Stability of *Arthrobacter* D-xylose isomerase to denaturants and heat

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There was no inactivation of Mg^{2+} -containing *Arthrobacter* D-xylose isomerase up to 1 h in 0–8 M-urea at 22 °C, but over this range there was rapid reversible dissociation into fully active dimers with a midpoint around 4 M-urea, as shown by gradient urea gels with an activity stain, and by ion-exchange chromatography and gel filtration in urea buffers. These dimers must have the A–B* conformation, since the tetramer could dissociate into A–A*, A–B or A–B* dimer conformations, but only residues across the A–B* interface contribute to the active site. The kinetics of inactivation of the Mg^{2+} -containing enzyme in 8 M-urea at higher temperatures suggest a partially unfolded Mg–A–B* dimer intermediate with 50 % activity, followed by irreversible inactivation coincident with the appearance of unfolded monomer. In 0–4 M guanidinium chloride, a similar reversible dissociation into active dimers occurs, but activity falls, suggesting that A–A* and/or A–B dimers might 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. The apoenzyme is least thermostable ($t_{1/2}$ at 80 °C, pH 7, = 0.06 h) but Mg^{2+} stabilizes strongly ($t_{1/2}$ = 5.5 h) and Co^{2+} even more so. Competitive inhibitors or substrates provide a small further stabilization, but this effect is more marked at 80 °C, pH 5.5. Together with a marked decrease in optimum pH with temperature, this allows batch isomerizations of glucose under these conditions that produce clean but sweeter syrups.

INTRODUCTION

Previous studies of *Arthrobacter* D-xylose isomerase include its purification and properties (Smith *et al.*, 1992), 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 Mg^{2+} -containing enzyme at 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 & Hartley, 1992). As a guide to improvements that might be made by protein engineering to its thermostability as a commercial glucose isomerase for production of high-fructose syrups, the stability to denaturants and/or heat in the presence of various ligands has been studied.

The tertiary structure reveals that the tetramer is composed of two crystallographically independent dimers, denoted as A–B and A*–B* in Fig. 1(a) (Henrick *et al.*, 1989). Each subunit contains two domains: the main domain (residues 1–327) is a parallel stranded α – β barrel, and the C-terminal domain (residues 327–394) is a loop structure consisting of five helical segments that is involved in intersubunit contacts. The active site lies in a deep pocket near the C-termini of the β -strands of the barrel domain and includes Phe-25 from an adjacent subunit (Fig. 1d).

It appeared likely that unfolding might involve subunit dissociation. The tetramer could theoretically dissociate into three possible dimer conformations, named A–A* (Fig. 1b), A–B (Fig. 1c) and A–B* (Fig. 1d). However, only the latter configuration preserves the important aromatic cluster of Trp-15 (A), Phe-93 (A), Trp-136 (A) and Phe-25 (B*) that is assumed to shield the anionic transition state from water during the isomerization step of the hydride ion transfer mechanism (Collyer *et al.*, 1990).

Therefore the subunit structure and enzyme activity was studied in various concentrations of urea, guanidinium chloride or SDS. Crystallographic (Collyer *et al.*, 1990) and enzyme-kinetic (Rangarajan & Hartley, 1992) studies of ligand binding

show that Mg^{2+} and Co^{2+} are the preferred activator ions and hence the thermostability was studied in the presence or absence of these.

EXPERIMENTAL

Unless otherwise stated all materials and methods were as in Smith *et al.* (1991).

Materials

D-Xylose isomerase was purified from cells of *Arthrobacter* strain NRRL B3728 according to the method of Smith *et al.* (1991). Samples of apoenzyme, Mg^{2+} -enzyme or Co^{2+} -enzyme were prepared from this as described by Rangarajan & Hartley (1992).

Methods

Enzyme assays in the presence or absence of urea, guanidinium chloride or SDS. Assay solutions (1 or 2 ml) generally contained D-fructose (1.25 M) and 10 mM- MgCl_2 in 0.1 M-Tris/HCl buffer, pH 8.0, containing 0–8 M-urea, 0–6 M-guanidinium chloride or 0–4 % (w/v) SDS. Solutions were preincubated at 30 °C for 45 min in a water-bath regulated by a Braun Thermomix regulator. Reaction was initiated by adding enzyme (15–100 μg as appropriate) in the same buffer. Portions (100 μl) were added at suitable time-intervals to 100 μl of 15 % (w/v) trichloroacetic acid to stop the reaction, and then 5 ml of 'GOD-PERID reagent' in 0.1 M-phosphate buffer, pH 7, was added. The absorbance at 610 nm, using a reagent blank, was measured after 25 min at room temperature. The glucose formed was calculated from a calibration curve. Activity is expressed as units/mg of enzyme where 1 unit = 1 μmol of glucose formed/min.

Gel electrophoresis in the presence of urea. Electrophoresis in the presence of 8 M-urea, pH 8.8, was performed in 7.5 % polyacrylamide gels according to the method of Marshall & Inglis (1986).

Urea-gradient gel electrophoresis (Creighton, 1979) was car-

Abbreviation used: TEMED, N,N,N',N'-tetramethylethylenediamine.

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Fig. 1. α -Carbon backbones of the tetramer and possible dimer conformations of D-xylose isomerase (adapted from Henrick *et al.*, 1989)

(a) The α -carbon backbones of the tetramer, showing the 222 symmetry axes, viewed down one of the aligned barrel axes. The arrows show the positions of D-sorbitol in the four active sites: (b) the A-A* dimer, related by the crystallographic 2-fold symmetry axis; (c) the asymmetric A-B dimer, related by 2-fold symmetry; (d) the non-crystallographically related 2-fold A-B* dimer, in the same view as (a). The arrows show the positions of D-sorbitol and the side chain of Phe-25 from the adjacent subunit in each active site.

ried out in 7.5% polyacrylamide gels. Two sets of glass plates (13.5 cm $\times$ 17.5 cm) were assembled in a slab-gel casting apparatus (Pharmacia GSC-2) with spacers 1.5 mm thick. Then 35 ml of solution 1, 2 ml of 1% (w/v) aqueous ammonium persulphate and 40 μ l of *NNN'*-tetramethylethylenediamine (TEMED) were placed in the reservoir [solution 1 = 37 ml of 30% (w/v) aqueous acrylamide/0.8% (w/v) *NN'*-methylene-

bisacrylamide, 32 ml of 1.75 M-Tris/HCl, pH 8.8, and 81 g of Ultrapure urea made up to 148 ml with distilled water]. The urea gradient was formed by using a BRL 250 ml gradient-former with 95 ml of solution 1 in the mixing compartment and 95 ml of solution 2 in the reservoir [solution 2 = 37 ml of 30% (w/v) aqueous acrylamide/0.8% (w/v) *NN'*-methylenebisacrylamide, 32 ml of 1.75 M-Tris/HCl, pH 8.8, 79 ml of distilled

water, 2 ml of 1% (w/v) aqueous ammonium persulphate and 50 μ l of TEMED]. Using a peristaltic pump, a gradient from 9 M to 0 M-urea was formed from the bottom to the top of the gel and 50 ml of solution 2 was layered on top. When the gel had set, the plates were inserted at right angles to the urea gradient in a Pharmacia GE-2/4 gel-electrophoresis apparatus containing 4 litres of electrode buffer (0.025 M-Tris/0.192 M-glycine). The protein [150 μ g in 75 μ l of sample buffer (15 mM-Tris/HCl buffer, pH 6.5/25% (v/v) glycerol/0.1% (w/v) Bromophenol Blue) was applied as a band on top of the gel and electrophoresis was performed at 150 V for 3.5 h at either 37 °C or 62 °C. Gels were then immediately stained for 5–10 min with 0.03% (w/v) Coomassie Blue G-250/0.59 M-perchloric acid. Protein bands were clearly visible and the gels could be stored in this solution indefinitely with no increase in background. They could subsequently be stained with Coomassie Brilliant Blue. D-Xylose isomerase activity in gels was detected as described by Smith *et al.* (1991).

Ion-exchange chromatography in the presence of urea. DEAE-Sephacel columns (9.5 cm $\times$ 0.7 cm internal diameter) were equilibrated at room temperature with freshly prepared solutions of 50 mM-Tris/HCl buffer (pH 8)/10 mM-MgCl₂ containing 0–8 M-urea. Samples of D-xylose isomerase [10 mg in 4.75 ml of 50 mM-Tris/HCl buffer (pH 8)/10 mM-MgCl₂ containing 0–8 M-urea] were incubated at 22 °C for various times and applied to these columns. The proteins were eluted over a period of 3 h with a gradient of 0–0.5 M-NaCl in the same buffer and the specific conductivity, absorbance at 280 nm and xylose isomerase activity of the fractions were measured.

Determination of molecular mass by gel filtration. (a) A Sephacryl S-300 column (26 cm $\times$ 1.6 cm internal diameter) was equilibrated and eluted at 22 °C with 10 mM-MgCl₂/50 mM-Tris/HCl, pH 8, containing 0, 1, 4 or 6 M-urea. The void volume was determined with Blue Dextran, and the total volume with L-tyrosine. The column was calibrated with BSA dimer, BSA, ovalbumin, thaumatin I and RNAase A; in the absence of urea, yeast alcohol dehydrogenase and β -amylase were also used. The elution positions were located by monitoring the absorbance at 280 nm and also by SDS/PAGE of the peak fractions.

(b) A Toyo Soda TSK-G 3000 SW column (30 cm $\times$ 7.5 mm internal diameter) was equilibrated and eluted at 22 °C with 10 mM-magnesium acetate/50 mM-Tris/acetate, pH 8, containing 0–4 M-urea or 0–1 M-guanidinium chloride. The column was calibrated with Blue Dextran, L-tyrosine, BSA dimer, BSA, ovalbumin and carbonic anhydrase; in the absence of urea or guanidinium chloride, apoferritin, β -amylase, yeast alcohol dehydrogenase and phosphorylase *b* (muscle) were also used. Protein samples (0.5 mg) preincubated for 30 min in 0.2 ml of the same buffer were applied and the elution positions were located by monitoring the absorbance at 280 nm.

Fluorimetry. Fluorimetric measurements were made using a Perkin-Elmer MPF-44A spectrofluorimeter. Excitation wavelength was 280 nm and emission spectra were recorded. The intensities determined at 310 nm and 335 nm were used in the calculations.

Measurement of thermostability. 'Melting point' experiments were performed in evacuated and sealed glass tubes (47 mm $\times$ 5.5 mm internal diam.) pretreated by washing with chromic acid, then exhaustively with distilled water and baking at 400 °C for 4 h. Triplicate portions (100 μ l) of D-xylose isomerase (1 mg/ml) in 5 mM-MgCl₂/20 mM-potassium phosphate buffers of the desired pH were heated in these tubes for 10 min in a water-bath regulated by a Braun Thermomix regulator. The tubes were then cooled in ice and later assayed for isomerase activity with either D-fructose or D-xylose as substrate at 30 °C (Smith *et al.*, 1991).

More accurate kinetic assays of thermal denaturation used Tris/HCl buffers, since even though the pH of phosphate buffers is essentially temperature independent, they cause precipitation of some of the bivalent metal ions under some of the conditions used. The pH of 0.5 M-Tris/HCl buffers was adjusted at 22 °C, by using a value of Δ pH = $-0.028/^\circ\text{C}$ (Good *et al.*, 1966), to give a pH of 7 or 8 at the temperature of the experiment. Buffers (0.1 M) containing the desired additives (10 mM-MgCl₂, 2 mM-CoCl₂ or 10 mM-xylitol) were incubated in stoppered glass tubes or cryotubes at the appropriate temperature in a water-bath regulated by a Braun Thermomix regulator for a minimum of 1 h. A small volume of concentrated D-xylose isomerase solution in equivalent buffer was then added to the hot buffer to give a final concentration of enzyme of 0.74 mg/ml. After thorough mixing, 50 μ l portions were withdrawn at various times into Eppendorf tubes precooled to 4 °C. Enzyme activity was measured with either D-xylose or D-fructose as substrate at 30 °C (Smith *et al.*, 1991). For the apoenzyme, the 50 μ l portions were added to an equal volume of 30 mM-MgCl₂/0.1 M-Tris/HCl, pH 8, at 4 °C, and D-xylose isomerase activity was measured after incubating overnight at 4 °C.

RESULTS

Effects of urea on enzyme activity and subunit structure

The Mg²⁺-containing enzyme showed no loss of activity when incubated for 1 h at 22 °C in 0–8 M-urea/10 mM-MgCl₂/100 mM-Tris/HCl, pH 8, and assayed in the same buffer, with or without urea.

The Mg²⁺-containing enzyme was subjected to electrophoresis at room temperature in 7.5% polyacrylamide gels at pH 8.8, with or without 8 M-urea and the gels were stained for enzyme activity and protein. In the absence of urea, only one band of active tetramer was seen. However, in the presence of 8 M-urea the major band of active protein had much lower mobility and a minor even slower band of inactive protein was also seen (results not shown). This suggested that the active band may be the dimer of D-xylose isomerase and the minor slower band may be the denatured monomer.

This phenomenon is more revealingly illustrated when Mg²⁺-enzyme samples are subjected to urea-gradient PAGE at 37 °C and stained for either protein or D-xylose isomerase activity (Fig. 2). As the urea concentration increases, the mobility changes from that corresponding to native tetramer to that corresponding to putative dimer, with a midpoint around 4 M-urea, and all of the protein band stains for D-xylose isomerase activity. This is consistent with a rapid reversible equilibrium between tetramer and fully active dimers. However, at concentrations of urea above 7 M, a band of inactive monomer begins to appear; the irreversible monomerization is greater and occurs at lower concentrations of urea when the gradient gel electrophoresis is performed at 62 °C.

To confirm this hypothesis, the Mg²⁺-enzyme was subjected to gel filtration on Sephacryl S-300 in buffers containing zero, 1, 4 or 6 M-urea. Fig. 3 shows the elution positions (K_{av}) with respect to M_r markers: the deduced M_r values are 182000 without urea, 135000 in 1 M-urea, 107000 in 4 M-urea and 81000 in 6 M-urea. It was not possible to determine the molecular size in higher concentrations of urea as no suitable markers were available. This confirms that urea readily promotes a rapid reversible dissociation of the tetramer (M_r 173336) into fully active dimers, with midpoint between 1 and 4 M-urea.

These conclusions were reinforced by incubating enzyme samples (0.7 mg/ml) for 30 min at 30 °C in 0–8 M-urea/10 mM-MgCl₂/50 mM-Tris/HCl, pH 8, and assaying D-fructose iso-

Fig. 2. Gradient-urea PAGE of Mg^{2+} -containing D-xylose isomerase

Urea-gradient (0–9 M) electrophoresis of D-xylose isomerase at pH 8.8 in 7.5% acrylamide gels (a) at 37 °C and (b) at 62 °C. Gels were stained for protein (top) or D-xylose isomerase activity (bottom). The gel at 62 °C stained for activity is not shown. Arrows alongside the gels indicate the direction of migration of protein. The numbers at the top of the gels indicate the molarity of urea across the gradient.

Fig. 3. Effects of urea on subunit structure of D-xylose isomerase

The effect of urea on subunit structure was determined by gel filtration on Sephacryl S-300 columns in buffers containing 0, 1, 4 and 6 M-urea as described in the Experimental section. $\square$, Position of elution of M_r markers; $\blacksquare$, position of the D-xylose isomerase peak. K_{av} , the partition coefficient between the liquid phase and the gel phase, $= (V_e - V_o)/(V_t - V_o)$ where V_o = void volume, V_t = total volume of the gel and V_e = elution volume of the protein.

merase activities in the same buffer, with or without urea. Similar samples were immediately separated on DEAE-Sephacel columns (9.5 cm $\times$ 0.7 cm diameter) equilibrated at 22 °C with urea-containing buffers of the same composition and eluted with a gradient of 0–0.5 M-NaCl. Fig. 4 shows the elution profiles superimposed on the gradient of effluent conductivity. The shift in peak position reflects rapid reversible equilibrium between tetramer (eluted at 27 mS) in the absence of urea and predominantly dimer (eluted at 13 mS) in 8 M-urea; again the midpoint is around 4 M-urea. In 8 M-urea, a small peak of the

Fig. 4. DEAE-Sephacel chromatography of the Mg^{2+} -containing D-xylose isomerase in urea buffers

D-Xylose isomerase in 0.05 M-Tris/HCl, pH 8, buffers containing 0.01 M-MgCl₂ and 0–8 M-urea was subjected to chromatography on DEAE-Sephacel columns (9.5 cm $\times$ 0.7 cm internal diameter) equilibrated with urea buffers of the same composition and eluted with a gradient of 0–0.5 M-NaCl. —, Incubated in 0, 2, 3.5, 5 or 8 M-urea for 1 h at 22 °C; ----, incubated in 8 M-urea for 3 days;, incubated in 8 M-urea for 7 days.

Fig. 5. Effects of urea on D-xylose isomerase activity

Mg^{2+} -containing enzyme (0.73 mg/ml) was incubated in 0.1 M-Tris/HCl (pH 8)/0.01 M-MgCl₂ containing from 0 to 8 M-urea and then assayed against 1.25 M-fructose in similar buffers (□) or in buffers containing no urea (■). The areas under the curve show the composition of tetramer, dimer and monomer and their specific catalytic activities at various urea concentrations.

denatured inactive monomer eluted at 5 mS is also seen. When the 8 M-urea samples were stored at 22 °C for 3 days (broken line) or 7 days (dotted line) the dimer peak decreased and the denatured monomer peak increased, showing a slow irreversible inactivation.

Fig. 5 shows the areas under the dimer/tetramer or monomer peaks in Fig. 4 together with the specific catalytic activities of the samples at various urea concentrations. The denatured monomer peak was dialysed exhaustively versus several volumes of 50 mM-Tris/HCl buffer (pH 8)/10 mM-MgCl₂ at 22 °C and applied to a column (9.5 cm $\times$ 0.7 cm internal diameter) of DEAE-Sephacel equilibrated in the same buffer at 22 °C. Approx. 30% of the material eluted from the column at 31 mS was inactive, and the remainder could not be recovered from the column even after

Fig. 6. Thermal inactivation of D-xylose isomerase in the presence of urea

First-order plots for thermal inactivation of D-xylose isomerase in the presence of urea. Experiments were conducted at pH 7 (at the temperature of the experiment). Enzyme activity remaining was measured with either D-xylose or D-fructose as substrate at 30 °C. ○, 6 M-urea; △, 8 M-urea + xylitol; □, 8 M-urea + sorbitol; ■, 8 M-urea alone.

washing with 1.5 M-NaCl. It is clear that the dimer is fully active and the monomer is not.

Thermostability in the presence of urea

The stability of the enzyme at pH 7 in 0–8 M-urea at 60 °C was measured in the presence of various ligands. There was no loss of

Table 1. Half-lives for inactivation in urea above 60 °C

[Urea] (M)	Temperature (°C)	Buffer (pH 7) containing:			<i>t</i> _{1/2} (h)
		Mg ²⁺ (10 mM)	Xylitol (10 mM)	Sorbitol (100 mM)	
5	60	+	–	–	10.1
6	60	+	–	–	3.6
7	60	+	–	–	1.3
8	60	+	–	–	0.8
8	60	+	–	–	0.6
8	60	–	–	–	0.5
8	60	+	+	–	0.15
8	60	+	+	–	2.7
8	60	+	–	+	1.0
8	60	+	–	+	2.0
8	60	+	–	+	1.1
8	70	+	–	–	0.02
8 (pH 6.5)	70	+	–	–	0.03

activity of the Mg²⁺–enzyme in 0–4 M-urea over a period of 6 h. In 5 M-urea the slow loss of activity followed first-order kinetics with a single rate constant, but in 6 M-urea and above, the first-order plots of loss of activity were biphasic, corresponding to a slow rate followed by a faster rate (Fig. 6). The transition from the slow to the fast rate occurred when there was around 50 % activity remaining. In 8 M-urea in the presence of the competitive inhibitors xylitol or sorbitol, similar biphasic first-order plots were seen (Fig. 6). By contrast, the apoenzyme undergoes rapid inactivation in 8 M-urea at 60 °C giving a monophasic first-order plot.

Table 1 gives a summary of the half-lives calculated from first-order rate constants for inactivation in urea under different conditions. For the Mg²⁺-containing enzyme at 60 °C, the ‘slow rate’ governs the whole inactivation process in 5 M-urea, but, as the urea concentration increases, a ‘fast rate’ above 50 % inactivation emerges, and this ‘fast rate’ increases until it comes

Fig. 7. Effect of guanidinium chloride on activity and subunit structure of D-xylose isomerase

(a) Mg²⁺-containing enzyme (0.73 mg/ml) was incubated at 30 °C for 30 min in 0.1 M-Tris/HCl, pH 8/0.01 M-MgCl₂ containing from 0 to 6 M-guanidinium chloride and then assayed against 1.25 M-D-fructose in similar buffers (■) or in buffers containing no guanidinium chloride (□). Also shown are results obtained when samples were assayed in buffers without guanidinium chloride after incubation periods of 4.5 and 24 h at 30 °C. (b) Mg²⁺-containing enzyme (0.73 mg/ml) was incubated at 30 °C for 30 min in 0.05 M-Tris/acetate, pH 8/0.01 M-magnesium acetate containing 0, 0.12, 0.5 and 1 M-guanidinium chloride and loaded on a Toyo Soda TSK-G-3000 SW gel filtration column (30 cm × 7.5 mm internal diameter) equilibrated in similar buffers. Open symbols indicate the position of elution of *M_r* markers and closed squares the position of the D-xylose isomerase peak.

Fig. 8. Effect of SDS on activity and subunit structure of D-xylose isomerase

(a) Mg^{2+} -containing enzyme (0.73 mg/ml) was incubated in 0.1 M-Tris/HCl, pH 8/0.01 M- MgCl_2 containing from 0 to 4% (w/v) SDS and then assayed against 1.6 M-fructose in similar buffers (■) or in buffers containing no SDS (□). (b) Mg^{2+} -containing enzyme was incubated in 0.015 M-Tris/HCl (pH 6.5)/1% (w/v) SDS and subjected to SDS/PAGE in 12.5% acrylamide gels. Lane 1, 25 °C, 30 min; lanes 2–5, 60 °C, 30 s, 1, 2 and 5 min respectively; lane 6, 100 °C, 2 min. Gels were stained for D-xylose isomerase activity and with Coomassie Blue. The position of elution and M_r of marker proteins are indicated alongside the gel stained for protein. t, d and m refer to tetramers, dimers and monomers respectively.

to dominate the kinetics of inactivation in 8 M-urea. Addition of the competitive inhibitors xylitol or D-sorbitol restores biphasic kinetics in 8 M-urea by decreasing both the 'slow rate' and the 'fast rate' to values otherwise seen in 6–7 M-urea.

The Mg^{2+} -containing enzyme inactivates in 8 M-urea at 60 °C about four times more slowly than the apoenzyme. Its inactivation rate increases over 20-fold between 60 and 70 °C, but a decrease from pH 7 to pH 6.5 decreases the latter effect.

Effects of guanidinium chloride on enzyme activity and subunit structure

Incubation in 0–6 M-guanidinium chloride at 30 °C for 30 min causes a similar reversible dissociation into dimers (Fig. 7a). When the activity was measured in buffers containing guanidinium chloride, there was 50% loss of activity from 0 to 1 M and the activity fell to 0% between 3 and 4 M-denaturant. However, when the same samples were assayed in buffer without denaturant, full activity was recovered in samples that had been incubated in 0–2 M-guanidinium chloride. If the D-xylose isomerase was incubated at 30 °C for longer periods of time, the percentage of such recoverable activity progressively decreased owing to irreversible conversion into denatured monomer, as seen in 8 M-urea.

H.p.l.c. gel filtration in the presence of 0–2 M-guanidinium chloride (Fig. 7b) showed that D-xylose isomerase steadily decreases in molecular size from 199 000 (0 M) and 166 000 (0.12 M) to 83 000 (0.5 M) and 81 000 Da (1 M). There was only

one peak of enzyme activity, showing that the tetramer is in rapid reversible equilibrium with the dimer between 0 and 1–2 M-denaturant. The fall in activity of 50% between 0 and 0.5 M-guanidinium chloride could be due to the presence of inactive A-A* and/or A-B dimers in the mixture. However, this conclusion is unsafe because the loss in activity could be due to displacement of the active-site Mg^{2+} by the high concentration of guanidinium ions.

Stability to SDS

The SDS stability of the Mg^{2+} -containing enzyme is also noteworthy. Incubation with 2% or 4% (w/v) SDS at 30 °C for 30 min had negligible effect on enzyme activity, irrespective of whether the enzyme was assayed in the presence or absence of SDS (Fig. 8a). Paradoxically, at lower SDS concentrations from 0.2 to 1%, approx. 25% of enzyme activity was lost. This effect was even more dramatic after similar incubations for 20 h. There was almost complete loss of activity between 0.2% and 1% SDS, but about 20% of the enzyme activity was retained in 2% SDS and 40% in 4% SDS.

Fig. 8(b) shows results obtained when D-xylose isomerase was treated with 1% SDS under progressively harsher conditions and subjected to PAGE on 12.5% acrylamide/SDS gels. Treatment for 30 min at 25 °C before PAGE gives predominantly active dimers, whereas heating at 60 °C for 1 min gives a mixture of dimers and monomers and heating at 100 °C for 2 min gives only inactive monomers.

Fig. 9. 'Melting points' of D-xylose isomerase in the presence or absence of ligands

D-Xylose isomerase in the apo form (Δ), Mg^{2+} -enzyme ($\circ$) and Mg^{2+} -enzyme-xylitol complex ($\square$) was heated at the desired temperature for 10 min in 20 mM-potassium phosphate buffer (a) pH 8 or (b) pH 5.5; samples were cooled on ice and enzyme activity remaining was assayed with either D-xylose or D-fructose as substrate at 30 °C. In the case of the apoenzyme, heated samples were incubated in buffer containing 30 mM- $MgCl_2$ for 20 h at 4 °C and then assayed for enzyme activity at 30 °C.

Does dilution promote subunit dissociation?

The activity of this enzyme is usually measured in assay solutions containing 10–100 μg of enzyme/ml. The above results suggested that it might dissociate at these dilutions to dimers and monomers with altered catalytic activity. Therefore comparative assays were performed in which enzyme at concentrations of 0.01 or 0.1 mg/ml was immediately diluted 50-fold into the assay solution; there was no change in rate during the assays and the specific activities were identical. Hence there is no evidence for inactive dimers or monomers.

In addition, h.p.l.c. gel filtration was performed at enzyme concentrations of 0.02–0.87 mg/ml (11.5×10^{-8} M to 5×10^{-6} M). In all cases, the enzyme was eluted as a single symmetrical peak and its position always corresponded to an apparent M_r of a tetramer. The resolution of the column was such that dimer formation up to 20% of the total protein would have had a significant effect on the elution position. This allows an estimate of 2.5×10^{-8} M as the upper limit for the dissociation constant of the tetramer-dimer equilibrium.

Fluorescence measurements of D-xylose isomerase also suggest that this dissociation constant is very low. The tertiary structure (Henrick *et al.*, 1989) shows that three of the five tryptophan residues in each subunit are located in the subunit interfaces, so subunit dissociation should affect the tryptophan fluorescence. However, the specific fluorescence intensity, a quantity highly sensitive to changes in the environment of tryptophans, showed no change over a 2.9×10^{-8} M to 9.2×10^{-7} M range of protein concentration. Hence subunit dissociation in the absence of denaturants appears to be negligible.

Thermostability

Fig. 9(a) shows the sharp 'melting points' of 73 °C for the apoenzyme, 81.5 °C for the Mg^{2+} -enzyme and 82 °C for the Mg^{2+} -enzyme-xylitol complex when incubated for 10 min at various temperatures in 5 mM- $MgCl_2$ /20 mM-potassium phosphate, pH 8. It should be emphasized that this represents irreversible inactivation, since assays conducted on similar samples up to 3 days after the treatment yielded identical results. At pH 5.5, the corresponding 'melting points' are 68 °C for the apoenzyme, 71 °C for the Mg^{2+} -enzyme and 77 °C for the Mg^{2+} -enzyme-xylitol complex (Fig. 9b).

The denatured protein does not refold after cooling. This was shown by heating apoenzyme (0.74 mg/ml) and Mg^{2+} -enzyme (0.74 mg/ml) at 80 °C for 1 h. The samples were then incubated with an equal volume of buffer containing 30 mM- Mg^{2+} for 20 h at 4 °C. Both the treated enzyme samples were subjected to h.p.l.c. gel filtration, native PAGE on 7.5% acrylamide gels, SDS/PAGE on 12.5% acrylamide gels and assay with D-xylose as substrate at 30 °C. As expected, the heated Mg^{2+} -enzyme (control) retained 100% activity whereas the heated apoenzyme showed no enzyme activity. The absorbance at 280 nm of the thermoinactivated apoenzyme was 15% higher than that of the active enzyme. Both samples gave identical bands on SDS/polyacrylamide gels; however, their behaviour in native gels was different. The control gave a sharp band which also

Fig. 10. Rates of inactivation of D-xylose isomerase incubated at 85 °C in the absence or presence of ligands

D-Xylose isomerase was incubated at 85 °C except where indicated, in (i) the apo form ($\circ$); (ii) in Mg^{2+} , either 10 mM (∇) or 20 mM ($\blacktriangle$); (iii) in 10 mM-xylitol and Mg^{2+} either 10 mM ($\triangle$) or 20 mM ($\blacktriangledown$); (iv) in Co^{2+} either 2 mM ($\square$) or 5 mM ($\blacksquare$). Enzyme activity remaining was measured at 30 °C with D-xylose as substrate. In the case of the apoenzyme, samples were incubated in buffer containing 30 mM- Mg^{2+} for 20 h at 4 °C and then assayed for enzyme activity.

Table 2. Half-lives for inactivation at various temperatures

Temperature (°C)	Buffer containing:			$t_{1/2}$ (h)
	Mg ²⁺ (10 mM)	Mg ²⁺ (20 mM)	Xylitol (10 mM)	
80	—	—	—	0.06
80	—	—	+	0.06
85	—	—	—	0.03
80	+	—	—	10.5
81	+	—	—	5.5
85	+	—	—	0.5
85	—	+	—	0.5
80	+	—	+	17.5
81	+	—	+	12
85	+	—	+	0.8
85	—	+	+	0.8

stained for enzyme activity whereas the inactivated enzyme gave a broad streaky band which did not stain for enzyme activity. The control Mg²⁺-enzyme gave a symmetrical peak of protein eluted at the expected tetramer position on h.p.l.c. gel filtration, but the thermoinactivated protein could not be eluted from the column. This behaviour is similar to that found for D-xylose isomerase monomer formed in the presence of 8 M-urea.

Rates of thermal inactivation

Relatively small changes in thermostability may be important in elucidating the results of protein engineering. A more accurate way to measure these is to determine the first-order rate constants of inactivation of enzyme incubated for various times at a fixed temperature. Fig. 10 shows rates for inactivation of the Mg²⁺- or Co²⁺-containing enzymes incubated at 80 °C or 85 °C in 100 mM-Tris/HCl, pH 7 (at the temperature of the incubation) containing 10 mM-Mg²⁺ or 5 mM-Co²⁺ respectively.

Table 2 shows the half-lives for inactivation derived from first-order rate constants for enzyme incubated at 80 °C or 85 °C in the presence or absence of various ligands. The half-life of the apoenzyme at 80 °C is only 3.5 min. Mg²⁺ alone provides significant stabilization, but xylitol alone has no effect. The ternary complex with Mg²⁺ and xylitol ($K_d = 3 \times 10^{-4}$ M) has

double the half-life at 80 °C of the Mg²⁺-enzyme complex ($K_d = 1 \times 10^{-4}$ M). The Co²⁺-enzyme lost no activity under comparable conditions over 18 h at 85 °C.

'High-fructose syrup production' at 80 °C, pH 5.5

Current processes for isomerization of glucose syrups to 'high-fructose syrups' use columns of stable immobilized D-xylose isomerases at 60 °C in the presence of Mg²⁺ and Co²⁺ ions at pH 7–8, their pH optima, but costs of immobilization and column operation add significantly to the product price. The preceding steps of starch hydrolysis to glucose are simple batch processes, so a much cheaper glucose isomerase used sequentially in the same vessel could cut costs. Moreover, since the fructose/glucose equilibrium increases with temperature (Takasaki, 1967), a higher-temperature isomerization would yield a sweeter syrup. However, it would then be necessary to operate at lower pH, since undesirable 'browning reactions' occur when glucose is heated at alkaline pH (Bucke, 1977).

This work has shown that *Arthrobacter* D-xylose isomerase is reasonably stable at 80 °C in the presence of 50 % (w/v) glucose and Mg²⁺ ions. Moreover, mechanistic studies (Rangarajan & Hartley, 1992) showed that its pH optimum decreases at higher temperatures, owing to the high temperature coefficient of ionization of His-219 which controls the turnover rate. It therefore seemed likely that it could be used for batch isomerizations of glucose syrups at 80 °C, pH 5.5.

Hence we performed a model batch isomerization at 80 °C, pH 5.5 in 50 % glucose, and made a comparison of the 'browning reaction' at this pH and at pH 7 by spectral analysis. Fig. 11(a) shows that the *Arthrobacter* enzyme in the presence of only 10 mM-Mg²⁺ ions is an excellent glucose isomerase. No inactivation of the enzyme was detected by reassaying the isomerized mixture at the end of the experiment. In these conditions, 1 g of the enzyme would convert 500 g of glucose into a mixture of 50 % glucose/50 % fructose in 1.5 h at 80 °C, pH 5.5 where the 'browning reaction' is only 10–15 % of that under the same conditions at pH 7 (Fig. 11b).

DISCUSSION

Effects of urea on enzyme activity and subunit structure

The aromatic cluster of Trp-15 (A), Phe-93 (A), Trp-136 (A) and Phe-25 (B*) appears to be an important feature of the active

Fig. 11. Batch isomerization of 50% (w/v) glucose at 80 °C, pH 5.5, and the 'browning reaction'

(a) 50% glucose in 0.1 M-Mes buffer, pH 5.5 (at 80 °C)/10 mM-MgCl₂ was incubated at 80 °C in a water bath in duplicate. The reaction was initiated by the addition of buffer to the 'control' tube and D-xylose isomerase to the 'experimental' tube to a final concentration of 0.74 mg/ml. Samples were withdrawn at different times into 15% trichloroacetic acid and the amount of glucose remaining was measured by the 'GOD-PERID' method and the amount of fructose was measured by the cysteine/carbazole method (Dische & Borenfreund, 1951) after suitable dilution. ●, Glucose; ▲, fructose. (b) 50% (w/v) glucose in 0.1 M-Mes buffer/10 mM-MgCl₂ (as above) and in 0.1 M-Tris/HCl buffer pH 7 (at 80 °C)/10 mM-MgCl₂ was incubated at 80 °C. Reaction was initiated by the addition of D-xylose isomerase to a final concentration of 0.74 mg/ml. The browning reaction was followed by measuring the absorbance of the reaction mixtures at 415 nm. ●, pH 7; ▲, pH 5.5.

site that is conserved in all known D-xylose isomerase sequences. In the hydride ion transfer mechanism (Collyer *et al.*, 1990), for which there is independent evidence (Lee *et al.*, 1990), it is assumed to shield the anionic transition state from water during the isomerization step. Since this cluster would be conserved only in A-B* dimers, it is probable that the fully active dimers formed in urea must all have the A-B* conformation.

The relatively facile dissociation into active A-B* dimers caused by urea contrasts with the remarkable stability of the enzyme in other respects. This suggests that the interactions of the A-A* and A-B interfaces are much weaker than at the A-B* interface. This is surprising because the A-A* conformation appears the most compact and conserves the salt bridge interactions of the helical tails with the β -barrel of their neighbours. The equilibrium between tetramer and active dimers must be reached fairly rapidly, since no separation of these species could be obtained on urea-gradient gel electrophoresis or on gel chromatography or ion-exchange chromatography in the presence of various urea concentrations. However, the tetramer seems always to be the predominant species in the absence of denaturants, since no observable effects were seen over a wide range of dilution.

In contrast, the dissociation of active dimers into inactive monomer is effectively irreversible. At 30 °C inactivation is slow, even in 8 M-urea, but occurs more rapidly and at lower urea concentrations at 60 °C. The unusual biphasic kinetics of inactivation of the Mg^{2+} -enzyme at 60 °C could be explained by the following inactivation pathway:

The effect of urea would be first to increase the concentration of Mg-A-B*-Mg dimers and then to promote dissociation of Mg^{2+} from these, perhaps by causing local reversible denaturation in one subunit as illustrated by the Mg-A-B* dimer above. Since the latter species would be 50% active, the 'slow rate' of inactivation would be that step. The 'fast rate' would be the conversion to A-B*, which then unfolds irreversibly to denatured monomers in a strongly temperature-dependent fashion. If so, protein engineering to strengthen interactions at the A-B* subunit interface should increase thermostability.

Analogous studies of urea denaturation have been made with other D-xylose isomerases. Kasumi *et al.* (1982) showed that incubation of D-xylose isomerase from *Streptomyces griseofuscus* S-41 with 8 M-urea at pH 7 for 1 h at 40 °C caused a loss of enzyme activity and a decrease in molar ellipticity; but both were restored by the removal of urea. They concluded that the enzyme retained an ordered conformation even in 8 M-urea, as seen with the *Bacillus coagulans* enzyme (Danno, 1973).

Luiten *et al.* (1989) concluded that prolonged exposure of the *Actinoplanes missouriensis* enzyme to 7 M-urea caused dissociation of the active tetrameric enzyme to inactive dimers, which they attribute to inactivation by cyanate generated from urea solutions on standing. They incubated that enzyme with increasing concentrations of cyanate in borate buffer for 16–24 days in the presence or absence of 5 M-cyanate-free urea. Treatment with cyanate alone did not cause dimerization, although native PAGE revealed a dose-dependent chemical modification of amino groups without apparent loss of enzyme activity. Incubation in 5 M-urea for 16 days followed by h.p.l.c. gel filtration did not indicate the presence of dimers, although native PAGE suggested the presence of dimers. However, simultaneous addition of cyanate and urea to the enzyme caused dissociation

of active tetramers to inactive dimers. They argue that the cyanate generated in 5 M-urea solutions might have chemically modified amino groups which could have weakened the dimer-dimer association to such an extent that dissociation could be brought about by the electric field applied during PAGE or that the combined influence of urea and the electric field caused dissociation of tetramers to dimers. However, these somewhat involved observations and interpretations do not reflect on our own experiments, where freshly prepared urea was used in 50 mM- or 100 mM-Tris/HCl buffers, pH 8; the latter would mop up any cyanate likely to be generated under the conditions of our experiments (Hagel *et al.*, 1971).

Effects of guanidinium chloride and SDS

Guanidinium chloride between about 0.2 and 1 M also promotes rapid reversible dissociation into dimers. The activity assayed in the presence of the denaturant drops rapidly over the range in which dimerization occurs to a constant level of 50% between 1 and 3 M-guanidinium chloride. If assays are conducted in the absence of the denaturant, full activity is recovered after 30 min incubation in up to 2 M-guanidinium chloride, but irreversible inactivation with the appearance of denatured monomers is seen at higher concentrations. However, even after 24 h in 2 M-guanidinium chloride, the enzyme retains 70% activity after dilution.

This suggests that guanidinium chloride promotes reversible dissociation to a mixture of active A-B* and inactive A-B and A-A* dimers, but this conclusion should be treated with caution. The guanidinium ions may decrease activity directly by competing with the catalytic Mg^{2+} ions. However, such direct inhibition would be concentration-dependent, which does not fit with the 50% plateau seen in the 1–3 M range of guanidinium ions.

SDS also promotes dissociation into active dimers, and the activity assays in the presence or absence of SDS suggest that these are predominantly A-B* dimers. However, the effects at increasing SDS concentrations are puzzling. In the range 0.2–1% SDS, the unfolding pathway appears to be via active dimers followed by inactive monomers, as in urea and guanidinium chloride. However, higher concentrations of SDS provide a relative stabilizing effect, perhaps by preventing A-B* subunit dissociation?

Thermostability

Studies of thermostability are generally made by incubating enzymes at various temperatures for a fixed time and measuring residual activity after cooling. Re-activation after cooling could be a problem, but in this case was shown to be negligible. Phosphate buffers are frequently used because their pH changes very little with temperature, but these are not buffers of choice for enzymes requiring metal ions for catalytic activity because of the limited solubility of many metal phosphates and the consequent change with temperature of free cation concentrations.

Nevertheless, the conventional 'melting points' of D-xylose isomerase determined in 20 mM-phosphate buffers in this way at two different pHs reveal significant stabilization by ligands (Fig. 9). The apoenzyme is least stable. At pH 8, substantial stabilization is provided by Mg^{2+} (the corresponding Co^{2+} -containing enzymes were not studied because of the poor solubility of cobalt phosphate) and the further presence of the competitive inhibitor xylitol improves the stability of the enzyme only very slightly. At pH 5.5, the apoenzyme is even less stable and Mg^{2+} improves the stability of the enzyme only moderately, whereas addition of xylitol now causes a dramatic improvement. At pH 5.5, the active-site carboxy groups that chelate the Mg^{2+} at site 1 are probably not fully ionized; addition of xylitol could be expected to strengthen Mg^{2+} binding and so push the

equilibrium to the high-pH form. Substrates also seem to provide even more significant thermostabilization, since no inactivation was seen after 5 h at the end of the model batch isomerization performed with 50% (w/v) glucose in 10 mM-Mg²⁺ at 80 °C, pH 5.5 (Fig. 11).

The more accurate first-order rate constants for inactivation in Tris/HCl buffers reinforce the above results and are even more revealing. The addition of Mg²⁺ alone to the apoenzyme provides significant stabilization, but xylitol alone has no effect; this shows that xylitol cannot bind to the apoenzyme (Rangarajan & Hartley, 1992). The ternary complex with Mg²⁺ and xylitol ($K_d = 3 \times 10^{-4}$ M) has double the lifetime of the Mg²⁺-enzyme complex ($K_d = 1 \times 10^{-4}$ M). This could be because Mg²⁺ at site 1 in the binary complex has two of the six co-ordination positions occupied by water molecules, whereas in the ternary complex these are replaced by O-2 and O-4 of the inhibitor.

The Co²⁺-containing enzyme lost no activity under comparable conditions over 18 h at 85 °C, we believe that this is because only one Mg²⁺ is bound whereas Co²⁺ binds to both site 1 and site 2 (Rangarajan & Hartley, 1992). This conclusion is reinforced by studies with the homologous and also thermostable *Streptomyces violaceoruber* enzyme (Callens *et al.*, 1986) where the Co²⁺-containing enzyme (K_d 2.5×10^{-4} M for site 1 and 3×10^{-7} M for site 2) is far more thermostable than the Mg²⁺-containing enzyme (K_d 10^{-5} M).

These results show that binding of active-site cations contributes greatly to the considerable thermostability of these enzymes. If thermal unfolding proceeds via reversible dimerization to A-B* dimers followed by kinetically irreversible conversion into random-coil monomers, as in urea or guanidinium chloride, protein engineering to strengthen interactions at the A-B* subunit interface should further increase thermostability. However, when thermal inactivation of D-xylose isomerase was measured in the presence of 10 mM-Mg²⁺ at 80 °C at two different concentrations of protein (0.09 mg/ml and 0.72 mg/ml; results not shown), the inactivation rate constants were identical. If inactivation occurs as a result of mono-

merization, the rate should be higher at the lower protein concentration. This suggests that dissociation of the enzyme may not play an important role in thermoinactivation in the absence of denaturants.

The research was funded by the SERC (Science and Engineering Research Council) Protein Engineering Initiative of the U.K.

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Received 4 November 1991/17 January 1992; accepted 23 January 1992