Arthrobacter D-xylose isomerase: protein-engineered subunit interfaces

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Mutants of *Arthrobacter* D-xylose isomerase were constructed in which one or two disulphide bridges or additional salt bridges were introduced at the A–A* subunit interfaces. These showed no change in enzyme activity or stability compared with the wild-type enzyme. However, a Tyr²⁵³ mutant in which a disulphide bridge was introduced at the A–B* subunit interface showed reduced thermostability that was identical in both oxidized and reduced forms, and also reduced stability in urea. X-ray-crystallographic analysis of the Mn²⁺-xylitol form of oxidized Y253C (the Tyr²⁵³ → Cys mutant) showed a changed conformation of Glu¹⁸⁵ and also alternative conformations for Asp²⁵⁴, which is a ligand to the Site-[2] metal ion. With fructose, Mg²⁺-Y253C has a

similar K_m to that of the wild-type, and its V_{max} is also similar below pH 6.4, but declined thereafter. In the presence of Co²⁺, Y253C has lower activity than wild-type at all pH values, but its activity also declines at alkaline pH. These results suggest that electrostatic repulsion from the new position of Glu¹⁸⁵ causes Asp²⁵⁴ to move when His²¹⁹ is unprotonated, thereby preventing M²⁺ binding at Site [2]. These results also suggest that subunit dissociation does not lie on the pathway of thermal inactivation of D-xylose isomerase, but that movements of active-site groups are a trigger for conformational changes that initiate the unfolding process.

INTRODUCTION

Previous studies of *Arthrobacter* D-xylose isomerase include its purification and properties (Smith et al., 1991), the cloning and expression of the structural gene in either *Arthrobacter* or *Escherichia coli* hosts (Loviny-Anderton et al., 1991), the tertiary structure of the Mg²⁺-enzyme at 0.23 nm (2.3 Å) resolution (Henrick et al., 1989) and studies of the mechanism by X-ray crystallography (Collyer et al., 1990), molecular-dynamics simulation (Smart et al., 1992) and enzyme kinetics (Rangarajan and Hartley, 1992).

As a background to improvements that might be made by protein engineering to improve its thermostability as a commercial glucose isomerase for production of high-fructose syrups, the stability to denaturants and/or heat in presence of various ligands was also studied (Rangarajan et al., 1992). The tertiary structure reveals that the tetramer could dissociate into three possible dimer conformations, named A–A*, A–B and A–B* (Figure 1), and that residues from each subunit contribute to the active site which lies at the A–B* interface (Henrick et al., 1989). Urea at room temperature causes rapid reversible dissociation of the Mg²⁺-enzyme into fully active A–B* dimers with midpoint around 4 M urea. In 8 M urea at higher temperatures these partially unfold to give an Mg–A–B* dimer intermediate with 50% activity, followed by irreversible inactivation coincident with the appearance of unfolded monomer. In 0–1 M guanidinium chloride, a similar reversible dissociation into active dimers occurs, but A–A* and/or A–B dimers seem also to be part of the mixture. Low concentrations of SDS also give active dimers, leading to unfolded monomers, but SDS above 1% (w/v) provides relative stabilization.

It therefore appeared that thermal unfolding might follow a similar pathway of dimerization followed by monomerization. In

that case, introduction by protein engineering of disulphide bridges or salt bridges at each of the subunit interfaces should strap these together and increase thermostability. The present paper reports the effect of such mutations on activity and thermostability.

MATERIALS AND METHODS

Unless otherwise stated, all materials and methods were as described by Smith et al. (1991) and Loviny-Anderton et al. (1991).

Figure 1 Subunit conformation and interface mutants of D-xylose isomerase

The four subunits, composed of a β -barrel (circle) and a C-terminal tail (oval) are separately shaded and labelled at their active sites. The approximate location of the four interface mutants are shown as: a, Y253C, A–B* disulphide bridge; b, A223C, A–A* disulphide bridge; c, L199C + A200C, pair of A–A* disulphide bridges; d, E203D + Q204R, pair of A–A* salt bridges.

Abbreviations used: Y253C, D-xylose isomerase mutant in which Tyr²⁵³ is changed to Cys (similarly with other mutants); ss, single-stranded; 6 × SSC, 0.09 M sodium citrate/0.9 M NaCl adjusted to pH 7 with NaOH; DTT, dithiothreitol; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); r.m.s., root mean square.

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Expression of the *Arthrobacter xylA* gene in plasmid pAXI 3A.00

The phagemid pTZ 19U (Mead et al., 1986) is convenient for protein-engineering work as the vector can be obtained as single-stranded DNA (ssDNA) on infection with a helper phage. Additionally the high copy number and strong promoter allow a high expression level of inserted genes. Plasmid pAXI 3A.00 is a construct featuring the *xylA* gene cloned into the *Sma*I site of the phagemid. *Escherichia coli* JM 101 transformants express the enzyme as 5–10% of soluble protein in cell extracts (Loviny-Anderton et al., 1991).

Site-directed mutagenesis

All mutant genes were made directly in plasmid pAXI 3A.00 using mutagenic oligonucleotides synthesized by Mr J. Knill-Jones (Department of Chemistry of this College) using an Applied Biosystems DNA synthesizer. After deprotection in ammonia overnight, they were purified on Pharmacia Mono-Q HR 5/5 columns, eluted with a gradient of 0–1.1 M NaCl in 0.1 M NaOH, phosphorylated as described by Zoller and Smith (1982) and stored as 5 pmol/ μ l stock solutions at -20°C .

Site-directed mutagenesis was performed using the phagemid *in vitro* Mutagenesis Kit (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.), which employs the method of Kunkel (1985).

Hybridization screening of transformants

Putative mutant colonies were plated on LB/ampicillin, and individual clones were lifted on to nylon membrane filters and lysed on Whatman 3MM paper pads soaked in 1.5 M NaCl/0.5 M NaOH for 7 min. The filters were neutralized on pads soaked in 1.5 M NaCl/0.5 M Tris/HCl, pH 7.5, air-dried and u.v.-irradiated for 3–5 min to fix the DNA.

Radiolabelled probes were made by incubating 5 μ l (25 pmol) of the oligonucleotide, 3 μ l of [γ - ^{32}P]ATP containing 30 μCi (Amersham International, Aylesbury, Bucks., U.K.), 2 μ l of T4 polynucleotide kinase, 5 μ l of kinase buffer [1 M Tris (pH 8.0)/100 mM MgCl_2 /70 mM dithiothreitol] and 36 μ l of sterile water at 37°C for 30 min, followed by incubation at 70°C for 10 min to inactivate the kinase. Non-incorporated radionucleotide was removed using Pharmacia Nap-5 columns according to the manufacturer's instructions (Pharmacia P-L Biochemicals, Milton Keynes, Bucks., U.K.).

Filters were prehybridized as described in Sambrook et al. (1989) for 1 h at 67°C before adding the labelled oligonucleotide. Annealing was carried out at 67°C for 20 min, followed by overnight cooling to room temperature. The filters were washed three times with $6\times$ SSC buffer (0.09 M sodium citrate/0.9 M NaCl adjusted to pH 7.0 with NaOH), dried on Whatman 3 MM paper, wrapped in clingfilm and autoradiographed at -70°C for 1–2 h. The washing temperature to distinguish wild-type and mutant colonies was chosen as described by Suggs et al. (1981). The identity of mutant colonies was confirmed by sequencing single-stranded phagemid DNA using the dideoxy-chain-termination method of Sanger et al. (1977).

Construction of mutant genes

The following mutagenic oligonucleotides were used (changed nucleotides are indicated in **bold**):

- 5' -CTCGATGAA-**ACAGCA**-GCCGTGGCC- 3' Leu¹⁹⁹ $\rightarrow$ Cys + Ala²⁰⁰ $\rightarrow$ Cys
- 5' -GTTCA**GGCCACA**-

CATCTGCTC- 3'	Ala ²²³ $\rightarrow$ Cys
3. 5' -GTCCTGGTCAC-
ACTTGATGCC- 3'	Tyr ²⁵³ $\rightarrow$ Cys
4. 5' -GTGCTCCAGCCGG-
TCGATGAAGGC- 3'	Glu ²⁰³ $\rightarrow$ Asp + Gln ²⁰⁴ $\rightarrow$ Arg

Purification of wild-type and mutant enzymes

Wild-type and mutant D-xylose isomerases were purified from *E. coli* transformants and assayed for isomerase activity as described by Smith et al. (1991), except that degassed buffers containing 1 mM dithiothreitol were used for enzymes purified in the thiol form. In all cases the behaviour and yields of the mutant enzymes resembled those with wild-type. The apoenzyme and Co²⁺-enzyme were prepared as described by Rangarajan and Hartley (1992).

Enzyme assays and kinetics

D-Xylose isomerase assays used the NADH-linked arabitol dehydrogenase assay method described by Smith et al. (1991). One unit of xylose isomerase activity is the amount of enzyme that converts 1 μ mol of D-xylose into D-xylulose in 1 min. Kinetic studies of the rate of formation of D-glucose from D-fructose in Mes, Mops or Hepes buffers were by a colorimetric GOD-PERID assay kit (BCL, Lewes, East Sussex, U.K.) containing glucose oxidase, peroxidase and diammonium 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate as described in Rangarajan and Hartley (1992). One unit of glucose isomerase activity is the amount of enzyme that forms 1 μ mol of D-glucose/min.

Values of apparent K_m of the Mg^{2+} -enzyme were derived from data with 0.04–3 M D-fructose at $30\pm 0.1^{\circ}\text{C}$, using non-linear regression analysis by R. J. Leatherbarrow's ENZFITTER program (Biosoft, Cambridge, U.K.). For simple measurements of maximal velocities of Mg^{2+} -D-xylose isomerase, 3.2 M D-fructose was used. Values of V_{max} obtained in this way were corrected for any variation in $K_{m(\text{app})}$ (Rangarajan and Hartley, 1992).

Electrophoresis and enzyme assays in the presence of urea

Electrophoresis in the presence of 8 M urea, pH 8.8, was performed in 7.5% (w/v) polyacrylamide gels by the method of Marshall and Inglis (1987) and D-xylose isomerase activity in gels was detected as described by Smith et al. (1991). Urea-gradient-gel electrophoresis (Creighton, 1979) and enzyme assays in the presence or absence of urea were carried out as in Rangarajan et al. (1992).

Measurement of thermostability

'Melting-point' experiments were performed in evacuated and sealed glass tubes in 5 mM MgCl_2 /20 mM potassium phosphate buffers at a concentration of 1 mg/ml enzyme at pH 6.5 or pH 8.0 as described by Rangarajan et al. (1992).

Determination of free thiol groups in mutant proteins

Mutant proteins purified in the thiol form were subjected to gel filtration at 4°C using Sephadex G-25 columns (10 ml) equilibrated in 0.05 M Tris/HCl (pH 8.0)/0.01 M MgCl_2 buffers to remove DTT (dithiothreitol). Portions containing 1 mg of protein in 50 μ l of buffer were incubated at 4°C for several days. The concentration of thiol groups remaining was measured. A 1 mg portion of protein was heated in 950 μ l of degassed 0.1 M phosphate buffer (pH 7.5)/0.001 M EDTA/1% (w/v) SDS at

95 °C for 5 min. Samples were cooled to room temperature, and 50 µl of 0.003 M Nbs₂ [5,5'-dithiobis-(2-nitrobenzoic acid)] in water was added and mixed thoroughly. The absorbance at 412 nm was measured after 15 to 20 min. Wild-type *Arthrobacter* D-xylose isomerase was used as a control.

X-ray crystallography

Metal-free crystals of the apo form of oxidized Y253C (Tyr²⁵³ → Cys) mutant enzyme were grown under similar conditions to those used for the native enzyme (Akins et al., 1986), and were of the same form as native crystals [space group P3₁21, *a* = 10.6 nm (106 Å), *c* = 15.3 nm (153 Å), with the asymmetric unit containing two subunits of the tetramer]. Crystals were initially harvested into 2.8 M (NH₄)₂SO₄ solution, saturated with thymol and buffered to pH 7.8 with 50 mM triethanolamine. Crystals were subsequently transferred to a similarly buffered solution containing 3.2 M ammonium sulphate/50 mM MnSO₄/5 mM β-mercaptoethanol, saturating thymol and 1.0 M xylitol, which is an acyclic polyol analogue of D-xylose/xylulose and a potent inhibitor of the enzyme. Following a 48 h soak, a single crystal was mounted directly from the soak solution into a capillary tube, which was maintained at 21 °C during the diffraction experiment. X-ray-diffraction measurements to 0.25 nm (2.5 Å) spacing were recorded on film at the synchrotron radiation source, Daresbury, as previously described (Henrick et al., 1989). The CCP4 suite of programs was used for data processing and reduction.

An initial molecular model was constructed by removing the substrate and the side-chain atoms beyond Cβ of residue 253 from each subunit of a model previously refined by Collyer et al. (1990). A difference electron-density map at 0.25 nm (2.5 Å) calculated using Fourier coefficients $F_{Y253C} - F_{wild-type}$ and wild-type phases allowed manual improvement of the starting model. Co-ordinate refinement was carried out using the restrained least-squares of refinement program PROLSQ (Hendrickson and Konnert, 1980) and was alternated with rounds of manual intervention. Refinement proceeded until all uninterpretable difference electron density was less than four times the root-mean-square (r.m.s.) deviation from mean density.

The crystallographic merging *R*-factor is defined as:

$$\frac{\sum_h \sum_i I_{h,i} - I_h}{\sum_h \sum_i I_{h,i}}$$

where $I_{h,i}$ is the *i*th measurement of the intensity of reflection *h* and I_h is the mean of these measurements.

RESULTS

Structural modelling of subunit-interface mutants

The target was to increase interactions at the subunit interfaces to reduce dissociation into dimers and monomers that appeared to lie on the unfolding pathway (Rangarajan et al., 1992). A search of refined protein structures using the IDITIS structure and sequence database (Thornton and Gardner, 1989) revealed that the distance between the β-carbons of disulphide bridges is generally in the range 0.36–0.4 nm (3.6–4.0 Å). The D-xylose isomerase crystal structure (Henrick et al., 1989) was scanned to find inter-subunit β-carbon distances of this magnitude. Then disulphide bridges were modelled at those sites that had plausible dihedral angles and minimum steric constraints with adjacent structures. Three strong candidates emerged, whose approximate locations are shown in Figure 1 and whose structures are shown in Figure 2.

(1) Leu¹⁹⁹ → Cys and Ala²⁰⁰ → Cys (L199C + A200C)

These provide two bridges at the A–A* interface with minimum steric constraints.

(2) Ala²²³ → Cys (A223C)

This residue lies on a symmetry axis, so could form a disulphide bridge in which the two additional sulphur atoms are comfortably accommodated at the A–A* interface.

(3) Tyr²⁵³ → Cys (Y253C)

This lies on a symmetry axis at the A–B* interface and elimination of the bulky aromatic side chains allows ample room for the two S atoms. However it leaves more of a hole than the other two.

(4) Glu²⁰³ → Asp and Gln²⁰⁴ → Arg (E203D + Q204R)

There are already several buried salt bridges that appear to stabilize the subunit interfaces (Henrick et al., 1989): two at the A–B* (B–A*) interfaces (Asp²³–Arg¹³⁹ and Asp¹⁴⁵–Arg²⁴⁹) and three at the A–A* (B–B*) interfaces (Asp¹⁰⁷–Arg³⁷⁵, Glu³⁵²–Arg¹¹² and Asp³⁶⁰–Arg¹¹²). It was observed that the above would provide an additional A–A* salt bridge shown in Figure 3.

Purification of the mutant proteins

The procedure was essentially identical with that described by Smith et al. (1991) for purification of the wild-type enzyme from *Arthrobacter* cells. Each *E. coli* TG2 host was grown to stationary state in 60-litre batch fermenters of 2 × TY (tryptone/yeast extract) + ampicillin medium. Cell-free extracts prepared by lysozyme treatment of 20–50 g samples of these cells were used in each purification. The yields were very similar for each mutant, corresponding to 7–14 % of the protein in the cell extract. The two disulphide-bridged mutants, L199C + A200C and A223C, and the salt-bridge mutant, E203D + Q204R, had activities towards D-xylose and D-fructose identical with those of the wild-type enzyme (Rangarajan and Hartley, 1992). The other disulphide mutant Y253C is discussed in greater detail below.

Formation of disulphide bridges

The three thiol-containing mutants formed disulphide bridges spontaneously when left over 2–3 days in the gel-filtration buffer (10 mM MgCl₂/50 mM Tris/HCl, pH 8.0) at 4 °C in the absence of thiol-reducing agents. The rate of formation of these bridges was determined by Nbs₂ titration of samples of the denatured proteins during such incubation, showing it to be essentially complete after 72 h. The mobility of the denatured oxidized forms on SDS/PAGE clearly shows that they are dimers, as expected (results not shown).

Thermostability of the A–A* mutant proteins

Previous studies showed that the rate of thermal inactivation of apo-(D-xylose isomerase) decreased in the presence of ligands Mg²⁺ and Mg²⁺ + xylitol (a competitive inhibitor) (Rangarajan et al., 1992). Therefore, in these studies, the effect of high fructose concentrations on the rate of thermal inactivation was measured by sampling (Rangarajan et al., 1992). A 1 ml portion of 20 mM phosphate buffer (pH 6.5)/5 mM MgCl₂/25 % (w/v) fructose was layered with 200 µl of liquid paraffin and incubated at 76 °C for 30 min. Concentrated D-xylose isomerase solution was added

Figure 2 Structures of proposed inter-subunit disulphide bridges

(a) Stereoscopic view of Leu¹⁹⁹ → Cys + Ala²⁰⁰ → Cys mutant with the disulphide bridge; (b) shows the same as in (a) from a different angle, together with the network of intra-subunit salt bridges of the helices of the two subunits; (c) stereo view of Ala²²³ → Cys mutant with the disulphide bridge; (d) shows the same view enlarged (monoscopic).

to a final concentration of 5.4 mg/ml and mixed thoroughly. Aliquots of 20 μ l were removed at different time intervals and immediately diluted into 200 μ l of cold 20 mM phosphate buffer (pH 6.5)/5 mM MgCl₂. To measure D-xylose isomerase activity remaining, 10 μ l aliquots were immediately added to 1 ml of D-xylose isomerase assay solution (Smith et al., 1991) maintained at 30 °C. The inactivation rate constant (k) was calculated from the decrease in enzyme activity (v) from the initial value (v_0) by fitting the data to the equation:

$$v = v_0 e^{-kt}$$

using a non-linear regression analysis program (ENZFITTER). The half-lives (in min) were: wild-type, 453 ± 7 ; L199C + A200C,

450 ± 20 ; A233C, 433 ± 9 ; E203D + Q204R, 426 ± 10 . Hence, within experimental error, the thermostability of each mutant enzyme was indistinguishable from that of the wild-type.

The Tyr²⁵³ → Cys mutant enzyme (A-B*)

This mutant enzyme was chosen for more detailed studies because its position in the structure appears to be important for the conformation of groups in the active site. Tyr²⁵³ is adjacent to Asp²⁵⁴, which co-ordinates the metal ion at site [2] which is responsible for the isomerization step (Collyer et al., 1990) and close to Asp²⁵⁶, which H-bonds to Asn²⁴⁶ and forms a salt bridge with Lys²⁹⁴. The aromatic ring of Tyr²⁵³ appears to lock this

Figure 3 Structure of the proposed A-A* salt bridge

(a) Stereoscopic view of the wild-type enzyme and (b) stereoscopic view of Glu²⁰³ → Asp + Glu²⁰⁴ → Arg mutant, together with the proposed salt bridge (shown as dotted lines).

Figure 4 pH-dependence of K_m and V_{max} of the Y253C mutant enzyme for D-fructose

Assays were at 30 °C in 0.1 M buffers [pH 5.50–6.50 (Mes); pH 6.75–7.90 (Mops); pH 8.1–8.3 (Hepes)] containing 10 mM Mg^{2+} . Values of $1/K_m$ (●) obtained against 0.04–3 M D-fructose by non-linear regression analysis fit a theoretical single ionization curve for a group with $pK_a = 7.17 \pm 0.2$ (ENZFITTER program; Biosoft, Cambridge, U.K.). Values of V_{max} (▲) were determined by assays against 3.2 M D-fructose and are corrected by using the observed K_m values. The broken lines show the pH-dependence of $K_{m(app)}$ and V_{max} of the wild-type (wt) enzyme under similar conditions (Rangarajan and Hartley, 1992).

section of the chain into the active-site conformation; hence some degree of distortion of active-site groups could be expected.

During the purification of the Y253C mutant enzyme, it was observed that enzyme activities measured using either D-xylose or D-fructose as substrate at pH 8.0 were far lower than the wild-type enzyme or any of the other mutants. They were of the order of 10–15% for both oxidized and reduced forms of the Mg^{2+} -enzyme (enzyme activities of the reduced enzyme were measured in deaerated solutions, as the assays could not be performed in the presence of reducing agents). A detailed study was therefore

made of the kinetic parameters of this mutant as a function of pH.

pH-dependence of K_m and V_{max} of the oxidized Y253C Mg^{2+} -enzyme

Figure 4 shows the values of $K_{m(app)}$ obtained from assays at 30 °C in saturating concentrations of Mg^{2+} (10 mM) at various fructose concentrations over a range of pH values. The decrease in $1/K_m$ at acid pH fits a single ionization constant of $pK_a = 7.17 \pm 0.2$ compared with a pK_a of 6.80 ± 0.07 for the wild-type enzyme (Rangarajan and Hartley, 1992). The value of K_m at pH 8 was comparable with that of the wild-type enzyme.

Values of V_{max} in the above experiments were unreliable because variable amounts of enzyme had been used, so the values shown in Figure 4 were obtained at 30 °C, and similar Mg^{2+} concentrations, using 3.2 M D-fructose, which is in large excess of the $K_{m(app)}$ at pH 7.5 (0.12 M), and are corrected at lower pH using the observed $K_{m(app)}$ values. Figure 4 shows that V_{max} below pH 6.4 is similar to that of the wild-type enzyme, but declines rapidly at higher pH.

The pH-dependence of the fructose isomerase activity of the Co^{2+} form of the oxidized mutant enzyme in the presence of 2 mM Co^{2+} was also investigated as shown in Figure 5, though in this case K_m and V_{max} were not determined separately. Although 3.2 M fructose was used, one cannot assume that the curve represents V_{max} at all pH values, since the $K_{m(app)}$ of the wild-type is very high (1.7 M) for this substrate (Rangarajan and Hartley, 1992). Nevertheless it is clear that the activity is much lower than for the wild-type at all pH values, in contrast with the Mg^{2+} mutant enzyme, but there is again a decline at alkaline pH resulting in a lower pH optimum.

Stability of Tyr²⁵³ → Cys (A-B*) and the A-A* salt-bridge mutant enzymes

The Glu²⁰³ → Asp + Glu²⁰⁴ → Arg mutant has two new salt bridges in close proximity between subunits A-A* and showed behaviour identical with the wild-type enzyme. It was included

Figure 5 pH-dependence of activity of the Co^{2+} -Y253C mutant enzyme for D-fructose

Assays were at 30 °C against 3.2 M D-fructose in buffers at various pH values, as in Figure 4, containing 2 mM Co^{2+} . The broken line shows the activity of wild-type (wt) enzyme under similar conditions, calculated from the data of Rangarajan and Hartley (1992).

Table 1 'Melting points' of native and mutant D-xylose isomerases in the presence or absence of ligands

Samples (1 mg/ml) in 20 mM potassium phosphate buffers were incubated for 10 min at various temperatures in sealed tubes and then cooled on ice. The 'melting point' is the temperature at which isomerase activity of the samples (or reconstituted Mg^{2+} -holoenzymes) was reduced by half.

D-Xylose isomerase species	pH	Ligands present in the buffer		'Melting point' (°C)
		Mg^{2+} (10 mM)	Xylitol (10 mM)	
E203D + Q204R	8.0	—	—	73
	8.0	+	—	81
	8.0	+	+	81.5
Y253C (Oxidized for reduced)	8.0	—	—	58
	8.0	+	—	70
	8.0	+	+	71.5
Wild-type	6.5	+	—	69
	6.5	+	—	78

together with the Tyr²⁵³ → Cys enzyme for more detailed comparisons with wild-type enzyme.

Table 1 shows the 'melting points' of the mutant D-xylose isomerases at pH 6.5 and pH 8.0 in the presence and absence of ligands. The salt-bridge mutant has thermostability identical with that of the wild-type for both apoenzyme (73 °C), Mg^{2+} -enzyme (81 °C) and Mg^{2+} -enzyme + xylitol (81.5 °C) (Rangarajan et al., 1992). However, the Y253C mutant has much lower melting points at both pH 8 and pH 6.5, approx. 10 °C less for the Mg^{2+} -enzymes and Mg^{2+} -xylitol-enzymes and 15 °C for the apoenzymes. It is interesting to note that the thermostability of both oxidized and reduced forms is the same.

The urea stability of the salt-bridge mutant was also very similar to that of wild-type, whereas the Y253C mutant was less stable. Wild-type and mutant D-xylose isomerases in the Mg^{2+} -form (0.7 mg/ml) were incubated for 1 h at 30 °C in 0–8 M-urea/10 mM MgCl_2 /0.1 M Tris/HCl, pH 8.0, and assayed using 1.25 M fructose as substrate in similar buffers. Figure 6 shows

Figure 6 Urea-stability of the Y253C and salt-bridge mutant enzymes

Mg^{2+} -enzymes (0.73 mg/ml) were incubated in 10 mM MgCl_2 /0.1 M Tris/HCl, pH 8.0, containing from 0 to 8 M urea at 30 °C for 1 h and then assayed against 1.25 M D-fructose in similar buffers. ●, Wild-type; ○, A-A* salt bridge, Glu²⁰³ → Asp + Gln²⁰⁴ → Arg; ▲, Tyr²⁵³ → Cys, oxidized (Ox.) form; △, Tyr²⁵³ → Cys, reduced (Red.) form.

the specific catalytic activities of the samples at various urea concentrations. In this case the reduced Y253C enzyme is slightly more stable than the oxidized form.

These conclusions were confirmed by gel electrophoresis in 8 M urea at pH 8.8. Treatment of the Mg^{2+} -A-A* mutant enzyme with 8 M urea at pH 8.0 at 25 °C for 30 min prior to PAGE gives a mixture of active dimers and inactive denatured monomers, similar to the wild-type enzyme. However the Tyr²⁵³ → Cys mutant enzyme (oxidized form) gives denatured dimers and only a small proportion of active dimers (results not shown). Heating of wild-type and mutant enzymes at 60 °C for 1.5 h in 8 M urea, pH 8.0, gives only denatured monomers when PAGE is performed in the presence of β-mercaptoethanol.

Urea-gradient (0–9 M) PAGE also revealed some differences in stability. The behaviour of the salt-bridge mutant was in all cases identical with that of wild-type (Rangarajan et al., 1992) and all the mutants behave like wild-type when urea-gradient electrophoresis is performed at 25 °C. However, when the electrophoresis is performed at 62 °C, the wild-type and salt-bridge mutant undergo irreversible denaturation above 6.7 M urea, whereas the oxidized Tyr²⁵³ → Cys mutant denatures above 5.7 M urea and the reduced Tyr²⁵³ → Cys form above 5.9 M urea (results not shown).

X-ray crystallography of the Tyr²⁵³ → Cys mutant enzyme

The final X-ray data set for the Tyr → Cys mutant crystal comprised 32015 unique reflections from a total of 71670 observations [91% of the unique data to 0.25 nm (2.5 Å) resolution] with a merging *R*-factor on intensities of 6.1%. The refined model has a crystallographic *R*-factor of 15.7% for all data to 0.25 nm (2.5 Å) resolution, and contains 6577 atoms with r.m.s. deviation from standard bond lengths of 0.0019 nm (0.019 Å). Superposition of the crystallographically independent monomers provides an estimate of the accuracy of the refined model; the r.m.s. separation of all α-carbon atoms is 0.024 nm (0.24 Å).

The initial difference Fourier map ($F_{\text{Y253C}} - F_{\text{wild-type}}$, α wild-type) at 0.25 nm (2.5 Å) showed a significant peak of electron density between the Cβ atoms of the mutated residue 253 on subunits A and B*. The residues were replaced with cysteines and a right-handed disulphide bridge was modelled. A difference map

Figure 7 Electron-density for the disulphide bridge in the Y253C mutant enzyme

A stereoscopic view of the electron density for the disulphide bridge formed in the Tyr → Cys mutant D-xylose isomerase is shown. The density, contoured at 4 and 8 σ , was calculated to 0.25 nm (2.5 Å) resolution using Fourier coefficients $F_o - F_c$ and phases calculated from a model in which residue 253 was truncated to an alanine for ten cycles of refinement. The unlabelled crosses are ordered solvent molecules.

Figure 8 Superposition of wild-type and Y253C mutant enzymes

The structures are viewed down the non-crystallographic dyad axis at the A-B* subunit interface. The wild-type model (structure 15 in Collyer et al., 1990) is drawn with thin lines and the mutant with thick lines. All equivalent α -carbon atoms were overlapped as described in the text.

Figure 9 Active site of the Tyr²⁵³ → Cys mutant D-xylose isomerase

The electron density was calculated at 0.25 nm (2.5 Å) resolution using Fourier coefficients $F_o - F_c$ and is contoured at the 4 σ level. Density is observed for the alternative conformation of Asp²⁵⁴.

($F_o - F_c$, α c) calculated after the completion of refinement is shown superimposed on the mutant structure in Figure 7. The resulting atypical disulphide dihedral angles are $\chi_1 = -71^\circ$, $\chi_2 = -65^\circ$, $\chi_3 = 157^\circ$, $\chi_4 = -51^\circ$, $\chi_5 = -75^\circ$, and the distance between the α -carbons of the two cysteines is 0.453 nm (4.53 Å). Superposition of the refined Tyr → Cys A-subunit on to the

corresponding wild-type subunit results in a r.m.s. distance between α -carbons of 0.018 nm (0.18 Å) and reveals only minor differences in the main chain in the vicinity of the disulphide bridge. Significant differences between the superposed structures are restricted to the mutated residue 253, the contiguous residue Asp²⁵⁴ and the side-chain of Glu¹⁸⁵, which adopts a mean χ_2 value of 44° (wild-type mean $\chi_2 = -158^\circ$) (Figure 8).

Electron-density maps show xylitol bound at full occupancy in the extended conformation. Metal site [1] is octahedral and binds the inhibitor, as previously observed by Collyer et al. (1990). Mn²⁺ is not observed with unitary occupancy at site [2]. Instead, electron densities above 4 σ in a difference map ($F_o - F_c$) are observed both at sites [2] and [2'] (Collyer et al., 1990). Both sites are modelled with manganese at half occupancy and the mean refined temperature factors for Mn[2] and Mn[2'] are 0.37 and 0.15 nm² (37 and 15 Å²) respectively. The distribution of metal between alternative positions in the vicinity of site [2] has also been noted by Whitlow et al. (1991) and by Jenkins et al. (1992). Asp²⁵⁴ is modelled with its carboxylate oxygens co-ordinating metal [2] in a bidentate fashion, as commonly observed by Collyer et al. (1990). Electron densities for Asp²⁵⁴ indicate an alternative low-occupancy position for this residue. After refinement, the only remaining electron density above the 4 σ level in difference Fourier maps is for this alternative conformation of Asp²⁵⁴ (Figure 9).

DISCUSSION

Several pieces of evidence indicate that the four mutant proteins have the structures modelled in Figure 2 and Figure 3. They were purified in similar fashion and yield to wild-type enzyme, and were tetramers with D-xylose- and D-fructose-isomerizing activities. The disulphide bridges formed spontaneously and quantitatively on standing in air at 4 °C, and the electrophoretic mobilities of the oxidized denatured proteins corresponded to dimers.

The thermostability of the three A-A* mutant enzymes, Ala²²³ → Cys, Leu¹⁹⁹ → Cys + Ala²⁰⁰ → Cys, and Glu²⁰³ → Asp + Gln²⁰⁴ → Arg, in the presence of Mg²⁺ was indistinguishable from that of the wild-type. [All the half-lives are lower than those obtained by Rangarajan et al. (1992), probably because the experiments were performed in phosphate buffers at low Mg²⁺ concentrations instead of Tris/HCl and high Mg²⁺.] This is most surprising, since the wild-type tetramer dissociates reversibly and quantitatively into fully active A-B* dimers in the presence of low concentrations of urea or SDS (Rangarajan et al., 1992), indicating that interactions at the A-A* and A-B subunit interfaces are much weaker than at the A-B* interface. This, too, is surprising, because hypothetical A-A* dimers appear relatively compact compared with A-B or A-B* dimers, where the long C-terminal tails lack contacts with the N-terminal β -barrel and appear to be very flexible (Henrick et al., 1989).

In 8 M urea, these A-B* dimers decay directly to unfolded inactive monomers, suggesting that reversible dimerization followed by irreversible monomerization should also lie on the thermal unfolding pathway (Rangarajan et al., 1992). The disulphide bridge in mutant Ala²²³ → Cys and the two bridges in mutant Leu¹⁹⁹ → Cys + Ala²⁰⁰ → Cys covalently link the A-A* subunits, which would prevent formation of A-B* dimers. Mutant Glu²⁰³ → Asp + Gln²⁰⁴ → Arg has an A-A* salt bridge additional to the three already present, which should strengthen the A-A* interface, yet it too is unchanged in thermostability. Dimerization at the A-A* interface would break these salt bridges and expose many buried residues that would at first sight be potential unfolding points at high temperatures. Hence one

can conclude that thermal unfolding does not primarily involve exposure of the A–A* interface, and by inference of the A–B interface, since this appears to be also weak. Irreversible thermal unfolding must therefore be in the tetramer.

The fact that none of the A–A* mutants showed *reduced* thermostability is equally significant. In all the A–A* thiol mutants, the disulphide bridges formed readily and spontaneously in air and the enzymic properties were unchanged in both the oxidized and reduced forms. The modelling showed minimal steric distortions for the A–A* interface mutants, but replacement of Tyr²⁵³ leaves space around the Cys²⁵³ residue that could increase flexibility in a region of the chain that seems important to control the conformation of active-site residues.

However, the Tyr²⁵³ → Cys mutant in the presence of 10 mM Mg²⁺ has fructose isomerase activity identical with that of the wild-type at pH 6.4, but showed only 10–15% activity of the wild-type enzyme at pH 8. The K_m values were similar to those of the wild-type, but showed a slight pH shift, corresponding to a shift in pK_a of His⁵³ from 6.80 to 7.17 (Rangarajan and Hartley, 1992). V_{max} values were also similar to those of the wild-type below pH 6.4, but decreased sharply at higher pH values. The V_{max} of the wild-type enzyme fits an ionization curve corresponding to an apparent pK_a of 6.0 for His²¹⁹. These results suggested that there is no change at the M²⁺-binding site [1], where ring-opening of the sugar substrate takes place, but that at high pH there is distortion of M²⁺-site [2] where isomerization occurs (Collyer et al., 1990; Rangarajan and Hartley, 1992).

Further light is shed by the X-ray structural study of Y253C crystals in the presence of Mn²⁺ and xylitol at pH 7.8. Of the residues making up the active site, the carboxylate side chain of Glu¹⁸⁵ is turned away from the active site and adopts a conformation that would be sterically unacceptable in the wild-type enzyme (Figure 8). The space vacated by Glu¹⁸⁵ may give Asp²⁵⁴ greater conformational freedom, which would account for the alternative low-occupancy position observed for this residue (Figure 9). Since Asp²⁵⁴ is one of the ligands to the metal ion at site [2], it is reasonable to assume that this alternative conformation arises from a small proportion of the molecules in which Mn²⁺ is absent at this position.

The activities and pH-dependence of the Y253C mutant enzyme can be explained if one assumes that the perturbation of Asp²⁵⁴ is triggered by electrostatic repulsions from the new position of Glu¹⁸⁵, following deprotonation of His²¹⁹ at alkaline pH, which would increase the negative charge in the Site [2] region. Mg²⁺ binds to Site [1], and the kinetics of the ring-opening step, reflected in the $K_{m(app.)}$ values, are unaffected by the mutation, except for a slight increase in the pK_a which reflects the ionization of His⁵³. However Mg²⁺ binds to Site [2] only after the ring-opening step (Rangarajan and Hartley, 1992) and the movement of Asp²⁵⁴, triggered by deprotonation of His²¹⁹, would decrease this binding still further, so V_{max} would decline. Mn²⁺ binds strongly to Site [2], so the X-ray structure of the mutant enzyme crystals soaked in 50 mM MnSO₄ at pH 7.8 shows that most of the Asp²⁵⁴ residues are in their wild-type position, but the alternative position will probably predominate in the Mg²⁺ form. The much reduced activity of the Co²⁺ form of the mutant enzyme suggests that it has reduced Site [2] affinity for Co²⁺ at all pH values, but the decline in activity above pH 6.9 again suggests that ionization of His²¹⁹ decreases this still further.

Glu¹⁸⁵ is conserved in other xylose isomerase enzymes (Henrick et al., 1989) and, in the wild-type enzyme, its carboxylate side chain is directed towards active-site residues Lys¹⁸² and Asp²⁵⁴, a ligand to the metal at site [2]. The role of the equivalent Glu¹⁸⁶ in xylose isomerase from *Actinoplanes missouriensis* has been studied by van Tilbeurgh et al. (1992) by mutating it to Gln. This

Gln mutant has an altered metal specificity and a pH optimum that resemble those observed with the Y253C mutant. Mg²⁺ binds with reduced affinity at site [2], and the mutant has correspondingly low activity with this cation. In the presence of Mn²⁺ at pH 6.8 the active site of their mutant is essentially identical with that of the wild-type. However, the activity declines rapidly at alkaline pH, as in the Y253C mutant, with both Mg²⁺ and Mn²⁺. These results can again be ascribed to movements of Asp²⁵⁴ in consequence of a changed electrostatic environment at the active site. Alternative positions of Asp²⁵⁴ have been observed in the wild-type enzyme (Collyer et al., 1990), and van Tilbeurgh et al. (1992) have proposed that positional shifts of this residue may be integral to the xylose isomerase mechanism, facilitating both the movement of metal at Site [2] and the shuttling of a proton between the substrate O2 and O1.

The thermostability of the Tyr²⁵³ → Cys mutant enzyme is identical in both oxidized and reduced forms, but significantly lower than that of the wild-type in the presence or absence of ligands, at both pH 6.5 and 8.0. It is also slightly less stable in 8 M urea and in urea-gradient PAGE at 62 °C, undergoing irreversible denaturation above 5.7 M urea in both oxidized and reduced forms, compared with 6.7 M urea for the wild-type. In contrast the A–A* salt-bridge enzyme is indistinguishable from wild-type in all these respects, as are the A–A* disulphide mutants in all properties investigated. Taken together with the unchanged activity and thermostability of thermolysin fragments of this enzyme, which also retain tetrameric structure but lack all the C-terminal helical tails (Siddiqui et al., 1993), this suggests that the irreversible thermal unfolding step begins in the tetramer and is triggered by movements of charged groups in the active site. This conclusion is supported by the finding that the Glu¹⁸⁶ → Gln mutant of van Tilbeurgh et al. (1992) in the Mg²⁺ form also has much reduced thermostability.

Other D-xylose (glucose) isomerases have tertiary structures very similar to that of the *Arthrobacter* enzyme (Henrick et al., 1989), notably those from *Actinoplanes missouriensis* (Rey et al., 1988), *Streptomyces olivochromogenes* (Farber, 1987) and *Streptomyces rubiginosus* (Carrell et al., 1984, 1989; Whitlow et al., 1991). Since the amino acid sequences of the *A. missouriensis* (Amore & Hollenberg, 1989) and *S. rubiginosus* enzymes (Wong et al., 1991) differ by 32 and 35% respectively from the *Arthrobacter* sequence (Loviny-Anderton et al., 1991), it would not be surprising if their thermostabilities and unfolding pathways were different. However, a similar attempt to engineer thermostable mutants of the *A. missouriensis* enzyme (Quax et al., 1991) yielded results very similar to our own. Conservative mutations were made designed to stabilize helix H2 (Ala⁷³ → Ser or Gly⁷⁰ → Ser + Gly⁷⁴ → Thr or a triple mutant thereof) or to replace Lys by Arg in salt bridges (Lys²⁵³, A–B* or Lys²⁹⁴, intra-subunit). In view of the very steep ‘melting curves’, half-lives at 85 °C ranging from 1.5 to 4.2 h were negligibly different from that of the wild-type (2.9 h). In contrast, mutations of Lys²⁵³ → Gln or Lys²⁹⁴ → Gln were predictably very unstable by destroying the above salt bridges. Hence it is probable that subunit interfaces are not primary determinants of thermostability in this enzyme also, except when drastic mutations introduce a new ‘weak point’ at the A–B* interface.

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