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Evidence for an essential histidine residue in D-xylose isomerases

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Diethyl pyrocarbonate inactivated D-xylose isomerases from *Streptomyces violaceoruber*, *Streptomyces* sp., *Lactobacillus xylosus* and *Lactobacillus brevis* with second-order rate constants of 422, 417, 99 and 92 $M^{-1} \cdot min^{-1}$ respectively (at pH 6.0 and 25 °C). Activity was completely restored by the addition of neutral hydroxylamine, and total protection was afforded by the substrate analogue xylitol in the presence of either Mg^{2+} or Mn^{2+} according to the genus studied. The difference spectra of the modified enzymes revealed an absorption maximum at 237–242 nm, characteristic for *N*-ethoxycarbonylhistidine. In addition, the spectrum of ethoxycarbonylated D-xylose isomerase from *L. xylosus* showed absorption minima at both 280 and 230 nm, indicative for modification of tyrosine residues. Nitration with tetranitromethane followed by diethyl pyrocarbonate treatment eliminated the possibility that modification of tyrosine residues was responsible for inactivation, and resulted in modification of one non-essential tyrosine residue and six histidine residues. Inactivation of the other D-xylose isomerases with diethyl pyrocarbonate required the modification of one (*L. brevis*), two (*Streptomyces* sp.) and four (*S. violaceoruber*) histidine residues per monomer. Spectral analysis and maintenance of total enzyme activities further indicated that either xylitol Mg^{2+} (streptomycetes) or xylitol Mn^{2+} (lactobacilli) prevented the modification of one crucial histidine residue. The overall results thus provide evidence that a single active-site histidine residue is involved in the catalytic reaction mechanism of D-xylose isomerases.

INTRODUCTION

D-Xylose isomerases (D-xylose ketol-isomerase, EC 5.3.1.5) are currently used in the industrial production of high-fructose corn syrup from starch via isomerization of D-glucose to D-fructose. Furthermore, efforts are being made to express the D-xylose isomerase gene in yeasts. This will permit the isomerization of D-xylose to D-xylulose and further conversion into ethanol in a single organism (Schellenberg *et al.*, 1983; Wilhelm & Hollenberg, 1984).

According to Schray & Rose (1971), the interconversions D-xylose $\rightleftharpoons$ D-xylulose and D-glucose $\rightleftharpoons$ D-fructose act via a *cis*-enediol open substrate intermediate (Scheme 1), analogous to the mechanism reported for phosphoglucose isomerase (Rose & O'Connell, 1961). Considerable efforts have been made to identify the catalytic residues of the latter enzyme. Dyson & Noltmann (1968) proposed general acid catalysis of ring-opening by a protonated lysine residue while a deprotonated histidine residue is assumed to function as a base abstracting the activated C-2 hydrogen and affecting its transfer to C-1. In contrast with those authors, O'Connell & Rose (1973) suggested that a glutamate residue mediates the proton transfer in the isomerization process. A mechanism with three functional groups has been proposed by Shaw & Muirhead (1976) in which the ring-opening step constitutes a charge-relay process: a positive charge originating from an acid group is first transferred to a carboxylate group followed by transfer to a nucleophile.

In contrast with phosphoglucose isomerase, D-xylose isomerases require bivalent metal ions such as Mg^{2+}, Co^{2+} or Mn^{2+} for activity. The distinct role of the bivalent metal ions may be that of an electrophile polarizing the carbonyl group or may be associated with the active conformation of the enzyme, or both.

In the present paper we report on the modification of several D-xylose isomerases by DEP, a histidine-acylating reagent (Miles, 1977; Lundblad & Noyes, 1984), by TNM, a tyrosine-selective reagent (Riordan & Vallee, 1972), and by PMSF, commonly employed as inhibitor of serine proteinases (Gold, 1967; Worku *et al.*, 1984), but also reported to be active in serine-modification studies of other enzymes (Whitaker & Perez-Villasenor, 1968; Kumar, 1975; Inoue *et al.*, 1978). Our results provide evidence for the involvement of a single histidine residue in the catalytic reaction mechanism of the D-xylose isomerases investigated.

MATERIALS AND METHODS

Materials

D-Sorbitol dehydrogenase from sheep liver and NADH were from Boehringer, Mannheim, Germany. D-Xylose and xylitol were products 'for Biochemistry' from E. Merck, Darmstadt, Germany. DEP and TNM were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A., PMSF was from Sigma Chemical Co., St. Louis, MO, U.S.A., and NH_2OH was purchased from Serva, Heidelberg, Germany. All other chemicals were of analytical grade.

Production and enzyme purification

Streptomyces violaceoruber (L.M.G. 7183), *Lactobacillus xylosus* (N.R.R.L. B-4449) and *Streptomyces* sp. (A.T.C.C. 21132) were cultivated as reported by Callens *et al.* (1985*a*), Yamanaka & Takahara (1977) and Takasaki *et al.* (1969) respectively. The D-xylose isomerases produced were purified according to the procedure of Callens *et al.* (1985*b*). D-Xylose isomerase from *Lactobacillus brevis* (A.T.C.C. 8287) was a gift from

Abbreviations used: DEP, diethyl pyrocarbonate; TNM, tetranitromethane; PMSF, phenylmethanesulphonyl fluoride.

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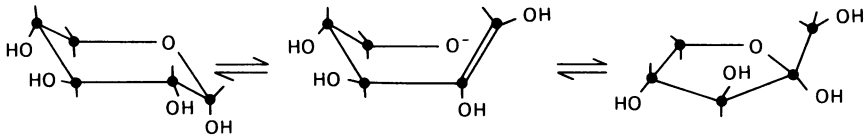


Scheme 1. Interconversion of D-xylose and D-xylulose

Professor K. Yamanaka (Tsukuba University, Japan). Protein concentrations of the purified D-xylose isomerases (tetramer) were determined at 280 nm with a Uvikon 810 spectrophotometer (Kontron), by using $A^{1\%}_{1\,cm} = 10.0$ (Callens *et al.*, 1985*b*).

Enzyme assay

Enzyme activity was measured by the coupled D-xylose isomerase/D-sorbitol dehydrogenase assay method (Callens *et al.*, 1986; Kersters-Hilderson *et al.*, 1987). The standard assay mixture (1 ml) contained 50 mM-D-xylose, 0.15 mM-NADH, 1.0 unit of D-sorbitol dehydrogenase, 0.03–0.05 unit of D-xylose isomerase, 10 mM-$MgSO_4$ (streptomycetes), 0.5 mM-$MnCl_2$ (*L. brevis*) or 1.0 mM-$MnCl_2$ (*L. xylosus*), and 0.01 M-triethanolamine/HCl buffer, pH 8.0 (streptomycetes), or 0.01 M-Mes/NaOH buffer, pH 6.3 (lactobacilli).

Metal ion concentrations and pH of buffers correspond to the optima of the investigated D-xylose isomerases (Sanchez & Smiley, 1975; Yamanaka, 1975; Yamanaka & Takahara, 1977; Callens *et al.*, 1986; W. Vangrysperre, H. Kersters-Hilderson & M. Callens, unpublished work).

Reaction of D-xylose isomerases with DEP

The concentration of commercial DEP was determined from the increase in absorbance at 242 nm (ϵ 3200 $M^{-1}\cdot cm^{-1}$) after reaction with 10 mM-imidazole in 0.05 M-sodium/potassium phosphate buffer, pH 7.5 (Ovádi *et al.*, 1967). The reagent was freshly diluted with ice-cold ethanol before each experiment. Modification was carried out by incubating D-xylose isomerase with DEP in 0.05 M-sodium/potassium phosphate buffer, pH 6.0, at 25 °C. The final concentration of ethanol in the reaction mixture (100 μl) never exceeded 5% (v/v) and had no effect upon enzyme activity and stability. Samples (10 μl) were taken at various time intervals and quenched by rapid addition and dilution with ice-cold 0.01 M-triethanolamine/HCl buffer, pH 8.0 (streptomycetes), or 0.01 M-Mes/NaOH buffer, pH 6.3 (lactobacilli). Enzyme activity was determined as described above. Recovery of enzyme activity after inactivation by DEP was assayed with neutral 0.5 M- or 0.1 M-hydroxylamine at 25 °C. The difference absorption spectra of DEP-modified versus native D-xylose isomerase were obtained with a Uvikon 810 double-beam spectrophotometer (Kontron) equipped with thermostatically controlled (25 °C) copper cuvette holders. Two standard cuvettes both containing D-xylose isomerase were used. DEP, dissolved in ethanol, was added to the sample cuvette after the addition of an equal volume of ethanol to the reference cuvette. The kinetics of modification were followed by differential absorption at the respective absorption maxima (237–242 nm). The amount of *N*-ethoxycarbonylhistidine per D-xylose isomerase monomer was calculated by using 3200 $M^{-1}\cdot cm^{-1}$ as the molar absorption coefficient (Ovádi *et al.*, 1967).

Reaction of D-xylose isomerases with TNM

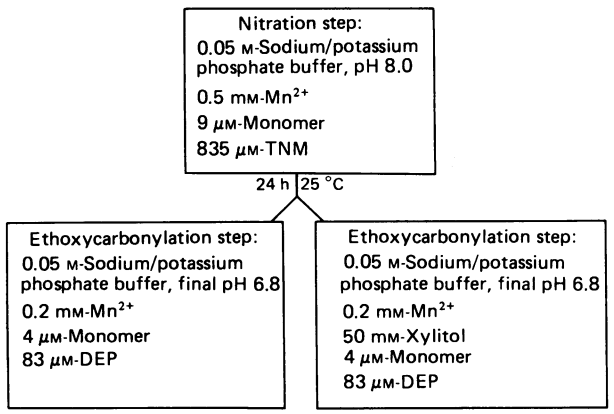
Nitration was generally carried out in 0.05 M-Tris/HCl buffer, pH 8.0, at 25 °C. The D-xylose isomerases (5–10 μM-monomer) were incubated with an ethanolic solution of TNM to give a molar excess of 9–116-fold over monomer. TNM was added to both sample and reference cuvettes, the first containing D-xylose isomerase and the latter containing only buffer. The reaction progress was followed by monitoring the absorbance at 428 nm (ϵ 4100 $M^{-1}\cdot cm^{-1}$) (Sokolovsky *et al.*, 1966). Samples (10 μl) were taken at various time intervals and the activity was determined as described above.

Nitration of D-xylose isomerase from *L. xylosus* followed by ethoxycarbonylation with DEP

The nitration reaction was carried out in a 2 ml cuvette at 25 °C in 0.05 M-sodium/potassium phosphate buffer, pH 8.0. The activity (in 10 μl samples) and the degree of nitration (absorbance at 428 nm) were monitored for 24 h. After completion of the tyrosine modification, DEP treatment was started, as presented in Scheme 2.

Reaction of D-xylose isomerases with PMSF

D-Xylose isomerase (0.7–0.9 μM-monomer) was incubated with 1 mM-PMSF (freshly diluted in ethanol) for 2 h in 1 ml of 0.05 M-sodium/potassium phosphate buffer, pH 6.0, at 37 °C. The final concentration of ethanol was 10% (v/v) and had no effect upon enzyme activity. Samples (100 μl) were withdrawn at time intervals and activity was determined as described above.



Scheme 2. Nitration of D-xylose isomerase from *L. xylosus* followed by ethoxycarbonylation with DEP

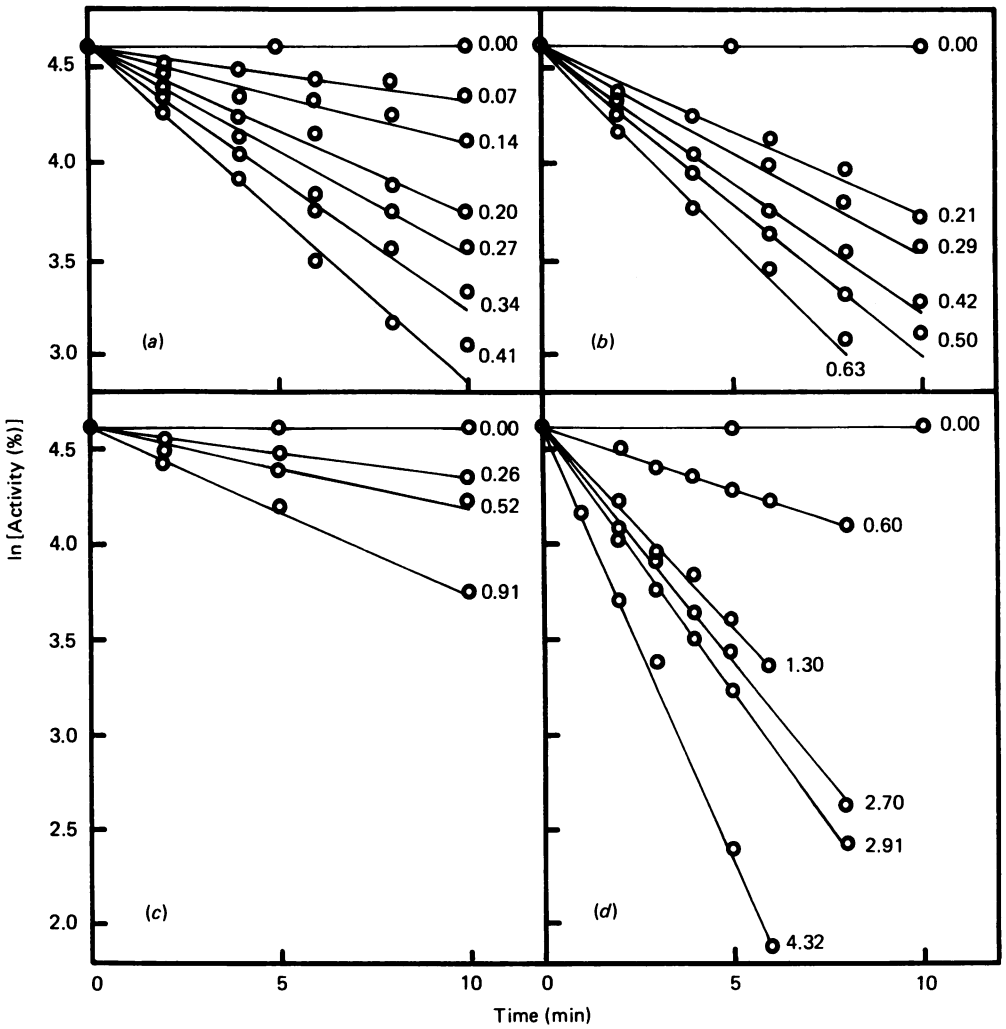


Fig. 1. Inactivation of D-xylose isomerases by DEP

D-Xylose isomerases from *S. violaceoruber* (*a*), *Streptomyces* sp. (*b*), *L. brevis* (*c*) and *L. xylosus* (*d*) (13 μM-, 20 μM-, 5 μM- and 20 μM-monomer respectively) were incubated with different amounts of the reagent in 0.05 M-sodium/potassium phosphate buffer, pH 6.0, at 25 °C. Samples were withdrawn at various time intervals for measurement of the residual activity as described in the Materials and methods section. The values on the slopes indicate DEP concentrations (mM).

RESULTS AND DISCUSSION

Kinetics of DEP inactivation of D-xylose isomerases

Incubation of D-xylose isomerases from *S. violaceoruber*, *Streptomyces* sp., *L. xylosus* and *L. brevis* with DEP at 25 °C in 0.05 M-sodium/potassium phosphate buffer, pH 6.0, in accordance with Ovádi *et al.* (1967), resulted in rapid loss of activity. The DEP treatment did not effect the subunit structure, as confirmed by polyacrylamide-gel electrophoresis (results not shown). Plots of logarithm of remaining activity versus time at various concentrations of DEP resulted in pseudo-first-order kinetics (Fig. 1). The linearity of the plot of $k_{obs.}$ (min^{-1}) versus DEP concentration (Fig. 2) indicated that a reversible DEP–enzyme complex was not formed before the inactivation process. The second-order rate constants for inactivation of D-xylose isomerases from *S. violaceoruber*, *Streptomyces* sp., *L. xylosus* and *L. brevis* were calculated to be 422, 417, 99 and 92 $M^{-1} \cdot min^{-1}$ respectively. These constants are sufficiently high and of the same order of magnitude as those found for other catalytically essential histidine residues (Lundblad & Noyes, 1984) and much larger than those observed for reactive lysine, cysteine or tyrosine residues (Holbrook & Ingram, 1973; Wells, 1973). The plot of $\log k_{obs.}$ versus log[DEP] (Levy *et al.*, 1963) yielded reaction orders with respect to reagent of 1.1, 1.0, 0.9 and 1.0 mol/mol respectively (results not shown), indicating that there is only one essential residue per monomer. The effect of modification on $k_{cat.}$ and K_m of partially modified D-xylose isomerase from *S. violaceoruber* (37% residual activity) was determined from plots of $[S]/v$ versus [S] (Hanes, 1932). The decrease in maximal velocity was proportional to the extent of modification, whereas the K_m value was unaltered (results not shown). This points to an all-or-nothing event, i.e. partially inactivated D-xylose isomerase contains a mixture of fully active and fully inactive enzyme. Treatment of the modified proteins from *S. violaceoruber* or *Streptomyces* sp. with 0.5 M-NH_2OH resulted in fast and complete re-activation within 4 min and 1 min respectively. In contrast, D-xylose isomerases from *L. xylosus* and *L. brevis* required

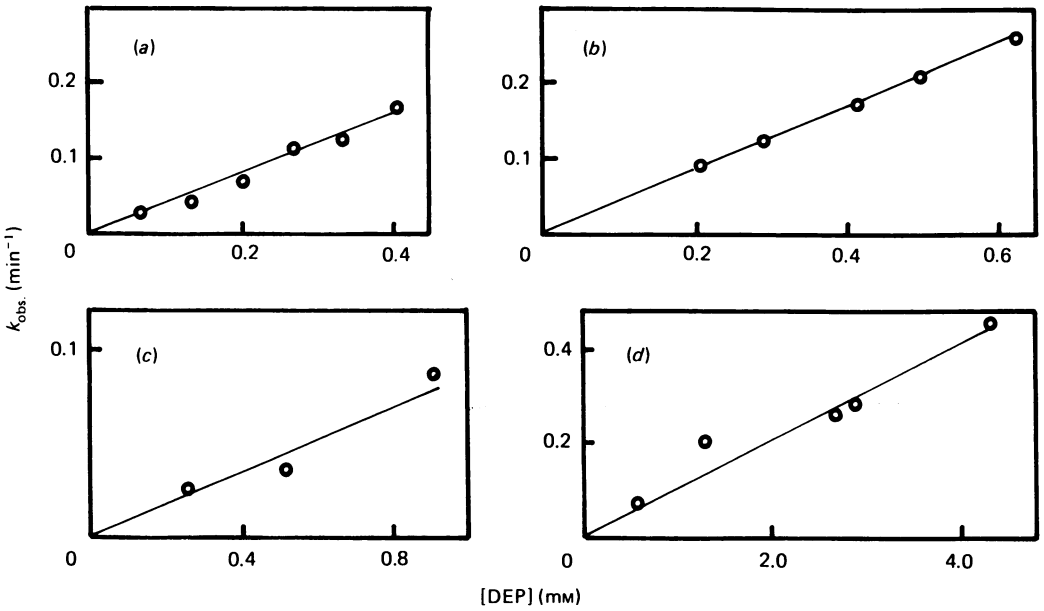


Fig. 2. Effect of DEP concentrations on the inactivation rate constants for D-xylose isomerases

The pseudo-first-order rate constants were obtained from the data in Fig. 1.

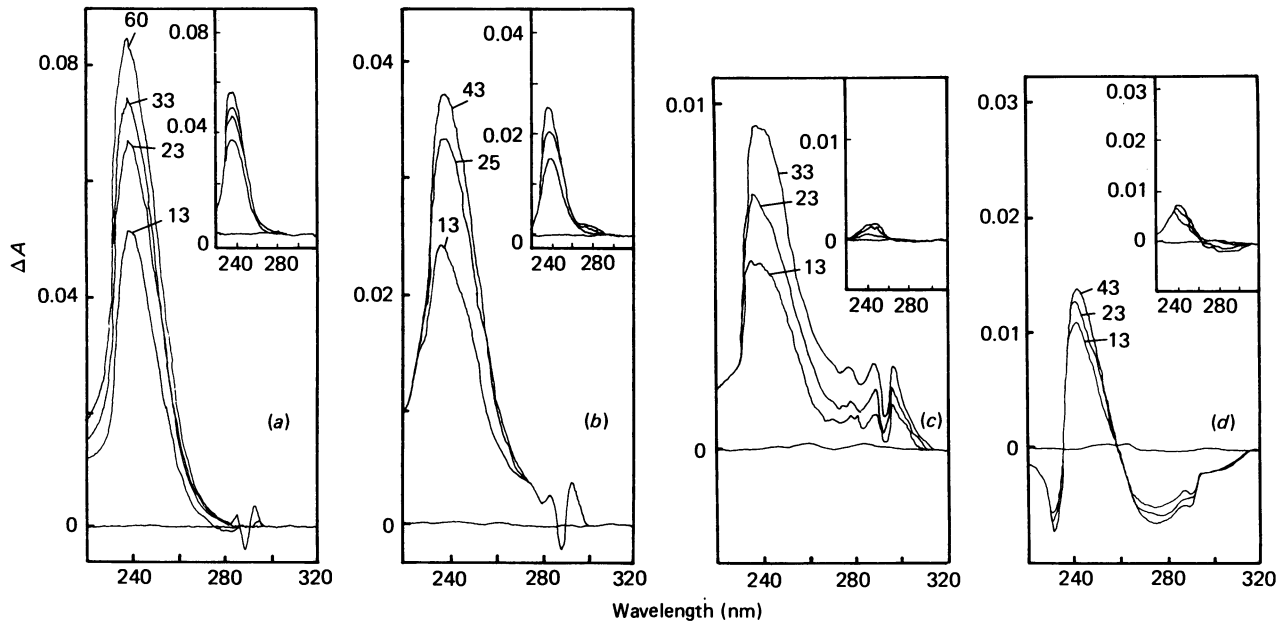


Fig. 3. Difference spectra for modification of D-xylose isomerases by DEP

D-Xylose isomerase from *S. violaceoruber* (*a*), *Streptomyces* sp. (*b*), *L. brevis* (*c*) and *L. xylosus* (*d*) (8 μM-, 6 μM-, 4 μM- and 5 μM-monomer respectively) was mixed with DEP (230, 127, 74 and 101 μM respectively) in 0.05 M-sodium/potassium phosphate buffer, pH 6.0, at 25 °C. Modification of D-xylose isomerases from *L. brevis* and *L. xylosus* was carried out in the presence of 0.5 mM- and 1.0 mM-Mn^{2+} respectively. Difference spectra were recorded at various time intervals; the values on the spectra indicate time (min). Insets: difference spectra for modification by DEP in the presence of 50 mM-xylitol + 10 mM-Mg^{2+} (streptomycetes), 50 mM-xylitol + 0.5 mM-Mn^{2+} (*L. brevis*) or 50 mM-xylitol + 1.0 mM-Mn^{2+} (*L. xylosus*), recorded at the same time intervals.

5 h and 22 h treatment with 0.5 M-NH_2OH respectively (in the presence of 1.0 mM- and 0.5 mM-Mn^{2+} respectively) before complete reversal of activity was observed. Addition of Mn^{2+} ions was necessary to avoid denaturation of the latter enzymes by NH_2OH. Since NH_2OH deacylates only ethoxyformylated histidine, serine and tyrosine residues, the inactivation of D-xylose isomerases by DEP cannot be due to the modification of thiol groups or lysine residues (Miles, 1977). In fact the essentiality of thiol groups had earlier been excluded by Callens *et al.* (1985*b*) for D-xylose isomerase from *S. violaceoruber*; also a study with different lysine-specific reagents eliminates the possibility that lysine residues are essential groups [in contrast with Callens *et al.* (1986)].

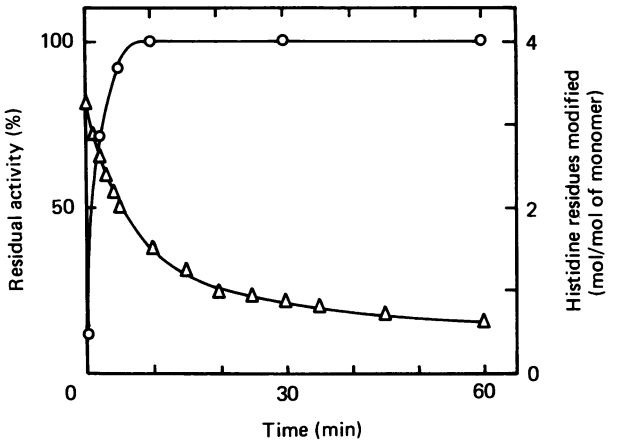


Fig. 4. Reversal of DEP modification of D-xylose isomerases with NH_2OH

D-Xylose isomerase from *S. violaceoruber*, modified by treatment of DEP for 1 h (see Fig. 3*a*), was treated with 0.1 M-NH_2OH. Residual activity (○) and the number of ethoxycarbonylated histidine residues (△) were determined as decribed in the Materials and methods section.

Furthermore, since incubation with 1 mM-PMSF for 2 h at 37 °C did not affect the enzyme activity at all, serine groups are not involved.

Characterization of DEP-modified residues of D-xylose isomerases

The difference spectra of the DEP-modified versus the untreated D-xylose isomerases showed an absorption maximum at 237–242 nm (Fig. 3), characteristic for ethoxycarbonylation of histidine residues (Ovádi *et al.*, 1967). The fact that de-ethoxycarbonylation by NH_2OH was accompanied by complete restoration of original activity (Fig. 4) reinforced the evidence for histidine modification at the active site and eliminated the possibility of gross conformational changes. No decrease in the difference spectra at 230 and 280 nm, indicative of acylation of tyrosine groups (Burstein *et al.*, 1974), was observed, except for D-xylose isomerase from *L. xylosus* (Fig. 3*d*). From the decrease in absorption at 278 nm (ϵ 1310 $M^{-1} \cdot cm^{-1}$), modification of 0.8 mol of tyrosine residues/mol of monomer was calculated for the latter enzyme.

The minor change in u.v. absorbance, showing a maximum at 293 nm and a minimum around 288 nm (Figs. 3*a*, 3*b*, 3*c* and 8), is due to the disturbance of tryptophan (De Boeck *et al.*, 1984) by treatment with DEP. The disturbance disappears on de-ethoxycarbonylation with NH_2OH (results not shown) and when modification is carried out in the presence of active-site protectors (insets to Figs. 3*a*, 3*b*, 3*c* and 8). This may be indicative of a tryptophan residue being located close to the essential histidine group. Further work is needed to find out whether this tryptophan residue plays an essential role in the catalytic process or in maintaining the active-site conformation.

Effect of ligands on DEP inactivation of D-xylose isomerases

The relative effects of the substrates D-xylose and D-glucose, the substrate analogue xylitol, the activating metal ions Mg^{2+}, Mn^{2+} and Co^{2+} or the non-activating

Table 1. Effect of ligands on the rate of inactivation of D-xylose isomerases by DEP

D-Xylose isomerases from *S. violaceoruber* (13 μM-monomer), *Streptomyces* sp. (20 μM-monomer), *L. brevis* (5 μM-monomer) and *L. xylosus* (20 μM-monomer) were preincubated with ligand (5 min) before the addition of DEP (0.20, 0.50, 0.91 and 1.30 mM respectively). Residual activity was determined as described in the Materials and methods section. Protection (%) is expressed as $[k_{obs.}$ (unprotected) $- k_{obs.}$ (protected)$/k_{obs.}$ (unprotected)$] \times 100$.

Addition	Protection (%)			
	S. violaceoruber	*Streptomyces* sp.	*L. brevis*	*L. xylosus*
None	0	0	0	0
0.2 M-D-Xylose	20	10	–	0
1.0 M-D-Xylose	25	–	–	–
0.5 M-D-Glucose	11	–	–	14
50 mM-Xylitol	55	24	–	10
10 mM-Mg^{2+}	−48	−38	–	−13
100 mM-Mg^{2+}	−47	–	–	–
50 mM-Xylitol + 10 mM-Mg^{2+}	100	100	–	94
0.5 mM-Mn^{2+}	–	–	−211	–
1.0 mM-Mn^{2+}	−108	−124	–	−472
50 mM-Xylitol + 0.5 mM-Mn^{2+}	–	–	100	–
50 mM-Xylitol + 1.0 mM-Mn^{2+}	76	68	–	100
1 mM-Co^{2+}	55	57	13	19
10 mM-Co^{2+}	73	–	–	–
50 mM-Xylitol + 1 mM-Co^{2+}	68	–	–	22
1 mM-Ca^{2+}	81	100	58	54
50 mM-Xylitol + 1 mM-Ca^{2+}	98	88	75	85
10 mM-Mg^{2+} + 1 mM-Co^{2+}	−15	–	–	–
10 mM-Mg^{2+} + 10 mM-Co^{2+}	53	–	–	–

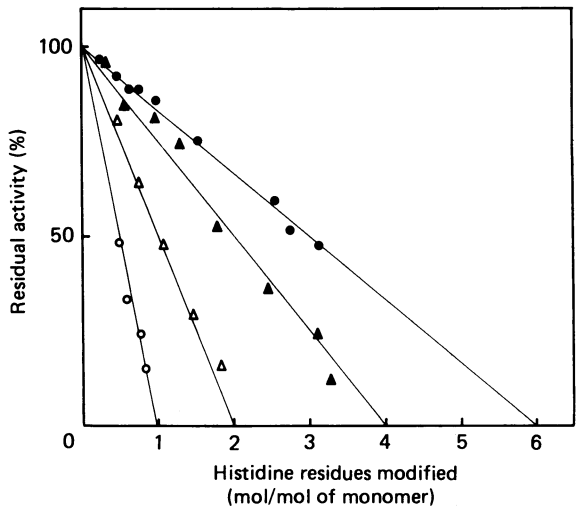


Fig. 5. Correlation between the number of histidine groups modified by DEP and the residual D-xylose isomerase activity

The plots for D-xylose isomerases from *L. brevis* (○), *Streptomyces* sp. (△) and *S. violaceoruber* (▲) were obtained from the data in Figs. 3(*a*), 3(*b*) and 3(*c*). The plot for D-xylose isomerase from *L. xylosus* (●) was obtained from the data in Fig. 8.

but competing Ca^{2+} ion, and of other ligand combinations on the rate of reaction between DEP and the essential histidine residue were investigated (Table 1). The ligands were added before the addition of DEP. As shown in Table 1, little effect on the inactivation rate was obtained with the substrates D-xylose or D-glucose or with the substrate analogue xylitol. The metal ions Mg^{2+} and, to a higher degree, Mn^{2+} increase the inactivation rate, whereas Co^{2+} and, in a more effective way, Ca^{2+} protect the enzyme against modification by DEP. These changes in histidine reactivity may be ascribed to differences in metal co-ordination geometry and co-ordinating ligands, inducing different conformational changes at the active site. As such, the enzyme–Mn^{2+} and enzyme–Mg^{2+} conformations seem to enhance the accessibility to the catalytic histidine residue, whereas the enzyme–Ca^{2+} and to a lower degree enzyme–Co^{2+} conformations seem to block the access to it. Surprisingly, total or almost total protection was afforded by the substrate analogue xylitol in the presence of the inactivation-enhancing Mn^{2+} and Mg^{2+} ions. These synergistic effects support the idea of induced fit upon binding of xylitol in the presence of Mn^{2+} or Mg^{2+}, causing absolute shielding of the active-site histidine residue. In contrast, no striking effects were obtained with xylitol in the presence of the protecting Co^{2+} or Ca^{2+} ions.

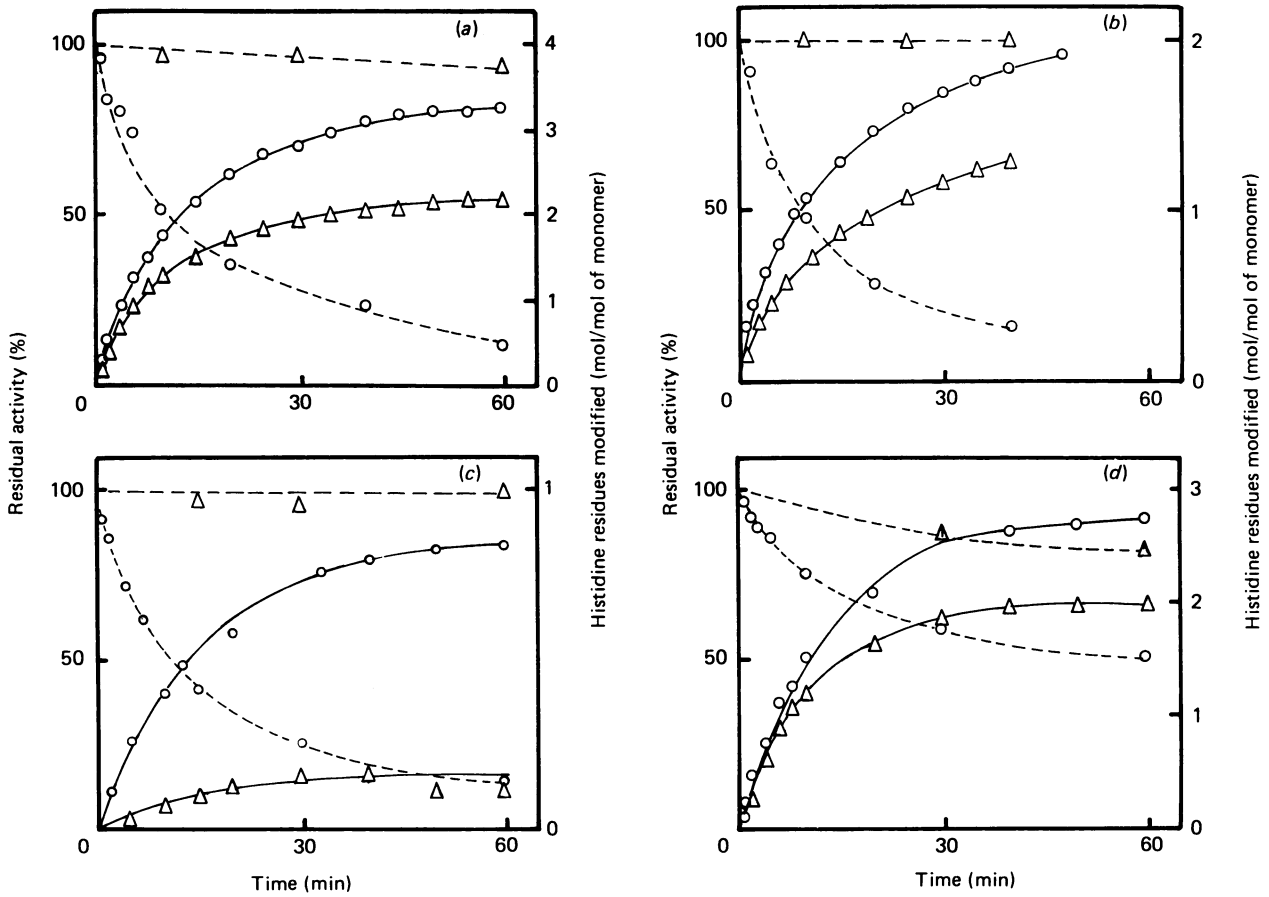


Fig. 6. Reaction of D-xylose isomerases from *S. violaceoruber* (*a*), *Streptomyces* sp. (*b*), *L. brevis* (*c*) and *L. xylosus* (*d*) with DEP in the presence (△) and in the absence (○) of protector

The conditions for modification were those as described in the legends to Figs. 3(*a*), 3(*b*), 3(*c*) and 8. Protecting ligands were 50 mM-xylitol in the presence of 10 mM-Mg^{2+} (streptomycetes), 0.5 mM-Mn^{2+} (*L. brevis*) or 1.0 mM-Mn^{2+} (*L. xylosus*). ----, Residual activity; ——, histidine residues modified.

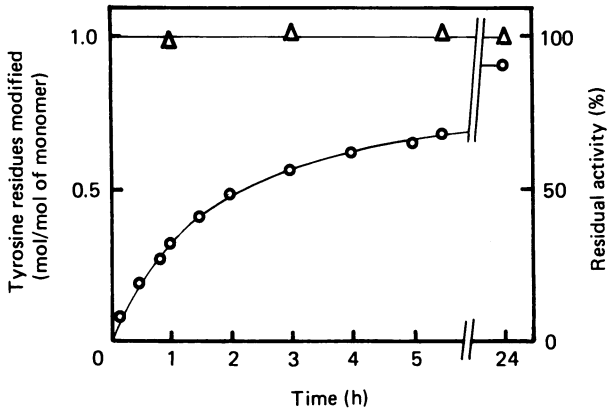


Fig. 7. Nitration of D-xylose isomerase from *L. xylosus* with TNM

D-Xylose isomerase (9 μM-monomer) was incubated with 0.84 mM-TNM in the presence of 0.5 mM-Mn^{2+} in 0.05 M-Tris/HCl buffer, pH 8.0, at 25 °C. The degree of nitration (○) was determined spectrophotometrically at 428 nm. Samples were withdrawn at various time intervals for measurement of activity (△) as described in the Materials and methods section.

Number of histidine residues essential for activity of D-xylose isomerases

D-Xylose isomerases from *S. violaceoruber*, *Streptomyces* sp. and *L. brevis*. Under the stipulated conditions (Fig. 3), approximately the same first-order rate constants were calculated for inactivation and modification of D-xylose isomerases from *S. violaceoruber*, *Streptomyces* sp. and *L. brevis* (0.077, 0.076 and 0.054 min^{-1} compared with 0.064, 0.074 and 0.064 min^{-1} respectively). The relationship between inactivation and extent of histidine modification is shown in Fig. 5. Extrapolation of the linear plot indicated that one of the 15 (*L. brevis*), two of the ten (*Streptomyces* sp.) or four of the ten (*S. violaceoruber*) histidine residues per monomer were modified before complete inactivation was achieved (W. Vangrysperre & J. Van Damme, unpublished work; Callens *et al.*, 1985*b*). Spectral analysis (insets to Figs. 3*a*, 3*b* and 3*c*) in the presence of protecting ligand (50 mM-xylitol in combination with 10 mM-Mg^{2+} or 0.5 mM-Mn^{2+}) (Figs. 6*a*, 6*b* and 6*c*) revealed that modification of one essential histidine residue was prevented; the other reactive histidine residues were modified without loss of activity. Analysis of the data by the method of Tsou (1962) led to the same conclusions.

D-Xylose isomerase from *L. xylosus*. In contrast with the other D-xylose isomerases, Fig. 3(*d*) indicates that histidine as well as tyrosine residues of the *L. xylosus* enzyme were modified by DEP treatment. Since the positive peak arising from ethoxycarbonylhistidine is very close to the negative peak arising from tyrosine acylation, anomalously small changes in apparent modified histidine residues resulted (Burstein *et al.*, 1974). In addition, the $k_{obs.}$ values for modification and inactivation were not identical, $k_{inact.}$ being estimated to be almost twice $k_{modif.}$ (results not shown).

In order to avoid the reaction of DEP with tyrosine residues, the enzyme was nitrated with TNM before the modification by DEP. The results are presented in Fig. 7.

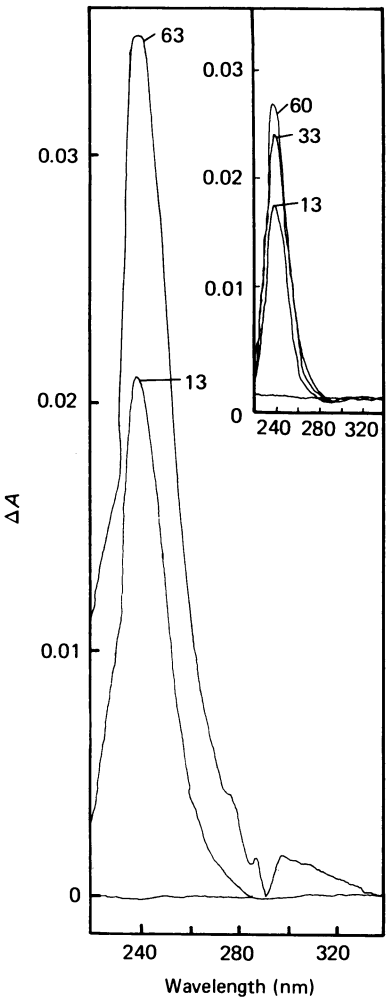


Fig. 8. Difference spectrum for DEP modification of nitrated D-xylose isomerase from *L. xylosus*

Conditions were as described in the Materials and methods section. The values on the spectra indicate time (min). Inset: DEP modification of the nitrated enzyme in the presence of protector.

Incubation of the enzyme (24 h) with TNM (9–116 molar excess) resulted in nitration of approx. 1 mol of tyrosine residue/mol of monomer, without loss of activity. Addition of different ligands was without effect. Following the nitration of this single tyrosine residue the nitrated enzyme was further treated with DEP. The resulting difference spectrum showed an absorption maximum at 242 nm, as expected for ethoxycarbonylhistidine. In contrast with Fig. 3(*d*), the absorption minima at 230 and 280 nm now disappeared (Fig. 8) and nearly identical $k_{obs.}$ values were obtained for inactivation (0.040 min^{-1}) and modification (0.044 min^{-1}) by DEP. The relationship between inactivation and modification (Fig. 5) further indicated that zero percentage activity was correlated with modification of six out of the 16 (W. Vangrysperre & J. Van Damme, unpublished work) histidine residues per monomer. In the presence of 50 mM-xylitol plus 0.2 mM-Mn^{2+} (inset to Fig. 8), modification of one histidine residue was prevented (Fig. 6*d*), pointing to an essential histidine residue at the active site, as concluded above for the other D-xylose isomerases.

Nitration of D-xylose isomerases from *S. violaceoruber*, *Streptomyces* sp. and *L. brevis* with TNM did not influence the enzyme activity, although one or more tyrosine residues were modified (results not shown), indicating that tyrosine residues are not essential for D-xylose isomerase activity.

In summary, the existence of an essential histidine residue in four D-xylose isomerases has been demonstrated. Previous studies concerning the reaction mechanism of phosphoglucose isomerase established the requirement for a proton acceptor or nucleophile mediating the proton transfer in the isomerization process (Dyson & Noltmann, 1968; O'Connell & Rose, 1973). From the pH–activity curves of the investigated D-xylose isomerases (Sanchez & Smiley, 1975; Yamanaka, 1975; Yamanaka & Takahara, 1977; Callens *et al.*, 1986; W. Vangrysperre, H. Kersters-Hilderson & M. Callens, unpublished work) a non-dissociated histidine residue may be in accordance with the above statement. The observation that D-xylose isomerases from four species belonging to two different genera contain an essential histidine residue, strongly supports the idea that the specific histidine residue may be conserved among D-xylose isomerases in general.

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