Arthrobacter D-xylose isomerase: chemical modification of carboxy groups and protein engineering of pH optimum

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To try to lower the pH optimum, the carboxy groups of *Arthrobacter* D-xylose isomerase were coupled to glycylamide using a water-soluble carbodi-imide. In conditions that substituted all of the 59 carboxy groups in the denatured monomer, a maximum of 30 groups/monomer reacted in the native enzyme, whether in presence or absence of ligands, and the enzyme remained fully active and tetrameric throughout the coupling reaction. Purification by f.p.l.c. ion-exchange chromatography gave broad symmetrical peaks with increased pI, suggesting that the modified enzymes are essentially homogeneous. However, they are less stable than native enzyme in 8 M urea or on heating ('melting points' of 59° versus 73 °C for the apoenzymes and 67° versus 81.5 °C for the Mg²⁺-enzymes). Kinetic studies of the D-fructose isomerase activity at 30 °C showed that the glycyl-

amidylated enzyme had unaltered activation constant for Mg²⁺, and K_m was also similar to that of the native enzyme at pH 7.3, but increased rapidly at higher pH rather than remaining constant. V_{max} was constant from pH 6.2 to 8.0, suggesting a reduced pK_a for His-219, which controls V_{max} in the native enzyme (normally 6.0). Three mutants were constructed by protein engineering with a view to reducing the pH optimum of enzyme activity. Two of these, Glu¹⁴⁰→Lys and Asp¹⁸⁹→Lys, could be detected in crude extracts of *Escherichia coli* by SDS/PAGE, but could not be purified, whereas mutant Trp¹³⁶→Glu was produced as a tetramer in amounts similar to the wild-type enzyme. However, it did not show any enzyme activity and was less stable in 0–9 M urea gradient PAGE.

INTRODUCTION

Previous studies of *Arthrobacter* D-xylose isomerase included 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 both X-ray crystallography (Collyer et al., 1990), molecular-dynamics simulation (Smart et al., 1992) and enzyme kinetics (Rangarajan and Hartley, 1992). As background for protein-engineering improvements to improve its thermostability as a commercial glucose isomerase, the stability to denaturants and/or heat in presence of various ligands was also studied (Rangarajan et al., 1992).

The tetramer is composed of two crystallographically independent dimers, denoted as A–B and A*–B*, that could dissociate into three possible dimer conformations, namely A–A*, A–B and A–B*, and residues from each subunit contribute to the active sites which lie at the A–B* interface (Henrick et al., 1989). The main domain of each subunit (residues 1–327) is a parallel stranded α – β barrel, and the C-terminal domain (328–394) is a loop consisting of five helices linked by random coil that makes contact with the N-terminal domains of adjacent subunits.

At room temperature, urea causes rapid reversible dissociation of the Mg²⁺-enzyme into fully active A–B* dimers, with a midpoint at about 4 M urea. Guanidinium chloride (0–1 M) causes similar reversible dissociation into active dimers, but in this case A–A* and/or A–B dimers also seem to be part of the mixture. Low concentrations of SDS also give active dimers, leading to unfolded monomers. This suggested that subunit dissociation was a step on the pathway to thermal denaturation.

Hence altered proteins were constructed by protein engineering in which two of the three subunit interfaces were strengthened by disulphide bridges or an additional salt bridge (Varsani et al., 1993). However, the rates of thermal inactivation of most of these mutant proteins were indistinguishable from that of the wild-type enzyme, so subunit dissociation does not appear to lie on the pathway of thermal unfolding.

Therefore thermal unfolding must begin from a chain terminus or surface loop within the tetramer. Such a region might be susceptible to 'nicking' by proteinases (Fontana et al., 1986; Hartley, 1988), so the effects of various proteinases on the native enzyme in the presence of various ligands were studied at different temperatures (Siddiqui et al., 1993). The kinetics of nicking by thermolysin revealed a flexible Thr³⁴⁷–Leu³⁴⁸ loop that 'melts' at 25 °C and can then be nicked to give a 38 kDa tetramer lacking all C-terminal loops. At higher temperatures, further nicking between the H8 and H9 helices removes a further 20 C-terminal residues, yielding a 36 kDa tetramer. Both 'nicked tetramers' have unaltered enzyme activity and are only slightly less stable in urea at 62 °C. Moreover, the 38 kDa tetramer has an unaltered 'melting point', and the 36 kDa tetramer is only slightly less thermostable, which may be due to unravelling from the end of helix H8. Since elimination of all the C-terminal helices and many inter-subunit contacts has so little effect, one can conclude that the 'weak point' that controls the thermostability lies within the N-terminal β -barrel domain.

It would be useful to lower the alkaline pH optimum of D-xylose isomerase so as to prevent 'browning reactions' of sugars in the production of high-fructose syrups from glucose syrups at higher temperatures. The pH optimum is determined by the protonation of His²¹⁹, which prevents metal-ion binding at the Site 2 isomerization site (Rangarajan and Hartley, 1992). Re-

Abbreviations used: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride.

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removal of negatively charged surface carboxy groups might decrease the pK_a of His²¹⁹ and so reduce pH optimum. For example, acetylation of all surface lysine residues of trypsin raises the pK_a of the active-site histidine by 0.2 unit (Spomer and Wootton, 1971). With chymotrypsin, succinylation of 14 surface lysine residues increases surface negative charge by 28 units and raises the pK_a of the active-site histidine by 1 pH unit (Valenzuela and Bender, 1971). In contrast, modification of 13 negatively charged surface carboxy groups to positively charged amines lowers the pK_a of the active site from 7.0 to 6.1 (Fersht, 1985). Hence the present paper describes the effects of coupling the surface carboxy groups of *Arthrobacter* D-xylose isomerase to glycylamide.

An alternative approach would be to modify specific carboxy groups close to His²¹⁹ by protein engineering. In subtilisin, elimination of a single negative charge 1.3 nm (13 Å) (Asp⁹⁹) or 1.5 nm (15 Å) (Glu¹⁵⁶) away from the active-site His reduces its pK_a at ionic strengths below 0.1 by ~0.3 pH unit or 0.4 unit respectively (Thomas et al., 1985; Russell and Fersht, 1987). Changing both simultaneously to give a double mutant lowers the pK_a by 0.65 units (Russell and Fersht, 1987). Unfortunately most of the carboxy groups in D-xylose isomerase play an essential role in metal-ion binding or in forming inter- or intra-subunit salt bridges, and other surface carboxy groups are relatively distant from His²¹⁹. However Glu¹⁴⁰ is 1.26 nm (12.6 Å) away from His²¹⁹, and Asp¹⁸⁹ is 1.53 nm (15.3 Å) away from one site and 1.56 nm (15.6 Å) from another, so mutations of each of these to Lys were studied.

Another mutation of Trp¹³⁶→Glu was made to test the mechanistic hypothesis of Collyer et al. (1990). This lies close to the catalytic Lys¹⁸² and with Phe⁹³ and Phe²⁵⁵ (from the neighbouring subunit) forms part of an 'aromatic lid' that shields the presumed hydride ion intermediate from water in the hydride-ion mechanism. It was envisaged that this mutant might be inactive, but, if not, its pH optimum should be higher so its stability and properties are relevant to the substance of this paper.

EXPERIMENTAL

Unless otherwise stated, all materials and methods were as described by Smith et al. (1991) and Loviny-Anderton et al. (1991). Gel electrophoresis in SDS was in 12.5% polyacrylamide gels at pH 8.8 (Smith et al., 1991) and in 8 M urea in 7.5% polyacrylamide gels at pH 8.8 (Marshall and Inglis, 1986). Urea-gradient (0–9 M)-gel electrophoresis and measurements of thermostability were as in Rangarajan et al. (1992).

Enzyme purification and assays

D-Xylose isomerase was purified from cells of *Arthrobacter* B3728 according to Smith et al. (1991) and apoenzyme was prepared from this as described by Rangarajan and Hartley (1992). Enzyme activity using D-xylose and D-fructose as substrate was measured by the method of Rangarajan and Hartley (1992). Units of activity are the amounts of enzyme that convert 1 μmol of D-xylose to D-xylulose/min or of D-fructose to D-glucose.

Preparation of [¹⁴C]glycine methyl ester

The procedure of Darbre and Islam (1968) and Islam and Darbre (1969) was used. Methanolic HCl was prepared by adding four 1.8 ml aliquots of acetyl chloride (Analar grade, BDH Chemicals, Poole, Dorset, U.K.) to 15 ml of h.p.l.c. grade methanol,

precooled for 10 min in a solid-CO₂/propan-2-ol bath. After each addition, the solution was gradually warmed to room temperature, then cooled thoroughly before adding the next aliquot; it was finally left at room temperature for 1 h and then made up to 25 ml with h.p.l.c.-grade methanol.

[U-¹⁴C]Glycine (250 μCi; 5 ml, sp. radioactivity 114 mCi/mmol; Amersham International, Amersham, Bucks., HP7 9NA, U.K.) in aq. 2% (v/v) ethanol was freeze-dried in a Quickfit round-bottomed flask. Freshly prepared methanolic HCl (4 ml) was then added and the flask was sealed with a ground-glass stopper and Parafilm and incubated in an oven at 70 °C for 1.5 h. The methanolic HCl was removed by rotary evaporation and washing five times with methanol, with rotary evaporation between washes.

The dried [¹⁴C]glycine methyl ester in 250 μl of methanol was purified by descending Whatman no. 1 paper chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) for 24 h at room temperature. Glycine and glycine methyl ester were used as markers. After drying the chromatogram in a well-ventilated hood, a guide strip was stained with ninhydrin/cadmium acetate [85 ml of 1% (w/v) ninhydrin in acetone, mixed with 15 ml of 0.04 M cadmium acetate solution in aq. 50% (v/v) acetic acid] and subjected to autoradiography using Betamax Hyper Film (Amersham International, Amersham, Bucks. HP7 9NA, U.K.) for 24 h. The [¹⁴C]glycine methyl ester band was cut out, eluted with methanol at 4 °C, dried and sealed under vacuum, and stored at –20 °C.

Carboxy-group modification

D-Xylose isomerase was modified with glycylamide using water-soluble EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride] as the activating agent in 50 mM KH₂PO₄/K₂HPO₄, pH 5.7 (Hoare and Koshland, 1967).

The rate of incorporation of glycylamide into D-xylose isomerase, in the presence or absence of 5 mM MgCl₂ ± 5 mM xylitol, was measured as a function of EDC concentration. [¹⁴C]Glycine methyl ester (sp. radioactivity 114 mCi/mmol) was added to 1 M glycylamide in buffer solution to give a final sp. radioactivity of 35 μCi/mmol. An aliquot (10 μl) was diluted to 1 ml with distilled water for measurements for radioactivity, and of the initial amount of amino groups present (glycylamide + glycine methyl ester) by reaction with 2,4,6-trinitrobenzenesulphonic acid. Concentrated D-xylose isomerase was then added (final concn. 1 mg/ml) and reaction was initiated by adding solid EDC to a final concentration of 0.1 M, followed by two similar additions at 2 h intervals. Aliquots (10 μl) were withdrawn at different times, and residual D-xylose isomerase activity was measured. To measure the radioactivity incorporated into protein, 10 μl aliquots were diluted into 1 ml of 0.1 M Tris/HCl buffer, pH 7.5, to stop the reaction, and 100 μl samples were dialysed against 0.1 M Tris/HCl buffer (pH 7.5)/0.01 M MgCl₂ in a Pierce Microdialyzer System 100 (Pierce, Rockford, IL, U.S.A.), followed by dialysis against distilled water. Total ¹⁴C radioactivity in the dialysed 100 μl samples was measured by mixing with 10 ml of OPTI-PHASE HISAFE-3 Scintillation cocktail (Pharmacia-LKB, Milton Keynes, Bucks., U.K.) and counting in a Kontron Betamatic liquid-scintillation spectrometer. Total protein present in the dialysate was determined after acid hydrolysis followed by amino acid analysis.

Routine glycylamidylations of D-xylose isomerase of apoenzyme, Mg²⁺ enzyme (in the presence of 5 mM MgCl₂) or Mg²⁺-xylitol-enzyme (5 mM MgCl₂ + 5 mM xylitol) were performed as follows. Concentrated enzyme was diluted to 1 mg/ml in 1 M glycylamide/50 mM KH₂PO₄/K₂HPO₄ buffer, pH 5.7, with or

without ligands. The reaction was initiated by adding solid EDC to a final concentration of 0.1 M, and the temperature maintained at 22 °C with constant stirring. Two similar lots of EDC were added at 2 h intervals. At 2 h after the final addition of EDC, the modified D-xylose isomerase was dialysed against 50 mM Tris/HCl (pH 7.5)/10 mM MgCl₂ to remove excess reagents. Dialysed protein was treated with 1 M hydroxylamine, pH 7.5, for 6 h at 40 °C to regenerate modified tyrosine residues (Carraway and Koshland, 1968).

Native D-xylose isomerase and modified enzymes were subjected to f.p.l.c. anion-exchange chromatography using a 1 ml Mono-Q column (Pharmacia) equilibrated in 50 mM Tris/HCl (pH 9.0)/10 mM MgCl₂ at a flow rate of 0.5 ml/min. Enzyme was eluted with a gradient of NaCl from 0 M to 1 M.

Enzyme kinetics

The effect of Mg²⁺ on D-fructose isomerase activity was determined using 5 µM–20 mM Mg²⁺ and 3.2 M fructose (Rangarajan and Hartley, 1992). The apparent *K_m* at various pH values in Mes, Mops and Hepes buffers was derived by non-linear regression analysis of data with 0.04–3 M D-fructose at 30 ± 0.1 °C using the ENZFITTER data-analysis program (Biosoft, Cambridge, U.K.). For simple measurements of the *V_{max}*, for Mg²⁺-D-xylose isomerase, 3.2 M D-fructose was used and values were corrected for variation of *K_m*(app.).

Thermolysin nicking of glycinamidylated D-xylose isomerase

Thermolysin nicking experiments at different temperatures were performed as described by Siddiqui et al. (1993).

Site-directed mutagenesis

Mutant genes were made using the following mutagenic oligonucleotides synthesized by Mr. J. Knill-Jones (of the Chemistry Department of this College) using an Applied Biosystems DNA Synthesizer (changed nucleotides are indicated in **bold**):

- 1 5'-CGCTGCCCTTGCGCCCG-3' Glu¹⁴⁰→Lys
- 2 5'-GGAAGATCTTGCCGCGT-3' Asp¹⁸⁹→Lys
- 3 5'-GCCCCGCTTCCATGACGAA-3' Trp¹³⁶→Glu

After deprotection in ammonia overnight, they were purified on a Pharmacia Mono-Q HR 5/5 column by elution with a gradient of 0–1.5 M NaCl in 0.1 M NaOH, then phosphorylated as described by Zoller and Smith (1982). Site-directed mutagenesis was performed by the method of Kunkel (1985) in the single-stranded phage M13mp9, which contained the 1.9 kb *SalI*-(filled-in)-*Bss*HII *xylA* insert, from the pAX12 plasmid described in Loviny-Anderton et al. (1991). Mutants were screened by hybridization with washings at specific temperatures exactly as described by Varsani et al. (1993). The mutated *xylA* inserts were subsequently transferred into the vector pTZ 19U (Mead et al., 1986) as *SalI*–*EcoRI* fragments, and mutations were checked by sequencing (Sanger et al., 1977).

Each *E. coli* TG2 host was grown to stationary state in 2-litre flasks containing 500 ml of 2 × TY (Tryptone/Yeast Extract) + ampicillin medium. Cell-free extracts were prepared by lysozyme treatment of 30–50 g samples of these cells, dialysed and subjected to ion-exchange chromatography on DEAE-Sephacel columns eluted with a gradient of 150–500 mM NaCl. In each case, no enzyme activity was detectable in crude extracts. However, protein bands corresponding to the 43.3 kDa monomer band were observed in SDS/PAGE of crude extracts.

RESULTS AND DISCUSSION

Glycinamidylation of D-xylose isomerase

Each subunit of D-xylose isomerase contains 58 Asp + Glu residues and one C-terminal carboxy group (Loviny-Anderton et al., 1991). These were first modified under denaturing conditions (6 M guanidinium chloride) using 0.1 M EDC as the activating agent and 0.01–1.0 M glycine methyl ester or glycineamide as the nucleophile at 30 °C for 4 h in phosphate buffer of the desired pH. Guanidinium chloride does not interfere with the modification of carboxy groups by carbodi-imides (Hoare and Koshland, 1967). Table 1 shows that the degree of modification increases with increasing concentration of the nucleophile and with decreasing pH. A maximum of 58–60 glycine residues are incorporated at pH 4.75 with > 0.75 M glycine methyl ester or glycineamide. Results with glycine methyl ester or glycineamide were identical, since the *pK_a* values of their α-amino groups are very similar (7.66 and 7.93 respectively at 25 °C; Edsall and Wyman, 1958). Use of glycine methyl ester as the nucleophile has the advantage that the original charge on modified peptides can be restored by alkaline hydrolysis of the coupled methyl ester, allowing purification by 'diagonal paper electrophoresis' according to the principles of Hartley (1970). However, glycineamide was used in subsequent experiments with native enzyme to avoid hydrolysis of the methyl ester groups during purification and other manipulations, and at pH 5.7, since the enzyme is not very soluble at pH 4.75.

The native enzyme in either apo- (M1), Mg²⁺- (5 mM MgCl₂; M2) or Mg²⁺-xylitol- forms (5 mM MgCl₂ + 5 mM xylitol; M3) was allowed to react with 1 M glycineamide containing [¹⁴C]glycine methyl ester at pH 5.7 and three 2 h additions of solid EDC (to 0.1 M). Figure 1 shows the rate of uptake of glycineamide residues into the Mg²⁺-xylitol complex; the results with apoenzyme and Mg²⁺-enzyme were almost identical. About 12 or 13 carboxy groups per subunit were modified within 1 min after the first addition of EDC, and a total of about 30 carboxy groups per subunit were modified shortly after the second addition of EDC, with no further incorporation thereafter. Enzyme activity remained constant throughout the experiment.

Of the 58 Asp + Glu residues of D-xylose isomerase, 21 are fully exposed, 13 are partially buried and 24 fully buried as shown by solvent accessible area (Å²) (Henrick et al., 1989; Collyer et al., 1990). The carboxy groups in the active site are clearly not modified, since the enzyme activity is unchanged during chemical modification. Excluding these and others involved in intra- or inter-subunit salt bridges or that lie at the A–A* interface (D149 and D189), we are left with only 30 carboxy groups that are potentially modifiable (D8, E44, D61, E64, E66, E68, D74, D80, D124, E127, E131, E140, D162, D170,

Table 1 Carboxy-group modification of D-xylose isomerase under denaturing conditions

[Glycine methyl ester] or [glycineamide] (M)	pH...	No. of glycine residues incorporated/ subunit of D-xylose isomerase		
		4.75	5.7	6.5
0.01		10	12	8
0.10		41	33	18
0.50		49	45	20
0.75		60	47	26
1		58	52	38

Figure 1 Rate of modification of carboxy groups in D-xylose isomerase

D-Xylose isomerase (1 mg/ml) in 0.05 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 5.7, containing 5 mM MgCl_2 , 5 mM xylitol, 1 M glycine and $[^{14}\text{C}]$ glycine methyl ester (sp. radioactivity 35 $\mu\text{Ci}/\text{mmol}$) was incubated at 25 °C. Three additions of EDC to final concentration of 0.1 M were made at 2 h intervals, as indicated by arrows. At different times, aliquots were withdrawn in duplicate. Enzyme activity remaining was measured using D-xylose as substrate; aliquots were diluted 100-fold with 0.1 M Tris/HCl buffer, pH 7.5, to stop the reaction and subjected to exhaustive dialysis against 0.1 M Tris/HCl (pH 7.5)/0.01 M MgCl_2 . Total protein in the dialysis residue was determined by total hydrolysis followed by amino acid analysis. ^{14}C radioactivity in the residue was measured by liquid-scintillation counting. ■, D-Xylose isomerase activity remaining; ●, Number of glycine residues incorporated into each D-xylose isomerase subunit.

E203, E206, E238, D264, D299, D302, D306, D328, E233, E345, E352, D356, D364, E370, E374 and the C-terminal carboxy group). The results suggest that all, and only, these 30 have been modified and that the other 28 are inaccessible. Hence all active-site carboxy groups are protected, even in the apoenzyme, indicating that the bulky EDC molecule cannot enter the rather narrow substrate-binding pocket. Therefore the glycinamidylated apoenzyme (M1), Mg^{2+} -enzyme (M2) and ternary complex (M3) are identical.

Characterization of glycinamidylated D-xylose isomerase

Native or modified D-xylose isomerases M1, M2 and M3 were subjected to f.p.l.c. anion-exchange chromatography. The native enzyme was eluted at a NaCl concentration of 0.56 M, whereas M1, M2 and M3 were eluted at a much lower concentration of NaCl (0.2 M), indicating that the pI had increased considerably after carboxy-group modification. Only one broad symmetrical peak was obtained for all three proteins suggesting that they are fairly homogeneous species and confirming that an identical number of acidic groups have been modified in each case.

SDS/PAGE of native and modified D-xylose isomerases showed only one band of protein, in all cases migrating to the same position as native monomer. H.p.l.c. gel-filtration chromatography of M1, M2 and M3 was performed either in columns equilibrated in 0.2 M Tris/acetate (pH 7.5)/0.01 M magnesium acetate or in 0.05 M Tris/acetate (pH 7.5)/0.01 M magnesium acetate/40% ethylene glycol (Rangarajan et al., 1992). However, in no case was it possible to elute the proteins off the column, suggesting that the modified proteins are very hydrophobic.

Gel electrophoresis, in 8 M urea at pH 8.8, of native D-xylose isomerase and proteins M1 and M2 is shown in Figure 2. Native enzyme gives the expected pattern (Siddiqui et al., 1993) of an active dimer band and an inactive denatured monomer band. However, both M1 and M2 gave only a very slow moving band which did not show enzyme activity and probably corresponds to the denatured modified monomer; no band of enzymatically

Figure 2 Gel electrophoresis of native and glycinamidylated D-xylose isomerases in 8 M urea

Samples of native and purified modified D-xylose isomerases (M1 and M2) were treated with 8 M urea/0.025 M Tris/HCl buffer, pH 6.5, at 25 °C for 30 min or at 60 °C for 1.5 h prior to electrophoresis in 8 M urea gels, pH 8.8. The gel was cut in half and stained for protein (a) or D-xylose isomerase activity (b). Abbreviations: m, denatured monomers; D, active dimers; N, native enzyme; M1, modified apoenzyme; M2, D-xylose isomerase modified with EDC-glycinimide in the presence of 5 mM MgCl_2 . N*, M1*, and M2* refer to samples heated at 60 °C for 1.5 h prior to electrophoresis.

Figure 3 Effect of Mg^{2+} on D-fructose isomerase activity of native and modified D-xylose isomerases

Native and modified D-xylose isomerase (M2) apoenzymes (0.8 mg/ml) were preincubated at 30 °C for 5 h in 0.05 M Tris/HCl buffer, pH 8.0, containing 5 μM –20 mM Mg^{2+} and then assayed against 3.2 M D-fructose in similar buffers. Activity is expressed as a percentage of the maximum activity obtained. ■, Native enzyme; ●, Modified enzyme.

active modified dimer band was seen. Hence the modified proteins are much less stable to urea.

They are also less thermostable. The melting point of the apo- and Mg^{2+} -forms of glycinamidylated D-xylose isomerase and native D-xylose isomerase were compared at pH 6.5 as described in Rangarajan et al. (1992). The melting points of the modified enzyme were considerably lower (59° versus 73 °C for the apoenzymes and 67° versus 81.5 °C for the Mg^{2+} -enzymes).

Enzyme kinetics of glycinamidylated D-xylose isomerase

Elimination of 30 surface carboxy groups/subunit does not affect Mg^{2+} binding. Figure 3 shows the effect of Mg^{2+} on D-fructose isomerase activity of native and modified D-xylose isomerase (M2) at 30 °C at pH 8.0 with a substrate concentration of 3.2 M that is well in excess of the observed $K_{\text{m(app.)}}$ for the

Figure 4 pH-dependence of K_m for D-fructose of the modified Mg^{2+} -enzyme

The Mg^{2+} -enzyme (M2) was assayed at 30 °C against 0.04–3 M D-fructose in 0.1 M buffers (pH 5.80–6.50, Mes; pH 6.60–7.80, Mops; pH 7.90–8.30, Hepes) containing 10 mM Mg^{2+} . Values of K_m were obtained by non-linear regression analysis. The broken line refers to data for the native enzyme.

Figure 5 pH-dependence of V_{max} for D-fructose for modified Mg^{2+} -enzyme

The Mg^{2+} -enzyme (M2) was assayed at 30 °C against 3.2 M D-fructose in buffers of various pH values as in Figure 4, containing 10 mM Mg^{2+} . The apparent values of V_{max} at various pH values were corrected by using the values of K_m shown in Figure 4. The broken line is the curve obtained with the native enzyme.

native Mg^{2+} -enzyme. In both cases, Mg^{2+} shows an apparent activation constant of 60–80 μ M (Rangarajan and Hartley, 1992).

The $K_{m(app.)}$ of the enzyme is affected by surface glycinamidylation, particularly at alkaline pH. Figure 4 shows values from fructose isomerization assays at 30 °C in saturating Mg^{2+} (10 mM) at various substrate concentrations over a range of pH values. The value of $1/K_m$ at pH 7.3 is similar to that of the native enzyme, but decreases at acid pH and drastically above 7.30. The data do not fit a simple ionization curve (non-linear ENZFITTER program; Biosoft), in contrast with native Mg^{2+} -D-xylose isomerase, where K_m depends on a single ionizing group of pK_a 6.80.

The pH-dependence of V_{max} for the modified and native Mg^{2+} -enzymes was compared using 3.2 M D-fructose [which is in large excess of the native $K_{m(app.)}$, 0.12–0.3 M] and similar Mg^{2+} concentrations at 30 °C. The data in Figure 5 show that V_{max} remains constant between pH 6.0 and 8.0 in the modified enzyme,

whereas it depends on ionization of His²¹⁹ with pK_a 6.0 in the native enzyme (Rangarajan and Hartley, 1992). This suggests that the pK_a of His²¹⁹ is much reduced by the surface charge modification.

These kinetic data with D-fructose should not be over-interpreted, since $K_{m(app.)}$ for fructose is very high compared with that for D-xylose, or even D-glucose, and is a complex constant reflecting both substrate binding and the kinetics of the ring-opening step (Rangarajan and Hartley, 1992). The fact that D-xylose isomerase activity remained unchanged during glycinamidylation implies very little effect on enzymic activity (Figure 1). Moreover, the modified and native enzymes are similar at pH 7.3, and the apparently lower V_{max} (Figure 5) may reflect some inactivation during purification of the modified enzyme.

Nevertheless, the sharp increase in $K_{m(app.)}$ for fructose above pH 7.3 deserves comment. None of the carboxy groups in the immediate vicinity of the active site seem to have been glycinamidylated, as argued above, so all active-site carboxy groups and both histidine residues will be unprotonated at pH 8. The long-distance effects of reducing negative surface charges on $K_{m(app.)}$ imply a change in conformation of the Mg^{2+} -enzyme–D-fructose complex at alkaline pH.

Thermolysin nicking of glycinamidylated D-xylose isomerase

The reduced thermostability of the modified enzyme prompted a study of the pattern and kinetics of its nicking by thermolysin, since this technique reveals the location and degree of flexibility of surface loops (Siddiqui et al., 1993). The pattern of thermolysin nicking of the glycinamidylated enzyme was similar, but both nicking sites appeared to be more labile. There was no loss of isomerase activity of the modified enzyme over 10 h during incubation with thermolysin at 37 °C in 5 mM Mg^{2+} /2.5 mM Ca^{2+} /0.1 M Tris/HCl, pH 8.1, in the presence or absence of 10 mM xylitol (the enzyme will be in the Ca^{2+} -form under these conditions; Siddiqui et al., 1993). Analysis of the reaction mixture by SDS/PAGE during the incubation showed disappearance of the 43.3 kDa monomer and the transitory appearance of a 38 kDa fragment which was rapidly converted into a 36 kDa subunit. The rate of conversion of the 43.3 kDa band into the 38 kDa band was unaffected by the presence of 10 mM xylitol, and the first-order rate constant ($420 \times 10^{-3} h^{-1}$) was about twice the rate of nicking of the native enzyme under identical conditions ($190 \times 10^{-3} h^{-1}$; Siddiqui et al., 1993). The results suggest that the primary nicking site (Thr³⁴⁷–Leu³⁴⁸) is more mobile in the modified enzyme, and the secondary site between helices H8 and H9 even more so. However, the drastic modification in surface charge of the tetramer did not cause mobility in any other surface strands that are cleavable by the broad-specificity proteinase.

Protein-engineered mutants to alter pH optimum

It seemed possible that protein-engineered mutations to convert some of the surface carboxy residues into lysine or arginine might decrease the pH optimum of enzyme activity so as to prevent 'browning reactions' in commercial production of high-fructose syrups. Mutants in which Glu¹⁴⁰ and Asp¹⁸⁹ were converted to Lys were constructed, since a mutation where a non-essential negative charge is replaced by a positive charge should double the electrostatic effect at the active site.

The procedure used to purify these proteins was essentially identical with that described by Smith et al. (1991) for purification of the wild-type enzyme from *Arthrobacter* cells. Column fractions after ion-exchange chromatography were monitored for the presence of mutant D-xylose isomerase protein by A_{280} and by

Figure 6 Urea-gradient (0–9 M)-gel electrophoresis of D-xylose isomerases

Samples of native (a) and Trp¹³⁶→Glu mutant D-xylose isomerases (b) were subjected to 0–9 M urea-gradient electrophoresis at pH 8.8 in 7.5% acrylamide gels at 25 °C, and the gels were stained for protein. Arrows alongside the gels indicate the direction of migration of protein and the numbers indicate the molarity of urea across the gradient.

SDS/PAGE. No band corresponding to the 43.3 kDa monomer of D-xylose isomerase was present in column fractions of extracts of mutant proteins (Glu¹⁴⁰→Lys and Asp¹⁸⁹→Lys).

Hence both the Glu¹⁴⁰→Lys and Asp¹⁸⁹→Lys mutant proteins appear to be synthesized in the *E. coli* host, but incapable of folding correctly. In hindsight this can be explained (C. A. Collyer and D. M. Blow, unpublished work), since Glu¹⁴⁰ is involved in tight H-bonds to the main chain-NH-groups of residues 102 and 103, and conversion into Lys may disrupt the 100–107 loop between Strand 3 and Helix 3. Asp¹⁸⁹ is close to Lys²⁵² and Arg²⁴⁹ of a neighbouring subunit, though not salt-bridged to these. Conversion of the negative charge to a positive charge may therefore cause repulsions between the subunits.

The Trp¹³⁶→Glu mutant enzyme

This mutant was constructed, expressed and purified as above in order to test the mechanistic hypothesis of Collyer et al. (1990).

It gave a peak of protein that was eluted from the ion-exchange column at the same concentration of NaCl as the wild-type enzyme. SDS/PAGE of the peak fractions showed the presence of a band of protein corresponding to the 43.3 kDa monomer band. After ACA-34 gel filtration, the mutant protein was essentially pure, as shown by SDS/PAGE and N-terminal sequencing. The yield of Trp¹³⁶→Glu protein was comparable with that obtained for wild-type enzyme starting from a similar amount of cell paste.

However, the mutant protein was completely inactive. SDS/PAGE of mutant protein showed that the subunit was identical in size to the native 43.3 kDa monomer. H.p.l.c. gel filtration performed as described in Rangarajan et al. (1992) confirmed that the mutant enzyme was a tetramer (results not shown).

The Trp¹³⁶→Glu D-xylose isomerase was as stable as native enzyme to SDS treatment. Treatment of either with 1% SDS in Tris/HCl buffer, pH 6.5, at 25 °C for 30 min gave a mixture of dimers and monomers, whereas heating at 100 °C for 2 min gave only monomers (results not shown). Heating at 60 °C for different lengths of time gave a mixture of monomers and dimers, the proportion of dimers decreasing as incubation time was increased. However, it was less stable than native enzyme in urea. Electrophoresis in 0–9 M urea gradient gels at 25 °C (Figure 6) shows that the mutant enzyme is irreversibly denatured between 6 and 7 M urea to give monomer, whereas wild-type enzyme gives a smooth continuous band of protein under these conditions,

indicating reversible dissociation between tetramers and dimers (Rangarajan et al., 1992).

Attempts to reduce the pH optimum of other glucose isomerases by protein engineering have not been conspicuously more successful. Modifications of the cluster of active-site carboxy groups in the homologous D-xylose isomerase from *Actinoplanes missouriensis* involved in binding metal ions (Jenkins et al., 1992) predictably either prevented expression or produced inactive enzyme. Some mutants, such as Glu¹⁸¹→Gln, Glu²¹⁷→Ser, Asp²⁵⁵→Asn or Asp²⁵⁷→Asn retained activity, but this was very low. However, a mutation of Asp⁵⁷→Asn, whose carboxy group interacts with N^{δ1} or His⁵⁴ which catalyses ring-opening, somewhat surprisingly retained almost 60% activity towards xylose and 30% activity towards glucose, but the pH optimum was reduced by only 0.1 pH unit (Lambeir et al., 1992). Another mutant, Glu¹⁸⁶→Gln, did have an altered pH optimum in the Mn²⁺-enzyme form. However, this appears to be due to a rapid decline in activity towards xylose above pH 6; the activities at lower pH are similar to those of the wild-type. Moreover, the activity of the Mg²⁺-enzyme was negligible at all pH values. As suggested by Varsani et al. (1993), these apparently confusing results can be explained by a conformational change in the mutant enzyme stimulated by electrostatic repulsions at alkaline pH.

These results suggest that it will be difficult to achieve a significant change in pH optimum by changing a small number of carboxylic acid groups by protein engineering. There is a large number of charged residues at the active site that may act as a buffer against long distance electrostatic effects. However this work shows that a decrease in pH optimum can be achieved by glycinamidylating 30 surface carboxy groups, so this may prove to be commercially useful.

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