Biochem. J. (1993) **289**, 201–208 (Printed in Great Britain)

Arthrobacter D-xylose isomerase: partial proteolysis with thermolysin

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The pattern and kinetics of partial proteolysis of *Arthrobacter* D-xylose isomerase tetramer was studied in order to determine the flexibility of surface loops that may control its stability. It was completely resistant to trypsin, chymotrypsin and elastase at 37 °C, but thermolysin cleaved specifically and quantitatively at Thr-347–Leu-348 between helices 10 and 11 to remove 47 residues from the C-terminus of each 43.3 kDa subunit. At high temperatures, helices 9 and 10 were removed from each 38 kDa subunit to give a 36 kDa tetramer. The kinetics of nicking by thermolysin indicated that the Thr-347–Leu-348 loop is locked at low temperatures, but 'melts' at 25 °C and is fully flexible above 34 °C. The flexibility appears to be associated with binding of Ca^{2+} ions at the active site, since Co^{2+}, Mg^{2+} and xylitol protect in proportion to their ability to displace Ca^{2+}. The missing C-terminal helices make many intersubunit contacts that appear in the structure to stabilize the tetramer, but the properties of the purified nicked proteins are almost indistinguishable from the native enzyme. Both the 38 kDa tetramer and the 36 kDa tetramer are identically active and dissociate similarly in urea or SDS to fully active dimers, but the nicked dimers are slightly less stable to urea at 62 °C. In the Mg^{2+} form the thermostability of the 38 kDa tetramer is identical with that of the native enzyme, but the 36 kDa tetramer has a slightly lower 'melting point' (70 °C versus 80 °C), which may be due to unravelling from the end of helix 8. Since elimination of all the C-terminal helices and many intersubunit contacts has so little effect, one can conclude that the 'weak point' that controls the protein's thermostability lies within the N-terminal β-barrel domain.

INTRODUCTION

Previous studies of *Arthrobacter* D-xylose isomerase include its purification and properties (Smith et al., 1991), cloning and expression of the structural gene in either *Arthrobacter* or *Escherichia coli* hosts (Loviny-Anderton et al., 1991), the tertiary structure of the tetrameric Mg^{2+}-enzyme–xylitol and –sorbitol complexes at 0.23 nm (2.3 Å) resolution (Henrick et al., 1989) and elucidation of the mechanism by both X-ray crystallography (Collyer et al., 1990), molecular-dynamics simulation (Smart et al., 1992) and enzyme kinetics (Rangarajan & Hartley, 1992). The tertiary structure reveals that the tetramer is composed of two crystallographically independent dimers denoted as A–B and A*–B* (Henrick et al., 1989). Each subunit contains a main domain (residues 1–327), which is a parallel stranded α–β barrel, and a C-terminal domain (residues 328–394), which forms a loop structure away from the main domain consisting of five helical segments linked by random coil, and which is involved in intersubunit contacts (Figure 1).

The stability to denaturants and/or heat in the presence of various ligands led to the hypothesis that subunit dissociation was a step on the pathway to thermal denaturation (Rangarajan et al., 1992). Hence altered proteins were constructed by protein engineering in which the three subunit interfaces were strengthened by disulphide bridges or an additional salt bridge (Varsani et al., 1993). But the rates of thermal inactivation of these mutant proteins were indistinguishable from that of the wild-type enzyme, so subunit dissociation does *not* affect the rate-limiting step in thermal unfolding.

Hence thermal unfolding must begin from a chain terminus or surface loop within the tetramer. Such a region may be identifiable by its susceptibility to 'nicking' by proteinases (Fontana et al., 1986a; Hartley, 1988). Therefore the effects of various proteinases on *Arthrobacter* D-xylose isomerase was studied at different temperatures in the presence of various ligands.

EXPERIMENTAL

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

Enzyme purification and assays

D-Xylose isomerase was purified from cells of *Arthrobacter* strain N.R.R.L. B 3728 as described by Smith et al. (1991). Samples of apoenzyme, Mg^{2+}-enzyme, Co^{2+}-enzyme or Ca^{2+}-enzyme were prepared from this as described by Rangarajan and Hartley (1992). Apoenzyme and Mg^{2+}-enzyme free of phenylmethanesulphonyl fluoride was prepared by dialysing exhaustively versus the appropriate buffer lacking phenylmethanesulphonyl fluoride. Enzyme activity using D-xylose or D-fructose as substrate was measured as described by Rangarajan and Hartley (1992).

Gel electrophoresis

Electrophoresis under native conditions was performed in 7.5% polyacrylamide gels at pH 8.8, and in the presence of SDS in 12.5% polyacrylamide gels at pH 8.8, as described by Smith et al. (1991). Urea-gradient-gel electrophoresis was performed by the method of Rangarajan et al. (1992). Electrophoresis in the presence of 8 M urea at pH 8.8 was performed in 7.5% polyacrylamide gels as described by Marshall and Inglis (1986).

Coomassie Blue-stained SDS/polyacrylamide gels were scanned in lanes, from the top of the gel to the bottom, at 540 nm using a Shimadzu TLC scanner CS-930 connected to a Shimadzu data recorder/integrator DR-2. The reliability of the quantitative data depends on the specific staining of the native monomer

Abbreviations used: FAB, fast atom bombardment; PVDF, poly(vinylidene difluoride).

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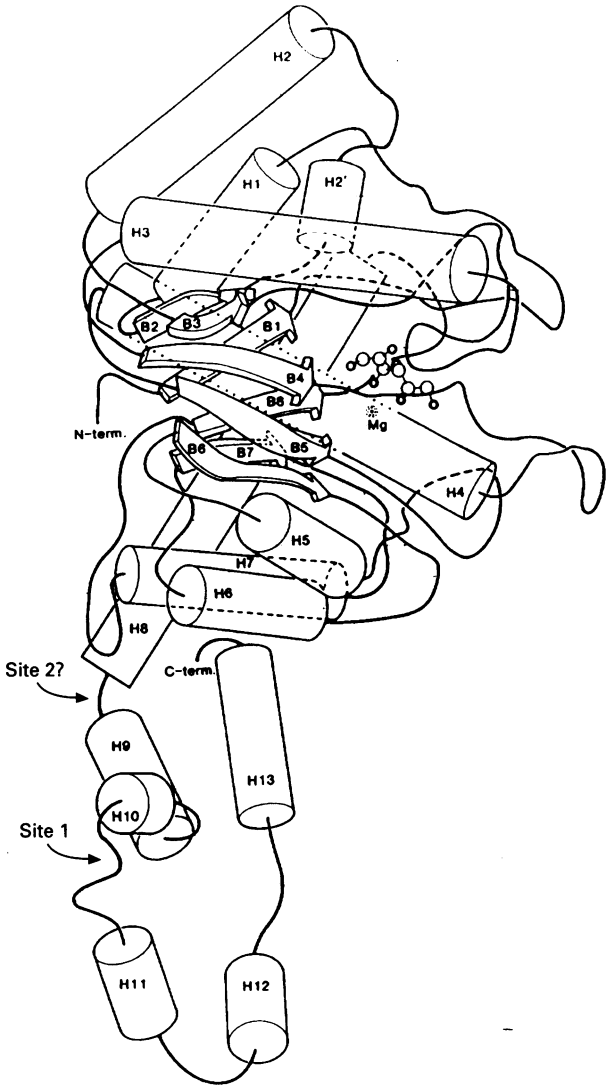

Figure 1 Subunit structure of D-xylose isomerase (adapted from Henrick et al., 1989)

The subunit structure of D-xylose isomerase showing the D-sorbitol-binding site. Helices are represented as cylinders and strands as twisted arrows. Helices H0 (residues 14–17) and H6′ (residues 217–220) have been omitted for clarity. Arrows indicate the first site and probable second site of thermolysin nicking. N-term., N-terminus.

and the nicked fragments being the same. In earlier experiments, lysozyme (15 μg) was routinely incorporated into all the samples before gel electrophoresis, and the intensities of the monomer and nicked bands were normalized to the lysozyme band. Results obtained in this way were almost identical with those obtained when lysozyme was excluded. Therefore lysozyme was not included in subsequent experiments.

Gel filtration by h.p.l.c.

A du Pont Zorbax GF-250 column (30 cm × 7.5 mm internal diam.) was equilibrated and eluted at 22 °C with 10 mM magnesium acetate/100 mM Tris acetate, pH 8.0. The column was calibrated with Blue Dextran, L-tyrosine, apoferritin, β-amylase, yeast alcohol dehydrogenase, phosphorylase *b* (muscle), BSA, BSA dimer and ovalbumin. The elution positions were located by monitoring the absorbance at 280 nm and also by SDS/PAGE of the peak fractions.

Treatment with proteinases

Elastase (porcine) and thermolysin from *Bacillus thermoproteolyticus* were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Chymotrypsin A4 (bovine pancreas) was purchased from Boehringer Mannheim, Lewes, East Sussex, U.K. Trypsin (bovine pancreas) was obtained from Worthington, Freehold, NJ, U.S.A. 'Nicking' experiments were usually performed with 5 mg/ml of D-xylose isomerase in 0.1 M Tris/HCl buffer, pH 8.1 (at the temperature of the experiment) containing various concentrations of Mg^{2+}, Co^{2+} or Ca^{2+} or xylitol, incubated at the desired temperature ($\pm$0.1 °C) in a water bath regulated by a Braun Thermomix B temperature regulator. The reaction was initiated by adding freshly prepared proteinase in a minimum of buffer to a final concentration of 0.5 mg/ml (proteinase/D-xylose isomerase ratio 1:10, w/w). Aliquots for enzyme assays were withdrawn at appropriate times and added immediately to the assay solution. For analysis by SDS gels, 5 μl aliquots were added to 10% (v/v) formic acid to stop the digestion immediately, but thermolysin reactions were stopped with EDTA at concentrations of 10 mM, 50 mM or 200 mM, depending on the concentration of metal ions used in the experiment. In earlier experiments, a thermolysin/D-xylose isomerase ratio of 1:100 (w/w) was used, but the reaction proved too slow, especially at low temperatures, and hence the ratio was increased to 1:10 (w/w).

Measurement of thermostability

'Melting-point' determinations of native and modified D-xylose isomerase were performed as described by Rangarajan et al. (1992).

N- and C-terminal sequencing of modified D-xylose isomerase

D-Xylose isomerase modified by proteinase was subjected to SDS/PAGE at pH 8.8 in 12.5% polyacrylamide gels (1.5 mm thick). Peptide bands were transferred to Immobilon poly(vinylidene difluoride) (PVDF) membrane (Millipore) by electroblotting overnight at a constant current of 50 mA with transfer buffer [18 mM Na_2CO_3/2 mM $NaHCO_3$/10% (v/v) methanol (h.p.l.c. grade)]. The Immobilon membrane was rinsed with Milli-Q water and stained for peptide/protein with 0.1% (w/v) Coomassie Blue R in aq. 50% (v/v) methanol for 10–15 min, then destained with methanol/water/acetic acid (9:9:2, by vol.), rinsed with Milli-Q water several times, and dried in air. The N-terminal sequence was determined by the automated gas-phase Edman method using a CI 4000 gas-phase sequencer.

The N-terminus of peptides released during digestion of D-xylose isomerase by thermolysin was determined manually by the dansyl method of Gray (1972). C-terminal sequencing of modified D-xylose isomerase was performed using carboxypeptidase Y, essentially as described by Klemm (1984).

M.s.

M.s. determinations of the native and 'proteolytically nicked' D-xylose isomerase were performed using the 'FAB (fast-atom-bombardment) mapping' strategy (Morris et al., 1983) on a high-field ZAB mass spectrometer by dissolving samples in aq. 5% (v/v) acetic acid and applying to a probe previously coated with 1 μl of glycerol. After loading the sample (2 μl), 0.5 μl of thioglycerol was added to maximize quasimolecular ion intensity. FAB was achieved by utilizing Xenon ions fired from an M-Scan FAB gun operating at 10 kV and 10 μA current.

The D-xylose isomerase samples were digested with CNBr in aq. 70% (v/v) formic acid at 25° for 5 h. The digests were freeze-dried and subjected to reversed-phase HPLC on a C_{18} Ultrasphere ODS column using a gradient of 0.1% trifluoroacetic acid/aq. 10–90% (w/v) acetonitrile over 100 min.

RESULTS

No cleavage by trypsin, chymotrypsin or elastase

D-Xylose isomerase showed no loss of activity when incubated at 37° for 120 h with trypsin, chymotrypsin or elastase at pH 8.1 in the presence of 5 mM $MgCl_2$. Aliquots of reaction mixture were analysed by SDS/PAGE and showed no change in molecular size of the D-xylose isomerase monomer on treatment with these three proteinases. This suggests that there are no mobile regions in D-xylose isomerase which can fit the specificities of trypsin, chymotrypsin or elastase.

Nicking of the Ca^{2+}-enzyme by thermolysin

Ca^{2+} is always present in commercial thermolysin preparations to increase its activity and thermostability. Its dependence on Ca^{2+} is a drawback for this purpose, since this is a powerful competitive inhibitor of Mg^{2+}, which is the preferred activator cation for the isomerase (Smith et al., 1991). Early experiments with thermolysin and D-xylose isomerase were conducted in volatile ammonium bicarbonate buffers containing 5 mM Mg^{2+} and 2.5 mM Ca^{2+}, so as to ease subsequent protein-chemistry analysis. However these proved equivocal, because of the poor solubilities of the magnesium and calcium (bi)carbonates. Therefore subsequent studies on the nicking of D-xylose isomerase by thermolysin were carried out in 0.1 M Tris/HCl pH 8.1 (at the temperature of the experiment using $\Delta pH = -0.028$ pH unit/°C; Good et al., 1966) containing 5 mM Mg^{2+} and 2.5 mM Ca^{2+}. As discussed below, the enzyme is likely to be in the Ca^{2+} form under these conditions.

There is no loss of isomerase activity over 118 h on incubation with thermolysin at 27 °C under these conditions. Analysis of the reaction mixture during the course of the incubation, by SDS/PAGE, showed the disappearance of the monomer of 43.3 kDa and the appearance of a band of approx. 38 kDa. At temperatures between 4 and 40 °C, thermolysin appeared eventually to convert all of the 43.3 kDa monomer into the 38 kD fragment. *Arthrobacter* D-xylose isomerase monomer has a molecular mass of 43.3 kDa a (Loviny-Anderton et al., 1991; Smith et al., 1991). However, on SDS/PAGE it consistently runs at a position of molecular weight ~ 52 kDa, which could be due to poor binding of SDS (because of its high content of acidic residues). The nicked fragment appears to have a molecular mass of 46 kDa on SDS/PAGE, but recalculation using the data above changes it to 38 kDa.

However, at temperatures greater than 37 °C, after all the 43.3 kDa monomer has been converted into the 38 kDa monomer, a band of approx. 36 kDa begins to appear on SDS/PAGE of the incubation mixture, and thereafter a variety of small peptides is seen. This suggests that, at higher temperatures, a new nicking site begins to emerge in the 38 kDa subunits, which eventually destabilizes the enzyme structure.

Incubation of D-xylose isomerase with thermolysin at 70 °C caused the loss of 50% enzyme activity in 4 h. There was no loss in activity of enzyme incubated under the same conditions in the absence of thermolysin during this period. Analysis of the incubation mixture at different times by SDS/PAGE showed that there was indeed an intermediate species of molecular mass 36 kDa, which was then rapidly digested into smaller peptides.

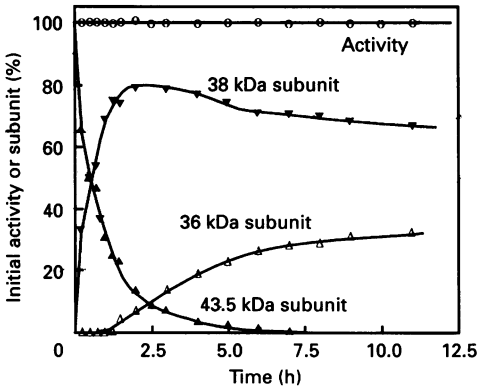

Figure 2 Effect of thermolysin nicking at 70 °C on activity and subunit structure

D-Xylose isomerase (5 mg/ml), in 5 mM $MgCl_2$/2.5 mM $CaCl_2$/0.1 M Tris/HCl, pH 8.1 (at the temperature of the experiment), was incubated with 0.5 mg/ml thermolysin at 70 °C. The reaction was initiated by the addition of thermolysin (0.5 mg) to 1 ml of D-xylose isomerase solution. Enzyme activity remaining was measured using D-xylose as substrate at 30 °C. Aliquots were subjected to SDS/PAGE in 12.5% acrylamide gels. Gels were stained for protein with Coomassie Blue and scanned in lanes from top to bottom, giving the percentage of 43.3 kDa and 38 kDa subunits.

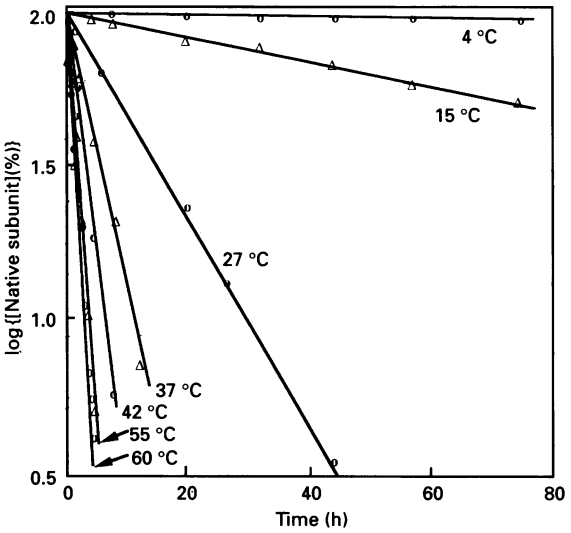

Figure 3 First-order plots for the disappearance of 43.3 kDa subunit

D-Xylose isomerase (5 mg/ml), in 5 mM $MgCl_2$/2.5 mM $CaCl_2$/0.1 M Tris/HCl, pH 8.1 (at the temperature of the experiment), was incubated with thermolysin (0.5 mg/ml) at the temperatures shown. At the times indicated, aliquots of reaction mixture were withdrawn into EDTA to stop the reaction and subjected to SDS/PAGE. Gels were scanned and the proportion of 43.3 kDa subunits present was measured.

Figure 2 shows that when the ternary complex of D-xylose isomerase, M^{2+} (M = bivalent metal) and the competitive inhibitor xylitol was incubated with thermolysin under similar conditions, it was first converted into 38 kDa monomer and then a 36 kDa subunit was generated over a period of 11 h. That species, which represents 35% of the total polypeptides stained in the gels, retains full activity. In order to convert all the 43.3 kDa subunits into 36 kDa fragments, the thermolysin/D-xylose isomerase ratio was increased to 1:1 (w/w) and the temperature decreased to 65 °C. Over a period of 8 h, all the 43.3 kDa subunits were converted into the 36 kDa subunits via the 38 kDa subunits.

Table 1 First-order constants for thermolysin nicking of D-xylose isomerase

The observed first-order constants (k) are for disappearance of native monomer during nicking of the enzyme (5 mg/ml) by thermolysin (0.5 mg/ml) in the presence of the indicated ligands at pH 8.1. The extrapolated rate constant (k') and the unfolding constant (K) are derived from these data as described in the Discussion section.

[Ligand] (mM)					Rate constant		
Ca^{2+}	Mg^{2+}	Co^{2+}	Xylitol	Temperature (°C)	$10^{-3} \times k$ (h^{-1})	$10^{-3} \times k'$ (h^{-1})	Unfolding constant (K)
2.5	5	–	–	4	1	18	0.06
				15	9	45	0.17
				27	70	100	1.25
				30	53	135	2.50
				37	192	–	–
				42	321	–	–
				48	424	–	–
				55	699	–	–
				60	821	–	–
2.5	30	–	–	42	184		
		–	–	48	322		
2.5	30	–	+	42	219		
		–	+	48	281		
2.5	100	–	–	42	94		
		–	–	48	168		
2.5	100	–	+	42	83		
		–	+	48	186		
0.5	5	–	–	42	106		
		–	+	42	78		
0.5	30	–	–	42	80		
		–	+	42	88		
0.5	100	–	–	42	59		
		–	+	42	66		
0.5	–	2	–	37	4		
	–	2	–	48	8		
	–	2	–	55	11		

Kinetics of thermolysin nicking

The effect of thermolysin on D-xylose isomerase in the presence of 5 mM Mg^{2+} and 2.5 mM Ca^{2+} at pH 8.1 was studied in the temperature range 4–60 °C. In all cases, aliquots of reaction mixture at different times were subjected to SDS/PAGE and the stained gels were scanned in lanes from the top to the bottom of the gel. The amount of the 43.3 kDa monomer band remaining was determined as a percentage of the total stain in that lane, and the rate of disappearance of the 43.3 kDa band was found in all cases to follow first-order kinetics (Figure 3).

The data at each temperature were used to construct first order plots using the Enzfitter software (Biosoft–Elsevier, Cambridge, U.K.) from which the rate constants (k) for the disappearance of the monomer were calculated, as shown in Table 1. In all cases, the rate of appearance of the 38 kDa band was exactly equal to the rate of disappearance of the 43.3 kDa band, i.e. to the rate of nicking.

Figure 4 is an Arrhenius plot for the variation of the rate constant of nicking with temperature in the range 4–60 °C, which shows two distinct linear regions with an inflexion corresponding to a temperature of 34 °C. Above this temperature the increase in rate is consistent with an increase in thermolysin activity, as seen for analogous Arrhenius plots for thermolysin acting on synthetic peptide substrates which are linear and have similar slopes in the temperature range 4–80 °C (Ohta et al., 1966; Matsubara, 1970; Heinrikson, 1977). However, at temperatures between 4 and 34 °C, the first-order rate constant for nicking increases almost 8-fold for an 11 °C rise in temperature (Table 1). This is consistent with a model in which the surface segment of chain that forms the 'nicking-site' 'melts' progressively between 4 and 34 °C, but is fully mobile above 34 °C.

Since the above results pertain to the Ca^{2+}-enzyme, nicking of the Mg^{2+}-enzyme was performed at temperatures between 42 and 60 °C in the presence of 2.5 mM Ca^{2+} + 30 mM Mg^{2+}, 2.5 mM Ca^{2+} + 100 mM Mg^{2+} or 2.5 mM Ca^{2+} + 30 mM Mg^{2+} + 10 mM xylitol and in the presence of 0.5 mM Ca^{2+} + 5 mM Mg^{2+}, 0.5 mM Ca^{2+} + 30 mM Mg^{2+}, 0.5 mM Ca^{2+} + 100 mM Mg^{2+} in the absence or presence of 10 mM xylitol; under these conditions, Mg^{2+} should displace Ca^{2+} (see the Discussion section). Increasing Mg^{2+} protected against thermolysin attack, and the presence of the competitive inhibitor xylitol increased the protection slightly (Table 1). In the presence of 100 mM Mg^{2+}, thermolysin showed only 70 % of its maximum activity (results not shown), probably owing to competition between Mg^{2+} and Ca^{2+}.

The effect of thermolysin on the Co^{2+}-enzyme was investigated in the presence of 2 mM Co^{2+} and 0.5 mM Ca^{2+} (the amount actually present in the commercial enzyme sample). Co^{2+} shows high affinity for both metal-binding sites in the active site of D-xylose isomerase (Rangarajan and Hartley, 1992) and cannot be displaced readily by other cations. There was no change in the 43.3 kDa monomer at 37 °C, even after 72 h (results not shown). Under similar conditions the monomer in the Ca^{2+}-enzyme is completely converted into the 38 kDa fragment. At temperatures between 37 and 60 °C, the monomer in the Co^{2+}-enzyme was slowly converted into a 38 kDa fragment, but first-order rate constants of nicking were an order of magnitude lower than those of the Ca^{2+}-enzyme (Table 1). Since 2 mM Co^{2+} inhibits thermolysin (results not shown), probably by competing with Ca^{2+}, the rates for nicking of the Co^{2+}-enzyme in the Arrhenius plot (Figure 4) are corrected for this effect. The Arrhenius activation energy of 44 kJ/mol is similar to that for the Ca^{2+}-enzyme above 34 °C (54 kJ/mol), as would be expected from the hypothesis discussed below.

Purification of modified D-xylose isomerases

D-Xylose isomerase treated with thermolysin at 15° for 80 h gave a protein (T-15) which contained an equimolar mixture of 43.3 kDa and 38 kDa subunits as seen on SDS/PAGE. Incubation with thermolysin at 37 °C for 30 h gave rise to a modified enzyme (T-37), which showed complete conversion of all four 43.3 kDa subunits into 38 kDa subunits. Both reaction mixtures (T-15 and T-37) were subjected to h.p.l.c. gel filtration, and the peaks corresponding to modified protein were used for further characterization.

To obtain pure 36 kDa tetramer, the D-xylose isomerase–M^{2+}–xylitol ternary complex was incubated with thermolysin [1:1 (w/w) ratio] at 65 °C for 11 h (TX-65). SDS/PAGE of the reaction mixture showed that all the 43.3 kDa subunits were then converted into 36 kDa subunits via the 38 kDa subunits. H.p.l.c. gel filtration of the reaction mixture gave a single peak corresponding to modified D-xylose isomerase, and this was used for further characterization.

Comparison of the properties of native and modified D-xylose isomerases

The purified T15, T37 and TX65 proteins were all tetramers [$(43.3\ kDa)_2(38\ kDa)_2$, $(38\ kDa)_4$ and $(36\ kDa)_4$ respectively] and showed the same specific activity towards D-xylose and D-fructose as the native enzyme [$(43.3\ kDa)_4$].

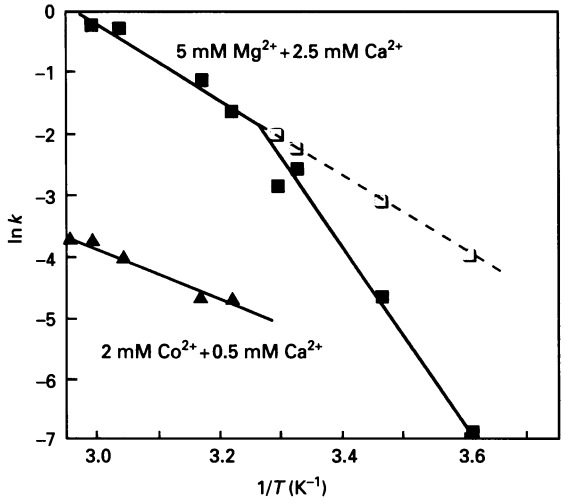

Figure 4 Arrhenius plot for nicking of D-xylose isomerase by thermolysin

First-order rate constants for the disappearance of 43.3 kDa subunit measured at different temperatures are plotted versus 1/*T*.

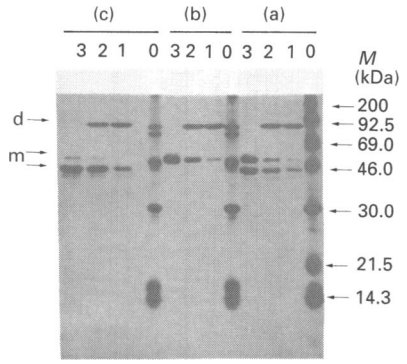

Figure 5 Effect of SDS on subunit structure of nicked D-xylose isomerases

The Mg^{2+}-forms of native or nicked enzymes [**a**, T 15, $(43.5\ kDa)_2(38\ kDa)_2$; **b**, native $(43.5\ kDa)_4$; **c**, T 37, $(38\ kDa)_4$] were incubated at 25 °C for 30 min (lanes 1) or 60 °C for 5 min (lanes 2) or 100 °C for 5 min (lanes 3) in 0.015 M Tris/HCl (pH 6.5)/1 % SDS (w/v) and subjected to SDS/PAGE in 12.5% acrylamide gels. Gels were stained for protein with Coomassie Blue. The position and molecular masses (*M*) of marker proteins (lanes 0) are indicated alongside the gel. Abbreviations: d, dimers; m, monomers.

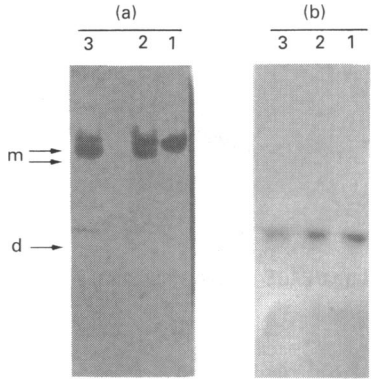

Figure 6 Gel electrophoresis of nicked D-xylose isomerase in 8 M urea

Samples of D-xylose isomerase (5 mg/ml) that had been exhaustively digested with thermolysin (0.5 mg) in 5 mM $MgCl_2$/2.5 mM $CaCl_2$/0.1 M Tris/HCl, pH 8.1, at 15 °C (T 15) or at 37 °C (T 37) or of native enzyme were treated with 8 M urea at 45 °C for 1 h and subjected to 8 M urea/PAGE at pH 8.8 in 7.5% acrylamide gels. Lanes 1, native enzyme; lanes 2, T 15; and lanes 3, T 37. The gel was cut in half and stained for protein (**a**) or D-xylose isomerase activity (**b**). Abbreviations: m, denatured monomers; d, active dimers.

All three nicked proteins showed similar mobility to native protein on native PAGE. This could be either because the folded 38 kDa tetramer adventitiously retains a similar charge/mass ratio, or because the cleaved peptide remains non-covalently associated with the rest of the protein molecule. This was tested by subjecting native and purified T-15 or T-37 proteins to SDS/PAGE in 8–20% acrylamide gradient gels, which can separate proteins and peptides in the range 50–2 kDa. No peptides in the molecular-mass range of 5 kDa were seen, suggesting that the 5 kDa peptide does not remain associated with the protein and is probably digested to smaller peptides. This conclusion was confirmed by dansylation of the reaction mixtures (Gray, 1972), which showed the presence of several dansyl-amino acids, indicating extensive digestive of the 5 kDa peptide.

Like the native enzyme, the nicked tetramers are very stable in SDS. In 1% (w/v) SDS, the native enzyme dissociates first into active dimers and then slowly into unfolded monomers (Rangarajan et al., 1992). Figure 5 shows that the native $(43.3\ kDa)_2(38\ kDa)_2$ and $(38\ kDa)_4$ tetramers are converted into their respective unfolded monomers only after 5 min incubation at 100 °C in 1% (w/v) SDS followed by SDS/PAGE; after 30 min at 25 °C or 5 min at 60 °C, strong bands of active dimers remain. Moreover the $(43.3\ kDa)_2(43.3\ kDa, 38\ kDa)$ and $(38\ kDa)_2$ dimers show identical electrophoretic mobilities.

The nicked Ca^{2+}-enzymes also show similar stabilities after gel electrophoresis in 8 M urea. Figure 6 shows that all three proteins give bands of active dimers with identical mobilities, plus bands of the 43.3 kDa and/or 38 kDa denatured monomers, which differ in mobility. *Arthrobacter* D-xylose isomerase dissociates reversibly into active dimers in 8 M urea (with a midpoint around 4 M urea) which are then slowly converted irreversibly into denatured monomers at 30 °C (Rangarajan et al., 1992) as shown by gel filtration in the presence of urea. Ion-exchange chromatography of the protein in 8 M urea separates the active dimer peak from the inactive monomer peak (Rangarajan et al., 1992), and gel electrophoresis of the two protein peaks in 8 M urea shows that the active dimer band has a higher mobility than the inactive monomer band. Gradient 0–9 M urea electrophoresis at 37 °C, followed by staining for D-xylose isomerase activity, also shows that there is rapid reversible equilibrium between tetramer and fully active dimer. 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 higher temperatures (see Figure 7).

The nicked 38 kDa tetramer is as thermostable as the native enzyme. In the Mg^{2+} form both have 'melting points' of 80 °C, but the 36 kDa tetramer 'melts' at 70 °C. Hence the removal of a further 20 residues from the C-terminus does affect thermostability.

In the presence of Mg^{2+} and substrate, the 38 kDa tetramer is as stable in 8 M urea as the native enzyme; no change in rate of isomerization of fructose was detected over 24 h at 25 °C. However, some slight decreases in stability of the 38 kDa and 36 kDa tetramers were seen using urea-gradient PAGE (Creighton, 1979). All the three proteins behave similarly at 37 °C, but at 62 °C the native enzyme undergoes irreversible denaturation above 6.5 M urea, the 38 kDa tetramer above 4.5 M urea and the 36 kD tetramer above 4 M urea (Figure 7).

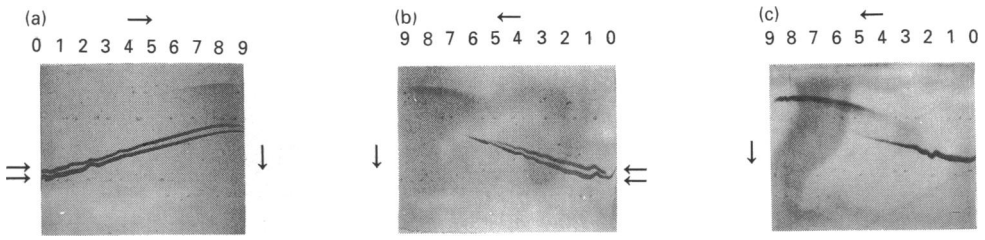

Figure 7 Gradient-urea/PAGE of native and nicked D-xylose isomerases

Samples were subjected to urea-gradient electrophoresis at pH 8.8 in 7.5% acrylamide gels in 37 °C (**a**) or at 62 °C (**b** and **c**) and the gels were stained for protein. Arrows indicate the direction of migration of protein, and the numbers indicate the molarity of urea across the gradient. (**a**) T 37 [$(38\ kDa)_4$] (upper band) and TX-65 [$(36\ kDa)_4$] (lower band) at 37 °C. The results obtained with native protein are not shown; (**b**) T 37 (upper band) and native (lower band) at 62 °C. (**c**) TX-65 protein at 62 °C.

Identification of the sites of nicking

Dansylation showed that serine was the N-terminal amino acid in both the native enzyme and purified 38 kDa tetramer (T37). After SDS/12.5% PAGE, the 38 kDa peptide was transferred to Immobilon PVDF membrane and subjected to automated gas-phase Edman sequencing for seven cycles. The N-terminal sequence was identical with that of D-xylose isomerase (Loviny-Anderton et al., 1991; Smith et al., 1991). Hence the site of nicking is at the C-terminal end of the molecule.

The purified 38 kDa subunit was treated with carboxypeptidase Y, and aliquots were removed at different times and the amino acids released were measured. The results were inconclusive.

The 36 kDa peptide was transferred to Immobilon PVDF membrane and subjected to automated gas-phase Edman sequencing for seven cycles. The N-terminal sequence was again identical with that of native D-xylose isomerase (Loviny-Anderton et al., 1991). This indicates that the new nicking site is also at the C-terminal end, probably in the 320–330 region of the chain.

M.s.

In order to identify the nicking point in the $(38\ kDa)_4$ D-xylose isomerase, samples of the nicked D-xylose isomerase and of the intact $(43.3\ kDa)_4$ isomerase were independently treated with CNBr. This cleavage was chosen after consideration of the positions of methionine in the sequence, and the anticipated loss of ~ 5 kDa C-terminal fragment from the $(43.3\ kDa)_4$ isomerase.

Table 2 Molecular ions and their predicted masses from a CNBr digest of D-xylose isomerase

Digestion of D-xylose isomerase with CNBr was performed as described in the text.

Amino acid residue no.	Expected $(M+H)^+$
1–87	> 3000
88–128	> 3000
129–135	706/724 (lactone/Hser*)
136–157	2335/2353 (lactone/Hser)
158–222	> 3000
223–312	> 3000
315–335	2414/2432 (lactone/Hser)
336–358	2292/2310 (lactone/Hser)
359–394	> 3000

* Homoserine and its lactone.

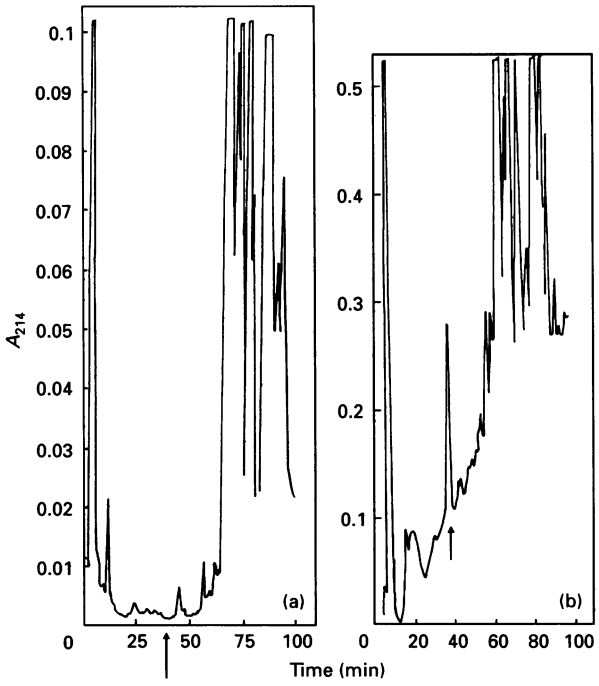

Figure 8 H.p.l.c. reversed-phase chromatography of CNBr digest of native and nicked D-xylose isomerases

Native D-xylose isomerase (**a**) and $(38\ kDa)_4$ nicked D-xylose isomerase (**b**) were digested with CNBr in aq. 70% (v/v) formic acid for 5 h at 25 °C. The reaction mixture was freeze-dried repeatedly after the addition of water and subjected to reversed-phase h.p.l.c. on a C_{18} Ultrasphere ODS column using a gradient of 0.1% trifluoroacetic acid/aq. 10–90% (v/v) acetonitrile over 100 min. The absorbance of the column effluent was monitored at 214 nm. The arrows indicate the position of fraction no. 29.

The h.p.l.c. chromatograms of the CNBr digests are shown in Figure 8. The mass range for m.s. screening of the fractions was set at 0–3000 Da after consideration of the expected masses of CNBr peptides some 5–6 kDa in from the C-terminus of the D-xylose isomerase molecule. The predicted masses and expected molecular ions are shown in Table 2.

Screening of the h.p.l.c. fractions obtained from the CNBr digest of the 38 kDa nicked fragment provided data at m/z 706 (residues 129–135, lactone), m/z 2335 (136–157, lactone) and m/z 2414 (315–335, lactone), but no evidence for the m/z 2292 anticipated for residues 336–358 (lactone).

However, screening of Fraction 29 in the h.p.l.c. chromatogram produced an intense quasimolecular ion at m/z 1268, which

shifted to m/z 1140 after a single step of Edman degradation of the sample. This showed that the peptide in fraction 29 (absent from the chromatogram of native D-xylose isomerase) had an N-terminal lysine or glutamine residue, and a computer search of the D-xylose isomerase sequence for a region of the molecule fitting the derivation of m/z 1268 by CNBr cleavage gives only one possible sequence, residues 336–347 (Lys-Thr-Ser-Gly-Val-Phe-Glu-Leu-Gly-Glu-Thr-Thr). The residue preceding the N-terminal lysine is methionine, and the Edman–m.s. data provides this unique sequence.

The thermolysin nicking point to produce the $(38\ kDa)_4$ D-xylose isomerase is thus shown to be between Thr-347 and Leu-348 in the sequence. Screening of the h.p.l.c. fractions of the CNBr digest of intact $(43.3\ kDa)_4$ D-xylose isomerase found no evidence of m/z 1268, thus confirming the thermolysin-cleavage data.

DISCUSSION

Kinetics of proteolysis of native proteins

Proteases can only cleave flexible surface segments or chain termini in native proteins, and the pathway of proteolytic cleavage can be represented as:

$$S_0 \overset{K}{\longleftrightarrow} S \xrightarrow[+E]{v_1} P_1 \xrightarrow[+E]{v_2} P_2 \xrightarrow[+E]{v_n} P_n \qquad (1)$$

where $(S_0 - S)$ represents the locked conformation and S the flexible conformation of the most susceptible peptide bond and $K = S/(S_0 - S)$ is the equilibrium constant between these. Since substrate concentrations are generally well below the protease K_m:

$$v_1 = \frac{k_{cat.}ES}{K_m} = \frac{k_{cat.}ES_0}{K_m} \times \frac{K}{1+K} \qquad (2)$$

Hence if $k_{cat.}/K_m$ for the proteinase is known (e.g. from studies with denatured proteins or synthetic peptides), the equilibrium constant for the local unfolding of the flexible loop (K) can be calculated (Hartley, 1988).

'All-or-none' proteolysis to small peptides, where v_1 is easily measured by loss of activity, will occur if the initial cleavages increase flexibility at adjacent nicking sites (v_2 in eqn. 1), leading to rapid unfolding (v_n in eqn. 1). 'Nicking' by proteinases will arise if there are few flexible regions and if nicking at one site does not increase flexibility of others, i.e. $v_1 \gg v_2 \gg v_n$ in eqn. (1). In that case nicked 'active fragments' may arise, but SDS/PAGE techniques can give accurate values of v_1.

Kinetics of proteinase nicking of *Arthrobacter* D-xylose isomerase

At 37 °C the Mg^{2+}-enzyme was completely resistant to trypsin, chymotrypsin and elastase; hence very few surface segments of the peptide chains in the Mg^{2+}-tetramer have significant flexibility at this temperature. Therefore thermolysin, a broad-specificity proteinase that is stable at higher temperatures in presence of Ca^{2+} (Ohta et al., 1966), was used to study the 'melting' of surface loops in the Ca^{2+}-enzyme at higher temperatures.

Thermolysin nicking of the native isomerase followed first-order kinetics and can be interpreted in terms of eqn. (2). Above 34 °C, the nicking site is fully flexible, so the increase in v_1 with temperature is due solely to increasing thermolysin activity. The apparent Arrhenius activation energy of 54 kJ/mol is consistent with values of 33 kJ/mol for thermolysin cleavage of casein (Matsubara, 1970) or 18 kJ/mol for cleavage of a synthetic peptide (Ohta et al., 1966). Below 34 °C a significant fraction of the nicking site is locked, so the apparent Arrhenius activation energy increases to 124 kJ/mol. Values for v_1 of a fully flexible nicking site at lower temperatures (k') can be obtained by extrapolating the high-temperature Arrhenius slope (Figure 4). Then eqn (2) yields $k'/k = 1 + 1/K$, from which the values of K shown in Table 1 can be calculated, indicating that the nicking site 'melts' at around 25 °C.

The nicking rates clearly depend on the type of metal ions bound at site 1 and site 2. Under standard conditions (5 mM Mg^{2+} and 2.5 mM Ca^{2+}) Ca^{2+} will be present at site 2, and a roughly equal mixture of Mg^{2+} or Ca^{2+} at site 1 (Rangarajan and Hartley, 1992). Above 30 mM Mg^{2+}, the Mg[1] Ca[2] will predominate, but, in presence of xylitol, the enzyme is probably in the Mg[1]–xylitol form, since xylitol prevents binding of M^{2+} at site 2 (Collyer et al., 1990). With 2 mM Co^{2+} and 0.5 mM Ca^{2+} the Co[1] Co[2] form will predominate.

The simplest explanation for these results is that thermolysin nicking occurs only when Ca^{2+} is present at both site 1 and site 2. When Mg^{2+}/Ca^{2+} ratios increase, nicking rates decrease sharply and xylitol decreases them still further, presumably by displacing Ca^{2+} from site 2. In 2 mM Co^{2+}/0.5 mM Ca^{2+}, the nicking rate is an order of magnitude lower than under the standard conditions, because the predominant species is Co[1] Co[2].

Site of thermolysin nicking in D-xylose isomerase

The site of thermolysin nicking in the 38 kDa tetramer is between Thr-347 and Leu-348. The N-terminus is unmodified, but FAB m.s. gave clear evidence for Lys-Thr-Ser-Gly-Val-Phe-Glu-Leu-Gly-Glu-Thr-Thr as the C-terminal peptide. The simplicity of the h.p.l.c. chromatogram in the region of this peptide and the absence of m.s. signals attributable to related sequences argue for relatively clean nicking rather than ragged C-terminal ends.

The Thr-347–Leu-348 bond is in a surface loop of the tetramer between helix H10 and helix H11 (Figure 1), distant from each active site, but close to the A–A* subunit interface where the C-terminal helical tail stacks on top of the β-barrel core of the adjacent subunit. In the Mg^{2+}-enzyme, Thr-347 and Leu-348 form strong H-bonds with Arg-108, Arg-111 in helix H3 and Glu-159 in helix H4 of the A* subunit. Hence this loop does not appear to be flexible, but flexibility may be induced by binding bulky Ca^{2+} cations in place of smaller Mg^{2+} or Co^{2+} ions in the active site of the adjacent subunit, which would trigger movements of helices H3 and/or H4.

The second nicking site that leads to the 36 kDa tetramer has not been precisely located, but plausibly lies in the sequence -^{326}Arg-Ala-Asp-Pro-Glu-Val^{331}- that forms a sharp surface turn between the end of the H8 helix (301–327) and the H9 helix (329–338) (Figure 1). Consideration of thermolysin specificity suggests the Arg–Ala or Glu–Val bonds as the most likely targets. These would become accessible to thermolysin only when H8 and H9 can uncoil; hence prior cleavage at 347–348 is an obvious requirement.

Properties of modified D-xylose isomerases

Despite the loss of about 50 or 70 residues from the C-terminus, each nicked species $[(43\ kDa)_2(38\ kDa)_2$, $(38\ kDa)_4$ and $(36\ kDa)_4]$ remained tetrameric, unaltered in isomerase activity and identical with native enzyme in PAGE mobility. The latter is puzzling, because the denatured nicked monomers had higher PAGE mobility in 8 M urea at pH 8.8 showing that charge/mass ratios are substantially different. Moreover, the 38 kDa tetramer

is almost indistinguishable from native enzyme in stability to temperature and urea.

The 38 kDa tetramer lacks helices H11, H12 and H13, which make many contacts with the core barrels of adjacent subunits at both the A–A* and A–B interfaces, but are not involved with the A–B* interfaces that contain the active sites. It is therefore understandable that the isomerase activity is unaffected, but surprising that removal of these helices and many of the A–A* and A–B subunit interactions should have negligible effect on stability either to heat or denaturants.

The 36 kD tetramer, which further lacks helices H9 and H10, was also an identically active tetramer and only slightly less stable to heat or urea. Again the unchanged activity is explicable, and the decrease in 'melting point' of the Mg^{2+}-enzyme may be due to uncoiling of the H8 helix from its new C-terminus. Since elimination of all the C-terminal helices and many intersubunit contacts has so little effect, one can conclude that the 'weak point' that controls the thermostability of the enzyme lies within the N-terminal β-barrel domain.

We thank J. Shaghaghi and C. Moon for performing some of the experiments, which formed part of short undergraduate and M.Sc. research projects. The research was funded by the SERC (Science and Engineering Research Council, U.K.) Protein Engineering Initiative.

Received 23 April 1992/23 July 1992; accepted 28 July 1992

REFERENCES

Collyer, C. A., Henrick, K. and Blow, D. M. (1990) J. Miol. Biol. **212**, 211–235
Creighton, T. E. (1979) J. Mol. Biol. **129**, 235–264
Fontana, A., Fassina, G., Vita, C., Dalzoppo, D., Zamai, M. and Zambonin, M. (1986) Biochemistry **25**, 1847–1851
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. and Singh, R. M. M. (1966) Biochemistry, **5**, 467–477
Gray, W. R. (1972) Methods Enzymol. **25**, 333–344
Hartley, B. S. (1988) Abstr. Int. Congr. Biochem. 14th Tu, S5-1, p.12
Heinrikson, R. L. (1977) Methods Enzymol. **47**, 175–189
Henrick, K., Collyer, C. A. and Blow, D. M. (1989) J. Mol. Biol. **208**, 129–157
Klemm, P. (1984) in Proteins (Walker, J. M., ed.), vol. **1**, pp. 243–259, Humana Press, Clifton, NJ
Loviny-Anderton, T., Shaw, P.-C., Shin, M.-K. and Hartley, B. S. (1991) Biochem. J. **277**, 263–271
Marshall, R. C. and Inglis, A. S. (1986) in Practical Protein Chemistry (Darbre, A., ed.), pp. 1–66, John Wiley and Sons, Chichester
Matsubara, H. (1970) Methods Enzymol. **19**, 642–651
Morris, H. R., Panico, M. and Taylor, G. W. (1983) Biochem. Biophys. Res. Commun. **117**, 299–305
Ohta, Y., Ogura, Y. and Wada, A. (1966) J. Biol. Chem. **241**, 5919–5925
Rangarajan, M. and Hartley, B. S. (1992) Biochem. J. **283**, 223–233
Rangarajan, M., Asboth, B. and Hartley, B. S. (1992) Biochem. J. **285**, 989–998
Smart, O. S., Akins, J. and Blow, D. M. (1992) Proteins Struct. Funct. Genet. **13**, 100–111
Smith, C. A., Rangarajan, M. and Hartley, B. S. (1991) Biochem. J. **277**, 255–261
Varsani, L., Cui, T., Rangarajan, M., Goldberg, J., Collyer, C. A. and Blow, D. M. (1993) Biochem. J., in the press