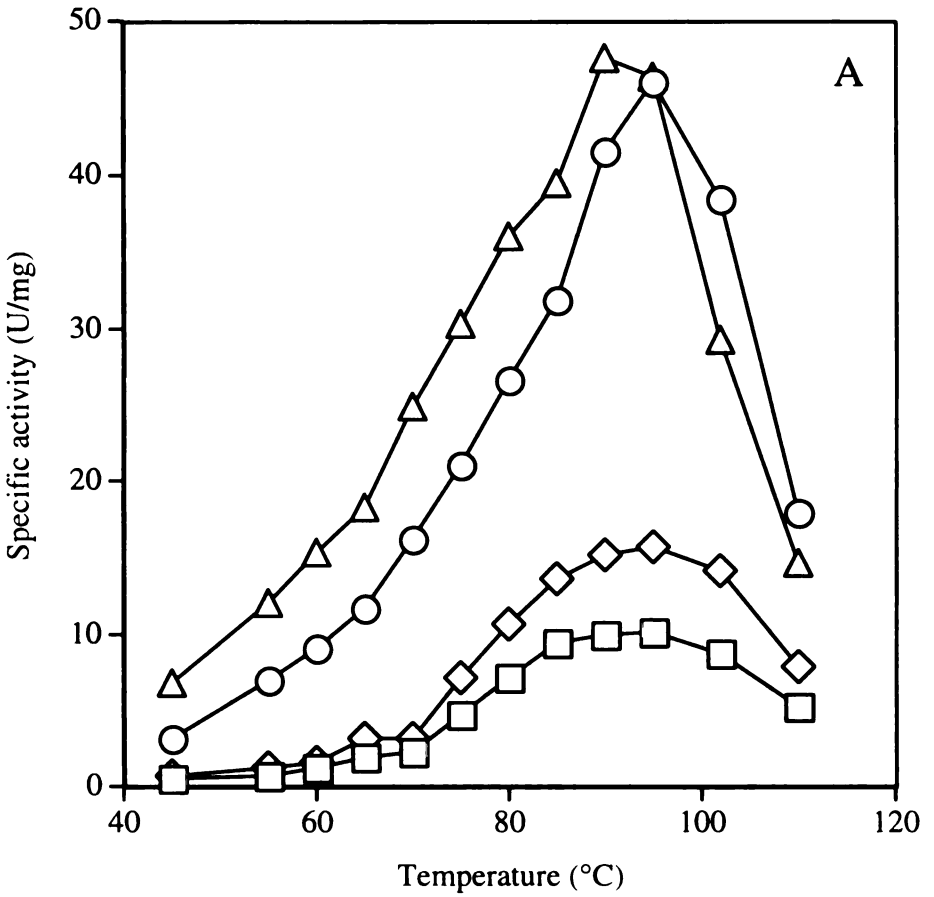

Figure 1: A) Effect of temperature on the specific activities of TNXI and its mutant derivatives on glucose at pH 7.0. (□): TNXI; (◊): TNXI V185T; (○): TNXI 3A2; (Δ): TNXI 1F1.

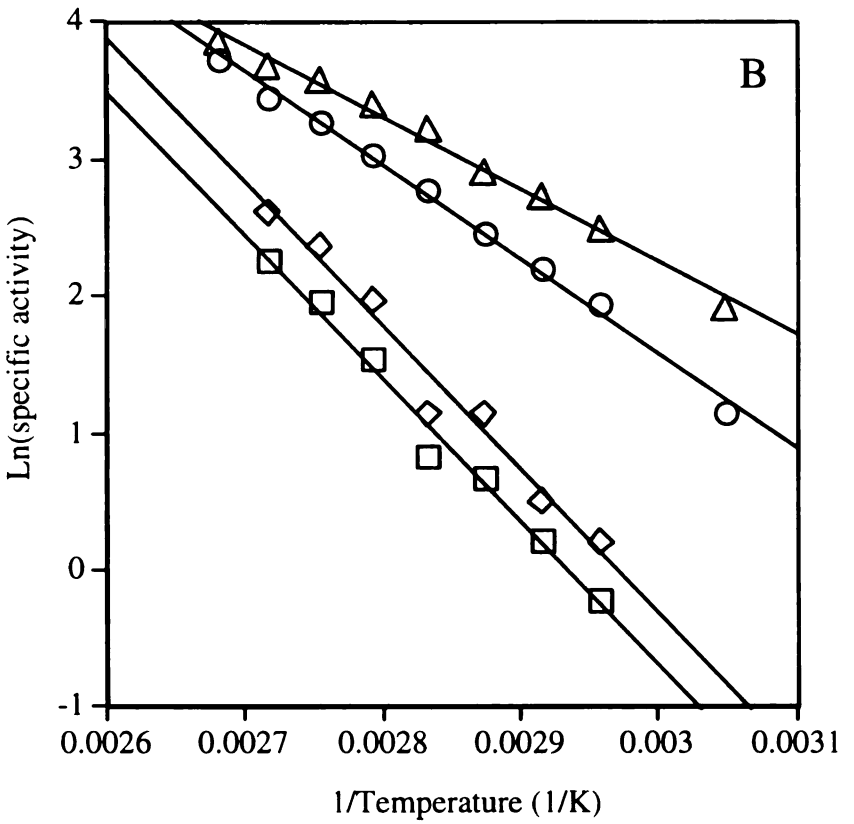

Figure 1: B) Ln (specific activity) versus 1/Temperature. All linear regressions had r^2 values above 0.97. (□): TNXI; (◊): TNXI V185T; (○): TNXI 3A2; (Δ): TNXI 1F1.

Activation energies (E_a) for activity of TNXI, TNXI V185T, TNXI 3A2, and TNXI 1F1 are 87, 87, 57, and 44 kJ/mole, respectively.

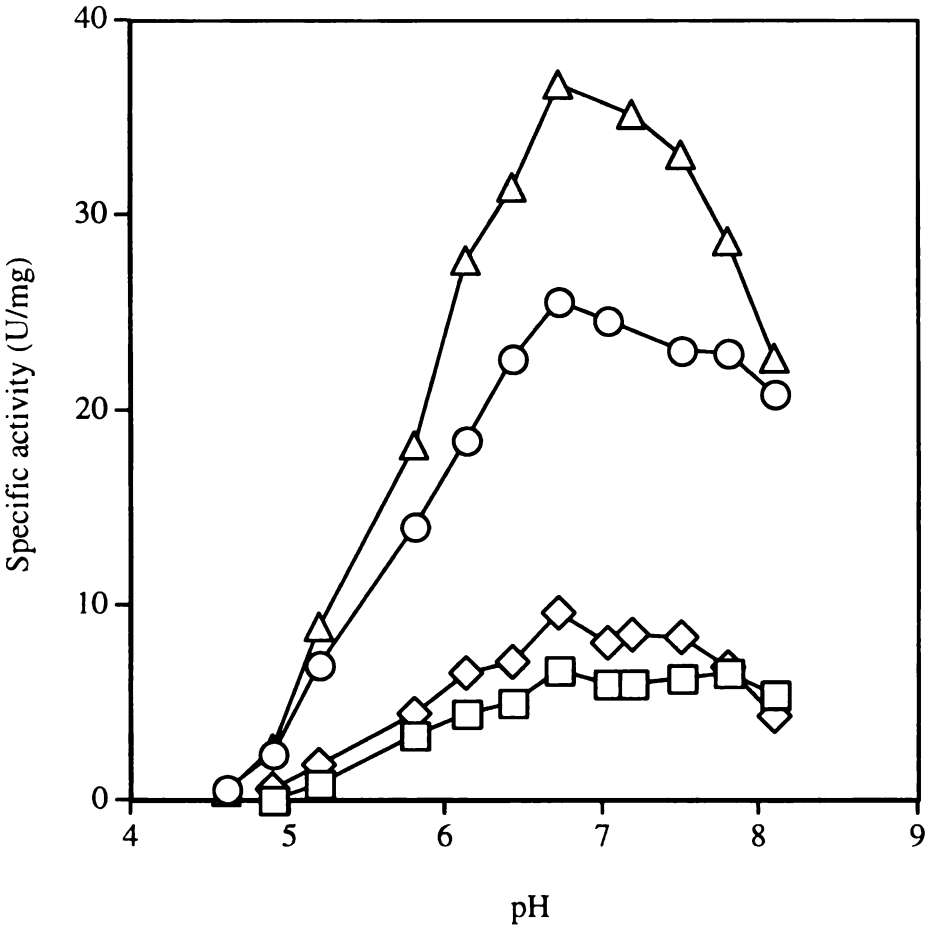

Figure 2: Effect of pH on specific activities of TNXI and its mutant derivatives on glucose at 80°C. (□): TNXI; (◊): TNXI V185T; (○): TNXI 3A2; Δ TNXI 1F1.

increase is so significant that 3A2 and 1F1 are more active at pH 5.5 than TNXI and TNXI V185T are at pH 7.0.

Kinetic parameters of TNXI 3A2 and 1F1: The kinetic parameters on glucose of TNXI V185T, 3A2, and 1F1 were compared in different conditions of temperature and pH (Table 1). In all conditions tested, TNXI 3A2 and 1F1 showed higher K_m and V_{max} values than TNXI V185T did. At pH 7.0 (both at 60°C and 80°C), TNXI 3A2 and 1F1's V_{max} values increased more significantly than their K_m's for glucose, yielding important increases in catalytic efficiencies (up to 2.3 fold for 1F1 at 60°C [pH 7.0]). At 80°C (pH 5.5), the increases in TNXI 3A2 and 1F1's V_{max}'s do not compensate for the major increases in their K_m for glucose (i.e., 3.0 fold for 3A2 and 4.6 fold for 1F1). In these conditions, TNXI 3A2 and 1F1 show catalytic efficiencies that are approximately half that of TNXI V185T. At 60°C (pH 5.5), TNXI 3A2's increase in V_{max} does not compensate for a poor glucose affinity (high K_m), resulting in a lower catalytic efficiency than that of TNXI V185T. Unlike TNXI 3A2, 1F1 has a higher catalytic efficiency on glucose than TNXI V185T does due to a dramatic increase (5 fold) in its V_{max} that surpasses the increases in its K_m (3.7 fold) in these conditions. Its 5-fold increase in V_{max} makes 1F1 a 1.7 fold more active enzyme at 60°C (pH 5.5) than TNXI V185T is at 80°C (pH 7.0).

Thermal stability of TNXI 3A2 and 1F1: To determine whether the mutations present in 3A2 and 1F1 affected the kinetic stability of the mutated enzymes, the residual activities of 3A2 and 1F1 were measured after heat treatment at 80°C (pH 7.0) and 80°C

Table 1: Kinetic parameters of TNXI and its mutant derivatives.[a]

Enzyme	80°C pH 7.0				80°C pH 5.5			
	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
TNXI V185T	22.5 ± 1.6	11.1 ± 0.1	564	25.1	59.3 ± 2.1	15.8 ± 0.4	806	13.6
TNXI 3A2	43.5 ± 2.4	24.9 ± 0.6	1268	29.1	180.3 ± 9.5	24.7 ± 0.4	1259	7.0
TNXI 1F1	52.0 ± 7.1	37.9 ± 2.7	1930	37.1	274.0 ± 94.0	35.3 ± 4.1	1798	6.6
	60°C pH 7.0				60°C pH 5.5			
	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
TNXI V185T	30.3 ± 18.4	3.9 ± 0.5	197	6.5	58.7 ± 14.5	3.8 ± 0.2	195	3.4
TNXI 3A2	54.5 ± 8.5	13.2 ± 0.7	672	12.3	298.5 ± 9.5	9.1 ± 0.5	465	1.6
TNXI 1F1	60.2 ± 5.6	17.4 ± 0.7	883	14.7	217.3 ± 4.5	19.0 ± 0.3	967	4.5

[a] Results are the means of three independent experiments. K_m and V_{max} values were obtained through direct fitting of the initial velocity data to the Michaelis-Menten equation.

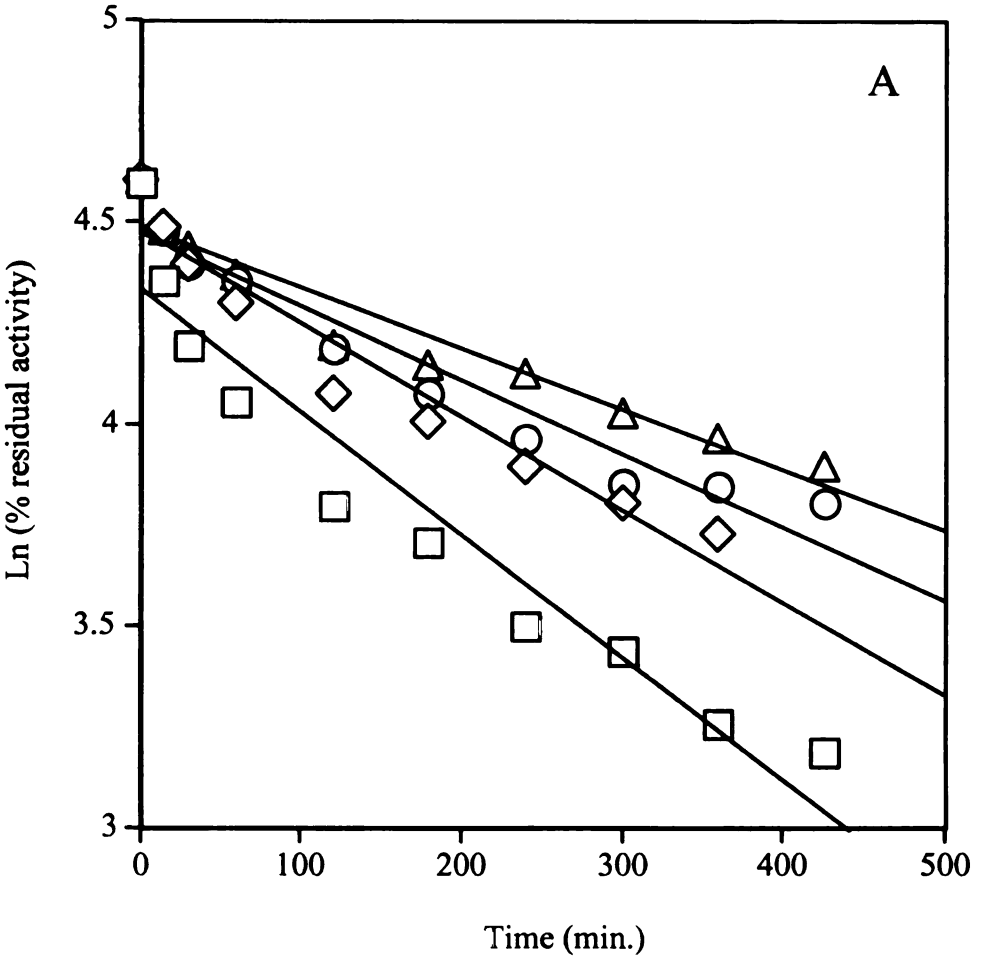

Figure 3: A) Inactivation curves of TNXI and its mutant derivatives at 80°C (pH 7.0). Symbols used are the same as in Figure 1. Half-lives of TNXI, TNXI V185T, TNXI 3A2, and TNXI 1F1 are 1.6 hr, 3.8 hr, 4.5 hr, 6.7 hr, respectively

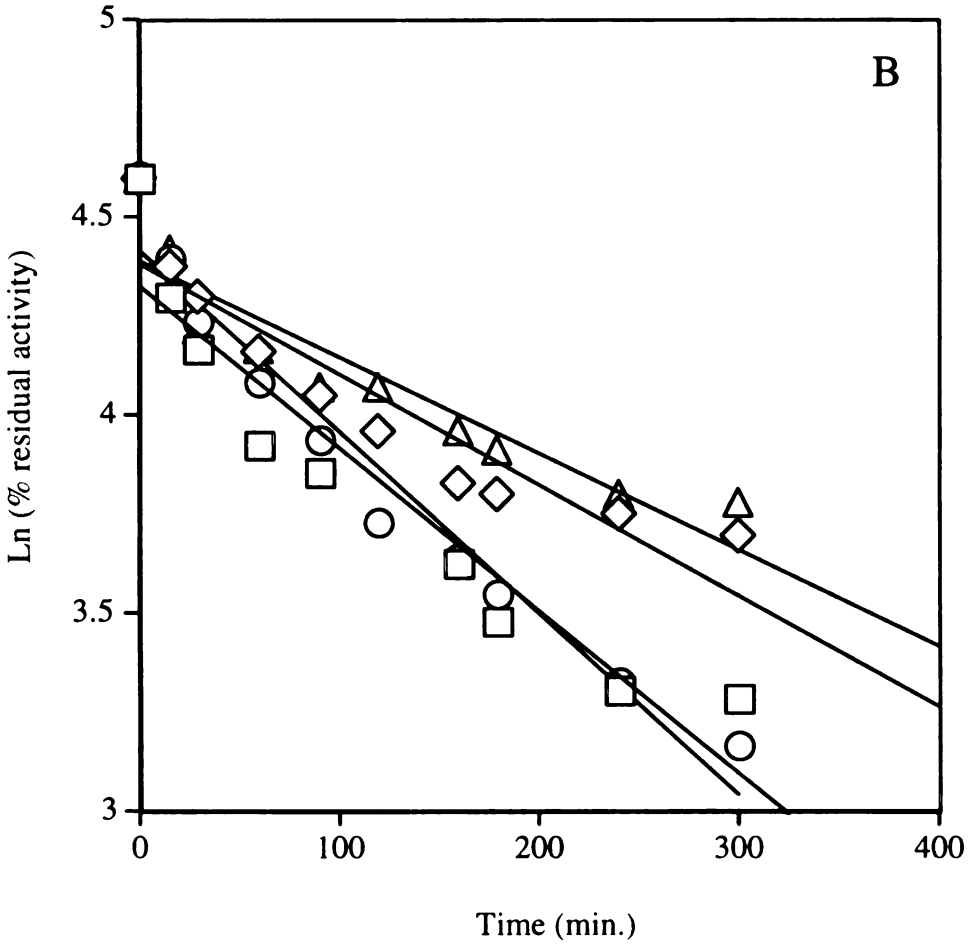

Figure 3: B) Inactivation curves of TNXI and its mutant derivatives at 80°C (pH 5.5). Symbols used are the same as in Figure 1. Half-lives of TNXI, TNXI V185T, TNXI 3A2, and TNXI 1F1 are 1.3 hr, 2.3 hr, 1.7 hr, and 3.0 hr, respectively.

(pH 5.5) for various lengths of time (Figure 3). Stability experiments performed with the metal-free enzymes in 10 mM MOPS (pH 7.0) containing 0.5 mM $CoCl_2$ showed that 3A2 and 1F1 (with $t_{1/2}$ of 4.5 hr and 6.7 hr, respectively) were kinetically more stable than TNXI ($t_{1/2}$ of 1.6 hr) and TNXI V185T ($t_{1/2}$ of 3.8 hr). At pH 5.5, 1F1 ($t_{1/2}$ of 3.0 hr) remained more stable than TNXI ($t_{1/2}$ of 1.3 hr) and TNXI V185T ($t_{1/2}$ of 2.3 hr); 3A2 was less stable ($t_{1/2}$ of 1.7 hr).

Amino acid substitutions in TNXI 3A2 and 1F1: The mutations present in 3A2 and 1F1 were identified by DNA sequencing. In addition to Val185Thr already present in TNXI V185T, 3A2 contained a single additional mutation, Leu282Pro. The Leu282Pro mutation is located in helix α_7 of the $(\alpha/\beta)_8$-barrel structure, at approximately 12-14 Å from the catalytic center (Figure 4). Helix α_7 itself is located at the surface of a monomer and at the interface of the dimer. Neither Leu nor Pro's sidechain can form hydrogen bonds with neighboring residues. Whenever a proline occurs in a peptide chain, it interrupts α-helices and creates a kink or bend (Lehninger, 1970). Detailed analysis of the Leu28Pro mutation modeled into the TNXI structure (Figure 5) suggests that Pro282's pyrrolidine ring (C_γ, and C_δ) is in close contact (in some cases ~1.7Å) with mainchain atoms of residues Phe278 and Gln279. With van der Waal's radii of 1.87 and 1.35 Å for C and O atoms, respectively, in proteins, optimal van der Waal interactions between carbon atoms of Pro282 sidechain and the mainchain C and O atoms of residues Phe278 and Gln279 would take place at approximately 3.2 Å to 3.7 Å. The unfavorable van der Waal contacts (clashes) probably lead to local conformational rearrangements. These

changes might, in turn, affect the active site structure and dynamics, the enzyme's interaction with the substrate, and probably inter-subunit interactions within the dimer.

1F1 contains the same two mutations as 3A2, plus mutation Phe186Ser. This last mutation is located in the active site, adjacent to mutation Val185Thr (Figure 4). Serine's sidechain is much less bulky than that of the original Phe. Residue 186's sidechain points into the active site cavity, and it is close to the bulky sidechains of residues Tyr184, Phe228, Phe262, and Leu229. The Phe186Ser mutation probably leads to a rearrangement of the neighboring residues. This change in local packing may in turn be responsible for the large increase in low temperature activity of mutant 1F1.

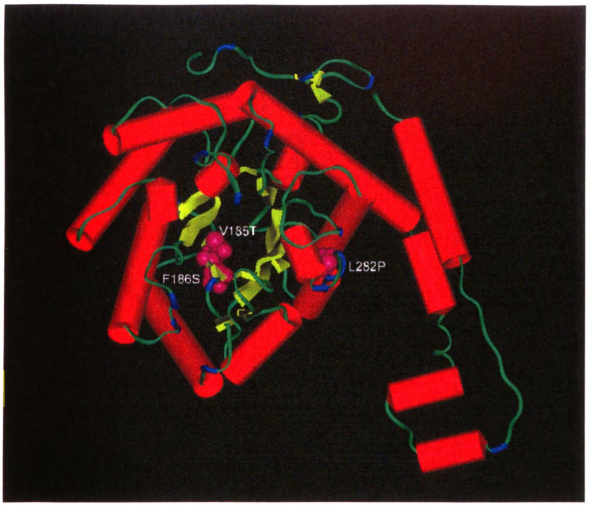

Figure 4: Three-dimensional model of the TNXI 1F1 monomer showing the positions of mutations V185T, F186S, and L282P.

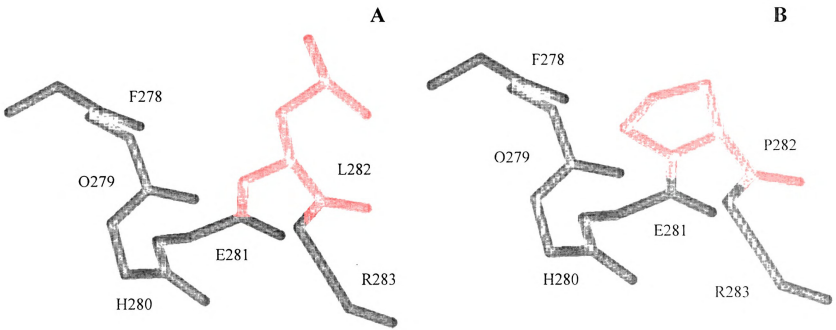

Figure 5: Three-dimensional model of part of the area surrounding TNXI 3A2 and 1F1's Leu282Pro mutation. Backbone structure of residues 278-283 is shown in light green. Leu282 (A) and Pro282 (B) sidechains are shown in red.

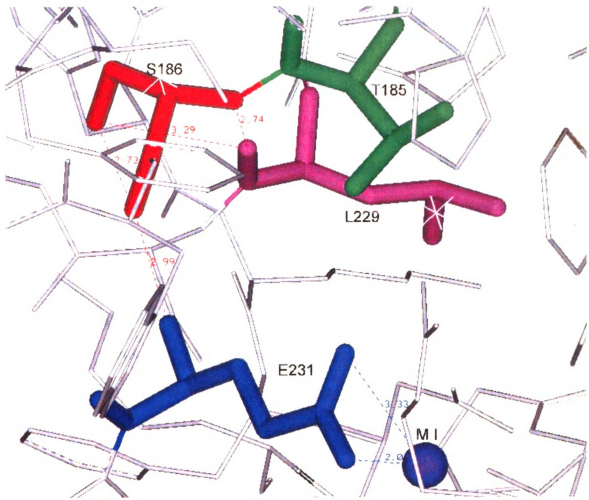

Figure 6: Three-dimensional model of part of the TNXI 1F1 active site showing hydrogen bonds among S186 (red), L229 (pink) and E231 (blue). E231co-ordinates Co^{2+} (purple ball) at the structural site (M1).

DISCUSSION

Thermostable enzymes are generally barely active at low temperature, but they are as active at their optimal growth temperature as their mesophilic counterparts (Zeikus and Brock, 1971; Varley and Pain, 1991). It was shown here that activity of a hyperthermostable enzyme at low temperature and low pH can be improved without a loss in its extreme stability. Our results have shown that the quality of the library of random TNXI mutants was sufficient to isolate mutants with increased activity at low temperature and low pH. Using two sequential rounds of random mutagenesis, we were able to obtain a TNXI mutant derivative, 1F1, showing high activity at low temperature and low pH. 1F1 is not only more active overall than its parental enzymes, but it is also more active especially at low temperature than its parental enzymes. Since 1F1 is more active at low temperature with a lower temperature optimum but more stable than the wild-type enzyme, we suggest that the molecular determinants of this enzyme's activity and thermal stability are in fact, not the same. This has also been previously observed in the study of *Bacillus subtilis* p-nitrobenzyl esterase, in which the laboratory-evolved mutant enzyme had a 14°C increase in T_m but still maintained its catalytic activity at low temperature (Giver *et al.*, 1998).

Recent studies (Aguilar *et al.*, 1997; Zavodsky *et al.*, 1998; Kohen *et al.*, 1999) suggested that it might be the reduced flexibility of thermostable enzymes that impairs their catalytic activity at low temperatures. Particularly striking is the potential of single point mutations to significantly increase low temperature activity. Recent studies of psychrophilic enzymes have suggested that, despite the many differences observed

between mesophilic and psychrophilic enzymes, single amino acid substitutions may be capable of conferring most psychrophilic characteristics (Somero, 1995; Feller and Gerday, 1997). Other studies using random mutagenesis and screening/selection succeeded in increasing the activity (by 3 fold at 20°C for *Pyrococcus furiosus* β-glucosidase and by 17 fold at 37°C for *Sulfolobus solfataricus* indole glycerol phosphate synthase) of hyperthermophilic enzymes at mesophilic temperatures with changes in temperature optima (Merz *et al.*, 2000; Lebbink *et al.*, 2000). One study even increased the catalytic efficiency of a mesophilic subtilisin at 10°C by 100 % (Taguchi *et al.*, 1999).

In our study, TNXI 1F1 was obtained with 4.5 and 2.2 fold increases in V_{max} at 60°C (pH 7.0) and 80°C (pH 5.5), respectively, with only 5°C lower optimal temperature compared to those of TNXI V185T. The Arrhenius plot of activity of TNXI and its mutants (Figure 1B) revealed that TNXI and TNXI V185T require higher levels of activation energy for their catalytic activities than either TNXI 3A2 or 1F1. The difference is more pronounced with 1F1 with an approximately 2-fold decrease in the E_a of activation compared with TNXI and TNXI V185T. The reduction of E_a of activity observed in TNXI 3A2 and 1F1 suggested improved dynamics and flexibility in the active site of the enzymes even at low temperature thus their activities at low temperature are vastly enhanced. Although TNXI V185T has improved catalytic efficiency on glucose compared to TNXI due to improved glucose binding affinity and higher catalytic rate (Sriprapundh *et al.*, 2000), its E_a of activity remained similar to TNXI's suggesting that its active site dynamics and flexibility remained unchanged. This observation is in good agreement with the assumption of cold-adapted thermophilic enzymes by Lonn *et al.*

(2002) that mutations underlying the adaptation of enzymes to temperatures lower than their optima allow a higher degree of flexibility in areas that move during catalysis. This, in turn, reduces the free energy of activation compared with the wild type enzymes. The higher flexibility in areas that move during catalysis increases the k_{cat} of the reactions catalyzed by the cold-adapted enzymes. A study of lactate dehydrogenases cold-adaptation (Fields and Somero, 1998) also found that mutations that increase flexibility in regions of the enzyme involved in catalytic conformational changes may reduce energy barriers to these rate-governing shifts in conformation and thereby increase k_{cat}. TNXI 1F1 has higher k_{cat} and K_m values than TNXI V185T does. This observation was rationalized in terms of localized increases in conformational flexibility; mutations that reduce the energetic barriers between different active site conformations (thus allowing for more rapid interconversion among them) will lead to higher values of k_{cat}. These same mutations, however, will allow the enzyme to populate conformations that bind substrate poorly more easily, leading to increases in K_m. Our present study provides obvious support for this hypothesis.

Our sequential random mutagenesis and screening approach with TNXI resulted in the identification of amino acid residues or local structural conformations that are critical for thermostability, metal-binding affinity, and low temperature/low pH catalytic activity. Two mutations were identified in TNXI 1F1 in addition to V185T, namely L282P and F186S. Leu282 is at the inter subunit interface of the enzyme dimer. While the L282P mutation improved the enzyme's low temperature and low pH activities, the detailed analysis of the modeled 3D structure of 3A2 revealed unfavorable van der Waal contacts between Pro282's pyrrolidine ring and the enzyme's backbone structure. These

unfavorable contacts probably lead to local conformational rearrangements and make the enzyme less stable (as observed in the shortened half-life at pH 5.5 compared to TNXI and TNXI V185T). The second mutation, F186S, is located in the active site, adjacent to Thr185. Since serine's sidechain is considerably smaller than phenylalanine's, this mutation would create a cavity or increase mobility in the active site of the enzyme resulting in a dramatic improvement of 1F1's low temperature activity. A potential extra strengthening H-bond between Ser186's sidechain and the Leu229 mainchain O (<3.2 Å) (Figure 6) is relatively close to E231. This E231 sidechain coordinates metal ion at the structural site (M1). This proposed extra H-bond in the F186S mutation might explain the increased kinetic stability of TNXI 1F1 that even surpassed that of TNXI.

There are a few evidences that demonstrated the effect of positions where mutations occur on activity and stability of laboratory and naturally evolved enzymes. A study of psychrophilic enzymes revealed that amino acid substitutions distant from the catalytic center or in the major substrate-binding site of enzymes could lead to cold-adaptation (Feller and Gerday, 1997). In the studies done by Lebbink *et al.* (2000), all mutants containing subunit interface substitutions were less stable and had lower temperature optima than the wild-type *Pyrococcus furiosus* β-glucosidase, suggesting that subunit interfaces also play an important role in thermoadaptation. Our results showed that even without selective pressure to maintain thermostability, it is possible to obtain a mutant thermozyme with a mutation in the active site that has comparable thermostability with the wild-type enzyme while its low temperature and low pH activity are vastly enhanced.

The only directed evolution approach (or random mutagenesis) performed on xylose isomerase that has been reported to date was by Lonn *et al.* (2002). The thermophilic type I *Thermus thermophilus* XI was subjected to one round of random PCR mutagenesis and screening for xylose isomerase activity at lower temperature than optimal. Three amino acid substitutions were identified as F163L in the C-terminal tail domain, and E372G/V379A in the $(\alpha/\beta)_8$ barrel domain. These mutant enzymes showed improved catalytic rate constants (k_{cat}) by up to nine times on both xylose and glucose with up to 26 times higher K_m values on xylose but relatively unchanged for glucose. All enzyme variants' relative activities on xylose are higher than the wild-type at low temperatures with lower thermostability. Kinetic analysis demonstrated that the increase in the relative activity in the enzyme variants for xylose at low temperatures was indeed caused by an increase in k_{cat} and not by a decrease in the K_m value. This suggests that the mutant enzymes did not acquire higher affinity for the substrate than the wild-type enzyme at lower temperatures. These results as well as ours suggested that amino acid substitutions either distant from or inside the catalytic center can lead to cold adaptation. The main difference between their work and ours was that we, in fact, were able to enhance the thermostability of our mutant enzyme while increasing its activity at low temperatures.

An alignment of different XIs (not shown) revealed that neither Pro282 nor Ser186 is present in any known XI. Because it is in the active site, mutation F186S could potentially have been rationally designed based on the structures of TNXI, TNXI V185T, and 3A2 in the presence and absence of substrate, and on modeling. In contrast, mutation L282P, in the middle of an α-helix, and 12 to 14 Å from the active site was completely

unpredictable, and could only be obtained through directed evolution. With a vast improvement in specific activity at 60°C, at pH 5.5, a higher catalytic efficiency on glucose than TNXI V185T in all conditions tested, and thermostability comparable to that of TNXI V185T, 1F1 could be an interesting candidate for industrial applications. Further study of 1F1's potential usefulness in conditions used in the industrial production of high fructose syrup in comparison with a commercially available glucose isomerase is underway.

ACKNOWLEDGEMENT

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CHAPTER V

BIOCHEMICAL COMPARISON OF STABILITY, ACTIVITY, AND FRUCTOSE SYNTHESIS BY AN INDUSTRIAL GLUCOSE ISOMERASE (GENSWEET™) VERSUS A LABORATORY-EVOLVED THERMO-ACID STABLE XYLOSE ISOMERASE (TNXI 1F1)

ABSTRACT

Biochemical properties and fructose productivities of a laboratory-evolved xylose isomerase from hyperthermophilic *Thermotoga neapolitana* (TNXI 1F1) and Gensweet™ (Genencor, Rochester, NY), a commercially available glucose isomerase from a genetically modified strain of *Streptomyces rubiginosus* were compared. TNXI 1F1 displayed higher catalytic efficiencies on glucose at low or high temperature and pH ranges and had greater thermal stability than Gensweet™ despite having similar temperature optima for activity. This greater thermal stability together with the superior kinetic parameters on glucose render TNXI 1F1 a genuine candidate for the industrial glucose isomerization process based on the lifetime fructose productivity estimation. At high temperature and neutral to alkaline pH, the Maillard browning reaction is a major concern in the resulting syrups. This was overcome by using TNXI 1F1 for fructose production at 90°C and pH 5.5-6.5.

INTRODUCTION

The production of high fructose corn syrup (HFCS) using immobilized glucose isomerase (GI) is considered one of the largest commercial enzymatic processes (Klibanov, 1983a; and Bhosale *et al.*, 1996). The last step of the process is the enzymatic isomerization of glucose into a mixture typically containing 42 % fructose with 51 % glucose and 7 % oligosaccharides (Visuri and Klibanov, 1987). A costly fructose enrichment step is then typically employed to increase the fructose concentration to 55 % level to give the same sweetness level as sucrose (at the same concentration of solids) for its major use (Bucke, 1981). The enzyme-catalyzed isomerization of glucose into fructose is performed in industrial bioreactors at 55-60°C where the half-life of GI is on the order of several weeks. Even a modest increase in the half-life of the enzyme will substantially reduce the cost of the HFCS production (Klibanov, 1983b).

Since most industrially employed GIs exhibit temperature optima in the range of 80-90°C (Hartley *et al.*, 2000), only insufficient operational stability precludes their use at these higher temperatures, which would be highly beneficial. Not only thermostability of commercial enzymes is the main limiting factor of such application at high temperature, but in high substrate concentrations, the Maillard browning reaction of the enzyme with reducing sugars (e.g. glucose and fructose) is also the dominant reason for enzyme inactivation. The Maillard reaction was considerably faster than other inactivation mechanisms (Visuri *et al.*, 1999). Theoretically, the Maillard reaction can be retarded at low temperature or low pH. Hence the use of highly thermostable GIs at higher temperature and lower pH than the current isomerization conditions would result

in more operational stability with reduced browning reactions. The proposed process would be ideal since previous liquefaction and saccharification steps were also performed at high temperature and low pH and minimal adjustment of isomerization condition would be required.

Xylose isomerase (XI) from the hyperthermophilic eubacterium *Thermotoga neapolitana* is one of the most thermostable characterized XIs (Vieille *et al.*, 2001). The gene encoding the enzyme was cloned, sequenced, and expressed in *Escherichia coli* (Vieille *et al.*, 1995). Its active site was also engineered by site-directed mutagenesis to increase its activity on glucose rather than its natural substrate, xylose (Sriprapundh *et al.*, 2000). The TNXI V185T mutant derivative is more active, more glucose efficient, and more stable than the wild-type TNXI. This enzyme's activity on glucose at low temperature and low pH was recently further improved by directed evolution resulting in the TNXI 1F1 derivative (V185T/L282P/F186S) with higher overall activity on glucose throughout the temperature and pH ranges compared to TNXI V185T (Sriprapundh *et al.*, submitted). Despite its higher activity at low temperature, TNXI 1F1 remains relatively as stable as TNXI V185T and is more stable than the wild-type TNXI. With such vast improvement in every biochemical aspect of TNXI 1F1, it would be interesting to compare its utility against a commercially available glucose isomerase to determine whether it can be genuinely considered for industrial applications.

In this study, the effect of temperature and pH on specific activity on glucose together with kinetic parameters and thermal stability in different incubation conditions were compared between TNXI 1F1 and Gensweet™, a commercially available glucose isomerase from a genetically modified strain of *Streptomyces rubiginosus*. Furthermore,

their fructose production in various combinations of pHs and temperatures as well as lifetime fructose productivities of each enzyme under these conditions were studied. We show that TNXI 1F1 has superior properties as an industrial glucose isomerase.

MATERIALS AND METHODS

Enzyme source: Gensweet™ SGI, a xylose isomerase derived from a genetically modified strain of *Streptomyces rubiginosus*, was provided by Dr. Jay Shetty of Genencor International (Rochester, NY) as a kind gift. SDS-gel electrophoresis showed that the enzyme was pure.

Protein Purification: TNXI 1F1 was purified using the procedure of Vieille *et al.* (1995) followed by an additional ion-exchange chromatography step. Partially purified enzyme was applied to a DEAE-Sepharose column (2.5x15 cm) equilibrated with buffer A, and the enzyme was eluted using a 500 ml linear 0–300 mM NaCl gradient in buffer A. The pooled fractions from the DEAE-Sepharose column were concentrated in a stirred ultrafiltration cell (MW cut-off 30 kDa) (Amicon, Beverly, MA) and dialyzed twice against buffer A. Concentrated, homogenous enzyme was dispensed and stored frozen at -70°C.

Glucose isomerase assays: TNXI 1F1 and Gensweet™ were assayed routinely with glucose as the substrate. The enzyme (1-1.5 mg/mL) was incubated in 100 mM MOPS buffer (pH 7.0) [or 100 mM sodium acetate buffer (pH 5.5)] containing 1 mM $CoCl_2$ and 0.4 mM glucose at 80°C for 10 min. The reaction was stopped by transferring the tube to an ice bath. The amount of fructose produced was determined by the modified resorcinol-ferric ammonium sulfate-hydrochloric acid method (Schenk and Bisswanger, 1998). To determine the effect of temperature on the activity of TNXI 1F1 and Gensweet™, the

holo-enzyme was incubated in the reaction mixture at the temperatures of interest in a heated water bath (45-95°C) or a heated oil bath (95-110°C) for 10 min. The effect of pH on activity was determined using the routine assay described above except that the MOPS buffer was substituted with 100 mM sodium acetate buffer (pH 4.3-5.8), 100 mM PIPES buffer (pH 6.1-7.0), or 100 mM EPPS buffer (pH 7.2-8.1). All pHs were adjusted at room temperature, and the $\Delta pKa/\Delta t$'s for acetate, PIPES, and EPPS (0, -0.0085, -0.011, respectively) (USB, Cleveland, OH) were taken into account for the results. To determine the kinetic parameters, assays were performed in the presence of 10-1,500 mM glucose, 50 mM MOPS (pH 7.0) and 1 mM $CoCl_2$. One unit of glucose isomerase activity is defined as the amount of enzyme that produces 1 μmol of fructose per minute under the assay conditions.

Thermal inactivation assays: To obtain the apo-enzymes (metal-free enzymes), the purified enzymes were incubated overnight at 4°C in 50 mM MOPS buffer (pH 7.0) containing 10 mM EDTA. They were then dialyzed twice against 50 mM MOPS buffer (pH 7.0) containing 2 mM EDTA, and they were finally dialyzed twice against 50 mM MOPS buffer (pH 7.0) without EDTA. $CoCl_2$ (0.5 mM) was added to the apo-enzyme and equilibrated at 4°C overnight before the thermoinactivation was initiated. The time course of irreversible thermoinactivation was measured by incubating the enzymes (0.1-0.2 mg/ml) in either 10 mM MOPS buffer (pH 7.0) or 10 mM sodium acetate (pH 5.5) at various temperatures for different periods of time in a heated water bath. Residual glucose isomerase activity was measured as described above at 80°C. The first order rate

constant, k, of irreversible thermoinactivation was obtained by linear regression in semi-log coordinates. Enzyme half-lives were calculated from the equation: $t_{(1/2)} = \ln 2/k$.

Differential Scanning Calorimetry (DSC): DSC experiments were performed on a Nanocal differential scanning calorimeter (Calorimetry Sciences Corp., Provo, UT) using a scan rate of 1°C/min. Samples were scanned from 25°C to 100°C. The apo-enzymes were scanned against 50 mM MOPS (pH 7.0). Enzymes containing both Mg^{2+} and Co^{2+} were dialyzed against buffer A, then scanned against the dialysis buffer as control.

Fructose production experiments: TNXI 1F1 and Gensweet™ (50 μg) were incubated in 1 ml reaction in capped 1.5 ml tubes containing 2.5 M glucose, 5 mM $MgSO_4$, and 50 mM of either MOPS (pH 7.0) or sodium acetate (pH 5.5) at various temperatures for up to 24 hours. The reactions were then stopped on ice and were assayed for fructose produced by the method described above. Browness of resulting syrups was monitored by maximal absorbance at 425 nm.

Modeled fructose productivity: Lifetime fructose productivity of TNXI 1F1 and Gensweet™ were estimated using the one phase inactivation mathematical model (Bandlish *et al.*, 2002). Kinetic and inactivation data for the soluble enzymes were used to eliminate variations due to non-optimal immobilization protocols and the potential influence of mass transfer limitations on immobilized enzyme kinetics. The final equations presented a derivative of parameters for GI kinetics that also considered the equilibrium between glucose and fructose:

$$\frac{P_{life}}{[E_0]} = \frac{[Glu]}{[Glu] + K_m} \frac{[k_{cat}]}{[k_D]}$$

$$\frac{P}{[E_0]} = \frac{[Glu]}{[Glu] + K_m} [1-\exp(-k_D t)] \frac{[k_{cat}]}{[k_D]}$$

Using these equations, enzyme productivities (kg fructose per kg enzyme) were calculated using representative data for the soluble enzymes. The calculations assumed 3 M glucose feed, a representative of an industrial HFCS production (Pedersen 1993; Godfrey and West, 1996). Enzyme productivity, *P*, is defined as the total amount of glucose converted to fructose per unit amount of enzyme during a period of time.

RESULTS

Effect of temperature and pH on TNXI 1F1 and Gensweet™ activities: The effect of temperature on TNXI 1F1 and Gensweet™ glucose isomerase activities is shown in Figure 1. Both enzymes have comparable specific activities on glucose in the temperature range of 45-75°C. The optimal temperatures of glucose isomerase activity of TNXI 1F1 and Gensweet™ are 90°C and 85°C, respectively. Although they have relatively the same activity at low temperature, TNXI 1F1 is much more active at its optimal temperature of activity with 47.6 U/mg compared with 30.9 U/mg of Gensweet™. Figure 2 demonstrated the effect of pH on glucose isomerase activities at 80°C of TNXI 1F1 and Gensweet™. The two enzymes have comparable activity over the pH range of 4.6 to 8.1 and retain more than 70 % of their optimal activities in the pH range of 6.1 to 8.0.

Thermal stability of TNXI 1F1 and Gensweet™: To determine the thermal stability of TNXI 1F1 and Gensweet™, the residual activities of TNXI 1F1 and Gensweet™ were measured after heat treatment at 60°C, pH 7.0 and 5.5 for various lengths of time (Figures 3, A and B). Investigations of the metal-free enzymes in buffer at saturated Co^{2+} concentration showed that at both pHs, TNXI 1F1 is far more superior in term of thermal stability when compared to Gensweet™ with half-lives of 115.5 and 38.5 hr at pH 7.0 and 5.5, respectively, compared to 2.9 and 1.7 hr for Gensweet™. The TNXI 1F1 is more stable than Gensweet™ by 1.6 and 1.36 orders of magnitudes at 60°C, pH 7.0 and 5.5, respectively. These results suggest that although Gensweet™ and TNXI 1F1 have almost the same temperature of optimal activity (T_{opt}), only TNXI 1F1 has extreme

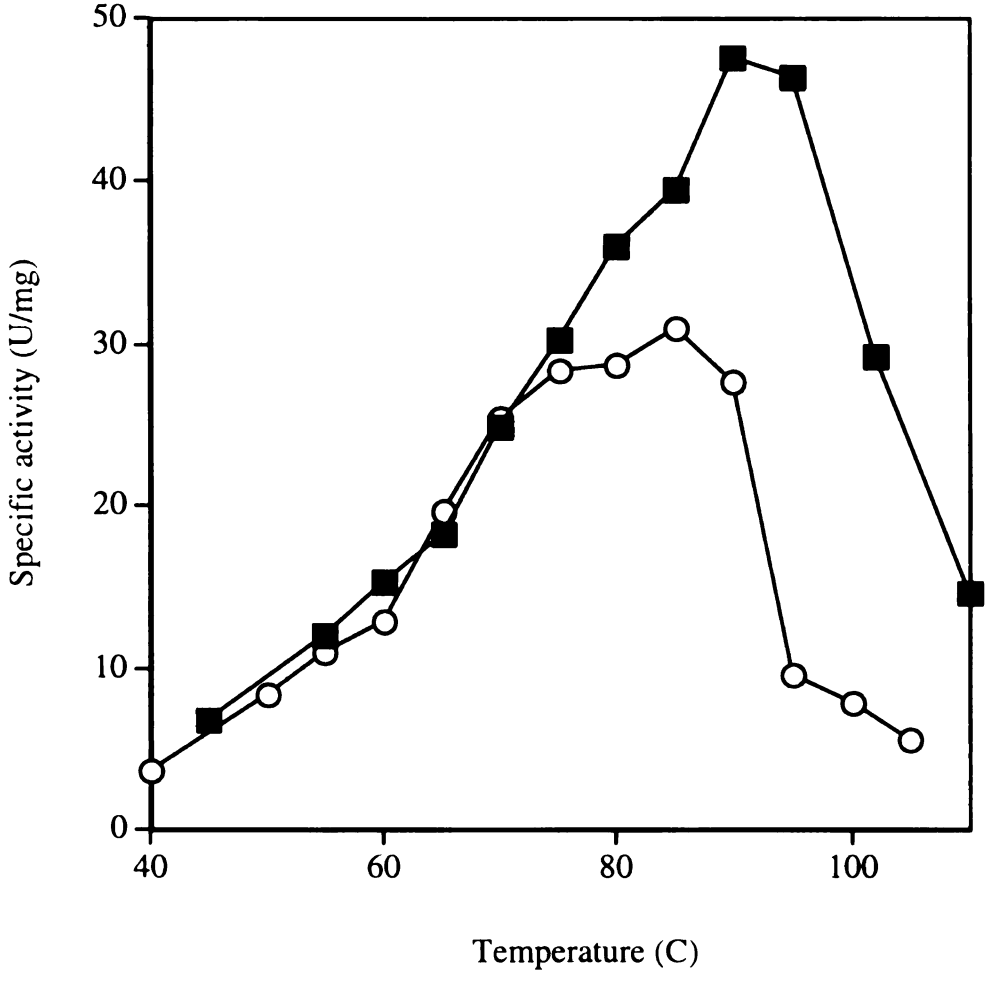


Figure 1: Effect of temperature on the specific activities of TNXI 1F1 (■) and Gensweet™ (○) on glucose at pH 7.0.

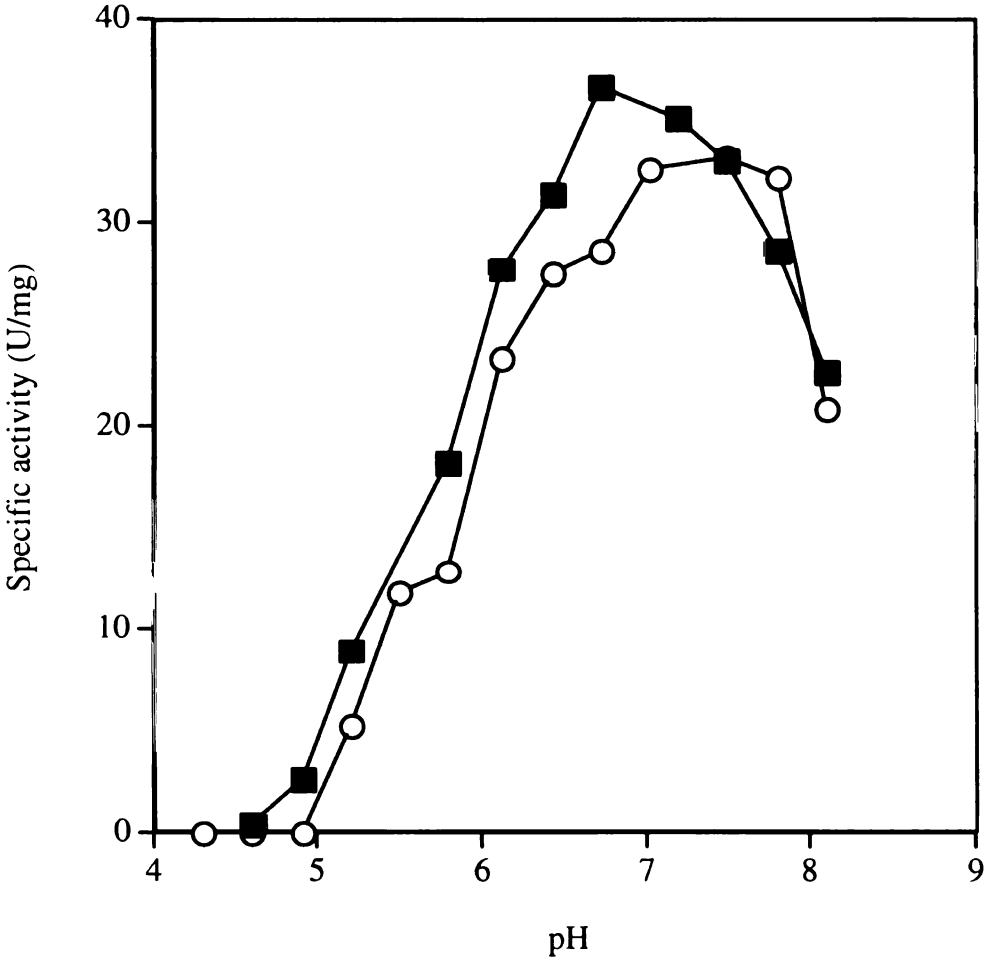


Figure 2: Effect of pH on the specific activities of TNXI 1F1 (■) and Gensweet™ (○) on glucose at 80°C.

thermostability. Gensweet™, on the other hand, is thermophilic but obviously not extremely thermostable.

TNXI 1F1 and Gensweet™'s melting temperatures (T_m) were determined by DSC in the presence and absence of metals (Figure 4, and Table 2). With the holo-enzymes, both scans revealed one thermal transition, at 107.3°C and 93.4°C, for the TNXI 1F1 and Gensweet™, respectively. With a higher T_m of ~14°C, TNXI 1F1 holo-enzyme is much more stable than that of Gensweet™ which is in good agreement with the results obtained from inactivation experiments. DSC of apo-enzymes of both enzymes also revealed one thermal transition 78.4°C and 76.1°C for the TNXI 1F1 and Gensweet™, respectively. The extent of T_m difference observed in apo-enzymes was not as pronounced as that in holo-enzyme forms

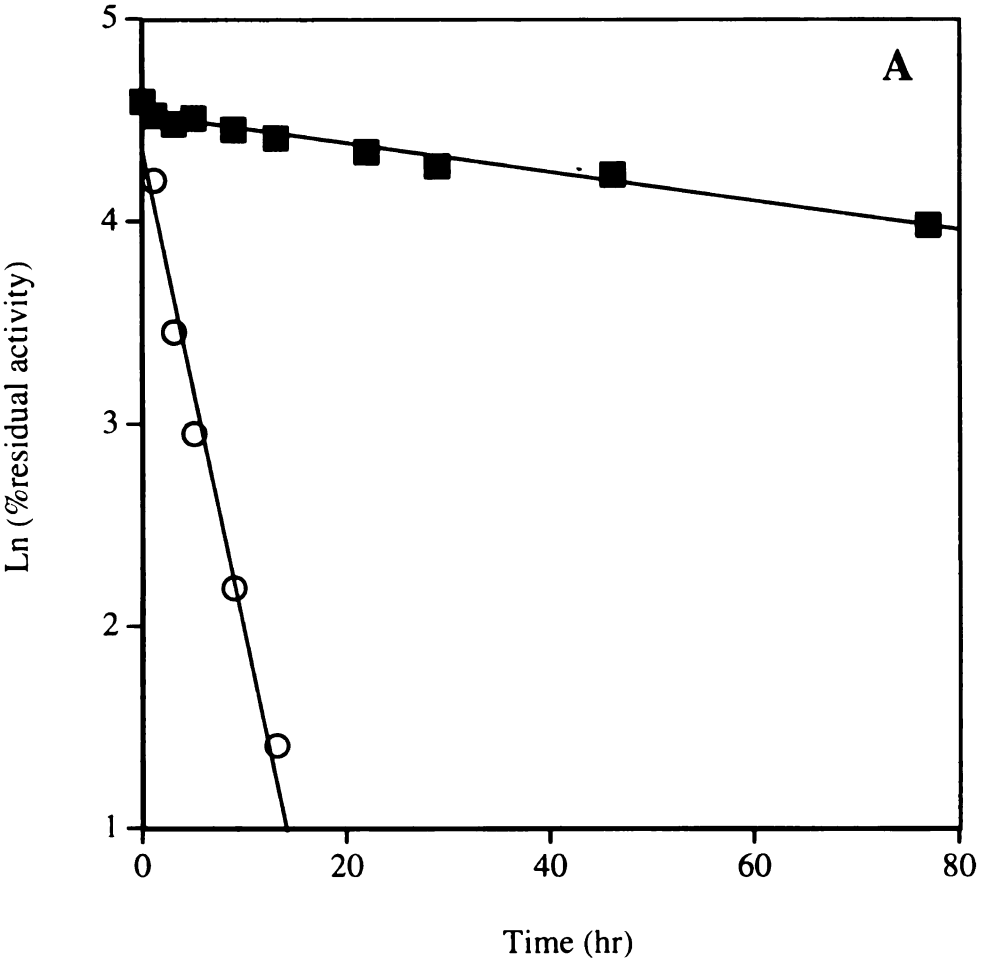


Figure 3: (A) Inactivation curves of TNXI 1F1 and Gensweet™ at 60°C (pH 7.0).

Symbols used are the same as in Figure 1.

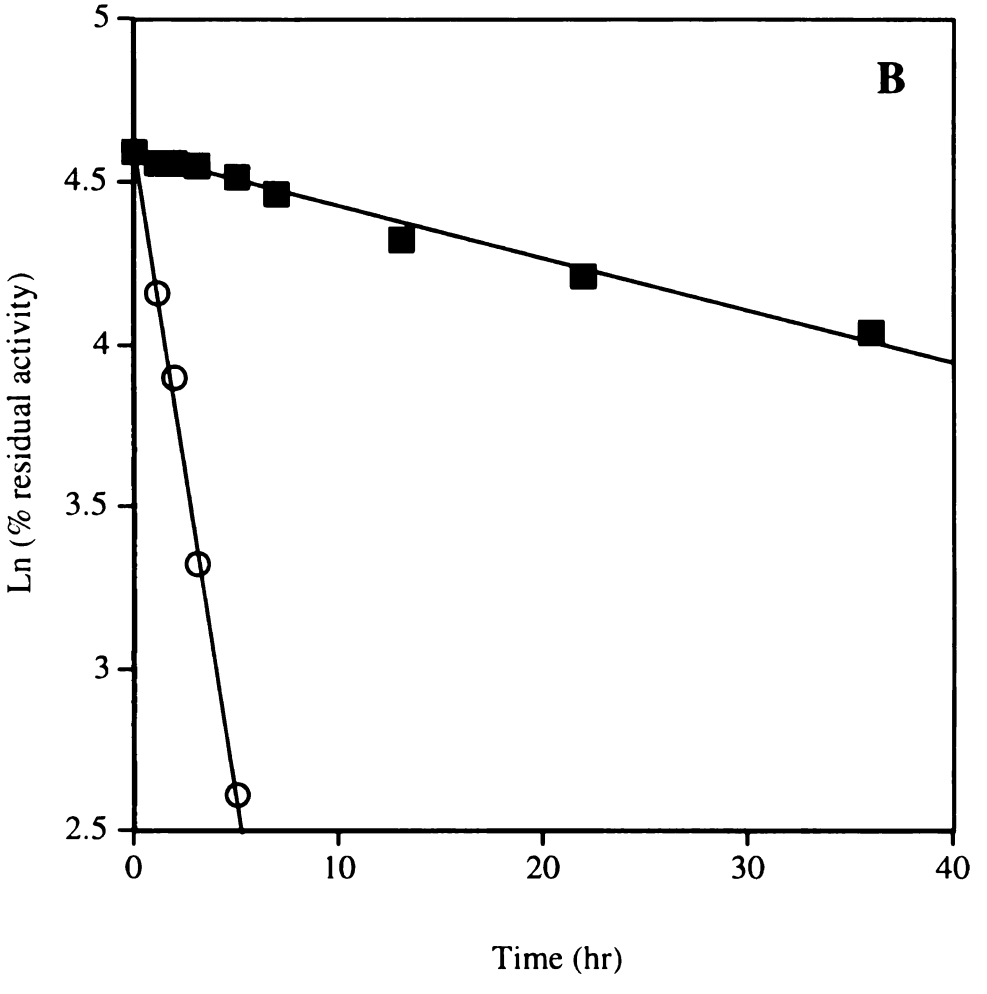


Figure 3: (B) Inactivation curves of TNXI 1F1 and Gensweet™ at 60°C (pH 5.5).

Symbols used are the same as in Figure 1.

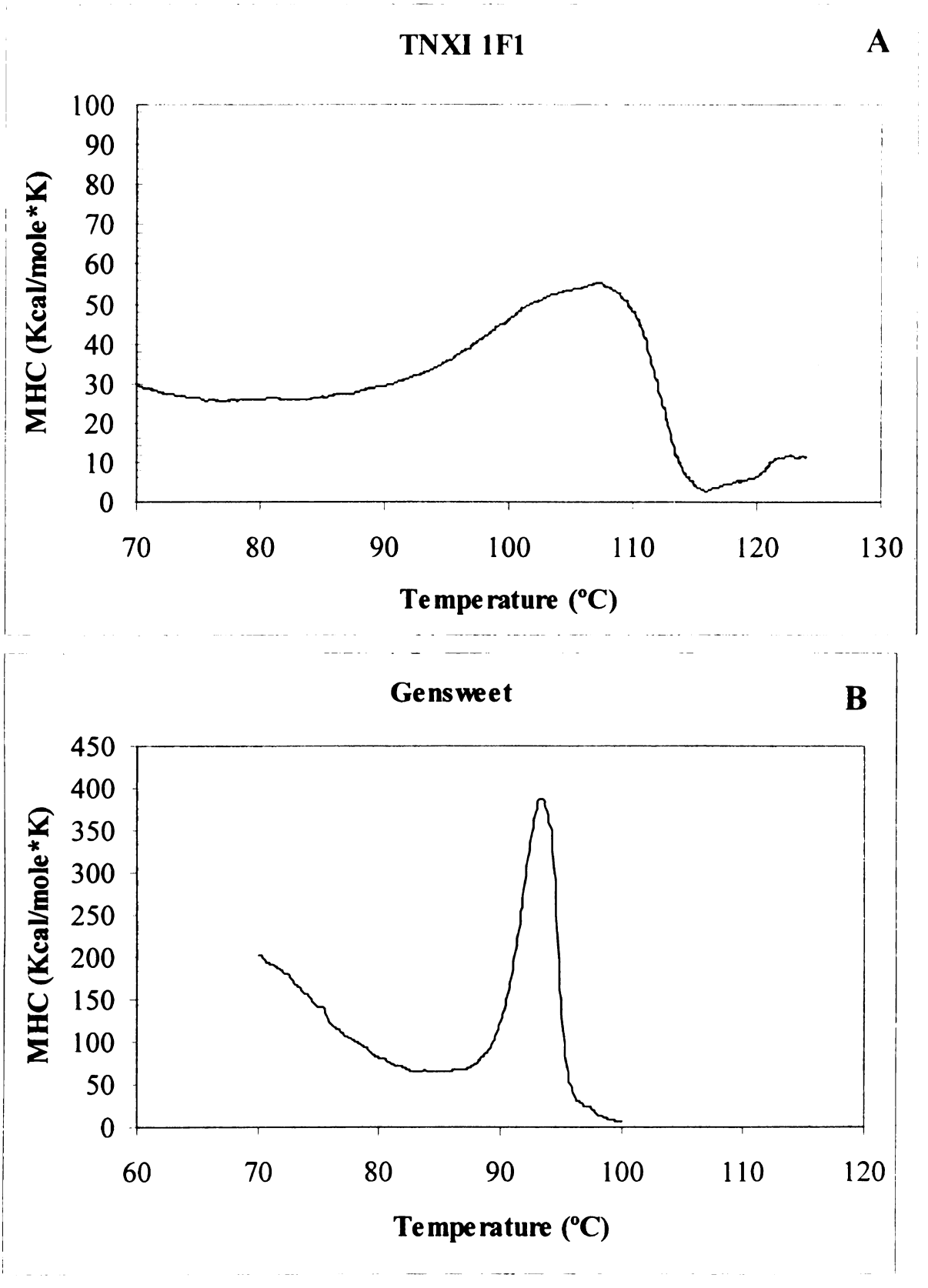


Figure 4: Thermal unfolding of (A) TNXI 1F1 and (B) Gensweet™ in the presence of 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$ followed by DSC.

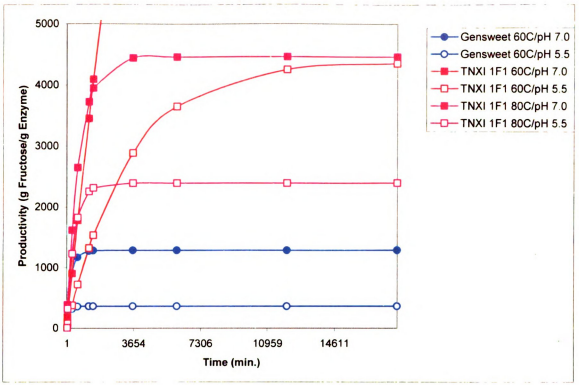
Kinetic parameters of TNXI 1F1 and Gensweet™: The kinetic parameters on glucose of TNXI 1F1 and Gensweet™ were compared in different conditions (Table 1). In all comparable conditions, Gensweet™ has higher K_m and V_{max} than TNXI 1F1 (except at 80°C, pH 5.5 in which TNXI 1F1's V_{max} is higher than that of Gensweet™). The difference in K_m of Gensweet™ and TNXI 1F1 is more pronounced than that of V_{max} resulting in worse catalytic efficiency (k_{cat}/K_m) on glucose for Gensweet™ than TNXI 1F1. The TNXI 1F1's superiority of glucose catalytic efficiency on glucose is more noted at pH 7.0 than at pH 5.5 and at higher (95°C) or lower (60°C) than both enzymes' optimal temperatures.

Modeled fructose productivity: The lifetime fructose productivity of both enzymes was estimated using the one phase inactivation model at various combinations of temperatures and pHs. The modeled time course of fructose productivity of TNXI 1F1 and Gensweet™ at various conditions is shown in (Figure 5 and Table 3). Fructose productivity of Gensweet™ at 80°C cannot be generated because no residual activity was detected at 80°C in buffer pH 7.0 and 5.5 after just 10 minutes. At 60°C, Gensweet™ produced a maximum amount of 1.3 and 0.4 kilogram (kg) fructose per gram (g) enzyme at pH 7.0 and 5.5, respectively. With lifetime fructose production of 30.5 and 4.4 kg fructose/g enzyme at pH 7.0 and 5.5, respectively, TNXI 1F1 yielded approximately 24 and 12-fold

Table 1: Comparison of glucose isomerization kinetic parameters of TNXI 1F1 vs. Gensweet™

Enzyme	pH 5.5				pH 7.0			
	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
95°C								
Gensweet™	ND*	ND	ND	ND	2264.3	65.9	2848	1.3
TNXI 1F1	ND	ND	ND	ND	184.6	60.6	3083	16.7
90°C								
Gensweet™	ND	ND	ND	ND	ND	ND	ND	ND
TNXI 1F1	354.8	53.1	2700	7.6	ND	ND	ND	ND
80°C								
Gensweet™	549.6	30.7	1327	2.4	490.4	64.8	2798	5.7
TNXI 1F1	274.0	35.3	1798	6.6	52.0	37.9	1930	37.1
60°C								
Gensweet™	1836.1	23.5	1014	0.6	1167.0	41.9	1808	1.6
TNXI 1F1	78.8	7.5	382	4.9	60.2	17.4	883	14.7

*ND: Not determined



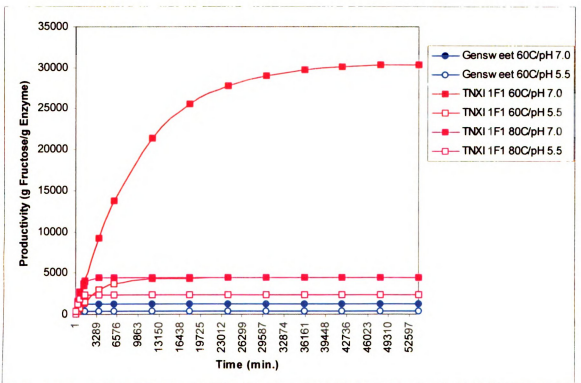
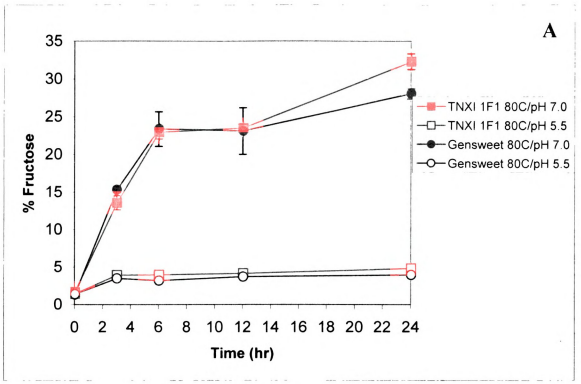


Figure 5: Estimated fructose productivity of TNXI 1F1 and Gensweet™ in different co nditions.

increases in fructose production compared to Gensweet™ under the same conditions. The main reason for superior fructose productivity by TNXI 1F1 over Gensweet™ is mainly due to its higher thermostability. It is also important to note that at 60°C the fructose production of Gensweet™ reached the maximum points before 24 hr whereas it took approximately 15 days at pH 5.5 and more than 30 days at pH 7.0 for TNXI 1F1 to reach its maximum production. At 80°C, TNXI 1F1 produced 4.5 and 2.4 kg fructose/g enzyme at pH 7.0 and 5.5, respectively. The fructose production for TNXI 1F1 at 80°C, both pH 7.0 and 5.5, reached the maximum points at approximately 2.5 days.

Fructose production experiments: Fructose production by TNXI 1F1 vs. Gensweet™ with 45 % glucose syrup at various combinations of temperatures and pHs was performed to study the effect of both temperature and pH on fructose conversion ratio (compared to glucose) and potential browning reactions with each enzyme (Figure 6, 7 and Table 3). To prove that higher isomerization yield of fructose may be achieved by increasing the reaction temperature, TNXI 1F1 and Gensweet™ were incubated with glucose syrups (pH 7.0 or 5.5) at 60, 80, and 90°C for up to 24 hr. At both pH, increases in fructose conversion was observed to be proportional to higher temperature for up to at least 24 hr in all cases with one exception. The syrup incubated with TNXI 1F1 at 90°C, pH 7.0 showed a higher conversion percentage for up to 6 hr after which the conversion rate remain relatively constant and its fructose conversion percentage was surpassed by that of the syrup incubated with TNXI 1F1 at 80°C, pH 7.0. The explanation for this event might be due to TNXI 1F1's relatively short half-life at high temperature above 85°C. The



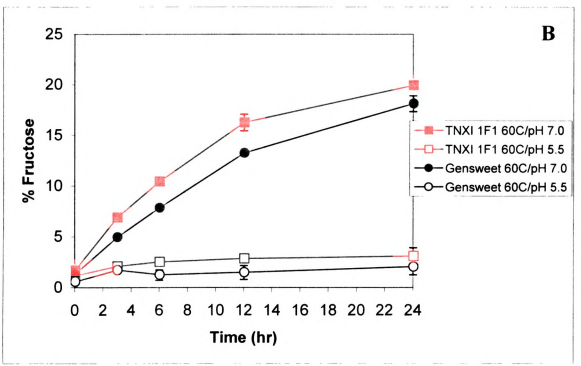


Figure 6: Experimental fructose conversion of TNXI 1F1 and Gensweet™ at pH 7.0 and 5.5. at 80°C (A) and at 60°C (B).

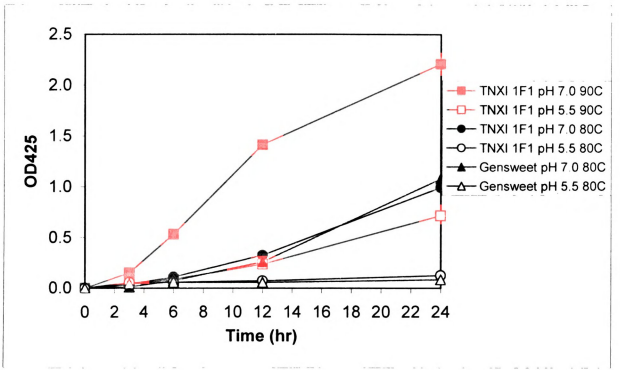


Figure 7: Browness of syrups from experimental fructose conversion of TNXI 1F1 and Gensweet™. The time course of browning reactions was monitored by maximal absorbance at 425 nm.

reactions were performed at two different pHs, 7.0 and 5.5, to also investigate the feasibility of prevention of browning the syrups that occurs at high temperature and pH. The brownness of the resulting syrups was monitored at the maximum wavelength of absorbency, which is 425 nm. As expected, the brownness of the syrups was observed to be most pronounced at the highest temperature tested (90°C) and pH (7.0). Syrups resulting from reactions at low pH (5.5) have dramatically fewer problems with browning.

Fructose conversion percentage was compared between TNXI 1F1 and Gensweet™. At 80°C, TNXI 1F1 compared favorably with Gensweet with slightly higher conversion percentage at 24 hr (32 % for TNXI 1F1 vs. 28 % for Gensweet™), which is possibly due to Gensweet™'s less stable nature at high temperature. However, at 60°C, TNXI 1F1 was obviously better than Gensweet at converting glucose to fructose with higher conversion rate throughout the time course of 24 hr.

DISCUSSION

The initial goal of this study was to compare biochemical and kinetic parameters as well as productivities of a laboratory-evolved xylose isomerase TNXI 1F1 and a commercially available glucose isomerase, Gensweet™ to ascertain that TNXI 1F1 can be genuinely considered for industrial glucose isomerization. Table 2 summarizes key properties of the two enzymes. Not surprisingly, TNXI 1F1 compares favorably with Gensweet™ in every aspect. The key factor that distinguishes the two enzymes was shown to be their thermal stability difference. Although TNXI 1F1 and Gensweet™ have almost the same apparent temperature optima (e.g., 90°C and 85°C, respectively), Gensweet™ is much less thermostable than TNXI 1F1 by more than one order of magnitude at 60°C at pH 7.0 or 5.5.

A mathematical model derived to account for the effect of temperature on reversible enzyme kinetics, inactivation rates, and the glucose-fructose chemical equilibrium (Bandlish *et al.*, 2002) was used to estimate their lifetime fructose productivity. Because K_m, k_{cat}, and k_D are based on soluble enzyme data, the effect of immobilization is not taken into account. However, these estimates provide useful information concerning the potential of the enzyme for HFCS production under optimal conditions. TNXI 1F1 has the lifetime fructose productivity at 60°C, pH 7.0 of 30.5 kg fructose/g enzyme whereas Gensweet™, which reached its maximum fructose conversion in less than a day due to its limited thermal stability, produced only 1.3 kg fructose/g enzyme. TNXI 1F1's estimated greater fructose productivity mainly resulted from both

Table 2: Comparison of thermal activity and stability properties of TNXI 1F1 vs. Gensweet™

Properties	Gensweet™	TNXI 1F1
T_{opt} (°C)	85	90
Optimal pH	7.5	6.7
T_m of Holo-enzyme (°C)	93.4	107.3
T_m of Apo-enzyme (°C)	76.1	78.4
$T_{1/2}$ at 60°C/pH 7.0 (hr)	2.9	115.5
$T_{1/2}$ at 60°C/pH 5.5 (hr)	1.7	38.5

greater thermal stability and better catalytic efficiency on glucose compared to those of Gensweet™.

Experimental fructose conversion was performed with 45 % glucose syrups incubated with 50 μg of either TNXI 1F1 or Gensweet™ in various conditions to simulate industrial conditions and also to study the effect of temperature and pH on browning reactions resulting from interactions of enzymes with reducing sugars. TNXI 1F1 has a slight edge in term of fructose conversion ratio in every condition tested compared to Gensweet™ with maximal conversion observed at 80°C, pH 7.0 (32 %). The browness of resulting syrups was monitored up to 24 hr at maximal absorbance of 425 nm. Browning of syrup occurs much more pronounced at 90°C, pH 7.0 and can be greatly reduced by either lowering the pH, the reaction temperature or both. It should also be noted that at 60°C, browness of the resulting syrup is marginal. When the enzyme concentration in the experiment was increased from 50 g to 100g and 1 mg, not only did we see more pronounced browning, but precipitates were also observed in the resulting syrups (data not shown).

A study of a glucose isomerase from *Streptomyces rubiginosus* by Visuri *et al.* (1999) suggested that in an industrial process, glucose isomerase inactivation is caused mainly by a Maillard-type browning reaction between the enzyme and the reactive substrates glucose and fructose resulting in inactive glycated protein complexes. From our data, we can speculate that using TNXI 1F1 at 60°C at pH 7.0 or even at slightly higher temperature or lower pH would result in sufficiently high fructose yield and productivity as well as reduced concern on Maillard browning reaction. Further detailed study of immobilized TNXI 1F1 and Gensweet™ on the production of high fructose

Table 3: Comparison of fructose production parameters of TNXI 1F1 vs. Gensweet™

Enzyme	Temp (°C)	pH	Productivity (10 hr) (g Fructose/g Enzyme)	Productivity (life) (g Fructose/g Enzyme)	Specific Activity (μmol Fructose/min./mg enzyme)	% Fructose produced (3 hr)	Browning (OD425 nm)
Gensweet™	80	7.0	ND*	ND	22	15.3	1.1
	80	5.5	ND	ND	11	3.5	0.1
	60	7.0	1169	1286	10	4.9	< 0.1
	60	5.5	358	364	ND	1.7	< 0.1
TNXI 1F1	90	7.0	ND	ND	48	19.3	2.2
	90	5.5	ND	ND	ND	0.7	0.7
	80	7.0	2648	4463	36	13.6	1.0
	80	5.5	1824	2391	13	3.9	0.1
	60	7.0	1778	30530	15	6.9	< 0.1
	60	5.5	720	4373	ND	2.1	< 0.1

*ND: Not determined

syrup in different conditions in underway to ascertain the potential use of TNXI 1F1 in industrial application.

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CHAPTER VI

ROLE OF METALS IN TNXI STABILITY AT EXTREMELY HIGH TEMPERATURES

ABSTRACT

TNXI and its directed evolution mutants' melting temperatures (T_m) were determined by DSC in the presence and absence of metals. With the holo-enzymes, all scans except TNXI 1F1 revealed two thermal transitions. Surprisingly, TNXI 1F1 showed only one thermal transition, at a slightly lower temperature than those of the wild-type TNXI and TNXI V185T second thermal transitions showing that 1F1 lacks the first thermal transition common in the wild-type TNXI, TNXI V185T, and TNXI 3A2 mutant. DSC of apo-enzymes of all TNXIs revealed only one thermal transition. The result suggested that the additional mutation that occurs in TNXI 1F1 mutant, Phe186Ser, is responsible for altered metal-binding property and also strongly enhances the metal requirement of TNXI as seen in a very low T_m of TNXI 1F1 apo-enzyme compared to those of the wild-type TNXI and TNXI V185T relative to their holo-enzymes. With a T_m difference of 28.9°C (approximately 2-fold higher that of TNXI) between apo- and holo-forms of TNXI 1F1, its extreme thermal stability is even more strongly metal-dependent than the wild-type TNXI.

INTRODUCTION

Xylose isomerase from hyperthermophilic eubacterium *Thermotoga neapolitana* (TNXI) is optimally active at a temperature of 95°C or above. Its *xylA* gene was cloned, sequenced, and expressed in *Escherichia coli*, which yielded a recombinant XI with catalytic characteristics identical to those of the native enzyme (Vieille *et al.*, 1995). Further study by gel filtration chromatography showed that the recombinant enzyme was both a homodimer and a homotetramer, with the dimer being the more abundant form (Hess *et al.*, 1998). The ratio of dimer to tetramer was approximately 20:1, based on total protein assay data (Bradford, 1976). The two forms had comparable stabilities when they were thermoinactivated at 95°C. Differential scanning calorimetry (DSC) revealed thermal transitions at 99 and 109.5°C for both forms suggesting that the association of the subunits into the tetrameric form may have little impact on the stability and biocatalytic properties of the enzyme (Hess *et al.*, 1998).

Typically for XIs, two divalent cations (Mg^{2+}, Co^{2+}, and Mn^{2+}) per monomer are required for catalytic activity and stability (Hess *et al.*, 1998) . The three metals activate TNXI with Co^{2+} being the best activating metal. Activity of the Mg^{2+}-TNXI and the Mn^{2+}-TNXI are approximately 40 and 16 %, respectively, of the activity observed with the Co^{2+}-TNXI. Also, the stabilization provided by metals to TNXI is metal specific: the Mn^{2+}-TNXI is significantly more stable than the Co^{2+}- and Mg^{2+}-TNXI (Vieille *et al.*, 2001).

The active site of TNXI has been previously engineered to improve its catalytic efficiency toward glucose (Sriprapundh *et al.*, 2000). The TNXI V185T is the most

efficient site-directed mutant with a 3.1 fold increase in its catalytic efficiency toward glucose and comparable kinetic properties on glucose and xylose. To further optimize this enzyme's potential industrial utility, directed evolution (sequential random mutagenesis and low temperature/low pH activity screening) was successfully applied to the TNXI V185T-encoding gene to obtain enzymes that have high glucose isomerase activity at low temperature and low pH. The best mutant enzyme, TNXI 1F1 (containing V185T, L282P, and F186S mutations), was dramatically more active than TNXI V185T at all temperatures and pHs tested. TNXI 1F1 is also more kinetically stable than TNXI and TNXI V185T. TNXI 1F1's enhanced stability is thought to be a result of additional H-bond formation between Ser186's sidechain and the neighboring L229 residue's mainchain structure. This, in turn, strengthens local conformation and the affinity of E231 co-ordination with the structural metal, hence improving the thermostability of the mutant enzyme.

In this study, the thermodynamic stability of TNXI, TNXI V185T and its directed evolution mutant derivatives, TNXI 3A2 and 1F1 were followed by DSC in both apo- and holo- forms to compare and contrast their unfolding behaviors with regard to metal ions requirement for their thermostability. We show here that increased thermal stability of xylose isomerase is associated with metal binding especially in TNXI 1F1 where the apo-enzyme was 30°C less stable than the holo-enzyme.

MATERIALS AND METHODS

Protein Purification: TNXI and its mutant derivatives were purified using the procedure of Vieille *et al.* (1995) followed by an additional ion-exchange chromatography step. Partially purified enzyme was applied to a DEAE-Sepharose column (2.5x15 cm) equilibrated with buffer A (50 mM MOPS pH 7.0, 5 mM $MgSO_4$, 0.5 mM $CoCl_2$), and the enzyme was eluted using a 500 ml linear 0–300 mM NaCl gradient in buffer A. The pooled fractions from the DEAE-Sepharose column were concentrated in a stirred ultrafiltration cell (MW cut-off 30 kDa) (Amicon, Beverly, MA) and dialyzed twice against buffer A. Concentrated, homogenous enzyme was dispensed and stored frozen at -70°C.

Differential Scanning Calorimetry (DSC): DSC experiments were performed on a Nanocal differential scanning calorimeter (Calorimetry Sciences Corp., Provo, UT) using a scan rate of 1°C/min. Samples were scanned from 25°C to 100°C. To obtain the apo-enzymes (metal-free enzymes), the purified enzymes in buffer A were incubated overnight at 4°C in 50 mM MOPS buffer (pH 7.0) containing 10 mM EDTA. They were then dialyzed twice against 50 mM MOPS buffer (pH 7.0) containing 2 mM EDTA, and they were finally dialyzed twice against 50 mM MOPS buffer (pH 7.0) without EDTA. The apo-enzymes were scanned against 50 mM MOPS (pH 7.0). Enzymes containing both Mg^{2+} and Co^{2+} were dialyzed against buffer A, then scanned against the dialysis buffer as control.

RESULTS

TNXI mutants' melting temperatures (T_m) were determined by DSC in the presence and absence of metals (Table 1). With the holo-enzymes (Figure 1), all scans except TNXI 1F1 revealed two thermal transitions. The TNXI wild-type and TNXI V185T went through thermal transitions at 101°C and then 110°C and 114.5°C, respectively. TNXI 3A2 went through these transitions at significantly lower temperatures (86.6°C and 101°C). Surprisingly, 1F1 showed only one thermal transition, at a slightly lower temperature than those of the wild-type TNXI and TNXI V185T second thermal transitions suggesting that 1F1 lacks the first thermal transition common in the wild-type TNXI, TNXI V185T, and TNXI 3A2 mutant.

DSC of apo-enzymes (Figure 2) of all TNXIs revealed only one thermal transition at 96.5°C, 96.9°C, 84.4°C, and 78.4°C for the wild-type, TNXI V185T, TNXI 3A2, and TNXI 1F1, respectively. The result suggested that the additional mutation that occurs in TNXI 1F1 mutant, Phe186Ser, is responsible for altered metal-binding property and also strongly enhances the metal requirement of TNXI as seen in a very low T_m of TNXI 1F1 apo-enzyme compared to those of the wild-type TNXI and TNXI V185T relative to their holo-enzymes. A T_m difference of 28.9°C between TNXI 1F1 apo- and holo- form was observed. This high T_m difference is approximately 2-fold higher than those observed in TNXI, TNXI V185T, and TNXI 3A2 (13.5°C, 17.6°C, and 16.6°C, respectively).

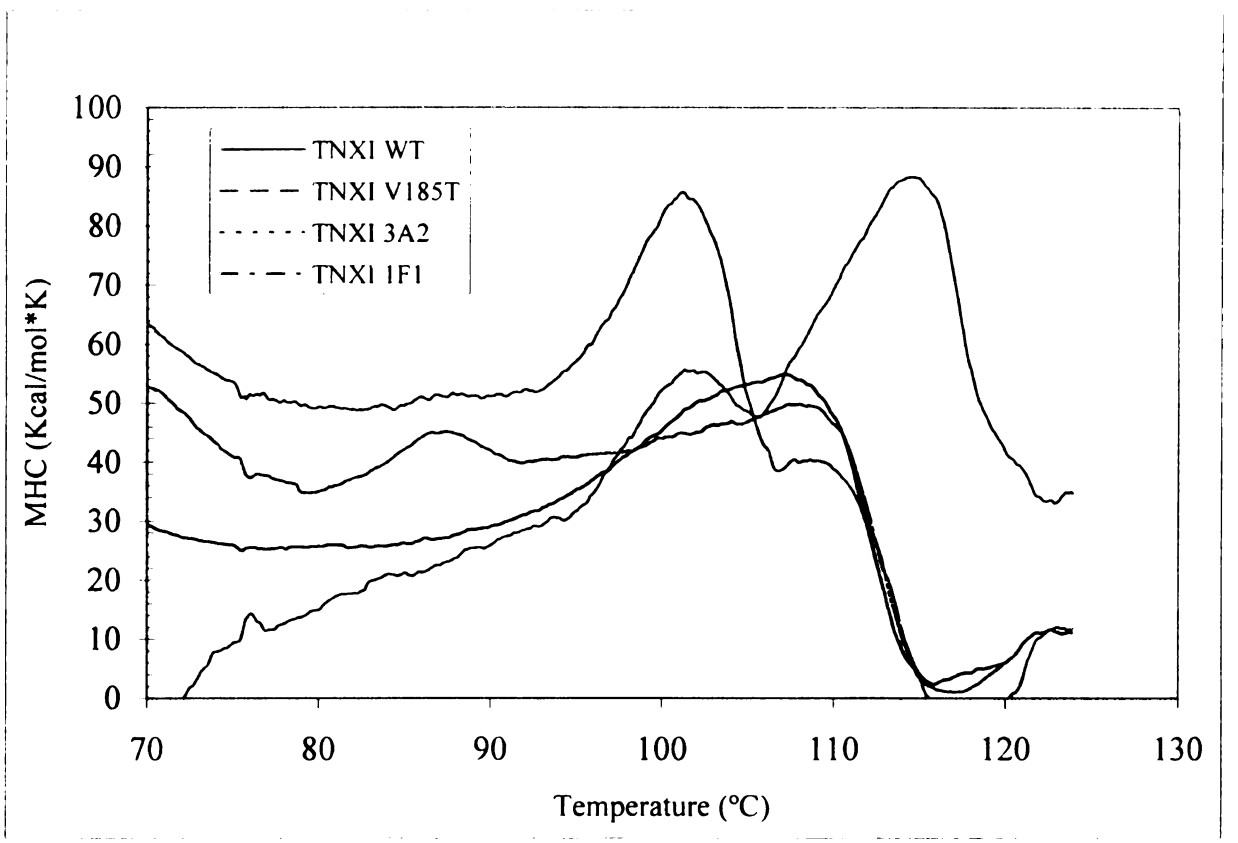

Figure 1: Thermal unfolding of the holo-forms of TNXI and its mutant derivatives in the presence of 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$ followed by DSC.

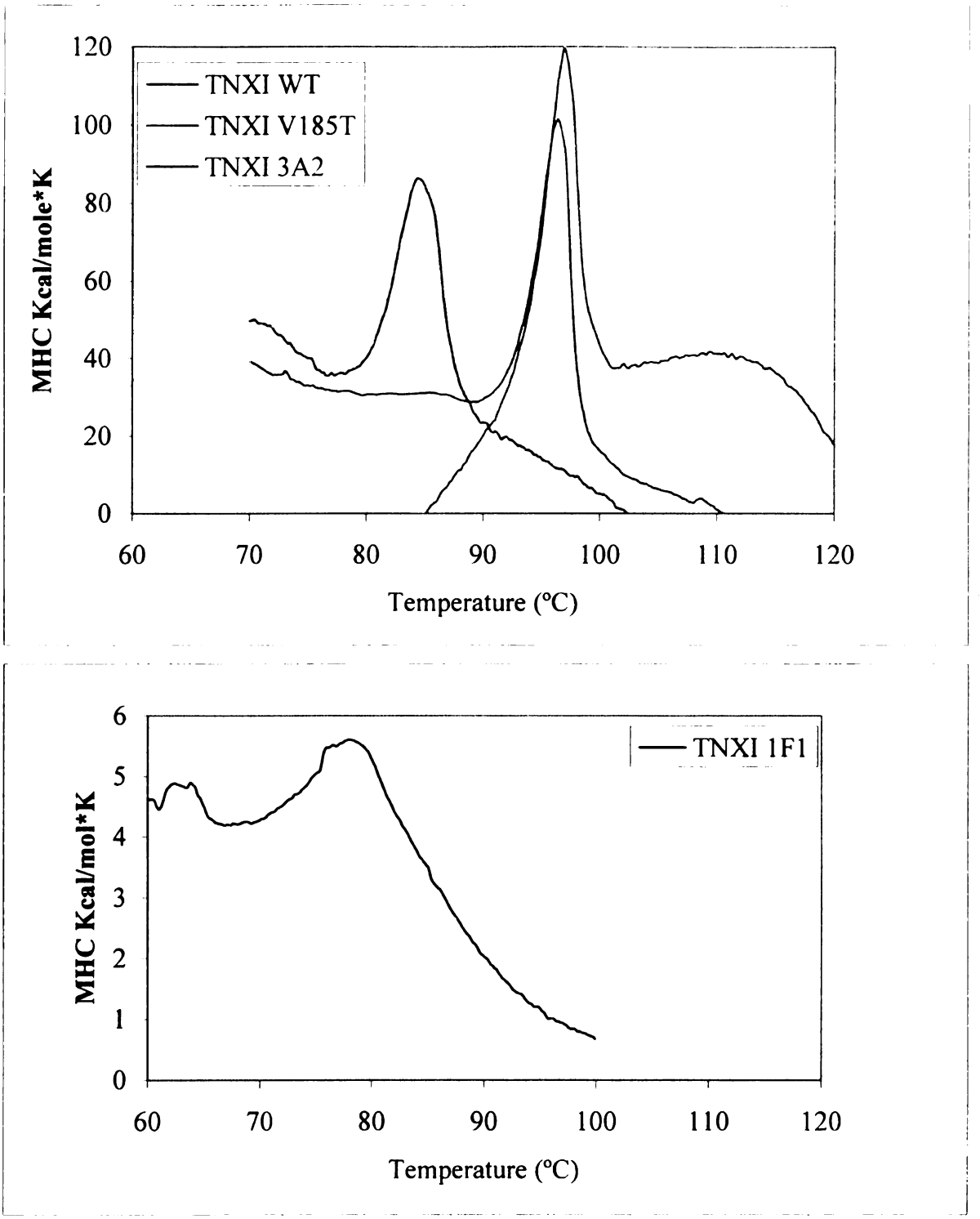

Figure 2: Thermal unfolding of the apo-forms of TNXI and its mutant derivatives followed by DSC.

Table 1: Melting temperatures (T_m) of TNXI and its mutant derivatives in the presence (holo-enzyme) and absence (apo-enzyme) of 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$ as determined by DSC.

Enzyme	Holo-enzyme		Apo-enzyme
	T_m1 (°C)	T_m2 (°C)	T_m (°C)
TNXI wild-type	101.2	110	96.5
TNXI V185T	101.3	114.5	96.9
TNXI 3A2	86.6	101	84.4
TNXI 1F1	N/A	107.3	78.4

DISCUSSION

Unfolding of TNXI and its mutant derivatives were followed by DSC. With the holo-enzymes, all scans except TNXI 1F1 revealed two thermal transitions, which is normal for TNXI (Hess et. al., 1998). These transitions could correspond either to the existence of two phases in TNXI inactivation (Hess and Kelly, 1999) or to the presence in the solution of a heterogeneous population of enzymes containing Mg^{2+} and Co^{2+} as ligand (s)(Hess *et al.*, 1998). Surprisingly, TNXI 1F1 showed only one thermal transition, at a slightly lower temperature than those of the wild-type TNXI and TNXI V185T second thermal transitions. This suggests that TNXI 1F1 probably lacks the first thermal transition common in the wild-type TNXI, TNXI V185T, and TNXI 3A2 mutant. DSC of apo-enzymes of all TNXIs showed only one thermal transition.

The result suggested that the additional mutation that occurs in TNXI 1F1 mutant, Phe186Ser, is responsible for altered metal-binding property and also strongly enhances the metal requirement of TNXI as seen in a very low T_m of TNXI 1F1 apo-enzyme compared to those of the wild-type TNXI and TNXI V185T relative to their holo-enzymes. A T_m difference of 28.9°C was observed between TNXI 1F1 apo- and holo- form. This high T_m difference is approximately 2-fold higher than those observed in TNXI, TNXI V185T, and TNXI 3A2 (13.5°C, 17.6°C, and 16.6°C, respectively). This evidently demonstrated that TNXI 1F1's extreme thermal stability is strongly metal-dependent, even more than those of TNXI, TNXI V185T, and TNXI 3A2. This is not surprising since the mutation is located in the active center close enough form a hydrogen bond to the mainchain carbonyl group of the Leu229 residue (Sriprapundh *et al.*, in

preparation). The neighboring Glu231 residue's sidechain co-ordinates (forms a hydrogen bond with) a structural metal (metal I) of the enzyme. So, the mutation would strengthen the local conformation that, in turn, affects the binding affinity of the structural metal and ultimately stabilize the enzyme.

The significance of cation binding in XI stability has not yet been examined closely (Vieille *et al.*, 2001). How ever some information is available on this issue. Site-directed mutagenesis has been used to partially fill a metal binding site with sidechain of an amino acid. These mutations to both metal-binding sites, M1 and M2, resulted in destabilized XIs. In *Streptomyces rubiginosus* XI, the mutation of His220 affected metal binding at M2, which is turn was responsible for destabilization (Cha *et al.*, 1994). A similar observation has been made for *Escherichia coli* XI: mutation of His271 (ligand to M2) significantly destabilized the enzyme (Batt *et al.*, 1990). Other point mutations triggering conformational changes in active site residues have also been found to destabilize XIs (Varsani *et al.*, 1993). Metal ions probably act to lock the active site in a stable conformation, which is lost as soon as the metal leaves the active site.

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CHAPTER VII

CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

From these research studies, we were able to identify molecular determinants responsible for the xylose isomerase enzyme from hyperthermophilic eubacterium *Thermotoga neapolitana* (TNXI)'s high thermostability at extremely high temperature by comparative thermostability and site-directed mutagenesis studies with less thermostable xylose isomerase from thermophilic eubacterium *Thermoanaerobacterium thermosulfurigenes* (TTXI). Despite their highly similar structures and amino acid sequences (70.4 % identity), no obvious differences in the enzyme structures can explain the differences in its stability compared to that of TTXI except for a few additional prolines and fewer Asn+Gln in TNXI. TNXI has 2 additional prolines in the Phe59 loop (Pro58 and Pro62) which helps forming another enzyme subunit's active site. When these 2 prolines in TNXI were substituted with the corresponding amino acids present in its less thermostable counterpart, TTXI, all the mutant enzymes showed significant loss in thermostability compared to the wild-type TNXI. These data confirmed the hypothesis that prolines indeed play important role in protein thermostability by reducing its entropy of unfolding. Introduction of these two prolines in TTXI resulted in one mutant enzyme (TTXI Q58P) with improved thermostability and the other (A62P), surprisingly with significantly lower thermostability. Analysis of the three-dimensional (3D) structure of TTXI and the predicted structure of its A62P mutant derivative indicated that a steric hindrance between Pro62-Cδ and Lys61-Cβ (2.92 Å) is responsible for the reduced thermostability of the mutant enzyme compared to the native TTXI.

We also successfully engineered TNXI's active site by site-directed mutagenesis to better fit glucose which is not its natural substrate. The V185T mutation in TNXI is the most efficient mutant derivative with a 3.1-fold increase in its catalytic efficiency toward

glucose as a consequence of an improvement in glucose binding affinity (low K_m) and a faster catalytic rate of the reaction (higher k_{cat}). This engineered "glucose" isomerase from a native xylose isomerase has now comparable kinetic properties on glucose and xylose.

To further optimize this enzyme's potential industrial utility, directed evolution (sequential random mutagenesis and low temperature/low pH activity screening) was successfully applied to the TNXI V185T-encoding gene to obtain enzymes that have high glucose isomerase activity at low temperature and low pH. The best mutant enzyme, TNXI 1F1 (containing V185T, L282P, and F186S mutations), was dramatically more active than TNXI V185T at all temperatures and pHs tested. Its high activities at low temperatures were due to significantly lower activation energies (44 kJ/mole) compared to that of TNXI and V185T (87 kJ/mole). TNXI 1F1 is also more kinetically stable than TNXI and TNXI V185T. TNXI 1F1's enhanced stability is thought to be a result of additional H-bond formation between Ser186's sidechain and the neighboring L229 residue's mainchain structure. This, in turn, strengthens local conformation and the affinity of E231 co-ordination with the structural metal, hence improving the thermostability of the mutant enzyme. Since the enzyme's low temperature activity can be significantly enhanced without loss in its thermostability, we suggest that the molecular determinants of this enzyme's activity and thermal stability are in fact, not the same.

TNXI 1F1 was also subjected to a comparative study of biochemical properties and fructose productivities with a commercially available glucose isomerase, Gensweet™ to further ascertain its properties to be used for industrial applications. TNXI 1F1

displayed higher catalytic efficiencies on glucose at low or high temperature and pH ranges and had greater thermal stability than Gensweet™ despite having similar temperature optima for activity. This greater thermal stability together with the superior kinetic parameters on glucose render TNXI 1F1 a genuine candidate for the industrial glucose isomerization process based on the lifetime fructose productivity estimation.

Directions of future research should include;

1) Directed evolution of TNXI 1F1 to obtain a mutant enzyme that has improved thermostability at extremely high temperature at lower pH. This would facilitate using such enzyme in HFCS production at very high glucose isomerization temperature (90-95°C) to obtain directly a 55 % fructose syrup without an additional fructose enrichment step currently employed.
2) TNXI 1F1 immobilization studies. Since Xylose isomerase are used in the industry as an immobilized form, studies of its properties, stability, and utility are encouraged.
3) Detailed study of role(s) of metals in thermostability of TNXI 1F1. Unlike TNXI and TNXI V185T, TNXI 1F1 holo-enzyme (containing Mg^{2+} and Co^{2+}) showed only one thermal transition followed by DSC. It would be interesting to address why it behaves such way and what is responsible for such event.