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Meng-Hsiao Meng

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M B Gaykasan'e
Major professor

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THE MECHANISM OF ACTION AND STRUCTURE/FUNCTION RELATIONSHIP OF D-XYLOSE ISOMERASE FROM *THERMOANAEROBACTERIUM THERMOSULFURIGENES*

By

Meng-Hsiao Meng

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ABSTRACT

THE MECHANISM OF ACTION AND STRUCTURE/FUNCTION RELATIONSHIP OF D-XYLOSE ISOMERASE FROM *THERMOANAEROBACTERIUM THERMOSULFUROGENES*

By

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The mechanism of catalytic reaction and the structure/function relationship of *Thermoanaerobacterium* D-xylose isomerase (E.C. 3.5.1.5) was studied by site-directed mutagenesis based on the available information of X-ray crystallographic structure of *Arthrobacter* and *Streptomyces* enzymes and on the comparison of amino acid sequence of D-xylose isomerases from different sources. Kinetic data indicated that isomerization and ring-opening occur at a concerted step and isomerization happens via hydride shift mechanism. His-101 acts as a hydrogen-bond acceptor to stabilize the transition state of the rate-limiting step. Asp-104 assists this function of His-101 by locking the imidazole ring in a tautomeric form convenient for acceptance of a hydrogen bond.

Steric hindrance by Trp-139 against the accommodation of glucose is the major mechanism for *Thermoanaerobacterium* D-xylose isomerase to discriminate between xylose and glucose. Sequential decrease in K_M for glucose was observed when Trp-139 was substituted by Tyr, Phe, Met, Leu, Val and Ala, in the order shown. Hydrophobic interactions between the hydrocarbon backbone of sugar and Trp-188 and Phe-145 provide strong binding energy for substrate binding. If the

architecture of the active site pocket was kept intact, reduction of the area of water-accessible hydrophobic surface enhanced the thermostability of enzyme.

Rate-determining step during the process of irreversible thermoinactivation of *Thermoanaerobacterium* D-xylose isomerase is the formation of incorrectly folded protein which is a monomolecular event. Besides the well known divalent cations such as Mg^{+2} and Co^{+2}, we found that monovalent cations, particularly K^{+} also enhance the thermostability of the enzyme.

To

My parents, wife and daughters

with love

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Finally I am most grateful to my wife Pai-Ying for her dedication and encouragement. I could not have undertaken this endeavor without the understanding of my wife Pai-Ying.

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ABBREVIATIONS

DEAE	diethylaminoethyl
DSC	differential scanning calorimetry
EDTA	ethylenediamine tetraacetic acid
EPR	electron paramagnetic resonance
GC	gas chromatography
HFCS	high fructose corn syrup
MES	2-(N-morpholino)propanesulfonic acid
MOPS	3-[N-morpholino]propanesulfonic acid
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
ENDOR	electron nucleic double resonance

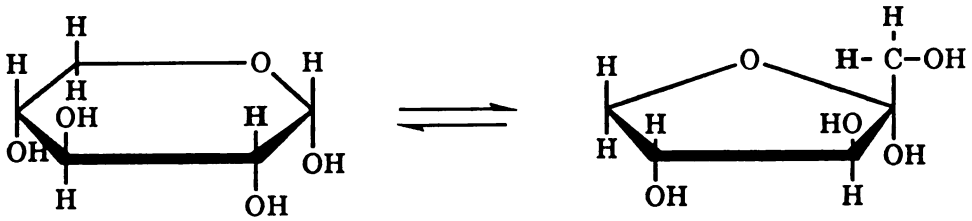
CHAPTER 1

GENERAL INTRODUCTION

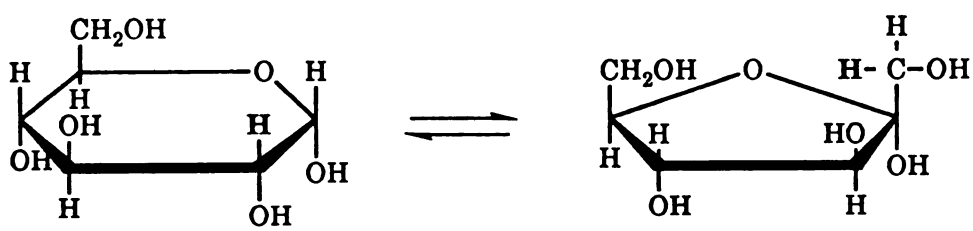
D-xylose isomerase (E.C. 5.3.1.5), often referred to as D-glucose isomerase, catalyzes the reversible isomerization of D-xylose to D-xylulose and D-glucose to D-fructose (Figure 1). The ability of catalyzing the latter reaction makes D-xylose isomerase an important industrial enzyme for the production of high fructose corn syrup (HFCS). In the United States alone, immobilized xylose isomerase is used to produce over 4 million tons of HFCS annually (Layman, 1986). The second major commercial interest in the enzyme is in the production of ethanol from xylose which is the predominant sugar in hemicellulose present in waste material of plant origin.

D-xylose isomerases have been isolated from many bacterial species, and the properties of these enzymes such as substrate specificity, divalent metal cation activation, optimum pH etc. have been intensely investigated, especially in the enzymes from *Streptomyces*, *Lactobacillus*, and *Bacillus* (Antrim et al., 1979). D-xylose isomerases require the presence of a divalent cation such as Mn^{+2}, Co^{+2} or Mg^{+2} for catalytic activity and thermostability. However, a specific cation that activates xylose isomerase from one organism may have no effect on xylose isomerase from a different organism. Moreover, a specific cation will often enhance the activity of xylose isomerase more toward one substrate than another. In all D-xylose isomerases studied to date D-xylose is a more favorable substrate than D-glucose, mainly due to the much lower K_M of D-xylose. The enzyme also

Figure 1: Schematic illustration of the reactions catalyzed by D-xylose isomerase.



xylopyranose xylulofuranose



glucopyranose fructofuranose

shows anomeric specificity in that it prefers to catalyze the isomerization of the α-anomer of the substrate. The enzymatic isomerization of D-glucose to D-fructose monitored by ^{13}C NMR spectroscopy showed that the α-D-glucopyranose and the α-D-fructofuranose are the reactive species for the enzyme (Makkee et al., 1984). During isomerization the proton at C2 position of the aldose is transferred to the 1-pro-R position of ketose without exchange with solvent (Bock et al., 1983).

Crystal structures of D-xylose isomerase, bound to divalent metal cations, substrate or inhibitors, from *Arthrobacter* species (Henrick et al., 1989; Collyer et al., 1990), *Streptomyces rubiginosus* (Carrell et al., 1989; Whitlow et al., 1991), *Streptomyces olivochromogenes* (Farber et al., 1989) and *Actinoplanes missouriensis* (Jenkins et al., 1992) have been solved. The enzyme is a homotetramer, each subunit consisting of a parallel α/ß barrel domain and an extended C-terminal tail interacting with neighboring subunit. The active site pocket is located near the C-terminal ends of ß strands of the barrel domain and includes residues from a second subunit. Two adjacent divalent metal ions are coordinated by the amino acid residues of the active site. The active site can be described as an amphipathic pocket with hydrophobic residues lining one side and hydrophilic residues the other (Figure 2).

Genes encoding D-xylose isomerase have been isolated from at least twelve different microorganisms. The alignment of amino acid sequences from five of them is shown in Figure 3. The one from *Thermoanaerobacterium*

Figure 2: Stereo structure of active site *Arthrobacter* D-xylose isomerase .

Substrate analog, α-5-thio-glucose (white), positioned in the middle of active site pocket, is in contact with His-53 (pink) through the axial C1-OH. Active site surface can be described as a amphipathic pocket with hydrophilic amino acids lining one side (yellow) and hydrophobic amino acids the other (green). Two metal cations (red cross) are coordinating to hydrophilic amino acid residues and substrate analog.

Figure 3: Comparison of amino acid sequences of D-xylose isomerases from different microorganisms.

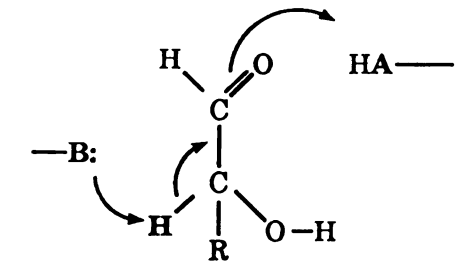
Boldfaced letters indicate residues changed in this work. S.r., *Streptomyces rubiginosus*; S.v., *Streptomyces violaceoniger*; A.m., *Ampullariella* strain 3876; Art., *Arthrobacter* strain B3726; T.t., *Thermoanaerobacterium thermosulfurigenes*; E.c., *E. coli*.

```
S.r. ..........  ..........  ..........  ....MNYQPT PEDRFTFGLW .TVGWQGRDP  25
S.v. ..........  ..........  ..........  .....SFQPT PEDKFTFGLW .TVGWQGRDP  24
A.m. ..........  ..........  ..........  ....MSVQAT REDKFSFGLW .TVGWQARDA  25
Art. ..........  ..........  ..........  ....MSVQPT PADHFTFGLW .TVGWTGADP  25
T.t. MNKYFENVSK IKYEGPKSNN P.YSFKFYNP EEVIDGKTME EHLRFSIAYW HTFTADGTDQ  59
E.c. MQAYFDQLDR VRYEGSKSSN P.LAFRHYNP DELVLGKRME EHLRFAACYW HTFCWNGADM  59

S.r. FGDAT.RRAL DPVESVQRLA ..........  ..ELGAHGVT FHDDDLIPFG SSDSER....  68
S.v. FGDAT.RPAL DPVETVQRLA ..........  ..ELGAYGVT FHDDDLIPFG SSDTER....  67
A.m. FGDAT.RTAL DPVEAVHKLA ..........  ..EIGAYGIT FHDDDLVPFG SDAQTR....  68
Art. FGVAT.RKNL DPVEAVHKLA ..........  ..ELGAYGIT FHDNDLIPFA ATEAER....  68
T.t. FGKATMQRPW NHYTDPMDIA KARVEAAFEF FDKINAPYFC FHDRDIAPEG DTLRETNKNL 119
E.c. FGVGAFNRPW QQPGEALALA KRKADVAFEF FHKLHVPFYC FHDVDVSPEG ASLKEYINNF 119

S.r. EEHVKRFRQA LDDTGMKVPM ATTNLFTHPV FKDGGFTAND RDVRRYALRK TIRNIDLAVE 128
S.v. ESHIKRFRQA LDATGMTVPM ATTNLFTHPV FKDGGFTAND RDVRRYALRK TIRNIDLAAE 127
A.m. DGIIAGFKKA LDETGLIVPM VTTNLFTHPV FKDGGFTSND RSVRRYAIRK VLRQMDLGAE 128
Art. EKILGDFNQA LKDTGLKVPM VTTNLFSHPV FKDGGFTSND RSIRRFALAK VLHNIDLAAE 128
T.t. DTIVAMIKDY LKTSKTKVLW GTANLFSNPR FVHGASTSCN ADVFAYSAAQ VKKALEITKE 179
E.c. AQMVDVLAGK QEESGVKLLW GTANCFTNPR YGAGAATNPD PEVFSWAATQ VVTAMEATHK 179

S.r. LGAETYVAWG GREGAESGGA KDVRDALDRM KEAFDLLGEY VTSQGYDIRF AIEPKPNEPR 188
S.v. LGAKTYVAWG GREGAESGGA KDVRDALDRM KEAFDLLGEY VTAQGYDLRF AIEPKPNEPR 187
A.m. LGAKTLVLWG GREGAEYDSA KDVSAALDRY REALNLLAQY SEDRGYGLRF AIEPKPNEPR 188
Art. MGAETFVMWG GREGSEYDGS KDLAAALDRM REGVDTAAGY IKDKGYNLRI ALEPKPNEPR 188
T.t. LGGENYVFWG GREGYETLLN TDMEFELDNF ARFLHMAVDY AKEIGFEGQF LIEPKPKEPT 239
E.c. LGGENYVLWG GREGYETLLN TDLRQEREQL GRFMQMVVEH KHKIGFQGTL LIEPKPQEPT 239

S.r. GDILLPTVGH ALAFIERLER PELYGVNPEV GHEQMAGLNF PHGIAQALWA GKLFHIDLN. 247
S.v. GDILLPTVGH ALAFIERLER PELYGVNPEV GHEQMAGLNF PHGIAQALWA GKLFHIDLN. 246
A.m. GDILLPTAGH AIAFVQELER PELFGINPET GHEQMSNLNF TQGIAQALWH KKLFHIDLN. 247
Art. GDIFLPTVGH GLAFIEQLEH GDIVGLNRET GHEQMAGLNF THGIAQALWA EKLFHIDLN. 247
T.t. KHQYDFDVAN VLAFLRKYDL DKYFKVNIEA NHATLAFHDF QHELRYARIN GVLGSIDANT 299
E.c. KHQYDYDAAT VYGFLKQFGL EKEIKLNIEA NHATLAGHSF HHEIATAIAL GLFGSVDANR 299

S.r. GQNGIKYDQD LRFGAGDLRA AFWLVDLLE. .....SAGY. .SGPRHFDFK PPRTE.DFDG 298
S.v. GQSGIKYDQD LRFGAGDLRA AFWLVDLLE. .....SAGY. .EGPRHFDFK PPRTE.DFDG 297
A.m. GQHGPKFDQD LVFGHGDLLN AFSLVDLLEN G.PDGAPAY. .DGPRHFDYK PSRTE.DYDG 303
Art. GQRGIKYDQD LVFGHGDLTS AFFTVDLLEN GFPNGGPKY. .TGPRHFDYK PSRTD.GYDG 304
T.t. GDMLLGWDTD .QFPTDIRMT TLAMYEVIK. .....MGGFD .KGGLNFDAK VRRASFEPED 351
E.c. GDAQLGWDTD .QFPNSVEEN ALVMYEILK. .....AGGFT .TGGLNFDAK VRRQSTDKYD 351

S.r. VWASAAGCMR NYLILKERAA AFRADPEVQE ALR...ASRL DELARPT..A ADGLQALLDD 353
S.v. VWASAEGCMR NYLILKERAA AFRADPEVQE ALR...AARL DQLAQPT..A ADGLEALLAD 352
A.m. VWESAKANIR MYLLLKERAK AFRADPEVQE ALA...ASKV AELKTPTLNP GEGYAELLAD 360
Art. VWDSAKANMS MYLLLKERAL AFRADPECQE AMK...TSGV FELGETTLNA GESAADLMND 361
T.t. LFLGHIAGMD AFAKGFKVAY KLVKDRVFDK FIEERYASYK DGIGADIVSG KADFRSL..E 409
E.c. LFYGHIGAMD TMALALKIAA RMIEDGELDK RIAQRYSGWN SELGQQILKG QMSLADL..A 409

S.r. RSAFEEFDVD AAAARGM.AF ERLDQLAMDH LLGARGAA 390
S.v. RTAFEDFDVE AAAARAAWPF ERLDQLAMDH LLGARG.. 388
A.m. RSAFEDYDAD AVGAKG.FGF VKLNQLAIEH LLGAR... 394
Art. SASFAGFDAE AAAERN.FAF IRLNQLAIEH LLGSR... 395
T.t. KYALERSQIV ....NKSGRQ ELLESILNQY LFAE.... 439
E.c. KYAQEH...H LSPVHQSGRQ EQLEMLVNHY LFDK.... 440
```

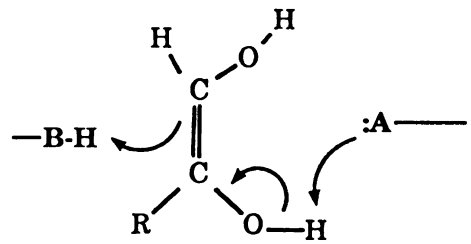
thermosulfurigenes was isolated by Lee, et al, (1990) in J. Gregory Zeikus's laboratory and has been studied in this work. Based on the comparison of the amino acid sequences, two classes of xylose isomerase can be identified with respect to the length of the monomer polypeptide chain and homologies in the substrate binding pocket. D-xylose isomerases isolated from *Streptomyces*, *Arthrobacter*, and *Actinoplanes* belong to one class whereas enzymes from *Clostridium*, *E. coli*, and *Bacillus* belong to a different class. All amino acid residues defining the active site pocket and believed to be involved in catalysis or substrate binding are conserved in D-xylose isomerase of both classes, with one exception. Met-87 of the *Arthrobacter* sequence is conserved throughout the enzymes of the first class whereas a Trp residue (Trp-139 in the *Thermoanaerobacterium* enzyme) is present, and conserved, in the enzymes of the second class (Figure 3).

Although extensive investigation of physicochemical properties and structure of enzymes from various source have been conducted, no single D-xylose isomerase has been studied in sufficient detail to provide experimental data that conclusively define the pathway of the catalytic reaction. In the past, a cis-enediol intermediate mechanism was proposed by analogy to the triosephosphate isomerase (TIM), which catalyzes a reaction of similar type, the isomerization of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate, via an cis-enediol intermediate (Rose et al., 1969; Rose, 1981, also see Figure 4). In this mechanism a base residue, near

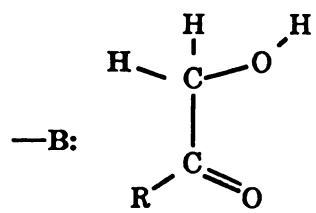
Figure 4: Schematic illustration of cis-enediol mechanism.



aldose



cis-enediol



ketose

the C2 hydrogen, attracts the proton from C2 of aldose, and subsequently has the substrate transformed to cis-enediol intermediate. Finally this proton is released to pro-R position at C1 of ketose from the same base residue. Crystal structure analysis performed by Carrell et al. with D-xylose isomerase from *Streptomyces rubiginosus* provided evidence supporting this mechanism (Carrell et al., 1989). It revealed a very close contact between histidine 54 (corresponding to histidine 101 in the enzyme from *T. thermosulfurigenes*) and C-1 of the substrate, suggesting that histidine 54 is the active-site base that attracts a proton from the substrate. It also showed that the mechanism-based inhibitor, 3-deoxy-C^3-fluoromethylene-D-glucose, a substrate analog, was turned over by the enzyme to give a product that alkylates this same histidine, reinforcing the theory of the base-catalyzed enolization mechanism. The role of metal ions in this mechanism was suggested to be the maintenance of the structure of the active site region.

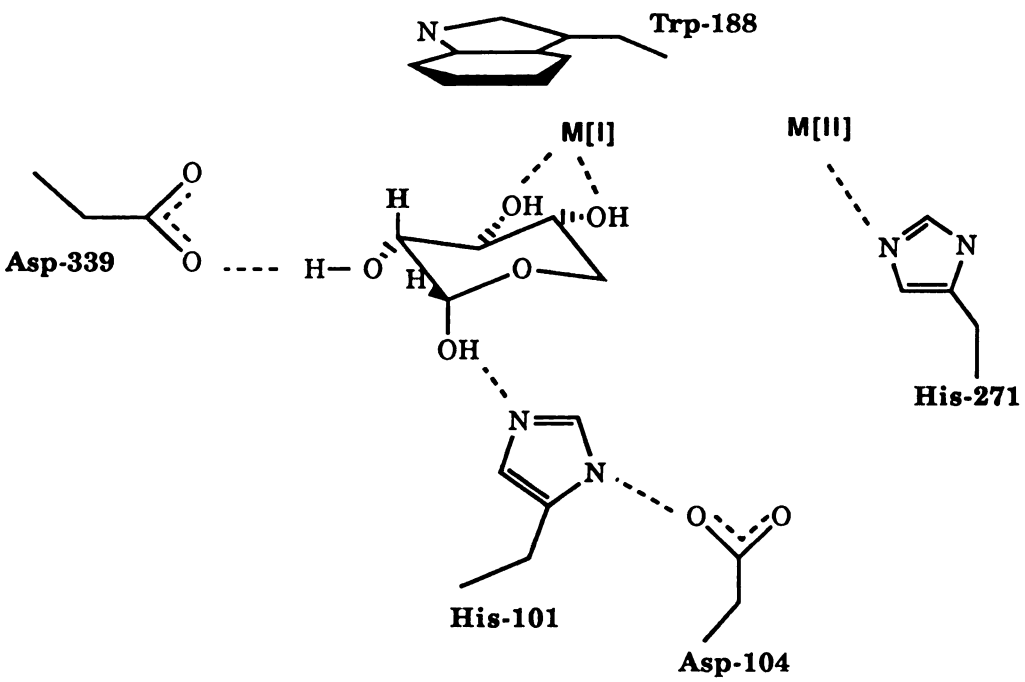
Recently, an alternative mechanism, the hydride shift mechanism, was proposed by Collyer et al. working on *Arthrobacter* enzyme (Collyer et al., 1990). They argued that Carrell has obtained a mixture of the cyclic and extended forms of D-xylose and that the suicide inactivator is not a good model for the binding of a closed-ring substrate (Collyer & Blow, 1990). In the crystal structure of *Arthrobacter* enzyme, bound to a extended linear form of substrate, C2 hydrogen points toward a very hydrophobic environment, consisting mainly of Typ-136, Phe-93 and Phe-25 (from a second subunit); whereas, C1 and C2 oxygens are

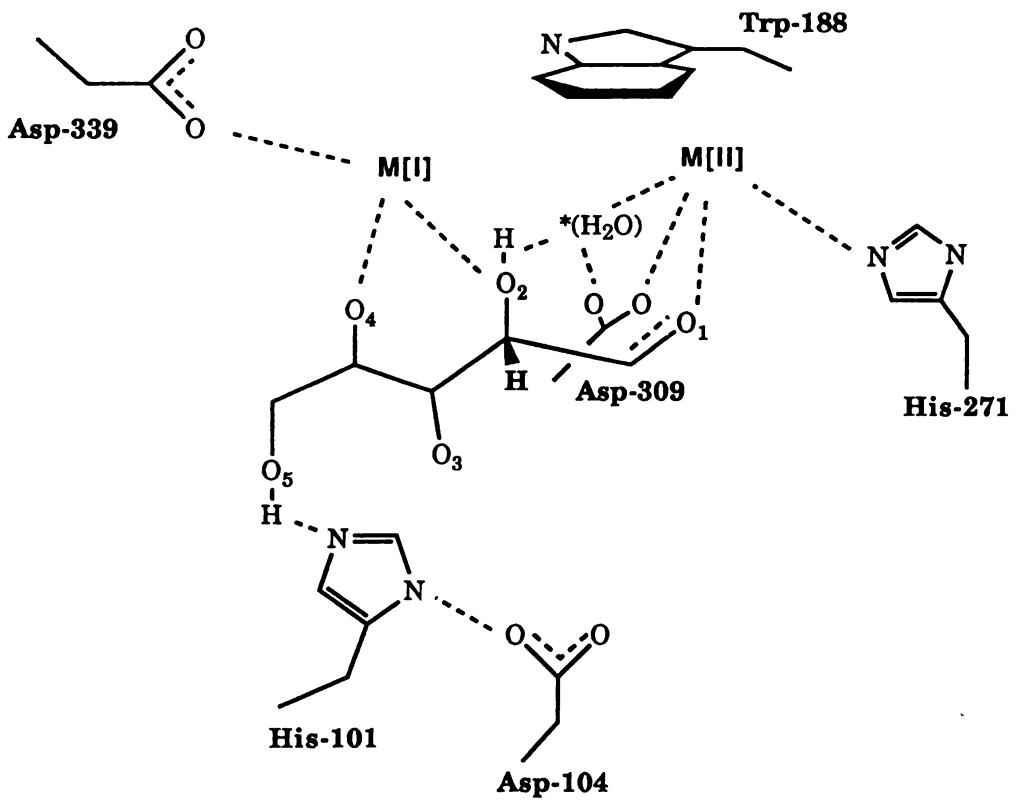
coordinating to a divalent metal ion, metal [II]. (The other metal ion coordinating to C2 and C4 oxygen of the linear form substrate was designated as metal [I], see Figure 5). Based on this, they proposed a metal-assisted hydride shift mechanism as the mechanism of isomerization. Collyer et al. also used a cyclic form of inhibitor, 5-thio-D-glucose, as a model compound to solve the crystal structure of the enzyme-cyclic form substrate complex. From crystal structures of various enzyme-substrate complexes Collyer et al. further proposed the mechanism for the entire reaction pathway (Collyer et al., 1990). Basically, it included five steps: (1) Binding of α-D-pyranose substrate to the enzyme, in which His-53 (corresponding the His-101 in *Thermoanaerobacterium* enzyme) hydrogen bonds to axial C1 hydroxyl group of α-D-pyranose. (2) ring-opening, catalyzed by His-53 which is assisted by Asp-56, by a mechanism analogous to the "charge-relay system" in chymotrypsin (Blow et al., 1969), (3) conformational rearrangement of substrate from pseudo-cyclic to an extended open chain form. (4) Hydride shift between C1 and C2, assisted by metal [II], (5) conformational rearrangement, ring-closure, and release of product.

Crystallographic studies performed by Whitlow et al. on the enzyme from *Streptomyces rubiginosus* suggested the same reaction mechanism with a slight modification in the hydride shift step (Whitlow et al., 1991). On the basis of 1.6 Å resolution of the structure, Whitlow et al. proposed that Asp-257 (corresponding to Asp-309 in *Thermoanaerobacterium* enzyme), acting as a base, works together

Figure 5: Schematic diagrams of the active site structure containing cyclic form and that containing linear form of the substrate.

These diagrams are derived from crystal structures of *S. rubiginosus* (Whitlow et al., 1991). However, the numbering of amino acid residues has been changed to those of the *Thermoanaerobacterium* enzyme on the basis of conservation of the primary amino acid sequence (Figure 3).


Trp-188
M[I]
M[II]
Asp-339
His-271
His-101
Asp-104


Trp-188
Asp-339
M[I]
M[II]
*(H2O)
Asp-309
His-271
His-101
Asp-104

with metal[II] to promote the hydride shift from C2 of aldose to C1 of ketose. The interactions of cyclic form and that of linear form substrate with the amino acid residues in the active site pocket are shown in Figure 5. Lately, crystallographic and kinetic studies on the wild-type and variant enzymes from *Actinoplanes missouriensis* suggested a similar mechanism as that proposed by Collyer et al. and Whitlow et al., although the base catalyst function of His-54 for ring opening was doubted. (Jenkins et al., 1992; Lambeir et al., 1992; van Tilbeurgh et al., 1992). Different authors studied crystals of the enzyme-substrate complex under different conditions and made different interpretation of the interaction of substrate with active site amino acids, leading to different conclusions about enzyme mechanism. Moreover, the crystal structure of an enzyme-substrate complex in fact only provide the information about the relative position between any particular functional groups; in other words, it can not prove the biological functions of amino acid residues. Therefore, more biochemical evidence are needed to elucidate the actual function of individual residues in order to define the catalytic pathway.

Protein engineering is a new developing technology combining recent advanced techniques in genetic engineering eg. site-directed mutagenesis, and enzymology. It is a powerful strategy to confirm the biological function of residues which other observations (structural, chemical or genetic) have implicated. It also offers a unique approach to determine experimentally the energetics of interactions between individual side chains and the various intermediates in a

enzyme catalyzed reaction pathway (Fersht et al., 1987). Protein engineering also provides a way to redesign proteins to fit specific requirements such as altered specificity (Wilks et al., 1988), increased turnover number (Russell & Fersht, 1987), different pH profile (Russell et al., 1987), or improved thermostability (Matsumura et al., 1988; Matthews et al., 1987). In this work protein engineering has been applied to study the catalytic mechanism of D-xylose isomerase from *T. thermosulfurigenes* and to improve its catalytic properties. To exploit a protein engineering approach, the *Thermoanaerobacterium* D-xylose isomerase gene has been overexpressed in *E. coli* and the enzyme has been purified to homogeneity through a heat treatment step, ion-exchange and gel filtration chromatography (Lee et al., 1990). Site-directed mutagenesis experiments have been guided by the X-ray crystal structure of D-xylose isomerase from *Arthrobacter* and *Streptomyces* and by amino acid sequence comparison. The specific aims of this study will be described in the following chapters.

Elucidation of the mechanism of reaction catalyzed by D-xylose isomerase. Although a wealth of information on the three-dimensional structure of D-xylose isomerase has been obtained to date, the exact mechanism of the isomerization reaction has not yet been conclusively defined. This work attempted to provide information on the functions of individual amino acid residues of the enzyme in the catalytic reaction. To distinguish between the proposals of Carrell et al. (1989), who have favored the cis-enediol mechanism and predicted the

function of the two metal ions in the maintenance of active site structure, and those of Collyer et al. (1990) or Whitlow et al. (1991), who proposed a hydride shift mechanism and implicated the metal [I] in the maintenance of the structure and metal [II] in the catalysis of the hydride-shift, several amino acid residues of the active site were substituted by site-directed mutagenesis of the xylose isomerase gene from *Thermoanaerobacterium thermosulfurigenes*. The properties of the resulting mutant variants of the enzyme were examined and compared to those of the wild type isomerase.

Understanding the mechanism of substrate preference for α-anomer. In aqueous solution, at equilibrium, D-glucose is a mixture of 64% β-anomer, 36% α-anomer and 0.02% of the free carbonyl form. The isomerization to fructose requires the transfer of a hydrogen atom between C1 and C2 atoms of the substrate. Since the reactive species for the enzyme is the α-anomer (Makkee et al., 1984), the mechanism of α-anomer recognition must be a property of the enzyme structure. The structures of *Arthrobacter* enzyme and of *Streptomyces* enzyme have both indicated that His-54 contacts the axial C1 hydroxyl group of α-pyranose. Based on this, it was proposed that the inability of the enzyme to perform the ring-opening reaction on the β-pyranose was the basis of the observed preference for the α-anomer (Collyer et al., 1990; Whitlow et al., 1991). In this work, site-directed mutagenesis has been performed on His-101/Asp-104 residue pair of the *Thermoanaerobacterium* enzyme (corresponding to His-54/Asp-57 in

Streptomyces enzyme) to test this hypothesis.

Elucidation of the structural basis for the discrimination between D-xylose (natural substrate) and D-glucose (industrial substrate). D-xylose isomerase from different sources has a higher specificity constant, k_{cat}/K_M, for xylose than for glucose. The difference, by one order of magnitude, in K_M, is the main cause of this. Difference in the structure of the two substrates is the presence of a methanolic group at the C-6 position in D-glucose. On the basis of three-dimensional structure of the active site in *Arthrobacter* enzyme it could be anticipated that three amino acid residues, Thr-89 (corresponding to Thr-141 in *Thermoanaerobacterium* enzyme), Met-87 (corresponding to Trp-139 in the *Thermoanaerobacterium* enzyme) and Val-134 (corresponding to Val-186 in the *Thermoanaerobacterium* enzyme), may cause steric hindrance for the binding of glucose. The effect of substitution of these amino acid residues on the kinetic constants of the enzyme were therefore examined.

Elucidation of the functional roles of the active-site aromatic amino acid residues in substrate binding. In *Arthrobacter* xylose isomerase the pyranose ring of the substrate is sandwiched between Trp-15 and Trp-136 (corresponding to Trp-49 and Trp-188 of the *Thermoanaerobacterium* enzyme, respectively). The indole ring of Trp-136, surrounded by Phe-93 and Phe-25 (from the neighboring subunit), interacts hydrophobically with the carbon backbone of the substrate pyranose ring. These Phe residues correspond to Phe-145 and Phe-60 of the

Thermoanaerobacterium enzyme, respectively. The role of the aromatic amino acid residues for binding the sugar has been demonstrated in maltose-binding protein (Martineau et al., 1990), arabinose-binding protein and galactose-binding protein (Quiocho et al., 1989). The role of the active site aromatic amino acids of *Thermoanaerobacterium* enzyme in substrate binding has been examined by site-directed mutagenesis in this work.

Enhancement of enzyme thermostability. The enzyme-catalyzed isomerization of glucose into fructose is carried out in industrial bioreactors at 60-65°C. An increase in the half-life of the enzyme would substantially reduce the cost of the HFCS production. Co^{+2} is the best protector against thermal inactivation for *Thermoanaerobacterium* D-xylose isomerase (Lee & Zeikus 1991). To enhance thermostability of the enzyme, site-directed replacement of active site amino acid residue was used to reduce the water-accessible hydrophobic surface area of the protein molecule and the effect of different salts on the enzyme stability has been tested.

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CHAPTER 2

MECHANISM OF REACTION CATALYZED BY D-XYLOSE ISOMERASE

ABSTRACT

Kinetic properties of the mutant xylose isomerase variants of the *Thermoanaerobacterium thermosulfurigenes* enzyme, obtained by substitution of His-101 by different amino acids, indicated that His-101 does not function as a general base. Instead, they suggested that this residue acts as a hydrogen bond acceptor to stabilize transition state of the rate-limiting step. His-101 is assisted in this function by the Asp-104 residue which locks the imidazole of His-101 in the tautomeric form most suitable for its hydrogen-bond acceptor function. The preference of the enzyme for the α-anomer form of the substrate depends on the presence of His-101/Asp-104 pair.

The primary isotope effect caused by the deuterated substrate, D-[2-^{2}H]glucose indicated that the rate-limiting step of the catalytic isomerization is the transfer of hydrogen between C1 and C2 of the substrate. The kinetic data obtained with the two anomeric forms of glucose as substrate indicated that hydride shift and ring opening occur as a concerted step. The effects of substitutions of the amino acid residues which are supposed to coordinate to the metal ions suggested that metal[I] contributes to the stabilization of the transition state whereas metal[II] may have a role in maintaining the active site structure. Asp-339, coordinating to metal[I], may act as a base to attract the proton from C2 hydroxyl group of the cyclic form of the substrate and facilitate the hydride shift from C2 to C1.

INTRODUCTION

The reaction catalyzed by D-xylose isomerase was initially proposed to proceed through the cis-enediol intermediate by analogy to the mechanism demonstrated for triosephosphate isomerase. Carrell et al. reported that His-54 has close contact to C1 oxygen of the linear substrate molecule in the crystal structure of *S. rubiginosus* enzyme, and proposed that His-54 is the general base required for the cis-enediol mechanism (Carrell et al., 1989). However, an opposite orientation of the linear substrate was observed in the crystal structure of the *Arthrobacter* enzyme, and no base close to C1 or C2 of the substrate was found in this structure (Collyer et al., 1990). Based on this observation, Collyer et al., proposed an alternative mechanism involving a C1-C2 hydride shift. This hydride shift model has received support from a series of X-ray structures of the enzymes from *Streptomyces rubiginosus* (Whitlow et al., 1991) and from *Actinoplanes missouriensis* (Jenkins et al., 1992). The function of His-54 was proposed to be the general base catalyst for the opening of the sugar ring and responsible for anomeric specificity of xylose isomerase due to its proximity to axial C1-OH, rather than to equatorial C1-OH of the substrate; therefore, only α-D-pyranose would be opened efficiently with the aid of this residue (Collyer et al., 1990; Whitlow et al., 1991).

Crystal structures have also revealed the positions of the two adjacent

divalent metal ion sites in the active pocket of the enzyme. In the model proposed by Collyer, et al. (1990), Whitlow, et al. (1991), and Jenkins, et al. (1992), metal[II] facilitates the hydride shift between C1 and C2, whereas metal[I] functions in maintaining the structure of the active site (The designation of these two metal cations refer to Figure 5 in Chapter I). The role of these two metal cations in the subunit of the homotetrameric xylose isomerase from *Streptomyces rubiginosus* has also been addressed by spectroscopic studies. The coordination sphere of the two metal-binding sites in the subunit has been probed by the investigation of the Co^{2+}-substituted enzyme using electronic absorption, circular dichroism and magnetic circular dichroic spectroscopy in the visible region (Sudfeldt et al., 1990). A high-affinity site (B site) with a distorted octahedral complex geometry and a low-affinity site (A site) with a distorted tetrahedral or pentacoordinated complex structure were demonstrated. The metal binds first to the B site and subsequently to the A site. Enzyme activity increased linearly upon addition of the metal to the A site. Moreover, displacement of Co^{2+} from the B site of the tetrameric enzyme by Cd^{2+} or Pb^{2+} to form the Pb_4/Co_4 derivative containing Co^{2+} in the A site reduced the activity fourfold whereas the Pb_4/Pb_4 species was completely inactive. These facts indicated that A site is involved in the catalytic process. In order to determine the ligand environment of these two metal binding sites the oxovanadium(IV) cation (VO^{2+}) was used as a spectroscopic probe for visible, EPR and electron nucleic double resonance (ENDOR) spectroscopy

(Bogumil et al., 1991). The visible absorption data and EPR parameters indicated that a nitrogen ligand was involved in the B site. The nitrogen coordination in B site was demonstrated by ENDOR and was assigned to a histidine residue. Crystal structure showed that metal[II] coordinates to a histidine residue, His-220, in *Streptomyces* enzyme (corresponding to His-271 in *Thermoanaerobacterium* enzyme). Therefore, B site identified in the spectroscopic studies is presumably the metal[II] site identified in the crystal structure and A site is the metal[I] site. Based on the crystallographic studies, metal[II] was predicted to catalyze the transfer of hydride, whereas spectroscopic studies suggested that metal[I] is the catalytic metal ion.

The gene encoding D-xylose isomerase of *T. thermosulfurigenes* was isolated and its complete nucleotide sequence was determined (Lee et al., 1990). The importance of His-101 for the catalysis was demonstrate by the fact that substitution of His-101 by phenylalanine inactivated the enzyme whereas its substitution by glutamine resulted in an enzyme still exhibiting 8% of activity. His-101 $\rightarrow$ Gln mutant enzyme was no longer inhibited by diethylpyrocarbonate, a histidine modifying agent that strong inhibits the activity of the wild type enzyme (Lee et al., 1990).

In this work, in order to gain further information on the functional roles of amino acid residues in the active site they have been individually substituted by site-directed mutagenesis of the xylose isomerase gene from *T. thermosulfurigenes*.

We have examined the catalytic constants of these mutant xylose isomerases with different substrates, including α- and β-anomers of glucose, and obtained evidence suggesting that (1) ring-opening and hydride shift occur at a concerted step. (2) Asp-104 helps maintain the position of the imidazole residue of His-101 and locks it in a tautomeric form which functions as a hydrogen-bonding acceptor to stabilize the transition state. (3) metal[I] stabilizes substrate and the transition state of rate-limiting step and may also stabilize the developing negative charge in the transition state by electrostatic force.

MATERIALS AND METHODS

Strains, Plasmids, and Chemicals. *E. coli* strain HB101 [F⁻*hsdS20 ara-1 recA13 proA12 lacY1 galK2 rpsL20 mtl-1 xyl-5*] (Boyer and Roulland-Dussoix, 1969) was used for expression of the *T. thermosulfurigenes* xylose isomerase gene present in the plasmid pCMG11-3 (Lee et al., 1990); *E. coli* strain TG1 [*thi supE hisD Δ(lac-proAB)*/F' *traD36 proA⁺B⁺ lacI^q lacZΔM15*] in conjugation with bacteriophage M13mp19 (Yanish-Perron et al., 1985) was used for oligonucleotide-directed mutagenesis and nucleotide sequence determination (Lee et al., 1990). α-glucose, β-glucose and D-[2-^{2}H]-glucose were from Sigma Chemical Co, St. Louis, MO. Bovine serum albumin standard was from Pierce. All chemicals were of reagent grade.

DNA Manipulation. Restriction endonucleases and other enzymes for DNA manipulation were from Bethesda Research Laboratories or from New England Biolabs. They were used according to the specifications of the manufacturers. The oligonucleotide-directed mutagenesis kit was from Amersham. The oligonucleotides used for site-directed mutagenesis were from Genosys, Woodlands, TX. Their sequences are shown in Table 1. Synthesis of mutant genes and selection for mutant clones were performed by the method of Sayers et al.(1988). Nucleotide sequences of the mutant genes were confirmed by the dideoxy chain

Table 1. Sequences of oligonucleotides used for site-directed mutagenesis

Mutation	sequence
His-101→Gln	5'-ACCGTATTTCTGCTTCC**A**<u>**A**</u>GATAGAGATATTGCC-3'
His-101→Asn	5'-GCACCGTATTTCTGCTTC<u>**A**</u>**AT**GATAGAGATATTGCC-3'
His-101→Glu	5'-CCGTATTTCTGCTTC<u>**G**</u>**A**<u>**G**</u>GATAGAGATATTGCC-3'
His-101→Asp	5'-CCGTATTTCTGCTTC<u>**G**</u>**A**<u>**T**</u>GATAGAGATATTGCC-3'
Asp-104→Asn	5'-GATAGA<u>**A**</u>**AT**ATTGCCCCTGAA-3'
Asp-104→Ala	5'-GATAGA**G**<u>**C**</u>**T**ATTGCCCCTGAA-3'
Asp-339→Asn	5'-CTCAACTTC<u>**A**</u>**AT**GCGAAAGT-3'
Asp-296→Asn	5'-GGATCGATT<u>**A**</u>**AC**GCAAATAC-3'
Asp-309→Asn	5'-TGGGATACA<u>**A**</u>**AT**CAGTTCCC-3'
Glu-232→Asp	5'-CAGTTCTTGATT**GA**<u>**T**</u>CCGAAGCCAAAGGAG-3'

New triplets are shown in bold face. Underlined nucleotides indicate the introduced mismatches.

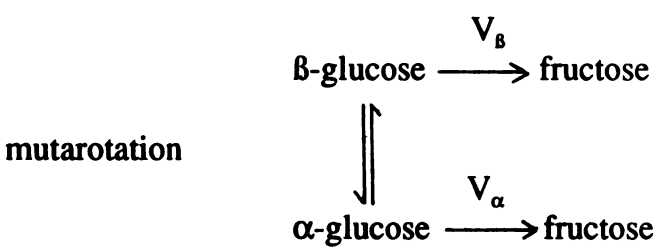
termination method (Sanger et al., 1977). The 1.4-kilobase *Eco*RI/*Bam*HI fragments containing the mutant genes were excised from the M13mp19 replicative form DNA, inserted into the vector pMMB67EH (Fürste et al., 1986), and introduced into *E. coli* strain HB101.

Protein Purification. Wild type and mutant xylose isomerases, expressed by *E. coli* HB101, were purified through a heat step of 75°C, DEAE-Sepharose and Sephacryl-300 chromatography as described previously (Lee et al., 1990), which gave enzymes homogeneous on SDS/PAGE. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Steady-State Kinetics. One ml of reaction mixture contained 20 mM MOPS (pH 7.0), 1.0 mM $CoCl_2$, substrate at concentrations of 0.3-2.5 K_m and enzyme 10-1500 μg. Reactions were run at 65°C for 30 min and product formed was determined by cysteine/carbazole/sulfuric acid method (Dishe & Borenfreund, 1951). Enzyme concentrations were adjusted such that less than 5% of the original substrate was converted in 30 min which allowed the determination of initial reaction velocities. Kinetic constants for α- and ß- anomer of glucose were determined with 0.3 - 1.5 mg of enzyme in 1.0 ml. Reactions were started by the addition of freshly dissolved substrate and run at 35°C for 1.5 min. Kinetic constants were determined from both Lineweaver-Burk and Eadie-Hofstee plots (Fersht, 1985). Each figure

of the kinetic constants shown in this study is represented as an average value from at least two independent experiments. We defined k_{cat} as turnover number per active site of enzyme at saturating substrate concentration, and determined it from the equation $k_{cat}[E]_o$=Vmax, where $[E]_o$=total active site concentration. For monitoring the dependence of activity on pH, reactions were run in 1 ml MES buffers (45 mM) containing 1.0 mM Co^{+2}. In the case of Asp-104 → Asn mutant, preincubation at 65°C was found necessary to attain maximal activity. For preincubation, this enzyme in MOPS buffer (pH 7.0) was kept at 65°C for 1 hour; then 200 μg of it was added to the incubation mixture containing MES buffer at the desired pH to start the reaction.

Correction for spontaneous mutarotation. In the determination of V_{max} and K_M for ß-glucose, the interference from α-glucose, formed by spontaneous mutarotation was considered since $K_{M(\alpha\text{-gluc})App} << K_{M(\beta\text{-gluc})App}$. If both of the anomers are initially present, fructose will arise from two different reactions:



The initial velocity of fructose formation from ß-glucose ($V_ß$) may be calculated by subtracting the initial velocity of fructose formation from α-glucose (that exists as impurity or is formed from ß-glucose by mutarotation), ($V_α$), from the apparent velocity of fructose formation ($V_ß + V_α$) determined experimentally. To account for the spontaneous mutarotation we have followed the change in optical rotation of ß-glucose under the same conditions as were used to determine enzymatic glucose isomerization (20 mM Mops, pH 7.0, 1.0 mM $CoCl_2$; 35°C; 1.5 min) with the Autopol II automatic polarimeter (Rudolph Research, Flanders, NJ). Since mutarotation is reversible and its rate constant is independent of the concentration of sugar over a wide range of concentrations at initial reaction conditions we have:

$$\frac{d[\alpha\text{-}gluc]}{dt} = \frac{-d[ß\text{-}gluc]}{dt} = k[ß\text{-}gluc] \qquad (1)$$

The reaction constant k calculated from the measurement of mutarotation and expressed in decimal logarithms and min^{-1} was 0.00727 ±0.0009. Since the spontaneous mutarotation rate is faster than the enzyme-catalyzed rate of glucose isomerization rate, we assumed that:

$$\left(\frac{d[\alpha\text{-}gluc]}{dt}\right)_{(Enz)} = \left(\frac{d[\alpha\text{-}gluc]}{dt}\right)_{(buffer)} = k[ß\text{-}gluc] \qquad (2)$$

The content of ß-glucose after 1.5 min of incubation under conditions used for enzymatic reaction was 96.6% of total. The content at 0 time, obtained by extrapolation of the mutarotation curve was 99.1%. As an approximation, therefore, we can consider that during the first 1.5 min [ß-gluc] = constant and

$$\frac{d[\alpha\text{-}gluc]}{dt} = const \qquad (3)$$

It is reasonable, therefore, to use the average content of α-glucose = 2.2%, present in the solution of ß-glucose during the initial 1.5 min of incubation with the enzyme, to calculate the apparent initial velocity of fructose formation from α-glucose, V_α, from the equation:

$$V_\alpha = \frac{V_{max(\alpha\text{-}gluc)}\,[\alpha\text{-}gluc]}{[\alpha\text{-}gluc] + K_{m(\alpha\text{-}gluc)}} \qquad (4)$$

in which we assume $V_{max(\alpha\text{-gluc})} = V_{maxApp(\alpha\text{-gluc})}$ and $KM_{(\alpha\text{-gluc})} = KM_{App(\alpha\text{-gluc})}$.

The velocity of fructose formation from ß-glucose at a given concentration of ß-glucose, $V_ß$, could thus be calculated and used to calculate $V_{max(ß\text{-gluc})}$ and $K_{M(ß\text{-gluc})}$.

To determine the catalytic constants, V_{max} and K_M, for α-glucose, the correction for

the interference from β-glucose must also be introduced. Using the method described above, we have determined the average content of β-glucose, present in the solution of α-glucose during the initial 1.5 min of reaction, to be 5.6%. The corrected $V_{max(\beta\text{-gluc})}$ and $K_{M(\beta\text{-gluc})}$ were used in the Michaelis-Menten equation to calculate the formation rate of fructose from β-glucose present as an impurity in the various concentrations of α-glucose. By subtracting V_β from total fructose formation rate, V_α at various concentrations of α-glucose could be obtained and thus be used to calculate the corrected $V_{max(\alpha\text{-gluc})}$ and $K_{M(\alpha\text{-gluc})}$. It was found that the corrections of catalytic constants for α-glucose are very small.

Conservation of deuterium between the 2-position of glucose and the 1-position of fructose. D-[2-^{2}H]-glucose was incubated overnight with wild-type enzyme at 60°C under the following condition: 20 mM MOPS buffer (pH 7.0), 1 mM Co^{2+}, 750 mM substrate and 0.45mg/ml enzyme. The enzyme was removed by passing the reaction mixture through a Centricon 10 membrane (10,000 mw cutoff, Amicon). In a glass vial a small amount of sodium borohydride, freshly dissolved in water, was added dropwise to 0.1 ml of the filtrate. After 1-hour at room temperature, the mixture was dried in a stream of nitrogen. A few ml of methanol/acetic acid (50:1) was then added to the residue and the mixture was vortexed and dried again. The addition of methanol/acetic acid and the drying was repeated several times. To the final dry residue 75 μl of acetyl anhydride and 75

μl of pyridine were added. Reaction was run at 85°C for 1.5 hours with vortexing every 15 min. The reaction mixture was dried and 100 μl of water was added to the residue. The product was then extracted with 200 μl-chloroform . The dry residue, after removal of chloroform, was redissolved in small amount of chloroform and injected into GC and GC-Mass spectrometer (Jeol JMS-AX505H). The column for GC separation was DB225. Temperature range was 200°C - 230°C and the temperature-rising speed 2°C/min.

RESULTS

Effect of mutation at His-101. His-101 was substituted with glutamine, asparagine, glutamate and aspartate. The kinetic constants of variant D-xylose isomerase are shown in Table 2. Such substitutions caused the decrease in k_{cat} but no significant change in K_M. At neutral pH the mutant enzymes exhibited 5-15% of activity of a wild type enzyme (expressed as *kcat*). Since glutamine and asparagine can not function as a base catalyst to open the ring of substrate, the observed properties of the mutant enzyme raise several questions: *(i)* What is the base that opens the ring in His-101 → Gln and His-101 → Asn mutant enzymes ? *(ii)* Is it possible that the activity observed in these mutant enzymes results from the reaction with the free aldehyde glucose present in the solution?

At equilibrium solution, the free aldehyde only represents 0.002% of total sugar. A big increase in K_{MApp} should be expected if this were the case. The insignificant change in K_M observed suggests that these two mutant enzymes use the same form of substrate as does the wild type enzyme. In other words, this data do not support the idea that His-101 acts as a base.

The change in energy of interaction, $\Delta\Delta G^{\neq}$, between the enzyme and transition state due to the mutation can be calculated from the equation given by the transition state theory (Fersht 1985):

Table 2: Kinetic constants of wild-type and His-101 substitution mutant D-xylose isomerases

Enzyme	pH7.0		pH5.5	
	kcat(s^{-1})	*KM*(mM)	*kcat*(s^{-1})	*KM*(mM)
WT	11.4	120	5.7	150
H101→Q	0.6	140	0.6	180
H101→N	1.9	170	1.9	250
H101→E	0.6	250	0.5	280
H101→D	0.9	200	0.8	370

Reaction was performed at 65°C.

$$\Delta\Delta G^{\neq} = -RT\ln(k_{cat}/K_M)_{mutant}/(k_{cat}/K_M)_{wild\ type}$$

This calculation suggests that a hydrogen bond between enzyme and transition state was lost in the mutant enzymes. When activity was measured at pH 5.5 the k_{cat} of the wild type enzyme dropped by approximately 50%, whereas no significant change was observed in mutant enzymes. At lower pH the imidazole group of His-101 becomes more protonated. This data suggest that the deprotonated form of imidazole group is required to provide a hydrogen bond to transition state. Therefore, His-101 might act as a hydrogen-bond acceptor to stabilize transition state. Side chains of Gln, Glu, Asp, and Asn may act as a hydrogen-bond acceptor. A difference in position of these side chains in the active center, as compared with the position of the His-101 imidazole, may be the reason for the lower k_{cat} observed in the mutant enzymes.

Deuterium isotope effect on catalytic constants. In order to identify the rate-limiting step during the isomerization reaction, deuterium isotope effects on the catalytic constants of both wild type and His-101 → Gln mutant enzyme were tested. The results are shown in Table 3. D-[2-^{2}H]glucose slowed the reaction rate of both wild type and mutant enzymes by a factor of approximately four. The results of this experiment suggest that the breakage of C2-H bond is involved in the rate-limiting step. They also indicate that His-101 → Gln mutant uses the same

Table 3: Deuterium isotope effect on catalytic constant, *kcat*, of wild-type and His-101 → Gln mutant enzyme

Enzyme	*kcat*(s^{-1}) D-glucose	*kcat*(s^{-1}) D-[2-^{2}H]-glucose	*kcat*(glucose) / *kcat*([2-^{2}H]-glucose)
WT	13	3.5	3.7
H101→Q	0.5	0.13	3.8

Reaction was performed at 65°C.

mechanism of catalysis as the wild type enzyme.

Effect of mutations on the anomer-specificity. The suggestion, based on crystallographic data, that His-54 (His-101 in the *Thermoanaerobacterium* enzyme), may be the base responsible for the opening of the ring (Collyer et al., 1990; Whitlow et al., 1991), was tested by site-directed substitution of this residue and determination of the kinetic constants with both anomeric forms of glucose for the mutant enzymes. The results are shown on Table 4. In the wild type enzyme both the catalytic constant, k_{cat}, and the substrate affinity (reflected by K_M) for β-glucose are approximately fivefold lower than those for α-glucose. When His-101 was substituted by Asn (which can not act as a base), $k_{cat(\alpha\text{-}gluc)}$ dropped to 12% of its wild type value whereas the $k_{cat(\beta\text{-}gluc)}$ did not change significantly. K_M remained essentially unchanged for either of the anomers. Thus, the substitution His-104 → Asn has substantially decreased the preference of the mutant enzyme for the α-anomer. The ratio of catalytic efficiency between α and β anomer was reduced more than ten-fold, from 27 to 2.5. This indicated that His-101 does not act as a base; however, it still confers the anomeric specificity on xylose isomerase by increasing the $k_{cat(\alpha\text{-}gluc)}$. As indicated by the determination of the primary isotope effect observed with D-[2-^{2}H]glucose as substrate, the constant contributing most to the k_{cat} would be the rate constant of the transfer of hydrogen atom between C1 and C2 of substrate. The hydride shift was assumed to take place on the open-

Table 4: Comparison of kinetic constants of variant D-xylose isomerase between α-D-glucose and β-D-glucose

Enzyme	α-D-glucose		β-D-glucose		$kcat/KM_{(\alpha)}$
	$kcat(s^{-1})$	KM(mM)	$kcat(s^{-1})$	KM(mM)	$kcat/KM_{(\beta)}$
WT	1.30±0.03	24±1	0.25±0.03	136±12	27
D104→N	0.65±0.01	33±1	0.14±0.02	204±18	29
H101→N	0.15±0.01	30±2	0.26±0.03	130±16	2.5
D104→A	0.08±0.01	45±2	0.27±0.01	275±20	1.8

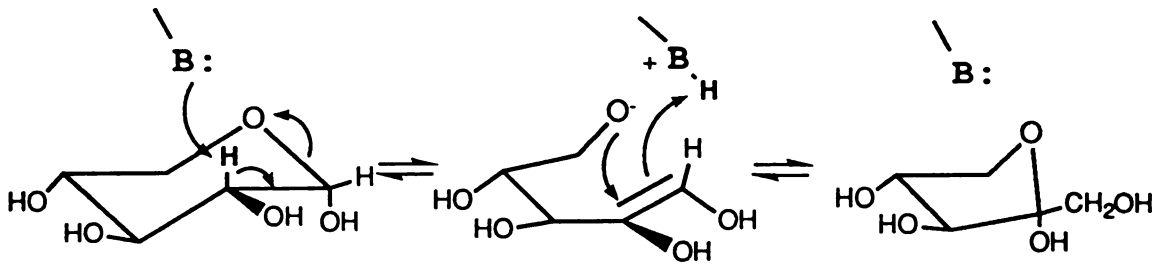
Reactions were started with freshly preparated substrate and run at 35°C for 1.5 min.
The figures have been corrected as described in Materials and Methods.

chain form of the substrate (Collyer et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992). If this were the case, $k_{cat(\beta\text{-gluc})}$ should have been of the same order of magnitude as the $k_{cat(\alpha\text{-gluc})}$ since stereo-anomers cease to exist when substrate is in the open-chained form. Moreover, these two catalytic constants should have been affected to the same extent by the substitution of Asn for His-101. Since there is no other amino acid residue with basic side chain close to equatorial C1-OH (ß-conformation) or axial C1-OH (α-conformation) position in the active pocket of xylose isomerase (Collyer et al., 1990; Whitlow et al., 1991), the pyranose ring could either be opened by a water molecule present in the active site of the His-101 → Asn mutant, or, more likely, the mechanism of ring-opening is different from the one that has been proposed. The last conclusion is based on the following arguments: (1) the observation of primary isotope effect in both wild-type and His-101 → Gln mutant enzymes with D-[2-^{2}H]-glucose suggested that the transfer of C2-hydrogen is the rate-limiting step (Lee et al. 1990; Smart et al., 1992). (2) mutant xylose isomerases in which His-101 has been substituted by residues unable to function as a base still exhibit catalytic activity equal to 10 - 14% of the wild type enzyme. (3) significant difference between $k_{cat(\alpha\text{-gluc})}$ and $k_{cat(\beta\text{-gluc})}$ suggests that the structure of the substrate immediately prior to the rate-limiting step is a cyclic structure rather than an extended open-chained structure. We would like to suggest, therefore, that isomerization and ring opening occur as a concerted step. Two concerted mechanisms may be proposed as shown in Figure 1. In one of

them, a base attracts the proton from the C2 carbon of the pyranose. This results in the formation of a cis-enediol intermediate and ring opening during the transfer of hydrogen. Two arguments may be raised against this mechanism: (1) no residue capable of acting as a general base has been observed near the C2 hydrogen in the available crystal structures; (2) no exchange of proton with the medium occurs during the isomerization reaction (Bock et al., 1983). In the second mechanism, a base attracts the proton from the C2-OH. This is followed by a hydride shift and ring opening. From the crystal structure of *Streptomyces* enzyme with 1.6 Å resolution (Whitlow et al., 1991), it follows that Asp-287 (corresponding to Asp-339 of *Thermoanaerobacterium* enzyme) is close to C2-OH. It is possible that this residue acts as a base in this catalytic reaction.

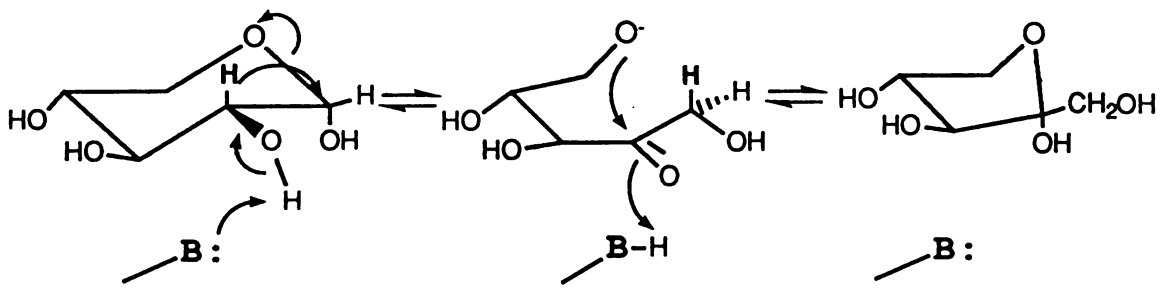
If the position of His-101 in the *Thermoanaerobacterium* enzyme is indeed equivalent to the position of His-53 of the *Arthrobacter* enzyme, its role would be hydrogen-bonding to the axial C1-OH, of the substrate in the α-pyranose form, and stabilization of the transition state. Asp-104 could assist this function by stabilization of the His residue. Substitution Asp-104 → Ala resulted in a drop of $k_{cat(\alpha\text{-gluc})}$ to about 6% of the wild-type value and a two-fold increase of K_M whereas the $k_{cat(\beta\text{-gluc})}$ remained unchanged (Table 4). This suggested that anomeric specificity depends not only on the presence of His-101, but also on the correct position of this residue. Without the hydrogen-bonding provided by Asp-104 the imidazole group of His-101 could rotate and take up positions that are unfavorable

Figure 1: schematic illustration of two possible mechanisms in which ring opening and isomerization could occur as a concerted step.



cis-ene-diol-intermediate

[I]



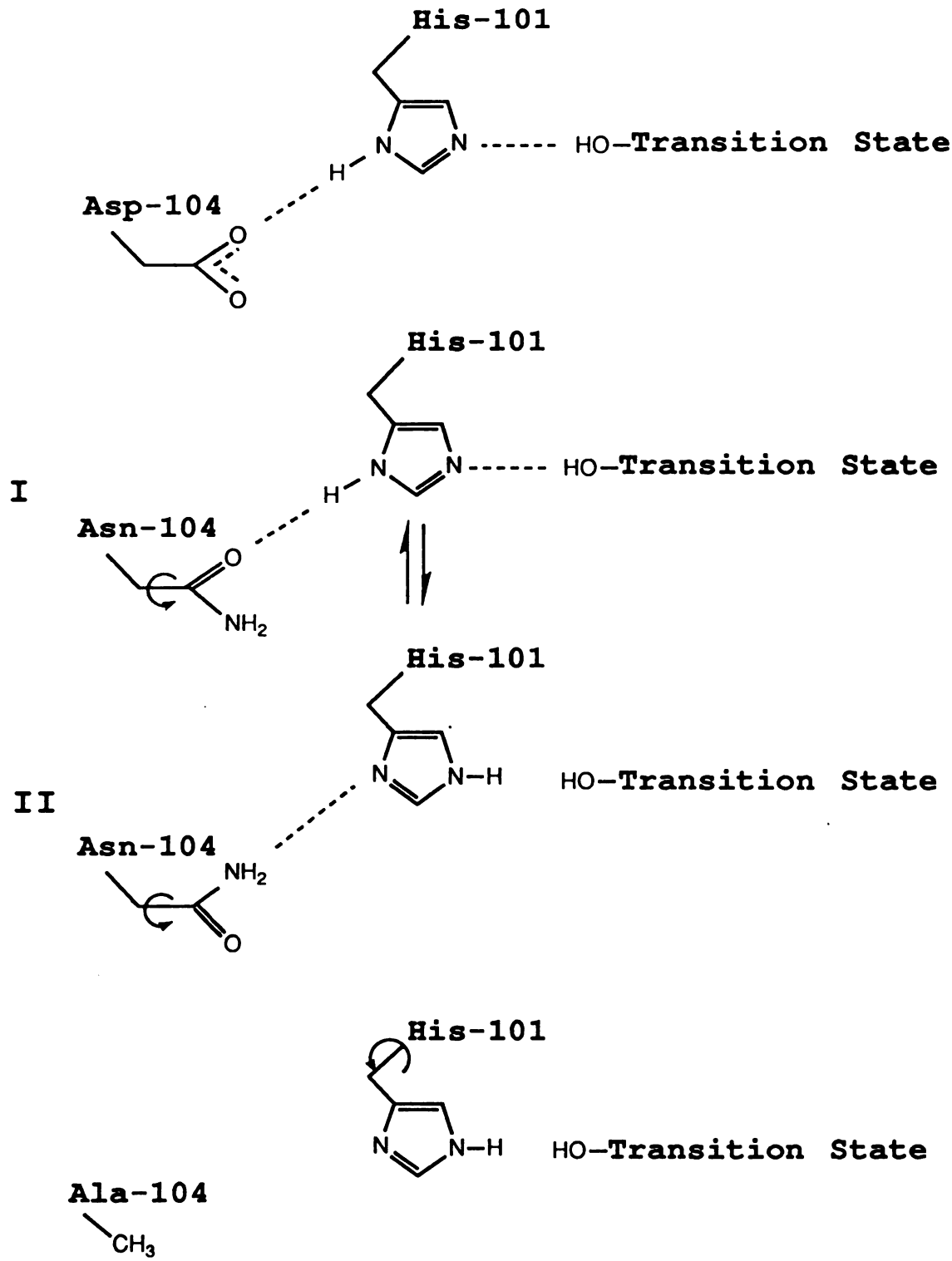
[II]

for the formation of hydrogen bond to the transition state. When, on the other hand, Asp-104 was substituted by Asn, k_{cat} dropped to approximately 50% of the wild type value for both anomers (Table 4). A hydrogen bond can be formed between Asn-104 and His-101, but only the oxygen of the amide group is a hydrogen-bond acceptor. In contrast to this, both oxygens of the carboxyl group in Asp-104 can be hydrogen-bond acceptors. Another function of Asp-104, therefore, seems to be the maintenance of His-104 imidazole group in a particular tautomeric form, most favorable for its function as a hydrogen-bond acceptor in the stabilization of the transition state (Figure 2).

Substitution of the metal-coordinating amino acids. In order to test the function of each metal cation in the active site pocket, Asp-309, Asp-296, Asp-339 and Glu-232 were substituted. Asp-309 is considered to bind the metal at position [II] whereas Asp-296, Asp-339 and Glu-232 bind the metal at position [I] (Figure 5 in Chapter I). Asp-257 in *Streptomyces* enzyme (corresponding to Asp-309 in *Thermoanaerobacterium* enzyme) also was proposed to be a base catalyst to initiate hydride shift occurring in linear form substrate molecule (Whitlow et al., 1991; van Tilbeurgh et al., 1992). Substitution of each of these aspartate residues with Asn resulted in mutant enzymes that still required Co^{2+} for maximal activity and for thermostability (measured as residual activity after 20 min at 75° C). This suggested that the metal binding site still exists in these mutants and that individual

Figure 2: Schematic illustration of the possible interaction between His-101, amino acid residue at position 104 and C1-OH of transition state.

Dashed lines indicate possible hydrogen bonds. The configuration of transition state is believed to be a cyclic form, and His-101 specifically interacts with its axial C1-OH.


His-101
HO–Transition State
Asp-104
His-101
HO–Transition State
I
Asn-104
His-101
HO–Transition State
II
Asn-104
His-101
HO–Transition State
Ala-104

mutations have brought about only local alterations such as perhaps changes in geometry of coordination due to the loss of a negative charge, the inability of $-NH_2$ in Asn to coordinate metal and a consequent change in the metal position. However, the exact structural changes in these mutants will have to be revealed by X-ray diffraction. Kinetic constants of the mutant enzymes are shown in Table 5. Asn-309 mutant enzyme exhibited approximately 20% of the wild type catalytic efficiency (k_{cat}/KM). This result argues against the hypothesis that Asp-309 might act as a base to initiate the hydride shift on the open-chain form of the substrate. It is consistent, however, with the supposition that Asp-309 or the metal[II] stabilizes the transition state. The substitution of Asn for either Asp-296 or Asp-339 caused drastic decrease in catalytic efficiency resulting from both the increase in K_M and decrease in k_{cat}. It seems, thus, that these two residues, or the metal[I], play an important role not only in the stabilization of the substrate but also in the stabilization of the transition state. The reduction of $kcat/KM$ by four order of magnitude in Asp-339 → Asn mutant enzyme consists with the hypothesis that Asp-339 may act as a base catalyst during catalysis of the enzyme. Glu-232 → Asp mutant enzyme lost solubility at temperatures above 45°C, and no activity was detected in this mutant enzyme. The metal binding site [I] probably has been destroyed in this mutant.

Dependence of the catalytic constant, k_{cat}, on pH. It has been suggested that in

Table 5: Kinetic constants of variant D-xylose isomerases towards xylose

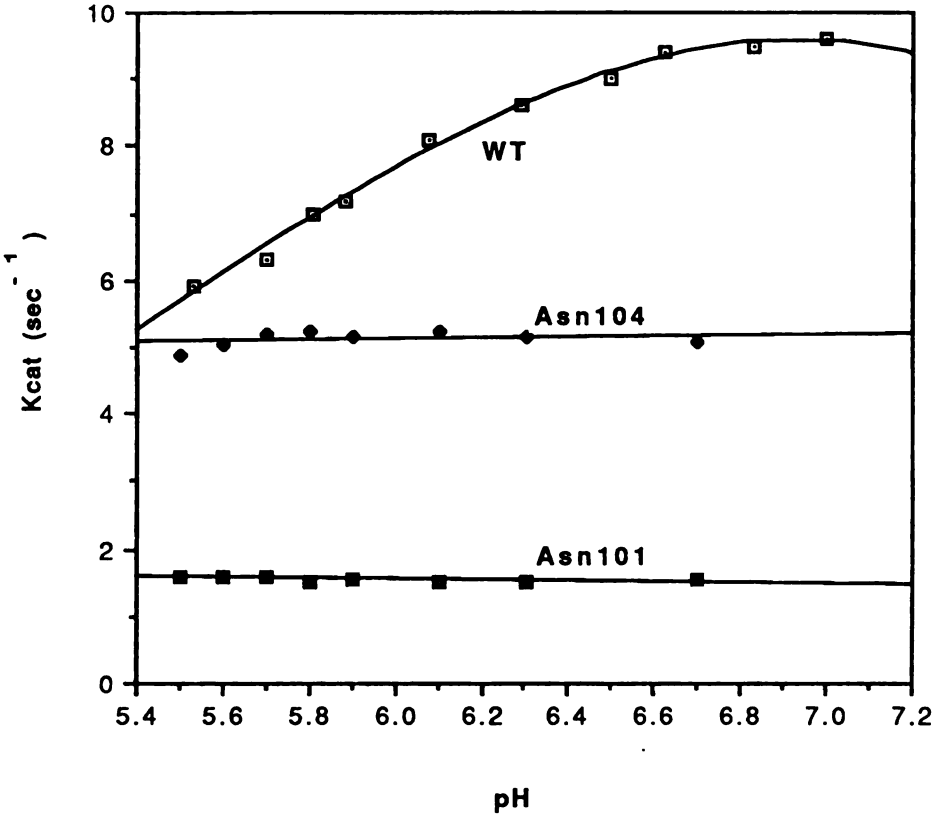
Enzyme	xylose		
	$kcat(s^{-1})$	KM(mM)	$\frac{kcat/KM_{(mutant)}}{kcat/KM_{(wild\text{-}type)}}$
WT	23±1	9.3±2.0	1
D309→N	2.7±0.1	5.8±1.0	0.2
D296→N	1.0±0.1	140±20	$28x10^{-4}$
D339→N	0.08±0.01	70±10	$4x10^{-4}$
E232→D[a]	no activity		

Reaction was performed at 65°C for 30 min.
a: E232→D mutant enzyme precipitated when temp. was above 45°C.

the xylose isomerase from *Thermoanaerobacterium* the protonation of His-101 is responsible for the decrease of V_{max} with the decrease of pH below 7.0 (Lee et al., 1990). It was considered possible, therefore, that the pK_a of enzyme-glucose complex, which can be estimated form the plot of k_{cat} vs. pH, might be lowered by the removal of a negative charge from the neighborhood of His-101. The dependence of k_{cat} on the pH for the wild-type enzyme and the two mutants, His-101 → Asn and Asp-104 → Asn is shown in Figure 3. Because the free enzyme is unstable at pH lower than 5.4, the activities were assayed between 5.5 and 7.0. From the equation $(k_{cat})_H=k_{cat}-(k_{cat})_H[H^+]/K_a$, the pK_a of the enzyme-glucose complex for wild-type enzyme was calculated to be 5.4. Although the pK_a for the mutant enzymes could not be determined exactly from the data obtained in this experiment, it must be far below 5.5 for each of the mutant enzymes. The apparent K_M did not change significantly for either the wild type or the mutant enzymes between pH 7.0 and pH 5.5. Thus, Asp-104 seems to contribute significantly to the overall negative-charge environment around His-101. If this charge is removed the apparent pK_a of the enzyme-glucose complex is lowered considerably.

Conservation of deuterium during the isomerization reaction. Isomerization of glucose performed in D_2O indicated that practically no exchange of deuterium occurred between the solvent and the product (Bock et al., 1983). Crystal structure of the *Arthrobacter* enzyme with 5-thio-α-D-glucose (Collyer et al, 1990) and of

Figure 3: Plot of pH dependence of *kcat*.

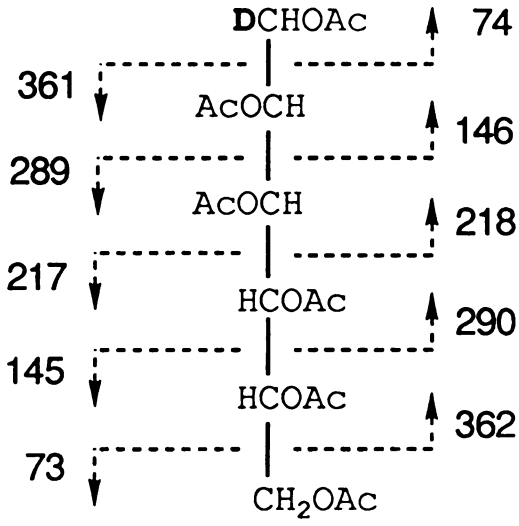

10
8
6
4
2
0
Kcat (sec^{-1})
WT
Asn104
Asn101
5.4
5.6
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2
pH

the *Streptomyces* enzyme with cyclic xylose (Whitlow et al., 1991) indicated that the hydrogen at C2 is positioned near a Trp side chain and is far from any residue with a potential base character. It seems to be very unlikely, therefore, that the isomerization proceeds by the mechanism [I] (Figure 2) involving an cis-enediol intermediate.

To provide further insights into the reaction mechanism we have evaluated the possibility of free hydrogen radical generation upon the breakage of the C2-H bond. To do this, we have reduced the reaction mixture with sodium borohydride to convert D-glucose to D-sorbitol and D-fructose to D-mannitol and D-sorbitol. These were acetylated and subjected to GC-Mass analysis. If the isomerization takes place by a hydride shift, then deuterium from the C2 atom of glucose will be quantitatively transferred to C1 atom of fructose. Consequently, deuterium will be present at C1 of all D-mannitol molecules. If the transfer of hydrogen would take place via an cis-enediol intermediate or by generation of free hydrogen radical, the conversion of deuterium would be lower than 100%. The structure of the ester derivatives of D-mannitol, derived from the isomerization of 2-[D]-D-glucose, is shown in Figure 4 in which the number along the dash arrow line indicates the molecular weight of the fragment generated after bombardment. If deuterium is present at C1 of all such derivatives, the ratio of fragment with molecular weight 362 to that with 361 should be close to unity, so will be the ratio of 290/289 and so on. The Mass spectrum of the ester derivatives of D-mannitol (Figure 5) shows

Figure 4: Structure of ester derivative of D-mannitol

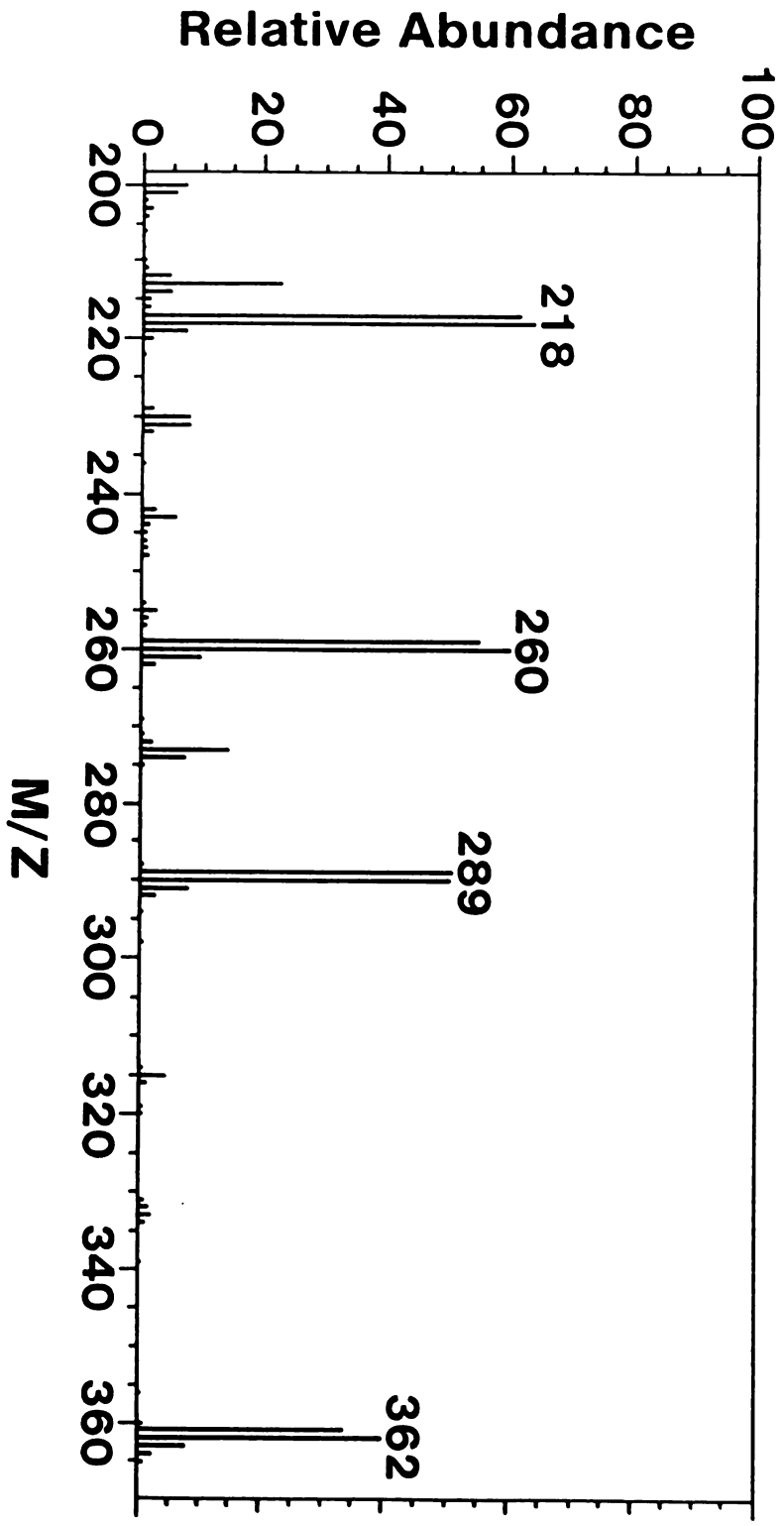
Number along the dashed arrow indicates molecular weight of the fragment after bombardment.



Ester-derivative of D-mannitol

Figure 5: Mass spectrum of ester derivatives of D-mannitol.

The mass range was selected between 200 and 370 for clarity of presentation.


Relative Abundance
0
20
40
60
80
100
200
220
240
260
280
300
320
340
360
M/Z
218
260
289
362

that the ratio of abundance of 361/362, 289/290 and 217/218 particles is very close to unity. This result strongly supports the conclusion that hydrogen transfer between C1 and C2 atoms of the substrate occurs by the hydride shift.

DISCUSSION

The models of xylose/glucose isomerization published to date postulated the opening of the α-pyranose ring as a necessary step prior the transfer of the C2 hydrogen. These models were supported by the finding of extended-chain species of the substrate in the crystal structures of the enzyme/xylose-xylulose complexes. These extended-chain substrates were interpreted as reaction intermediates (Carrell et al., 1989; Collyer et al., 1990). However, the extended-chain species of sugar, observed in the crystal structure, do not necessarily have to be reaction intermediates preceding the hydride shift. Since D-xylulose in aqueous solution contains 20.2% free ketose (Wu and Serianni, 1990), the extended species could be the free ketose of xylulose. In these models, the active site histidine (His-54 of *Streptomyces* enzyme or His-101 of *Thermoanaerobacterium* enzyme) was assigned as a base to either open the ring or attract the proton from C2 of the linear substrate. Effects of substitution of His-101 by Asn and Gln in *Thermoanaerobacterium* enzyme do not support the idea that His-101 acts as a base. Recently, Lambier et al., (1992) challenged the hypothesis that assigns to His-54 of *Actinoplanes missouriensis* isomerase (His-53 of *Arthrobacter*, His-101 of the *Thermoanaerobacterium* enzyme) the role of ring opening. They found that variant enzymes, obtained by substitution of His-54 by different residues capable

of hydrogen-bonding to the substrate, retained approximately 10% of wild type activity and acted by the same mechanism as the wild-type enzyme similarly to the mutants of the *Thermoanaerobacterium* xylose isomerase described in this work and our previous work (Lee et al., 1990). However, these authors still postulated the ring opening as obligatory step preceding the hydride transfer.

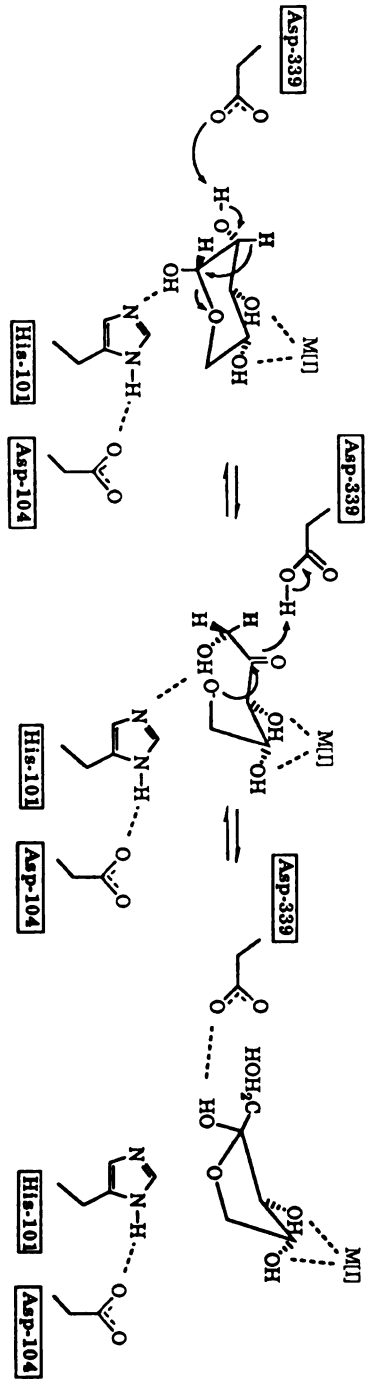
Kinetic data for the two anomers of glucose for the wild type and mutant enzymes, presented in this work, suggested that the substrate preceding the rate-limiting step is in the cyclic form. This conclusion is consistent with the results of crystallographic studies performed under steady-state conditions in a flow-cell (Farber et al., 1989). The electron densities observed by these authors indicated that the rate-limiting step was preceded by a cyclic form of the substrate. Our results of the isotope effect on the isomerization kinetics have indicated that hydrogen transfer is the rate-limiting step. We propose, therefore, that hydrogen transfer and ring opening are performed as a concerted step. We also provide further support for hydride shift mechanism of the hydrogen transfer. This support is based on the following arguments: (1) no exchange of the proton between the substrate and the medium takes place during the reaction; (2) deuterium at C2 of glucose is transferred to C1 of fructose with the efficiency close to 100%; (3) there is no basic amino acid residue, capable of attracting the proton, in the vicinity of the C2-H of the substrate.

Metal[I] coordinates to C3-OH and C4-OH of the α-pyranose (Collyer et al.,

1990; Whitlow et al., 1991). Although metal[I] does not seem to interact directly with C5-O of α-pyranose, the distance between metal[I] and C5-O could become shorter if a distortion of the transition state occurred. It is possible, thus, that metal[I] provides the electrostatic interaction to stabilize the developing negative charge at C5-O in the transition state.

Asp-339 of the *Thermoanaerobacterium* enzyme (Asp-287 of *Streptomyces*), coordinating to metal[I], also hydrogen-bonds to C2-OH of the α-pyranose (Whitlow et al., 1991). It could, therefore, attract the proton from the C2-OH to initiate the hydride shift. The drop of catalytic efficiency, by four orders of magnitude, upon substitution of this residue by Asn (Table 5) is consistent with this hypothesis. Based on all these data, we would like to propose a new model for the reaction catalyzed by xylose isomerase (Figure 6). In this model, His-101, locked at one tautomeric form by Asp-104, acts as a hydrogen-bond acceptor to stabilize the substrate as well as the transition state. Asp-339, acting as a base, attracts the proton from C2-OH. This facilitates the subsequent hydride shift from C1 to C2, and simultaneously induces the opening of the ring. Metal[I] stabilizes the substrate and transition state by coordination. It may also provide the electrostatic force to stabilize the developing negative charge at C5-oxygen. Metal[II] probably helps maintain the active site structure and affects the activity indirectly. The product is formed after the attack of the C5-oxygen on the C2 keto group and closing of the ring.

Figure 6: The proposed mechanism for the isomerization reaction catalyzed by D-xylose isomerase.


Asp-339
His-101
Asp-104
M[]
Asp-339
His-101
Asp-104
M[]
Asp-339
His-101
Asp-104
M[]

The exact three-dimensional structure of xylose isomerase from *Thermoanaerobacterium thermosulfurigenes* is not available at present. The inferences concerning the positions of amino acid residues, addressed in this dissertation are based on the known crystal structure of enzymes from *Arthrobacter* (Henrick, et al., 1989; Collyer and Blow, 1990; Collyer et al., 1990) and of *Streptomyces* (Farber et al., 1989; Whitlow et al., 1991) and on the conservation of several domains in the primary structure of xylose isomerases from many different sources, particularly in the region of the active site. Although the results presented in this study are consistent with the belief that the same mechanism of isomerization is functioning in the catalysis by several different xylose isomerases, it is possible that minor structural differences in the relative positions of different amino acid residues might lead to misinterpretation of the results provided by the kinetic data. It is important, therefore, that the conclusions presented here are confirmed by the determination of three-dimensional structure of appropriate mutant enzymes.

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CHAPTER III

MECHANISM FOR DISCRIMINATION BETWEEN XYLOSE AND GLUCOSE AND THE ROLE OF ACTIVE SITE AROMATIC AMINO ACIDS

ABSTRACT

The structural basis for substrate specificity of the thermophilic xylose isomerase from *Thermoanaerobacterium thermosulfurigenes* was examined by using predictions from the known crystal structure of the *Arthrobacter* enzyme and amino acid residue substitutions by site-directed mutagenesis of the *xyl*A gene of *T. thermosulfurigenes*. The locations of Met-87, Thr-89 and Val-134, which contact the C6-OH group of D-5-thio-glucose in xylose isomerase from *Arthrobacter* (Collyer et al., 1990) are equivalent to those of Trp-139, Thr-141 and Val-186 in the *Thermoanaerobacterium* enzyme. The comparison of kinetic data between wild type and mutant enzymes suggests that the major mechanism for discrimination between D-xylose and D-glucose in *T. thermosulfurigenes* enzyme is the steric hindrance between the indole group of Trp-139 and the C6-methanolic group of D-glucose. Sequential decrease in K_M toward glucose was observed when Trp-139 was substituted by Tyr, Phe, Met, Leu, Val or Ala, in the order shown. Although Thr-141 and Val-186 cause no steric hindrance against glucose binding, introduction of an additional hydrogen bond by replacing Val-186 with Thr also increased affinity toward glucose. Besides Trp-139, the functional roles of four other aromatic amino acids in the active site pocket have been examined. The indole group of Trp-188, which is presumed to be positioned parallel to the hydrophobic backbone of the sugar, and believed to be interacting with it, plays an

essential role in the binding of the sugar substrate. Trp-49, which presumably hydrogen bonds to Asp-339 coordinating to metal [I], helps in the binding of substrate to the enzyme by maintaining the structure of the active site. Reducing the area of water-accessible hydrophobic surface of the active site pocket by replacing Trp-139 with smaller hydrophobic amino acids or replacing Trp-49 with Arg enhanced the thermostability of the enzyme.

INTRODUCTION

Specificity of enzymes towards their substrates is determined in part by molecular residues that provide for binding of the substrate and which maintain substrate steric configuration in the active site. A variety of factors influence enzyme-substrate complementarity and catalytic efficiency including steric fit, charge interactions, hydrogen bonding and hydrophobic interactions (Craik et al., 1985). Until recently, the main strategy to reveal and study the molecular basis of these factors was to determine the three-dimensional structure of the enzyme-substrate complexes by X-ray crystallography. Redesigning proteins by engineering of their genes is now a viable approach that complements structural studies and enables determination of the effect caused by amino acid substitution on the function of mutant enzyme. Thus, substrate specificity has been altered by redesigning the structural frame of an enzyme (Wilks et al., 1988; Bone et al., 1989; Scrutton et al., 1990), its electrostatic network (Wilkinson et al., 1984; Wells et al., 1987; Dean et al., 1990; & Evnin et al., 1990) or its hydrophobic interaction with the substrate (Estell et al., 1989). Catalytic function of an enzyme can also be changed and regulated by modifications of the physical microenvironment of its catalytic site (Hurley et al., 1990; Higaki et al., 1990).

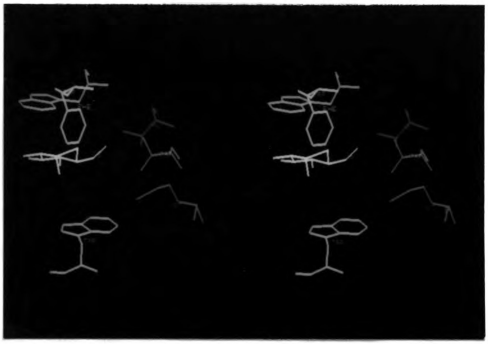
In all D-xylose isomerases studied to date D-xylose is a more favorable substrate than D-glucose, mainly due to the lower K_M of D-xylose. D-xylose and

D-glucose have identical configuration, except for the presence of an additional -CH_2OH group at the C6 position in the glucose molecule. This extra methanolic group must, therefore, be responsible for the differences in the K_M exhibited by xylose isomerase towards glucose versus xylose. According to the alignment of amino acid sequences from different D-xylose isomerases and the active site structures of D-xylose isomerases from *Streptomyces* and *Arthrobacter* (Figure 1 see also Collyer et al., 1990; & Whitlow er al., 1991), Trp-139, Val-186 and Thr-141 of *Thermoanaerobacterium* enzyme might cause such steric hindrance against glucose binding. Mutation at the codons specifying these amino acids have been performed to test this hypothesis.

Besides Trp-139, there are four more aromatic amino acids constituting the hydrophobic surface of the active site pocket. The role of the aromatic amino acid residues for binding the sugar have been demonstrated in maltose-binding protein (Martineau et al., 1990), arabinose-binding protein and galactose-binding protein (Quiocho et al., 1989). In this dissertation, the role of these aromatic amino acids in substrate binding and on thermostability of the enzyme have been examined.

Figure 1: Stereo analysis of the interaction between hydrophobic amino acids and α-5-thio-D-glucose in the active site pocket of *Arthrobacter* D-xylose isomerase.

Substrate analog is sandwiched between two indole groups (yellow). C6-OH of analog is close to residue of Val-134 (red), Thr-89 (green) and Met-87 (light blue). In *Thermoanaerobacterium* enzyme the corresponding amino acids are believed to be Val-186, Thr-141 and Trp-139, respectively.



MATERIALS AND METHODS

Strains, Plasmids & Site-directed mutagenesis. Were as described in Chapter II. The oligonucleotides used for site-directed mutagenesis are shown in Table 1.

Protein Purification. Wild type and most mutant xylose isomerases, expressed by *E. coli* HB101, were purified through a heat step of 75°C for 15 min, DEAE-Sepharose and Sephacryl-300 chromatography as described previously (Lee et al., 1990). Phe-145 → Lys, and Trp-188 → His mutant enzymes were heated at 60°C for 20 min, and Trp-139 → Lys was heated at 65°C for 30 min instead of 75°C. After purification through DEAE-Sepharose and Sephacryl-300 all mutant enzymes were homogeneous on SDS/PAGE. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard (from Pierce). The apoenzyme was obtained by dialysis of the homogeneous enzyme against 100-time volume of MOPS buffer (10 mM pH 7.0) with EDTA (10 mM) for 36 hours and then against MOPS without EDTA for another 36 hours. Dialysis buffer solutions were changed every 12 hours.

Steady-State Kinetics. Kinetic constants were determined as described in Chapter II.

Table 1: Sequences of oligonucleotides for site-directed mutagenesis

Mutation	sequence
Trp-139→Tyr	5'-ACGAAAGTTTTGT**AT**GGTACTGCGAAT-3'
Trp-139→Phe	5'-ACGAAAGTTTTGT**TT**GGTACTGCGAAT-3'
Trp-139→Met	5'-AAAGTTTTG**AT**GGGTACTGCG-3'
Trp-139→Leu	5'-CGAAAGTTTTG**CT**GGGTACTGCGA-3'
Trp-139→Val	5'-CGAAAGTTTTG**GT**GGGTACTGCGA-3'
Trp-139→Ala	5'-CGAAAGTTTTG**GC**GGGTACTGCGA-3'
Trp-139→Lys	5'-ACGAAAGTTTTG**AA**GGGTACTGCGAA-3'
Trp-49→Phe	5'-ATAGCTTAT**TTT**CACACTTTT-3'
Trp-49→Ala	5'-ATAGCTTAT**GC**GCACACTTTT-3'
Trp-49→Arg	5'-CTATAGCTTAT**AG**GCACACTTTT-3'
Trp-188→Asp	5'-AACTACGTATTC**GAT**GGTGGAAGAGAA-3'
Trp-188→His	5'-AACTACGTATTC**CAT**GGTGGAAGAGAA-3'
Trp-188→Lys	5'-ACTACGTATTC**AA**GGGTGGAAG-3'
Trp-188→Glu	5'-ACTACGTATTC**GA**GGGTGGAAG-3'
Phe-145→Lys	5'-TACTGCGAATCTT**AAA**TCCAATCCAAGAT-3'
Phe-60→His	5'-GAACAGATCAA**CA**TGGCAAAGCTA-3'

New triplets are shown in bold face. Underlined nucleotides indicate the introduced mismatches.

Kinetics of Irreversible Thermoinactivation. The time course of irreversible thermoinactivation of D-xylose isomerase was measured by incubating 0.6 ml enzyme solution (0.1mg/ml) in 10 mM MOPS buffer (pH 7.0 at 85°C) containing 50 μM $CoCl_2$ at 85°C for various periods of time and then determining the residual activity at 65°C. First order rate constants of irreversible thermoinactivation were obtained by linear regression in semilogarithmic coordinates.

RESULTS

The residues causing steric hindrance against glucose binding. In order to find out what is the mechanism for discrimination between the two substrates, Thr-141, Trp-139, and Val-186 were chosen as the target amino acids for substitution. We changed Thr-141 to Ser, Trp-139 to Phe and Tyr, and Val-186 to Thr, Ser, and Ala. The steady-state kinetic constants of mutant enzymes are shown in Table 2. The variant Thr-141 → Ser exhibited a two-fold lower catalytic efficiency, k_{cat}/K_M, toward glucose than wild type. It was considered, therefore, that Thr-141 does not cause steric hindrance against glucose binding in *Thermoanaerobacterium* xylose isomerase. In the Trp-139 → Phe variant an increase of the catalytic efficiency toward glucose but decrease toward xylose was observed, implying that the side chain of Trp-139 does cause a steric hindrance against the binding of glucose. The enlarged pocket in Trp-139 → Phe mutant enzyme presumably accommodates glucose better, but may be too large for xylose. Catalytic efficiencies for both glucose and xylose in Val-186 → Ala variant are practically the same as in the wild type enzyme. This indicates that the side chain of Val-186 does not cause steric hindrance against the binding of glucose. The increase in catalytic efficiency for glucose in the Val-186 → Thr mutant variant should be attributed, therefore, to the introduction of a new hydrogen bond between the enzyme and the substrate since both side chains of Thr and Val are of approximately the same size. Trp-139 →

Table 2: Comparison of kinetic constants of variant D-xylose isomerases for glucose and xylose

Enzyme	Glucose			Xylose		
	kcat(s^{-1})	*KM*(mM)	*kcat/KM*	*kcat*(s^{-1})	*KM*(mM)	*kcat/KM*
WT	11±2	110±8	0.10	23±1	9.3±1.8	2.5
W139→F	16±1	65±8	0.25	24±2	16±2	1.5
W139→Y	9.0±0.4	91±12	0.10	10±1	14±1	0.7
V186→T	15±2	91±7	0.16	23±1	9.8±1.8	2.3
V186→S	13±1	140±7	0.09	18±2	17±1	1.1
V186→A	9.0±1	100±10	0.09	31±3	14±2	2.3
T141→S	7.8±0.5	160±20	0.05	46±2	28±2	1.7
W139→F, V186→T	16±2	29±4	0.55	24±1	13±2	1.8
W139→F, V186→S	12±1	58±4	0.21	9.5±0.4	21±2	0.45

Reaction was performed at 65°C in 20 mM MOPS buffer (pH 7.0) containing 1 mM $CoCl_2$.

Tyr and Val-186 → Ser mutant variants have same level of specificity constant for glucose as the wild type, but the specificity constant for xylose is three- and two-folds lower, respectively. Introduction of an inappropriate hydrogen bonding in an enlarged substrate-binding pocket may by responsible for this decrease. Finally, two double mutant enzymes, Trp-139 → Phe/Val-186 → Thr and Trp-139 → Phe/Val-186 → Ser, were constructed to see whether the effects on the increase of catalytic efficiency for glucose are additive. Catalytic efficiency toward glucose increased further in Trp-139 → Phe/Val-186 → Thr due to a further decrease of K_M. The $\Delta\Delta G^{\neq}$ between Val-186 → Thr and the wild type enzyme, and between Trp-139 → Phe/Val-186 → Thr and Trp-139 → Phe were 1.5 and 2.2 $KJmol^{-1}$, respectively. This values are consistent with the presumption that the hydroxyl group of Thr-186 participates in a new hydrogen bonding between the enzyme and C6-OH of glucose.

To elucidate further the functional role of Trp-139 we have substituted this residue with a series of hydrophobic amino acids. As shown in Table 3, substitution of Trp-139 with residues having smaller side chains increased the catalytic efficiency for glucose while decreasing the efficiency for xylose. This confirms the conclusion that the bulky side chain of Trp-139 hinders the accommodation of glucose in the catalytic pocket of the *Thermoanaerobacterium* xylose isomerase.

Table 3: Kinetic constants of wild-type and active site aromatic amino acids substituted mutant D-xylose isomerases (I)

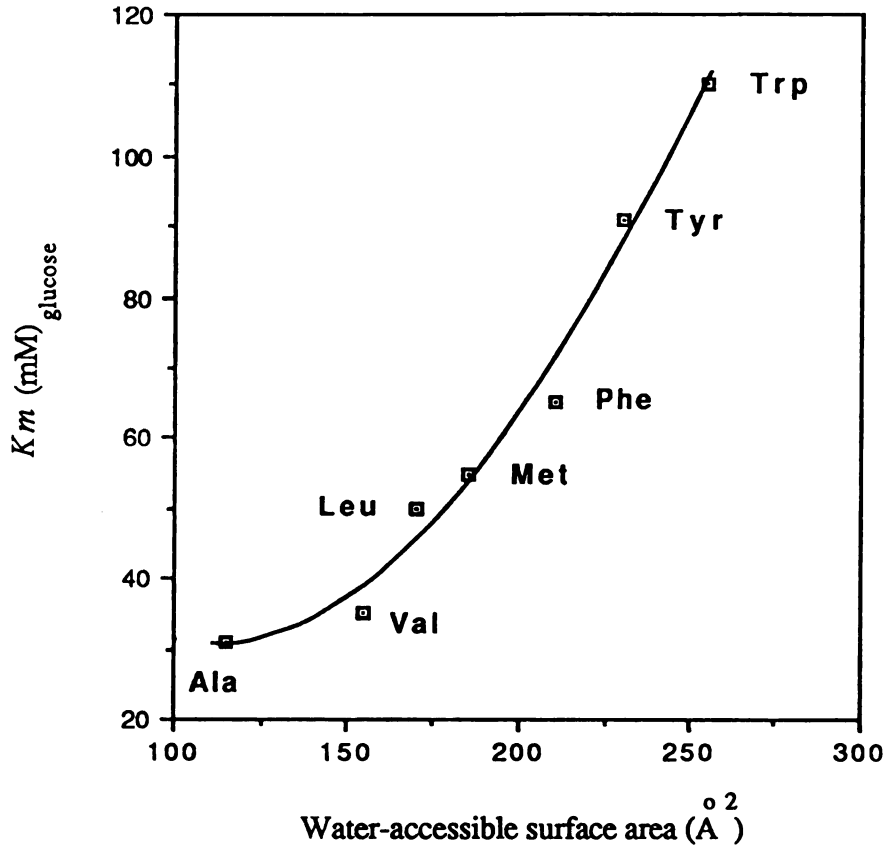
Enzyme	glucose			xylose		
	kcat(s^{-1})	*KM*(mM)	*kcat/KM*	*kcat*(s^{-1})	*KM*(mM)	*kcat/KM*
WT	11±2	110±8	0.10	23±2	9.3±1.8	2.5
W139→Y	9.0±0.4	91±12	0.10	10±1	14±1	0.7
W139→F	16±1	65±7	0.25	24±2	16±2	1.5
W139→M	11±1	55±2	0.20	10±1	9.2±0.2	1.1
W139→L	7.7±0.4	50±4	0.15	14±1	11±1	1.3
W139→V	4.5±0.2	35±1	0.13	7.7±0.1	6.4±0.1	1.2
W139→A	8.2±0.2	31±2	0.26	8.4±0.2	5.9±0.4	1.4
W139→K	3.1±0.2	21±1	0.15	3.5±0.1	5.4±0.7	0.7
W49→R	10±1.0	110±3	0.09			
W49→F	10±0.3	330±14	0.03			
W49→A	7.2±0.3	710±48	0.01			
F60→H	2.1±0.1	140±4	0.02			

Reaction was performed at 65°C.

Correlation between $K_{M(glucose)}$ and the Water Accessible Surface Area of the Side Chain of the Residue at position 139. As shown in Table 3, a progressive decrease in K_M for glucose was observed when Trp-139 was substituted by Tyr, Phe, Met, Leu, Val or Ala, whereas the $K_{M(xylose)}$ was changed more or less randomly. Figure 2 shows that there is a correlation between the $K_{M(glucose)}$ and the water-accessible surface of the side chain of the residue in position 139. This suggests that this side chain protrudes into the cavity of the active site pocket and this protrusion is the reason for the steric hindrance against glucose binding. This suggestion is consistent with the predictions based on the crystal structure of the active site of *Arthrobacter* enzyme (Collyer et al., 1990) in which Met-87 (corresponding to Trp-139 of *Thermoanaerobacterium* enzyme) is indeed in the proximity of C6-methanolic group of α-thio-D-glucose (Figure 1). Lys, whose side chain has the size comparable to that of Leu, falls out of the proportionality rule. Its placement in position 139 resulted in a mutant enzyme with the lowest K_M and k_{cat} toward both substrates. We believe that the $-NH_2^{\epsilon}$ group of Lys side chain may be responsible for this anomaly. There are several carboxyl groups coordinating to metal ions and/or hydrogen bonding to substrate in the immediate vicinity of the residue 139. The positive charge of Lys may interact with them, causing local structural changes, and, consequently confer upon the Trp-139 → Lys mutant enzyme properties different from those expected from a simple change in the hydrophobic side chain size. It is also possible that amino group of Lys forms

Figure 2: The correlation between K_m(glucose) and the water-accessible surface area of the side chain of amino acid at position 139 of the *Thermoanaerobacterium* xylose isomerase

The value of the accessible surface area of the side chain for each amino acid is from C. Chothia, *J. Mol. Biol.* **105**, 1-14, 1975.


120
100
80
60
40
20
100
150
200
250
300
Km (mM) glucose
Water-accessible surface area (A o 2)
Trp
Tyr
Phe
Met
Leu
Val
Ala

a hydrogen bond to the substrate and increases the affinity for both substrates in a way similar to that which has been observed in the case of Val-186 → Thr substitution.

The Role of Other Aromatic Amino Acids in the Active Site. Besides Trp-139, there are four other aromatic amino acid residues in the active site pocket. In *Arthrobacter* xylose isomerase the pyranose ring of the substrate is sandwiched between Trp-15 and Trp-136 (corresponding to Trp-49 and Trp-188 of the *Thermoanaerobacterium* enzyme, respectively). The indole ring of Trp-136, surrounded by Phe-93 and Phe-25 (from the neighboring subunit), interact hydrophobically with the carbon backbone of the substrate pyranose ring (Figure 1). These Phe residues correspond to Phe-145 and Phe-60 of the *Thermoanaerobacterium* enzyme, respectively. The $N^{\varepsilon 1}$ of the Trp-15 in the *Arthrobacter* enzyme hydrogen bonds to Asp-292 (corresponding to Asp-339 in *Thermoanaerobacterium*) which coordinates to metal ion[I] (Collyer et al., 1990). When Trp-49 of *Thermoanaerobacterium* enzyme was substituted by Phe or Ala, the $K_{M(glucose)}$ increased 3 and 6.5 folds, respectively, with slight decrease in the k_{cat} (Table 3). The loss of the hydrogen bond between Trp-49 and Asp-339 may be expected to affect the structure of the active site. A decrease in the binding affinity for the substrate is consistent with this expectation. Surprisingly, the substitution of Trp-49 with Arg did not result in appreciable change of either K_M

or k_{cat}. Introduction of the positive charge of Arg seemed not to change the active site structure. It is possible that the N^{ϵ} of Arg may superimpose on the $N^{\epsilon 1}$ of tryptophan and hydrogen bond to Asp-339 thus maintaining a structure of the active site close enough to the wild type to be functionally indistinguishable.

When Trp-188 was substituted by Lys, Asp, Glu or His none of the four mutant proteins exhibited any detectable catalytic activity toward glucose. With D-xylose as substrate, Trp-188 → Lys, Trp-188 → Asp and Trp-188 → Glu still did not show any activity but in the Trp-188 → His mutant a low activity could be detected. The protein solubility of Trp-188 → Glu and Trp-188 → His after incubation at 65°C was checked by applying the supernatant, after centrifugation, onto SDS-PAGE. The results showed that the protein still remains soluble. The lack of activity in these mutants must, therefore, be attributed to the loss of catalytic ability rather than the instability of proteins at 65°C. Although Trp-188 → His mutant showed activity towards xylose, the $K_{M(xylose)}$ was very high so that the catalytic constants could not be measured precisely at 60°C. However, it was found that K_M of this variant xylose isomerase is temperature dependent (Table 4). At 37°C $K_{M(xylose)}$ was 820 mM which is about 800 times higher than that of the wild-type enzyme whereas k_{cat} was lower only by a factor of 2 as compared with the wild-type xylose isomerase (Table 4). It is possible that the loss of catalytic activity in the Trp-188 → Lys, Trp-188 → Asp or Trp-188 → Glu mutant enzymes was also due to the inability of these proteins to bind the substrate. These data

Table 4: Kinetic constants of wild-type and active site aromatic amino acids substituted mutant D-xylose isomerases (II)

Enzyme	xylose		
	k_{cat}(s^{-1})	K_M(mM)	k_{cat}/K_M
WT(65°C)[a]	23±1	9.3±1.8	2.5
WT(37°C)[b]	1.0±0.1	1.1±0.1	0.9
F145→K(37°C)[b]	1.1±0.1	53±6	$2x10^{-2}$
W188→H(37°C)[b]	0.50±0.02	820±30	$6x10^{-4}$
W188→D	no activity		
W188→E	no activity		
W188→K	no activity		

a: Reaction was performed at 65°C.
b: Reaction was performed at 37°C.

indicate that the hydrophobic interaction between the indole ring of Trp-188 and the hydrophobic backbone of pyranose plays an important role in the binding of sugar substrates to the xylose isomerase catalytic site. Substitution of Phe-145 with Lys resulted in a fifty-fold increase of $K_{M(\text{xylose})}$ and an insignificant change in k_{cat} at 37°C (Table 4). This suggests that Phe-145 also plays an important role in substrate binding. Since the phenyl group of Phe-145 does not interact directly with the hydrophobic backbone of the substrate, the role of Phe-145 may be to maintain the indole group of Trp-188 in an optimal position for the interaction with the substrate. Substitution of Phe-60 with His did not change the $K_{M(\text{glucose})}$ significantly, but this mutant enzyme exhibited only 20% of k_{cat} in comparison with the wild type (Table 3). Phe-60 is involved in the association of monomers to form the active dimers. As is shown by SDS-PAGE, Phe-60 → His has stronger interaction at subunit interface of active dimer (see Figure 6 in Chapter IV). This indicates that the active site structure was changed somehow in Phe-60 → His enzyme, and this change must account for the change of kinetic constants. However, a crystal structure of the mutant enzyme is required to fully understand the mechanism of this defect.

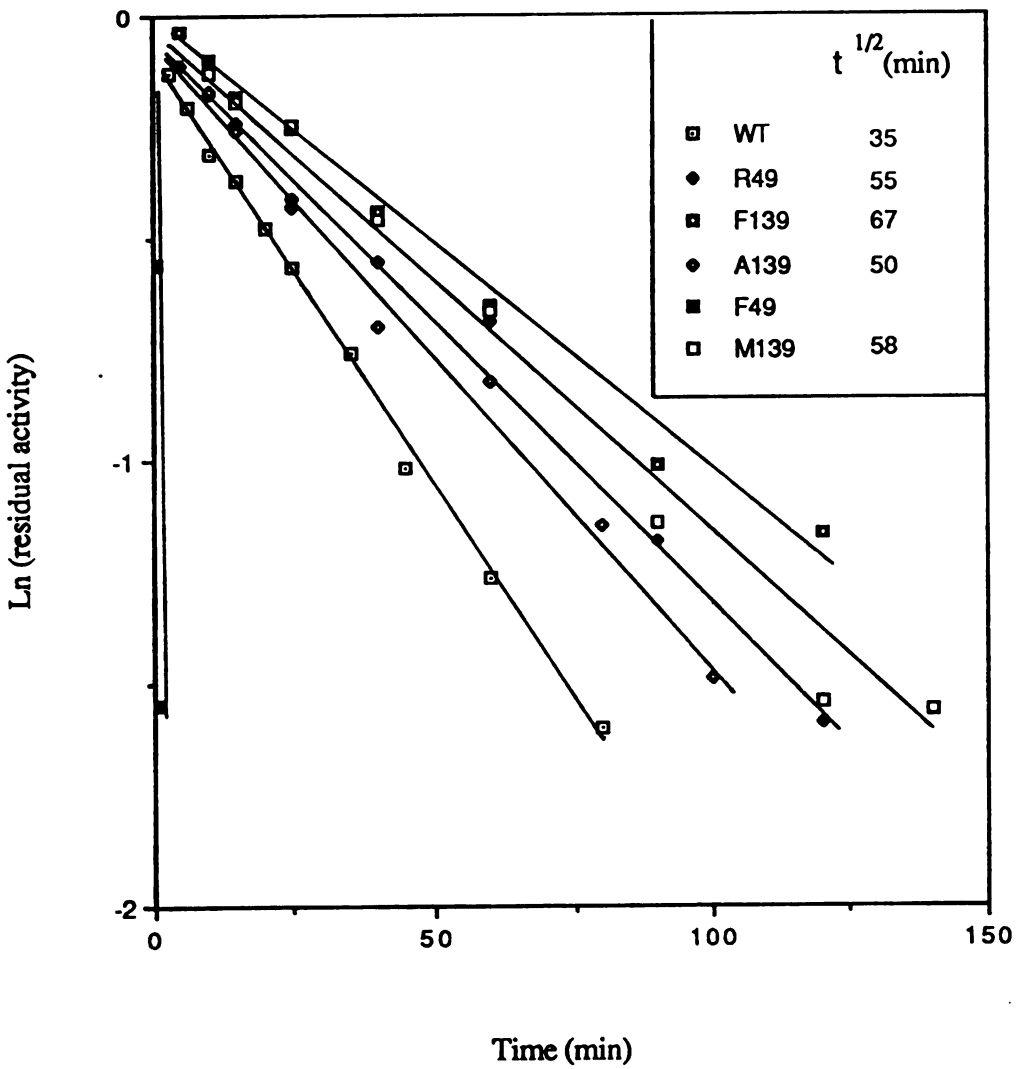
Effect of Mutations on the Thermostability of D-Xylose isomerase. D-xylose isomerase is an enzyme of considerable thermostability in aqueous solutions at neutral pH. This property has been of great advantage in the purification of this

enzyme when it was synthesized in *E. coli*. Incubation of crude cell extract at 75°C precipitated the majority of the host proteins while *Thermoanaerobacterium* xylose isomerase was left as soluble and active enzyme. Among the mutant proteins created in this study, Trp-139 → Lys, Trp-188 → His and Phe-145 → Lys were no longer resistant to the heat treatment at 75°C. This indicates that a disruption of the hydrophobic interactions between aromatic residues in the active site, by substituting them with charged residues, destabilizes the enzyme.

For comparison of the thermostability between wild type and mutant proteins we have determined the time course of inactivation at 85°C. The optimal concentration of $CoCl_2$ for these experiments was found to be 50 mM (data not shown). D-xylose isomerase did not follow the reversible two-state process of thermal inactivation, Native ⇌ Unfolded form. Upon prolonged incubation at 85°C enzymatic activity was lost progressively and was accompanied by precipitation of protein. This irreversible thermoinactivation process followed a first order kinetics (Fig. 3), and the reaction rate constant was independent of the initial protein concentration within the range 50-500 μg/ml (data not shown). From the reaction rate constant, the half-life for each variant xylose isomerase was determined. We tested the thermostability of Trp-139 → Phe, Trp-139 → Met, Trp-139 → Ala, Trp-49 → Arg, Trp-49 → Phe and Trp-49 → Ala. The mutant protein Trp-49 → Ala lost activity and precipitated immediately after heating at 85°C. Trp-49 → Phe was also very unstable with half-life less than 1.5 min. The gain in thermostability of Trp-

Figure 3: Time course of irreversible thermoinactivation of factitious variants of *Thermoanaerobacterium* xylose isomerase.

The half-life ($t^{1/2}$) of enzyme was determined from the equation: $t^{1/2}=(\ln 2)/k$, in which k is the first order rate constant of thermoinactivation.


0
-1
-2
0
50
100
150
Ln (residual activity)
Time (min)
t 1/2 (min)
WT 35
R49 55
F139 67
A139 50
F49
M139 58

49 → Arg mutant suggests again that N^{ε} of Arg may take the position of $N^{\varepsilon 1}$ of Trp-49. The increments in thermostability of Trp-49 → Arg were the same at pH 7.0 and pH 5.0 (data not shown), suggesting that the positive charge of Arg residue may not be the factor responsible for the increase in the thermostability of the mutant enzyme. The results of mutation at position 49 thus suggest that the hydrogen bond between Trp-49 and Asp-339 or/and the hydrophobic side chain provided by Trp-49 are involved in maintaining the active site structure for substrate binding (see effect on K_M in Table 3); this bond is also important in the resistance to thermoinactivation. Substitution of Trp-139 by smaller hydrophobic amino acids enhanced thermostability, indicating that the indole group of Trp-139 does not contribute to the maintenance of the active site structure. In fact, the bulky indole group of Trp-139 has adverse effect on protein thermostability.

DISCUSSION

Comparison of the catalytic efficiency, k_{cat}/K_M, for the two substrates indicated that D-xylose is a much better substrate than D-glucose, and this difference is due mainly to the differences in the Michaelis constant. One of the important mechanisms for *Thermoanaerobacterium* D-xylose isomerase to discriminate between xylose and glucose is the steric hindrance offered by the indole group of Trp-139 against the C6-methanolic group of glucose. When Trp-139 was substituted by amino acids with smaller side chains the catalytic efficiency for glucose increased. We believe that the enlarged active site pocket did not accommodate xylose as well as the pocket of the wild- type enzyme and this resulted in the decrease of the catalytic efficiency for xylose. The correlation of $K_{M(glucose)}$ with the water accessible surface area of the side chain of amino acid at position 139 indicates that this side chain protrudes into the solvent and constitutes the steric hindrance for the binding of glucose.

The indole group of Trp-188 is parallel to the hydrocarbon backbone of the pyranose ring and, in the *Arthrobacter* isomerase, it is in the hydrophobic interaction with this part of the substrate molecule. The 800-fold increase in $K_{M(xylose)}$ caused by the substitution of Trp-188 with His suggests that this hydrophobic interaction contributes very significantly to substrate binding. The indole group may also orient the pyranose ring in a position such that the hydroxyl

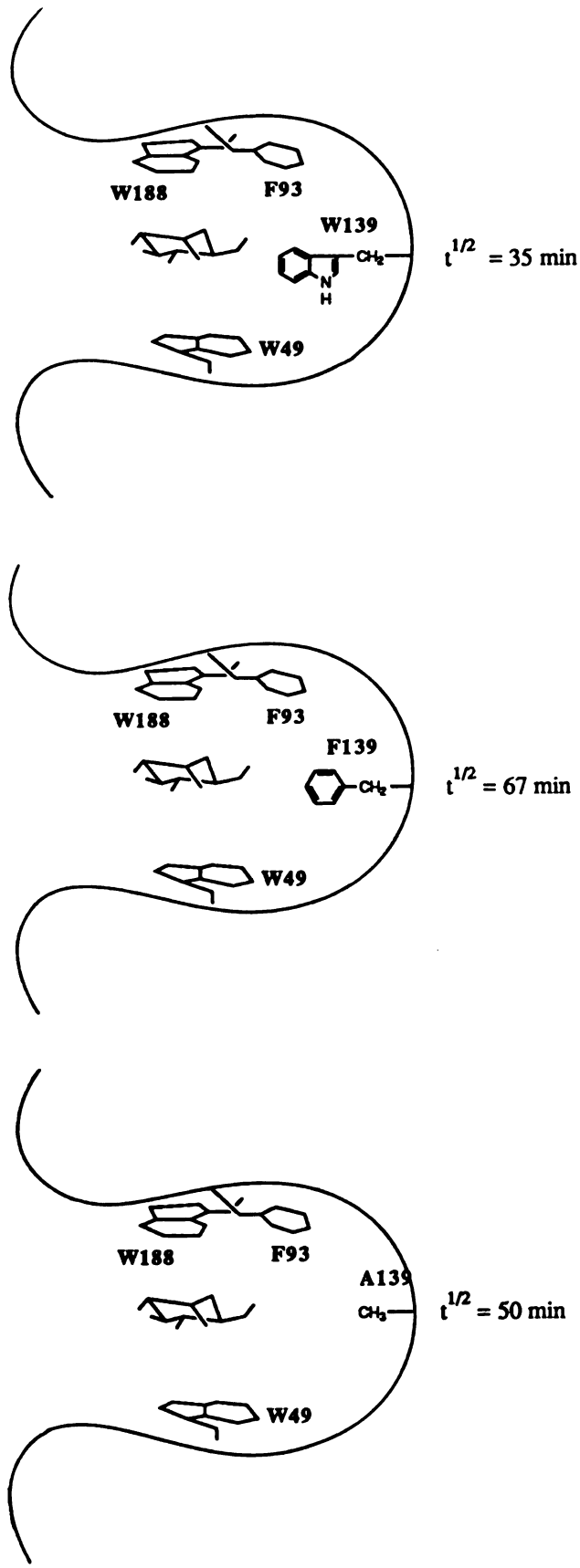
groups of substrate can hydrogen bond to the hydrophilic residues of the active site. In xylose isomerase from *Actinoplanes missouriensis* Trp-137 (corresponding to Trp-188 of *Thermoanaerobacterium* enzyme) has been changed to Phe and the K_M for xylose and glucose have been found to increase 4.4 and 2.7 fold, respectively (Lambeir et al., 1992). The importance of the hydrophobic interaction between tryptophan and sugar for substrate binding has also been found in maltose-binding protein of *E. coli*. Substitution of one of the tryptophan residues in the active site of the maltose binding protein by alanine increased dissociation constant of the enzyme-substrate complex, *Kd*, 67 fold, similar substitution of another tryptophan at this site resulted in a 300 fold increase in K_d (Martineau et al., 1990).

In this work substitution of Phe-145 with Lys increased the $K_{M(\text{xylose})}$ 50 fold. Predictions based on the structure of the *Arthrobacter* isomerase active center indicate that the phenyl group of Phe-145 is perpendicular to the indole group of Trp-188 (Figure 1). Phe-145 might help Trp-188 to maintain a proper position for the interaction with the substrate. Although $K_{M(\text{glucose})}$ increased in both Trp-49 → Phe and Trp-49 → Ala mutant proteins, Trp-49 → Arg exhibited kinetic constants similar to those of the wild type protein. We speculate that Arg-49 may hydrogen bond to Asp-339 which coordinates to metal [I] and thus provide the same function as Trp-49. The primary functional role of Trp-49, therefore, might be to hydrogen bond to Asp-339 and contribute to substrate binding indirectly.

Argos et al. (Argos et al., 1979) have proposed that protein thermal stability

is enhanced when the area of hydrophobic surface in contact with the aqueous solvent is reduced. The thermal stability of lactate dehydrogenase from *Bacillus stearothermophilus* has been enhanced by reduction of the area of a water-accessible hydrophobic surface (Wigley et al., 1987). In this work we examined thermostabilities of Trp-139 → Phe, Trp-139 → Met and Trp-139 → Ala mutant enzymes and found that all of them are more stable than the wild-type enzyme. Since the indole group of Trp-139 protrudes into the solvent and does not contribute to the architecture of the active site, replacement of Trp-139 with Phe, Met or Ala reduced the area of active site hydrophobic surface which is expected to expose to water (Figure 4). This is, therefore, responsible for the enhancement of thermostability in Trp-139 → Phe, Trp-139 → Met and Trp-139 → Ala mutant enzymes. Enhancement of the thermostability in Trp-49 → Arg could be brought about by the same mechanism since Arg-49 could presumably fulfill the function of hydrogen bonding to Asp-339 and thus leave the active site structure unchanged. This explanation is in good agreement with the properties of Trp-49 → Phe mutant which exhibited a reduced thermostability. Phenylalanine would be unable to hydrogen bond with Asp-339 and this substitution would, therefore, be expected to disturb the architecture of the active site. The reduction of thermostability in Trp-188 → His, Trp-139 → Lys and Phe-145 → Lys, observed in this work, may be due to the perturbation of the active site structure. Substitution of the hydrophobic residues involved in architecture of the active site with charged residues could

Figure 4: Schematic illustration of the reduction of hydrophobic surface area as Trp-139 changed to Phe or Ala.


W188
F93
W139
CH_2
N
H
W49
$t^{1/2}$ = 35 min
W188
F93
F139
CH_2
W49
$t^{1/2}$ = 67 min
W188
F93
A139
CH_3
W49
$t^{1/2}$ = 50 min

certainly be expected to destabilize this region of the protein.

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CHAPTER IV

EFFECT OF SALTS ON THERMOSTABILITY OF D-XYLOSE ISOMERASE: THE DOMINANT FACTOR GOVERNING THE PROCESS OF IRREVERSIBLE THERMOINACTIVATION OF *THERMOANAEROBACTERIUM* XYLOSE ISOMERASE

ABSTRACT

The kinetics of thermoinactivation of D-xylose isomerase from *Thermoanaerobacterium thermosulfurigenes* in aqueous solution was investigated. Here we report for the first time that besides well known divalent cations, monovalent cations, particularly K^+, also protects the enzyme against thermoinactivation. The kinetic data suggest that the rate of formation of incorrect conformation of the enzyme ("scrambled structure") is the dominant factor governing the process of thermoinactivation.

INTRODUCTION

D-xylose isomerase (E.C. 5.3.1.5), often referred to glucose isomerase, has been utilized to produce high fructose corn syrup for three decades. The industrial process for this enzymatic conversion is performed at 60-65°C; therefore, the enhancing half-life of this enzyme at elevated temperatures is desirable for practical applications. Xylose isomerase is a homotetramer; each subunit contains an α/β barrel domain. The active site pocket is located at carboxyl end of the β-strand, and involves amino acids from neighboring subunit (Henrick et al., 1989 & Whitlow et al., 1991). Therefore, the basic functional unit of xylose isomerase is a dimer. It was proposed that strengthen interaction in the interface of active dimer may enhance thermostability of the enzyme (Rangarajan, et al., 1992). Divalent cations, such as Co^{+2}, Mn^{+2} or Mg^{+2} are required for both the catalytic activity and the thermostability of D-xylose isomerase. The extent of the effects by these metal ions depends on the origin of the enzyme and the substrate (Chen, 1980). For the enzyme from *Thermoanaerobacterium thermosulfurigenes* Co^{+2} is the best protector (Lee & Zeikus, 1991). To find the optimal conditions for the stabilization of the *Thermoanaerobacterium* xylose isomerase we have examined the effect of different ions on thermoinactivation. The results have indicated some stages of the thermoinactivation process.

MATERIALS AND METHODS

Enzymes. The *Thermoanaerobacterium thermosulfurigenes* gene coding for D-xylose isomerase was expressed in *E.coli* HB101 cells as described previously (Lee et al., 1990). Mutant enzyme, Phe-60 → His, was created and expressed as described in Materials and Methods in Chapter III. The wild type and mutant enzymes were purified through the steps of heat treatment (75°C, 20 min), DEAE-Sepharose and Sephacryl-300 chromatography to the stage of homogeneity in SDS-PAGE (Lee et al., 1990). The metal-free enzyme was prepared by dialyzing the pure enzyme against 100 time volume of 10 mM MOPS buffer (pH 7.0) containing 10 mM EDTA; subsequently, dialyzing against 10 mM MOPS buffer (pH 7.0) to remove EDTA. Dialysis was performed at 4°C and the dialysis buffer was changed every 12 hours for three times. The metal-free enzyme was used for all the experiments. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard (from Pierce).

Buffer. MOPS buffer was used in all the experiments. Solution was adjusted at room temperature to the pH values that would result in pH 7.0 at the temperature of the experiments according to $\Delta pH/\Delta t$ of MOPS buffer which is -0.011 (Dawson, et al., 1986).

Kinetics of Irreversible Thermoinactivation. The time course of irreversible thermoinactivation of D-xylose isomerase was measured by incubating 0.6 ml enzyme solution (in the range of 0.05-0.5 mg/ml in 10 mM MOPS pH 7.0) at the desired temperature for various periods of time and then determining the residual activity at 65°C. In wild type enzyme the inactivation followed first order reaction and the rate constant, k, of irreversible thermoinactivation was obtained by linear regression in semilogarithmic coordinates. In the Phe-60 → His mutant enzyme the activity was persistent within the initial 20 minute then it followed first order inactivation. The rate constant of Phe-60 → His was calculated by taking the data from the first order reaction range. Half-life of enzyme was calculated from the equation: $t^{(1/2)}=(\ln 2)/k$.

Enzyme activity assay. Reaction was started by the addition of glucose (at final concentration of 800 mM) to enzyme solution and runing the reaction at 65°C for 30 min. Fructose formed was determined by cysteine/carbazole/sulfuric acid method (Dishe & Borenfreund, 1951); pure fructose was used as standard. Enzyme concentrations were adjusted such that less than 5% of the original substrate was converted within 30 min, which allowed the determination of initial reaction velocities.

SDS-Polyacrylamide gel electrophoresis. SDS-PAGE was carried out according

to the method of Laemmli (Laemmli, 1970) on 7% separation and 3% stacking gel. Protein samples were dissolved in 56 mM Tris buffer, pH 6.8, containing 9% glycerol and 0.1% SDS and were loaded on the gel without prior heating step at 100°C. After electrophoresis, the protein bands were stained with 0.1% Coomassie blue R-250 in 40% methanol and 10% acetic acid solution.

Scanning calorimetry. Ultrasensitive scanning calorimeter MC-2 (MicroCal, Inc. Northampton, MA) was used in this study. Enzyme samples, (1 mg/ml) in 10 mM MOPS buffer (pH 7.7 at room temperature), containing 50 μM Co^{+2} or 50 μM Co^{+2} + 10 mM KCl, were loaded into the sample cell. Buffer of the same composition without the enzyme was loaded into reference cell as blank. Samples were degassed before loading as described in the manual. The temperature rising rate was 60°C/hr. A buffer baseline was stored and subtracted from the displayed data to obtain the normalized excess-heat-capacity function (NEF) curve.

RESULTS

Irreversible thermoinactivation of *Thermoanaerobacterium thermosulfurigenes* D-xylose isomerase at pH 7.0. Upon heating at elevated temperature, e.g., 56°C, metal-free D-xylose isomerase lost activity progressively. This inactivation followed a first order reaction with $t^{(1/2)}$(half-life)=8 min. An overnight incubation of this inactivated enzyme at 4°C did not restore any of the lost activity. Co^{+2} was known as the best protective agent against thermoinactivation (Lee & Zeikus 1991). Incubation of xylose isomerase, in the presence of Co^{+2}, at pH 7.0 and 85°C also resulted in a progressive inactivation of the enzyme. Such inactivation was accompanied by a significant precipitation of the enzyme and was irreversible. The optimum concentration of Co^{+2} for thermal protection was 50 μM (Table 1). Two putative divalent metal binding sites were assumed in the active site pocket of *Thermoanaerobacterium* enzyme according to the crystal structures of enzymes from *Arthrobacter* and *Streptomyces* (Henrick et al., 1989, & Carrell et al., 1989). Occupancy of these two metal binding sites is believed to be responsible for the enhanced thermostability of the enzyme. Excess amount of Co^{+2} actually has an adverse effect and this may be due to the binding of excess Co^{+2} to other potential binding sites.

The events occurring during irreversible thermoinactivation of enzymes can be classified into (1) covalent changes such as hydrolysis of disulfide bonds,

Table 1: Effect of $CoCl_2$ on the half-life of D-xylose isomerase at 85°C

$CoCl_2$ (μM)	$t^{1/2}$ (min)
12.5	29
25	37
50	39
200	17
800	4

Aqueous enzyme solution (200μg/ml) in 10 mM MOPS buffer (pH 7.0 at 85°C) with various concentration of $CoCl_2$ was incubated at 85°C. Half-life of the enzyme in each concentration of $CoCl_2$ was calculated form the inactivation constant of the first ordered thermoinactivation curve.

peptide bonds and amides (asparagine and glutamine), (2) noncovalent changes such as aggregation of protein and formation of incorrect folded protein (Klibanov, 1983). In a preliminary test the optimum concentration of Co^{+2}, 50 μM, was found to be independent of the initial protein concentration. In order to test whether the precipitation (aggregation) is the dominant factor causing irreversible inactivation of the enzyme at 85°C in the presence of Co^{+2}, the thermoinactivation curves for the enzyme at different initial protein concentrations (50-500 μg/ml) were determined. The results are shown in Figure 1. The inactivation curves all obeyed the first order kinetics with correlation coefficients greater than 0.98. The almost identical inactivation constants (slopes of the lines) for different initial protein concentrations suggest that the dominant factor causing thermoinactivation is monomolecular event. The inactivation constant would have been dependent on the initial concentration if aggregation were the main factor governing thermoinactivation, since aggregation is a multiple molecular event.

The temperature is a critical parameter. Therefore, the temperature dependence of the rate constant of irreversible thermoinactivation of xylose isomerase in the presence of Co^{+2} was examined within the temperature range of 80-90°C. Arrhenius plot (Figure 2) shows an activation energy of 120 kcal/mol. This high activation energy is totally uncharacteristic of a covalent reaction (Tomazic, & Klibanov, 1988). Since aggregation and covalent changes of the enzyme are unlikely to be the dominant factor accounting for the irreversible

Figure 1: Effect of initial protein concentration on the inactivation process of D-xylose isomerase at 85°c

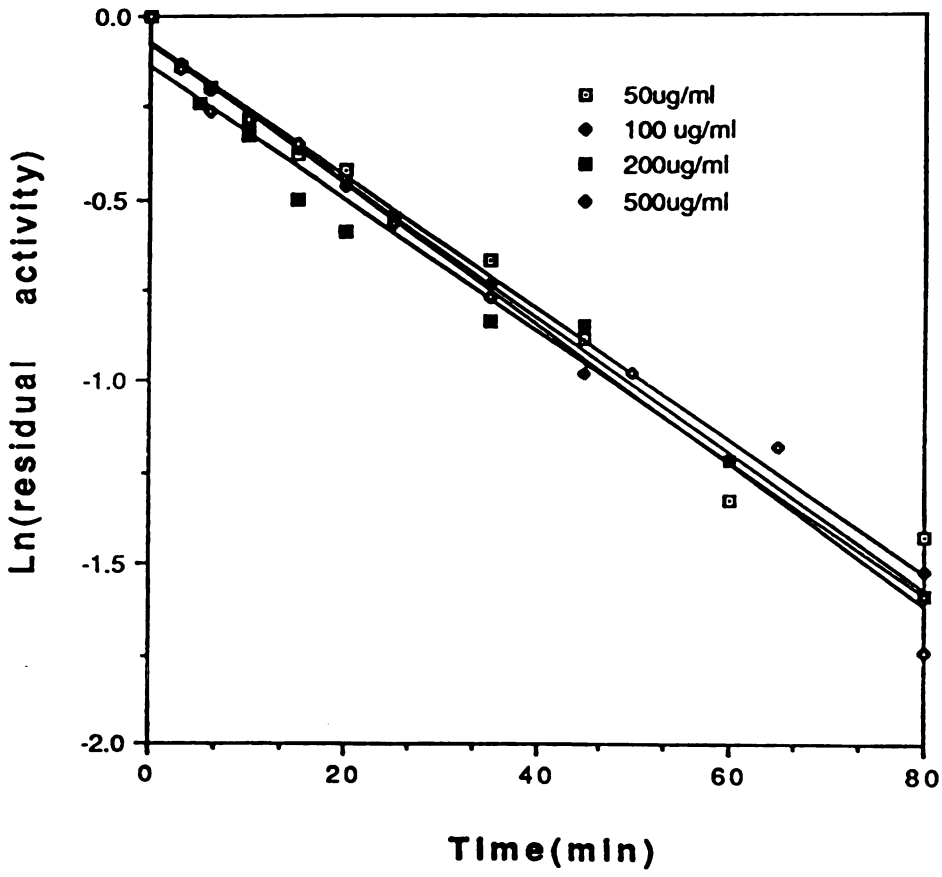
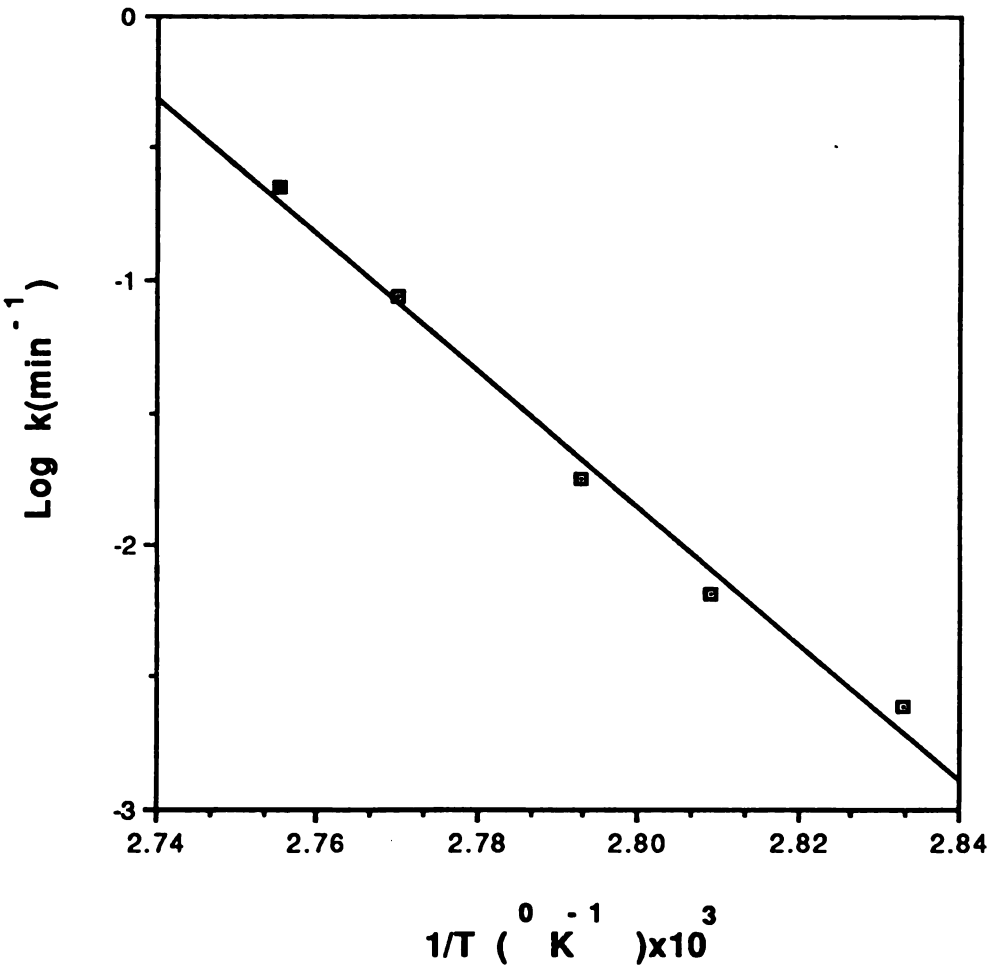

0.0
-0.5
-1.0
-1.5
-2.0
0
20
40
60
80
Ln(residual activity)
Time(min)
50ug/ml
100 ug/ml
200ug/ml
500ug/ml

Figure 2: Arrhenius plot of thermoinactivation of D-xylose isomerase

Thermoinactivation constant, *k*, of xylose isomerase at desired temperature was determined from slope of the irreversible thermoinactivation plot of the enzyme at that temperature. Temperature was in the range of 80-90°C.


0
-1
-2
-3
Log k(min^{-1})
2.74
2.76
2.78
2.80
2.82
2.84
1/T (^{0}K^{-1})x10^{3}

thermoinactivation of xylose isomerase, the main factor could be the formation of incorrectly folded enzyme which is followed by the precipitation of protein.

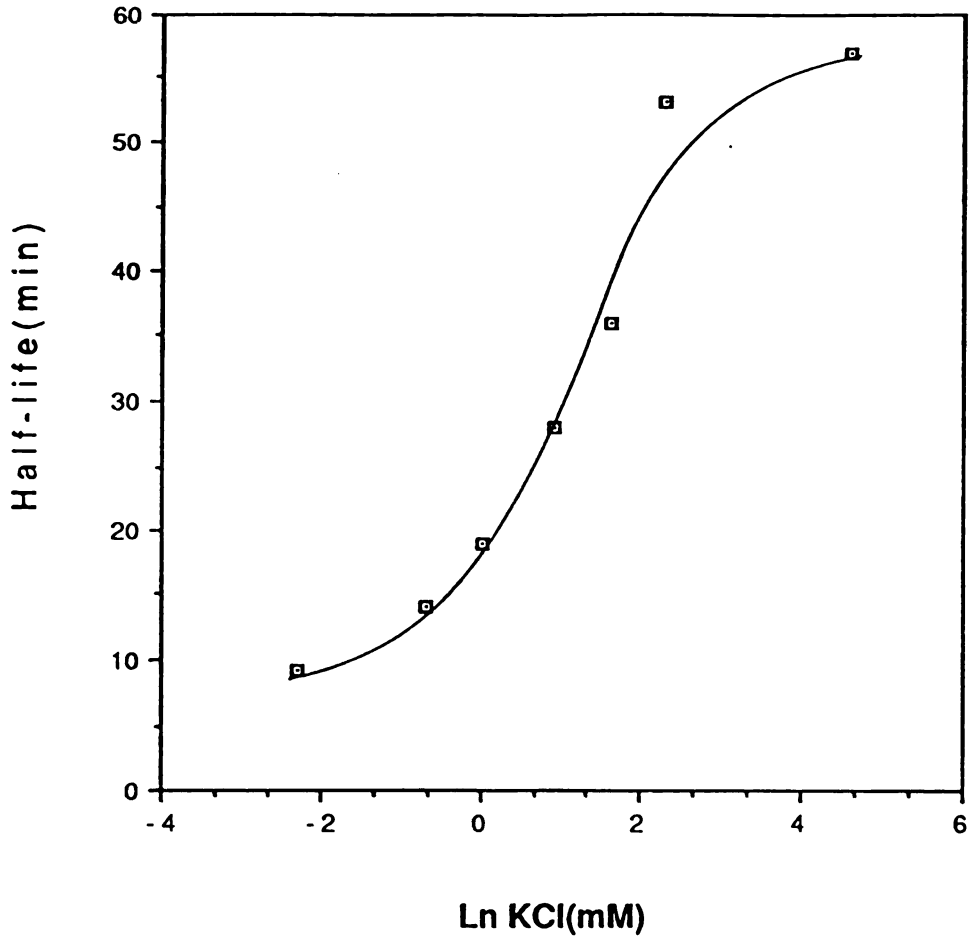
Effect of salts on thermostability of D-xylose isomerase. Based on the empirical observation that the enzyme in crude cell extract is more stable than in homogeneous buffer solution, we decided to test the effect of salts on thermostability of the enzyme. Xylose isomerase (100 μg/ml) in 10 mM MOPS buffer (pH 7.0) containing 50 μM Co^{+2} and various salts was incubated at 85°C for 45 min. The residual activities were determined and the results are shown in Table 2. Except for $Al_2(SO_4)_3$ and tetramethylammonium chloride, all salts added had positive effect on thermostability. However, the most prominent effects came from KCl, KNO_3, CsCl, $(NH_4)_2SO_4$, and NH_4Cl. This enhancement of thermostability could not be the result of a general salt effect because (1) different salts protected to different extent, e. g. the effect of K^+ was more prominent than that of Na^+, (2) As low as 10 mM of salts was enough for protection, (3) tetramethylammonium chloride did not show protection effect. If enhancement of thermostability were due to general salt effect, tetramethylammonium chloride should have had the same effect as K^+. The dependence of half-life of xylose isomerase on K^+ concentration at 88°C is shown in Figure 3. The relationship of K^+ and half-life of the enzyme at 88°C is sigmoid. Seven-fold enhancement in thermostability was achieved at 10-100 mM of K^+. The stabilizing effect of K^+ could also be demonstrated on the

Table 1: Effect of salt on thermostability of wild-type D-xylose isomerase

Salt	Residual activity (%)[a]
Control[b]	38
LiCl (10mM)	62
NaCl (10mM)	50
NaCl (100mM)	69
Na_2SO_4 (3.3mM)	42
KCl (10mM)	80
KCl (100mM)	84
KNO_3 (10mM)	82
CsCl (10mM)	79
$(NH_4)_2SO_4$ (3.3mM)	81
NH_4Cl (10mM)	79
$MgCl_2$ (3.3mM)	57
$Al_2(SO_4)_3$ (0.33mM)	15
Tetramethylammonium chloride (10mM)	33

Aqueous enzyme solution (100μg/ml) containing 50μM $CoCl_2$, 10mM MOPS buffer (pH 7.0 at 85°C) and various salt was incubated at 85°C for 45 min. Enzyme activities (before heating and after heating) were assayed at 65°C for 30 min as described in Materials and Methods.
a: Residual activity is expressed as the percentage of activity left after 85°C incubation. b: Control means what only contained $CoCl_2$ and MOPS buffer.

Figure 3: Effect of KCl on half-life of D-xylose isomerase at 88°C

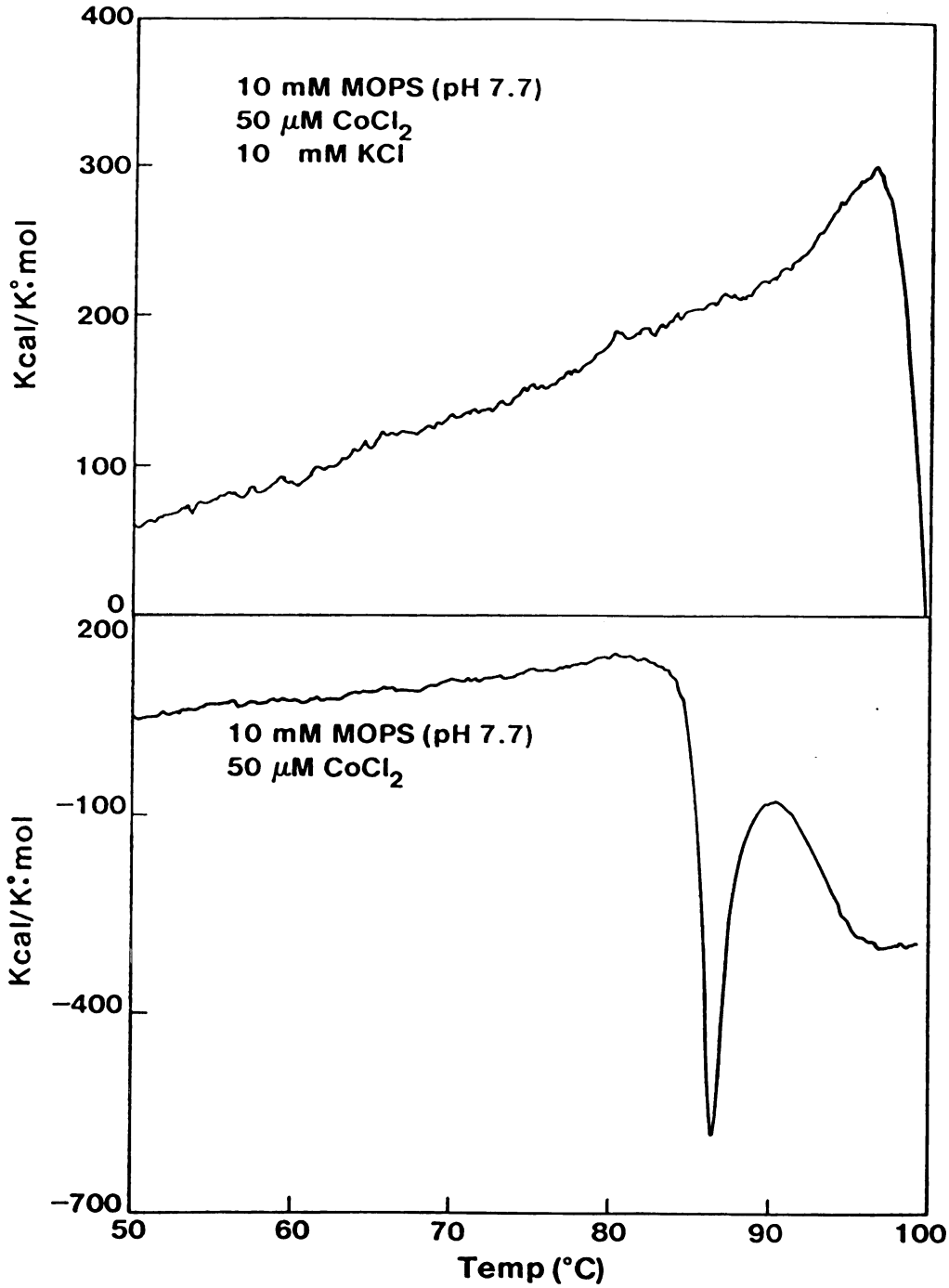

60
50
40
30
20
10
0
Half-life(min)
-4
-2
0
2
4
6
Ln KCl(mM)

apoenzyme (the enzyme containing no Co^{2+}). Half-life increased from 8 min to 75 min when the apoenzyme, in 10 mM MOPS buffer (pH 7.0), was incubated at 56°C in 100mM K^+.

To determine the changes of energy constants during the process of thermoinactivation, purified enzyme was subjected to scanning microcalorimetry. The temperature dependence of specific heat capacity is shown in Figure 4. Xylose isomerase, instead of showing a typical unfolding peak, precipitated at a certain temperature; therefore, it was impossible to calculate the changes of energy constants during themoinactivation. However, the precipitation temperature still provides some information about relative thermostability of the enzyme. In the presence of 50 μM of Co^{+2} the enzyme started to precipitate at 84°C; whereas, it became more resistent to heat in the presence of 10 mM of K^+ + 50 μM of Co^{+2} and started to precipitate at 96°C. Since K^+ is not expected to change the rate of covalent changes of the protein, the effect of K^+ on thermostability also suggests that the rate-determining event in the process of thermoinactivation is the formation of incorrectly folded protein.

Effect of substitution of Phe-60 by His on thermostability. A cluster of aromatic amino acids, Trp-15, Phe-93, Trp-136 and Phe-25 (from the neighboring subunit) is present in the active site pocket of xylose isomerase from *Streptomyces* (Whitlow et al., 1991) and *Arthrobacter* (Collyer et al., 1990). These amino acids

Figure 4: Differential scanning microcalorimetric plots of D-xylose isomerase


400
300
200
100
0
10 mM MOPS (pH 7.7)
50 μM CoCl2
10 mM KCl
Kcal/K°mol
200
−100
−400
−700
10 mM MOPS (pH 7.7)
50 μM CoCl2
Kcal/K°mol
50
60
70
80
90
100
Temp (°C)

are conserved in all known xylose isomerases. The corresponding amino acids in *Thermoanaerobacterium* enzyme are Trp-49, Phe-145, Trp-188 and Phe-60 (from the neighboring subunit). The hydrophobic interaction among these aromatic amino acids was postulated to be one of the important forces that keep the monomers associated in an active dimer. Rangarajan et al., working on stability of *Arthrobactor* xylose isomerase, proposed that strengthening the interactions at the interface of active dimer by protein engineering should increase thermostability (Rangarajan, et al., 1992). To test the significance of these putative hydrophobic forces at the interface of active dimer, Phe-60 of the *Thermoanaerobacterium* enzyme was changed to His. The activity of this mutant enzyme, Phe-60 → His, dropped to approximately 20% of the wild type enzyme (see Table 3 in Chapter III). Preincubation of Phe-60 → His mutant enzyme in 10 mM MOPS buffer at 85°C for 5 min increased the activity two-fold. The strength of active dimer was tested by incubation of the enzyme in 0.1% SDS at 50°C followed by SDS-PAGE. Surprisingly, it was found that the interaction of monomers in the dimer was stronger in Phe-60 → His mutant than in wild-type enzyme (Figure 5). The reason for this difference is difficult to interpret without crystal structures of the enzymes. Besides the cluster of aromatic amino acids, there are several hydrophilic amino acids, such as glutamic and aspartic acid that line up the active site surface. His-60 (from neighboring subunit) may interact with one of these hydrophilic amino acid residues. Time course of thermoinactivation of Phe-60 → His at 85°C is shown in

Figure 5: SDS-PAGE of wild-type and Phe-60 → His mutant xylose isomerase.

Enzyme (13 μg) was incubated in 0.1% SDS at 50°C for 0, 10, 20 min. Afterward, it was loaded into gel without prior boiling at 100°C. Maker is the high molecular weight standards from Biolab. Letter A, B and C represent wild-type, Phe-60 → His and Trp-139 → Phe, respectively. Number 1, 2 and 3 represent incubation time 0, 10 and 20 min, respectively.

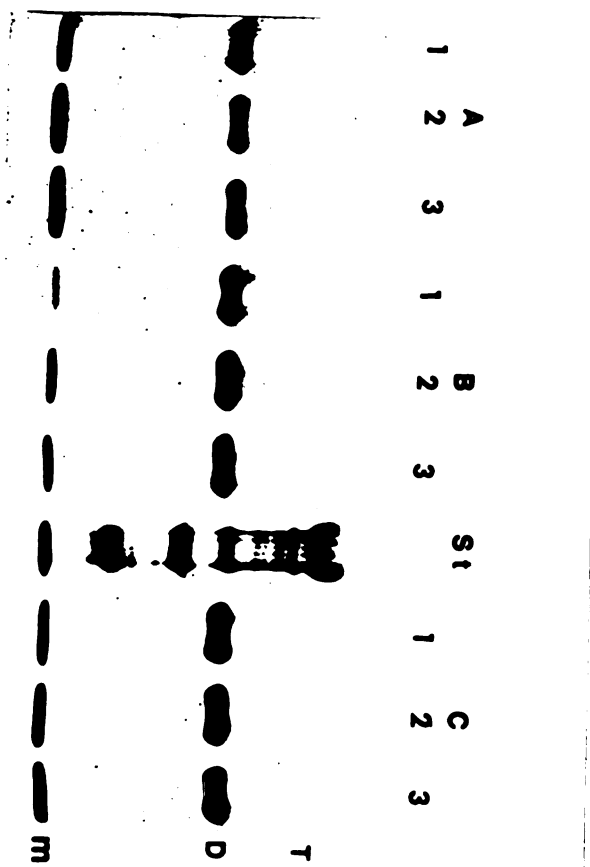
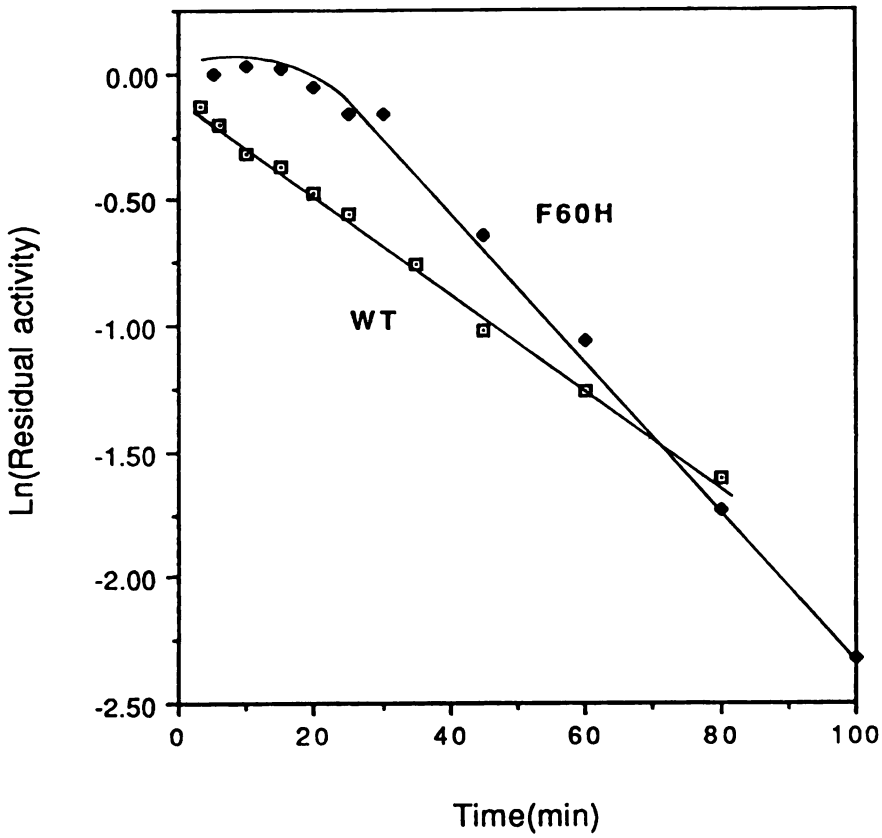

A
1
2
3
B
1
2
3
St
C
1
2
3
m
D
T

Figure 6. Unlike wild-type enzyme, this mutant enzyme did not obey the first order kinetics. It is resistant to 85°C during the initial 20 min then it follows the first order inactivation with a half-life 25 min. The stronger interaction of active dimer does not seem to slow down the rate of inactivation in the stage which proceeds with the first order kinetics. However, it does delay start of the process of thermoinactivation.

Figure 6: Time course of irreversible thermoinactivation of wild-type and Phe-60 →His mutant enzymes at 85°C.


0.00
-0.50
-1.00
-1.50
-2.00
-2.50
Ln(Residual activity)
F60H
WT
0
20
40
60
80
100
Time(min)

DISCUSSION

It has been well documented that the binding of divalent cations, such as Mg^{+2} and Co^{+2}, to xylose isomerase remarkably enhance stability of the enzyme against heat and other denaturing agents (Kasumi, et al., 1982; Callens, et al., 1988; Lee, & Zeikus, 1991; Gaikwad, et al., 1992; Rangarajan, et al., 1992). In this study we report for the first time that some monovalent ions, such as K^+, can enhance thermostability of *Thermoanaerobacterium* xylose isomerase and this stabilization is not due to a general salt effect. Such effect of K^+ was also shown on a commercial glucose isomerase (Spezyme GI); 100 mM KCl increased half-life of Spezyme (in the presence of 50 μM Co^{+2}) from 350 min to 590 min at 85°C.

Upon heating at elevated temperature *Thermoanaerobacterium* xylose isomerase undergoes an irreversible thermoinactivation that runs with the first order kinetics. The independence of inactivation constant on the initial protein concentration and a very high activation energy suggest that the rate-determining step during the process of thermoinactivation is the formation of incorrectly folded enzyme. K^+ ions probably bind to the enzyme and stabilize the correct folding. Substitution of Phe-60 with His made the active dimer more resistent to thermal denaturation. However, such stronger monomer interaction did not slow down the rate of thermoinactivation. This indicates that dissociation of monomers is not involved in the rate-determining event.

It is impossible to conclusively define a pathway describing the events during thermoinactivation process with this limited information. However, a simplified model is proposed based on results obtained in this work.

tetramer ⇌ active dimer ⇌ monomer → incorrect folded monomer → aggregation

Dissociations of the tetramer to active dimer and active dimer to monomer are reversible. The irreversible formation of incorrectly folded monomer from the native monomer is the rate-determining event and it is followed by aggregation of protein and precipitation. K^+ might bind to the protein and stabilize the correct form of monomer and, therefore, reduce the rate of formation of incorrectly folded polypeptide. Strengthened interaction at the subunit interface of the dimer could not slow down the overall rate of inactivation as indicated by Phe-60 → His mutant enzyme.

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CHAPTER V

CONCLUSION

To apply a protein engineering approach to elucidate the structure/function relationship of a protein, one of the most important requisites is the availability of structural information of this protein. Although the structure of xylose isomerase from *T. thermosulfurigenes* has not been available, the crystal structures of the enzyme from *Arthrobacter* and *Streptomyces* species were used as guidance to conduct site-directed mutagenesis in this study. Based on such strategy we were able to obtain following achievements : (1) proposed the mechanism of enzymatic reaction based on the understanding of the functions of active-site residues involved in catalysis and metal coordination. (2) elucidated the roles of active-site aromatic amino acids on substrate binding and understood the structural basis of the enzyme for discrimination between glucose and xylose. (3) created mutant enzymes which are more applicable to industrial process in that they are more resistant to thermoinactivation and have higher catalytic efficiencies, k_{cat}/K_M, toward glucose by rational redesign of the active-site pocket. However, such strategy has its limitation due to the rare homology of amino acids outside the active-site pocket so that mutation could only be performed at the conserved active-site amino acids. Crystallographic study of *T. thermosulfurigenes* xylose isomerase has been undertaken at the laboratory of D. M. Blow in Imperial College (London, England). The crystal structure of the enzyme will, hopefully, be available soon. Such information will be of great advantage on the following aspects: (1) to confirm the interpretations of mutational results of this research. (2) to broad the target sites for

further site-directed mutagenesis experiments such as that of enhancing thermostability or changing metal specificity of the enzyme.

Protein engineering has being used to increase the stability of enzymes. The common approaches include (1) reducing the difference in entropy between folded and unfolded protein, which in practice means reducing the number of conformations in the unfolded state (Matsumura et al., 1989; Matthews et al., 1987), (2) stabilizing the dipoles of α helices, (Nicholson et al., 1988) (3) increasing the number of hydrophobic interactions and packing ratio in the interior core (Sandberg & Terwilliger 1989). There is an example for enhancing thermostability of xylose isomerase by protein engineering. The enzyme from *Actinoplanes missouriensis* has been engineered to enhance its thermostability by substituting Arg for Lys-253 which is the major glycation site in the presence of high concentration of glucose (Quax et al., 1991). Due to the limited information regarding the structure of *T. thermosulfurigenes* xylose isomerase, the effort, in this study, for enhancing thermostability of the enzyme could only be focused on the mutations of active-site amino acids. With crystal structure of *T. thermosulfurigenes* enzyme at hand in the future, the battle field for site-directed mutagenesis will be able to expand to the whole structure.

Considering the applicability of *T. thermosulfurigenes* xylose isomerase, another challenging, also obligatory, objective will be the alteration of metal specificity. Wild-type *T. thermosulfurigenes* enzyme requires Co^{+2} for both

catalytic ability and thermostability. However, Co^{+2} is an environmental hazard and can not be used in food process. Mg^{+2} and Mn^{+2} can only partially fulfill the function of Co^{+2} (Lee & Zeikus, 1991). Alteration of the metal requirement from Co^{+2} to Mg^{+2} or to Mn^{+2} by engineering the metal binding sites could be feasible if the detail structure is provided.

Although *T. thermosulfurigenes* xylose isomerase (belongs to class II) is not the enzyme currently used for the production of high fructose corn syrup, the discoveries in this research are still applicable to the engineering of commercial xylose isomerases (belong to class I). We showed that the indole group of Trp-139 causes steric hindrance against glucose binding in *T. thermosulfurigenes* enzyme and a correlation between $KM_{(glucose)}$ and the size of hydrophobic side chain of amino acid in position 139 exists. Instead of tryptophane, a methionine presents in the corresponding position in class I xylose isomerase . We believe that $KM_{(glucose)}$ of class I enzymes can be reduced if this methionine is replaced with a smaller hydrophobic amino acid such as valine, or alanine. Also substitution of Val-134 with threonine may introduce a hydrogen bond between enzyme and glucose and therefore could be helpful for the decrease of $KM_{(glucose)}$ in class I enzymes. In respect of enhancing thermostability, reducing the area of water-accessible hydrophobic surface by substituting Arg for Trp-15 in class I enzymes may be is an useful approach.

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