Localization of the essential histidine and carboxylate group in D-xylose isomerases

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D-Xylose isomerases from different bacterial strains were chemically modified with histidine and carboxylate-specific reagents. The active-site residues were identified by amino acid sequence analysis of peptides recognized by differential peptide mapping on ligand-protected and unprotected derivatized enzyme. Both types of modified residues were found to cluster in a region with consensus sequence: Phe-His-Asp-Xaa-Asp-Xaa-Xaa-Pro-Xaa-Gly, conserved in all D-xylose isomerases studied so far. These results are consistent with the recently published X-ray data of the enzyme active centre from Streptomyces rubiginosus showing hydrogen bond formation between Asp-57 and His-54 which locks the latter in one tautomeric form. A study of the pH-dependence of the kinetic parameters suggests the participation of a histidine group in the substrate-binding but not in the isomerization process. Comparison of the N-terminal amino acid sequences of several D-xylose isomerases further revealed a striking homology among the Actinomycetaceae enzymes and identifies them as a specific class of D-xylose isomerases.

INTRODUCTION

D-Xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5) catalyses the interconversion of D-xylose with D-xylulose and of D-glucose with D-fructose. The enzyme is of considerable industrial importance in the production of high-fructose corn syrup, and also attracts attention in the conversion of xylan-containing biomass into ethanol.

D-Xylose isomerases from Actinomycetaceae have distinct catalytic and physicochemical properties. To function catalytically, a stoichiometry of 2 mol of metal/mol of monomer is required in contrast with a stoichiometry of 1 mol of metal/mol of monomer for the other isomerases (Callens et al., 1988; M. Callens, unpublished work). Their monomeric molecular masses of 43 kDa (Callens et al., 1985; Saari et al., 1987; Drocourt et al., 1988; Vangrysperre et al., 1989a) are lower than those reported for D-xylose isomerases from other strains, having an average value of 50 kDa (Schellenberg et al., 1984; Wilhelm & Hollenberg, 1985; Vangrysperre et al., 1989a). The metal-activating capacities further differ, Mg2+ being superior to Co2+ and Mn2+ with D-xylose isomerases from Actinomycetaceae (Takasaki et al., 1969; Callens et al., 1986) and Mn2+ or Co2+ being superior to Mg2+ with the other D-xylose isomerases (Yamanaka, 1968; Danno, 1970; Yamanaka & Takanaha, 1977; W. Vangrysperre, unpublished work). In addition, as concluded from the literature data, D-xylose isomerases from Actinomycetaceae exhibit a superior thermostability and have higher pH optima than the other ones. The close relationship among the Actinomycales enzymes will be confirmed in this paper by comparison and alignment of their respective N-termini.

Notwithstanding the apparent existence of two classes of enzymes, chemical modification studies, as earlier reported by some of us (Vangrysperre et al., 1988, 1989a), support the idea of a unique action pattern, since derivatization of one active-site histidine and carboxylate group abolishes the activity of all D-xylose isomerases studied so far. In order to elucidate the structure–function relationships of D-xylose isomerases in general, the amino acid sequences of the isolated active-site histidine- and carboxylate-containing peptides of D-xylose isomerase from Streptomyces violaceoruber, Streptomyces sp., Lactobacillus brevis and Bacillus coagulans will be compared. As far as possible these segments of primary structure will be aligned with the reported total sequences of D-xylose isomerase from Escherichia coli (Schellenberg et al., 1984), Bacillus subtilis (Wilhelm & Hollenberg, 1985), Ampullariella sp. (Saari et al., 1987) and Streptomyces violaceoniger (Drocourt et al., 1988). Based on the positions of their functional amino acid residues in the primary structure, and on a kinetic study as a function of the pH, their possible role in the reaction mechanism is discussed.

EXPERIMENTAL PROCEDURES

Materials

The organisms were cultivated, the enzymes purified and the apoenzymes prepared as described previously (Vangrysperre et al., 1989a). D-Xylose, D-glucose and xyitol (products ‘for biochemistry’), trifluoroacetic acid (TFA), cysteine hydrochloride, MgCl₂, 6H₂O, MnCl₂, 4H₂O and CoCl₂, 6H₂O were from Merck. Diethylpyrocarbonate (DEP) was purchased from Aldrich Chemical Co. Guanidinium

Abbreviations used: DEP, diethylpyrocarbonate; [¹⁴C]DEP, diethyl pyrocarbonate labelled in the carbonyl group; WRK, Woodward’s Reagent K (N-ethyl-5-phenylisoxazolium-3'-sulphonate); TFA, trifluoroacetic acid.

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chloride, Woodward's Reagent K (WRK), [14C]DEP (labelled in the carbonyl group; 5.1 mCi/mmole), glucose oxidase and D-fructose (cell culture tested) were from Sigma Chemical Co. Sorbitol dehydrogenase, subtilisin, papain, peroxidase and 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) ('ABTS') were obtained from Boehringer Mannheim. PD-10 Sephadex G-25M columns were from Pharmacia. Collodion bags (5M 13200) were obtained from Sartorius. The scintillation liquid was Insta-Gel II from Packard. Acetonitrile was purchased from Farmitalia Carlo Erba. All other chemicals were of analytical grade.

Assays
Protein concentrations were determined as reported by Vangrysperre et al. (1989a). D-Xylose isomerase activities were assayed by the coupled D-xylene isomerase/sorbitol dehydrogenase method (Vangrysperre et al., 1988, 1989a) for D-xylene as substrate, or by the glucose oxidase/peroxidase/2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) method (Werner et al., 1970) for D-fructose as substrate.

Histidine modification
Modification of D-xylene isomerase from Streptomyces sp. was performed according to the procedure described for D-xylene isomerase from S. violaceoruber (Vangrysperre et al., 1989b).

Modification of D-xylene isomerase from B. coagulans and L. brevis was carried out by incubating D-xylene isomerase (10 nmol of monomer) with 1 mM-[14C]DEP at 25 °C for 40 min in 0.05 m sodium/potassium phosphate buffer, pH 6.0 (50 μl). The inactivation was carried out in the presence of the inactivation-enhancing metal cofactor Mn2+ (1 mM) (Vangrysperre et al., 1988). As protecting ligand, 50 mM-xylitol (B. coagulans) (W. Vangrysperre, unpublished work) or the combination 50 mM-xylitol plus 1 mM-Mn2+ (L. brevis) (Vangrysperre et al., 1988) was used.

Carboxylate modification
The D-xylene isomerases (20 nmol of monomer) were incubated with 20 mM-WRK for 10 min in 400 μl of 0.5 m-sodium/potassium phosphate buffer, pH 6.0, at 25 °C. A second addition of 20 mM-WRK followed for another incubation period of 10 min. Excess reagent was then removed by gel filtration (Sinha & Brewer, 1985) on a PD-10 Sephadex G-25M column, equilibrated with the same buffer. The modified D-xylene isomerases were concentrated by ultrafiltration (collodion bags 5M 13200) to a final volume of 100–200 μl. Protection (75–100%) against inactivation was obtained using the following ligand combinations, added prior to the addition of WRK: 50 mM-xylitol plus 10 mM-Mg2+ (Streptomycescaceae), 50 mM-xylitol plus 10 mM-Mn2+ (L. brevis) or 0.5 mM-glucose plus 1 mM-Co2+ (B. coagulans).

Denaturation and proteolysis
DEP- or [14C]DEP-modified D-xylene isomerase was denatured by guanidinium hydrochloride and further digested with subtilisin as described previously by Vangrysperre et al. (1989b).

Denaturation and proteolysis of WRK-modified enzyme were obtained by treatment with 4 M-guanidinium chloride, 2 mM-EDTA, 5 mM-cysteine hydrochloride and papain (5%, w/v) for 90 min at 30 °C.

Reverse-phase h.p.l.c. of peptides
Chromatographic experiments were carried out using a reverse-phase h.p.l.c. system from Waters Associates. Column eluates were monitored both at 214 nm and at 238 nm (DEP-modified peptides) or at 340 nm (WRK-modified peptides). A Vyda 214 TP 54 column was used with two different solvent systems as earlier reported (Vangrysperre et al., 1989b): system I [12 mM-sodium phosphate buffer, pH 6.0 (solvent A)/12 mM-sodium phosphate buffer, pH 6.0, acetonitrile/water (7:3, v/v)] (solvent B) and system II [0.1% TFA/0.1% TFA in acetonitrile/water (7:3, v/v)]. Flow rates were 1 ml/min. The gradient slopes are illustrated on the Figures.

Measurements of 14C radioactivity
A SL 20 liquid scintillation spectrometer (Intertechnique) was used. Aliquots (100 μl) were counted for 14C in 10 ml of scintillation liquid. The measurements were corrected for quenching effects.

Automatic sequence determination
The amino acid sequences were determined with a gas-phase sequenator (Applied Biosystems Inc.) model 470A equipped with an on-line phenylthiobodyntaino acid analyser model 120A. The apparatus was operated according to the manufacturer's instructions.

Kinetic parameters
The kinetic parameters kcat (μmol·min⁻¹·unit⁻¹) and K (M⁻¹) for D-fructose were calculated from least squares fits of linear [S]/v versus [S] plots (Hanes, 1932).

RESULTS
Sequence comparison (partial) of D-xylene isomerases of different origin
We determined the amino acid sequences of the N-termini of D-xylene isomerase from S. violaceoruber, Streptomyces sp., B. coagulans, L. brevis and L. xylosus. The alignment is shown in Fig. 1. Comparison with the sequences reported for the E. coli (Schellenberg et al., 1984), B. subtilis (Wilhelm & Hollenberg, 1985), Ampullariella sp. (Saari et al., 1987) and S. violaceoniger (Drocourt et al., 1988) enzymes (Fig. 1) provides evidence for two distinct groups of D-xylene isomerases, in agreement with our conclusions drawn from a study of physicochemical and catalytic properties (see the Introduction). The most striking difference between the two classes of enzymes is the less extended N-terminal region characteristic for D-xylene isomerases from Actinomyctaceae (approx. 50 residues). This is in accordance with the reported molecular mass differences for the two enzyme classes (see the Introduction). The N-termini of D-xylene isomerases from Actinomyctaceae (Group I) further exhibit a very high degree of similarity. Alignment of the available complete amino acid sequences of D-xylene isomerase from Ampullariella sp. and S. violaceoniger (Dauter et al., 1989) further supports the conserved character of D-xylene isomerases from Group I. However, stretches of more limited similarity (Fig. 1, boxed) are also present among the partial (this work) and total available sequences of the two enzyme classes. As will be described in this paper, these conserved micro-environments of primary structure may be of crucial importance for the active-site structures.
Localization of essential groups in D-xylose isomerases

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<th>GROUP I</th>
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<th>S.vr.</th>
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Fig. 1. Alignment of the N-terminal regions and of the internal active-site histidine and carboxylate-containing peptides from different D-xylose isomerases

Group I: *Amycolatopsis* sp. (A. sp.) region 1–66; *S. violaceoniger* (S. vn.) region 1–66; *S. violaceoruber* (S. vr.) region 1–13 plus internal peptides; *Streptomyces* sp. (S. sp.) region 1–20 plus internal peptides. Group II: *E. coli* (E. c.) region 1–113; *B. subtilis* (B. s.) region 1–119; *B. coagulans* (B. c.) region 1–33 plus internal peptides; *L. brevis* (L. b.) region 1–48 plus internal peptides; *L. xylosus* (L. x.) region 1–48. The residues are aligned according to Dauter et al. (1989) or by using manual methods. Conserved amino acid residues (considering both groups) are boxed. Data from the present study including the N-terminus of D-xylose isomerase from *S. violaceoruber* previously reported by some of us (Callens et al., 1985), are printed in bold. Non-determined residues are presented by ?. One additional deletion (–) has been inserted in the N-terminal region of the available amino acid sequences of the *E. coli* and *B. subtilis* isomerases in order to maximize alignment with the N-terminus of the *L. brevis* enzyme. *Deduced peptide sequence.

Isolation of the active-site-histidine-containing peptides

Complete inactivation of D-xylose isomerase from *Streptomyces* sp. by DEP was accompanied by modification of two histidine side chains monomer. In contrast, modification of only one histidine residue/monomer was observed and full activity was retained when DEP-derivatization was carried out in the presence of the protecting ligand combination 50 mM-xyitol plus 10 mM-Mg²⁺. The resulting reverse-phase h.p.l.c. profiles were nearly identical, except for one peak shifting towards a more hydrophobic position upon active site protection (from retention time 35 to 31 min). The chromatographic patterns were in excellent agreement with the results obtained for D-xylose isomerase from *S. violaceoruber* (Vangrysperre et al., 1989b). The differential peptide (35 min) of the digest of the ethoxycarbonylated D-xylose isomerase from *Streptomyces* sp. was isolated, treated with NH₂OH and rechromatographed using solvent system II. This resulted in further purification of the peptide. The retention time (54 min) was identical to that reported for D-xylose isomerase from *S. violaceoruber* (Vangrysperre et al., 1989b). Automatic sequence determination of the purified peptide yielded: Gly-Val-Thr-Phe-His-Asp-Asp-Asp-Leu-Ile-Pro-Phe. This sequence is identical to the active-site-histidine-containing peptide from D-xylose isomerase from *S. violaceoruber* (Fig. 1).
The same approach was used for D-xylose isomerase from two more divergent bacterial strains, L. brevis and B. coagulans, but failed to permit spectrophotometric detection of the differential peptides. Probably these peptides, being present in low yield, were masked by the absorbance of other peptides. They were therefore labelled with [14C]DEP and isolated by their radioactivity. The results for D-xylose isomerase from B. coagulans are presented in Fig. 2. Identical reverse-phase h.p.l.c. elution profiles were obtained in the presence or absence of protector (Fig. 2a). In contrast, when samples from the collected 1 ml fractions were counted for the 14C radioactivity incorporated, a significant decrease in c.p.m. was observed for the fraction denoted XI-1 (19 min) when [14C]DEP derivatization was carried out in the presence of protecting ligand (Fig. 2b). Indeed, as shown in Fig. 2(a), the differential peptide could not be detected at 214 nm, in contrast to the experiments carried out with two D-xylose isomerases from Actinomycetaceae. This fact, together with the observed difference in retention time (19 min instead of 35 min), permits us to assume that the amino acid sequence of the active-site-histidine-containing peptide of D-xylose isomerase from B. coagulans will have a different composition than the peptides isolated of D-xylose isomerase from Actinomycetaceae. Rechromatography of the radioactive fraction (XI-1) with solvent system II yields the elution profile of Fig. 3(a). Only one of the peaks, referred to as XI-2 and showing a retention time of 34 min, was radioactive (Fig. 3b). Automatic sequence determination of peptide XI-2 yielded: His-Asp-Arg-Asp-Ile-Ala-Pro-Glu-Gly. The sequence is similar to a segment of primary structure of D-xylose isomerase from B. subtilis and E. coli (Fig. 1) and matches that of the active-site-histidine-containing peptide of D-xylose isomerase from S. violaceoruber and Streptomyces sp. The absence of two phenylalanine residues and probably the low peptide yield, caused by rapid decomposition of the carboxethoxhistidine group in this specific peptide, may explain the failure of the spectrophotometric detection both at 214 and at 236 nm.

A similar peptide profile was obtained for the L. brevis enzyme. In both solvent systems the radiolabelled peptide migrated in a position identical to that of the B. coagulans peptide (for the separation profile in solvent system II, see Fig. 4). The final peptide yields were very low and did not allow further amino acid sequence analysis. As a matter of fact the considerable loss of 14C-label (as CO2) eluted prior to the peptide peaks (Fig. 4b) suggests very rapid decomposition of the carboxethoxylate histidine group in 0.1% TFA (solvent system II) and, consequently, the low yield of the radioactive XI-2 fraction. However, the similarity of the results to those with the B. coagulans enzyme permits us to assume that this difference peptide represents the active-site-histidine-containing peptide from L. brevis isomerase and may display a similar sequence (Fig. 1).

**Isolation of the active-site-carboxylate-containing peptides**

D-xylose isomerase from S. violaceoruber, modified by WRK (in the absence or presence of protector), was denatured by guanidinium hydrochloride and digested with papain instead of subtilisin in order to obtain...
different reverse-phase h.p.l.c. patterns for WRK- and DEP-modified D-xylose isomerase. As recently observed (W. Vangrysperre, unpublished work) a satisfying peptide map could be obtained using papain in 4 M-guanidinium hydrochloride. The resulting elution patterns at 214 and 340 nm (detection of WRK-modified carboxylate groups) (Sinha & Brewer, 1985, 1986) are illustrated in Fig. 5. The 340 nm tracings show different carboxylate groups modified by WRK; complete inactivation of D-xylose isomerase from *S. violaceoruber* was accompanied by modification of five carboxylate groups/monomer (Vangrysperre et al., 1989a).

The reverse-phase h.p.l.c. patterns of the digests from modified and protected enzyme coincided except for one peak (XI-1; 25.5 min) which partially disappeared (broken lines) in the latter case at both 214 nm (Fig. 5a) and 340 nm (Fig. 5b). The fraction XI-1 was rechromatographed with solvent system II (Fig. 6) in order to isolate the fully purified WRK-derivatized peptide. The 214 nm tracings show several peptides, in contrast to the 340 nm tracings showing only two peaks. The latter peptides were isolated and sequenced. Fraction XI-2, corresponding to the sequence Ala-Phe-Glu-His-Leu-Asp-Glu-Leu-Ala, reveals considerable similarity to a segment of the C-terminus of the *Streptomyces violaceoniger* enzyme (Drocourt et al., 1988). Since the glutamate group of the XI-2 sequence is not conserved among the other isomerases, the identity of this fraction as the active-site-carboxylate-containing peptide is questionable. In contrast, fraction XI-3, corresponding to the sequence Val-Thr-Phe-His-Asp-Asp-Asp, coincides surprisingly well with the active-site-histidine-containing peptide (Fig. 1). The fact that both the functional histidine and carboxylate groups are closely located on the same conserved Phe-His-Asp-Xaa-Asp-Xaa-Pro-Xaa-Gly segment of primary structure (Fig. 1) point to its unique function in the catalytic process.

The reverse-phase h.p.l.c. patterns of WRK-modified D-xylose isomerase from *Streptomyces* sp. were quite analogous to the tracings at 214 and 340 nm, as presented in Fig. 5 for D-xylose isomerase from *S. violaceoruber* except that two peaks totally disappeared in the presence of protecting ligand (results not shown). Rechromatography using solvent system II did not result in further fractionation. Sequence analysis revealed that both peptides overlapped each other in the primary structure, the first corresponding to Gly-Val-Thr-Phe-His-Asp-Asp-Asp and the second to His-Asp-Asp-Asp [referred as (1) and (2) in Fig. 1].

Analysis of the WRK-modified peptide map from D-xylose isomerase from *B. coagulans* did not permit conclusive results. In contrast, successful results were obtained with WRK-modified D-xylose isomerase from *L. brevis*. The peptide maps for protected and unprotected enzyme again coincided, except for two peaks which partially disappeared in the presence of protector (results not shown). The amino acid sequence of one of both rechromatographed fractions corresponded to Xaa-Xaa-Tyr-Leu-Cys-Phe-His-Asp-Arg-Asp-Leu, showing again the unique His-Asp-Xaa region characteristic for all active-site-derivatized histidine- and carboxylate-containing peptides studied in this paper (Fig. 1).

### The pH variation of the kinetic parameters

To obtain more information concerning the possible role of the conserved Phe-His-Asp-Xaa-Asp-Xaa-Xaa-Pro-Xaa-Gly region, the pH variation of $k_{cat}$ (μmol·min⁻¹·unit⁻¹), $K$ (M⁻¹) and $k_{cat}/K$ ($10^{-3}$ × ml·min⁻¹·unit⁻¹) was investigated for D-xylose isomerase from

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**Fig. 4.** Reverse-phase h.p.l.c. absorbance (a) and radioactivity (b) elution profiles (solvent system II) of the radioactive difference fraction isolated from the subtilisin digest of [¹⁴C]DEP-modified D-xylose isomerase from *L. brevis*.

The difference fraction isolated from the reverse-phase h.p.l.c. pattern obtained with solvent system I (results not shown) was diluted with 0.1% TFA and immediately injected on to the column.

**Fig. 5.** Reverse-phase h.p.l.c. elution profiles of WRK-modified D-xylose isomerase from *S. violaceoruber* digested with papain.

Inactivation by WRK in the absence (-----) or presence (-----) of protector and proteolysis were performed as described under 'Experimental procedures'. Solvent system I was used. Detection was done at both 214 nm (a) and 340 nm (b).
B. coagulans using excess Co\(^{3+}\) (1 mm) as metal activator and D-fructose as substrate. A pH range 5–8 was chosen for reasons of enzyme stability and metal binding (lower limit) or to avoid precipitation of Co(OH)\(_2\) (higher limit). Identification of functional groups with pH values beyond this pH range is thus excluded.

The \(k_{cat}\), the \(K\) and \(k_{cat}/K\) values are shown in Fig. 7. Although \(k_{cat}\) and \(k_{cat}/K\) could represent different kinetic steps in the overall turnover sequence, the pH profiles (Fig. 7), in the pH range studied, may be consistent with a monoprotic model. Since the \(k_{cat}\) values are pH-independent, the pH variation of \(K\) \((M^{-1})\) could be easily analysed in terms of the protonation of a single group resulting in loss of activity and being implicated in substrate binding. From the \(K\) and \(k_{cat}/K\) versus pH curves, fitting to \(pK_a\) values of 5.72 and 5.65 respectively, the protonated group is supposed to be a histidine group.

For D-xylose isomerase from *Streptomyces* sp., using excess Mg\(^{2+}\) as cofactor, \(pK_a\) values of 6.68 and 6.77 respectively were obtained (results not shown).

The data of the pH study confirm the results from our chemical modification study (Vangrysperre et al., 1988) providing further proof for the essentiality of the histidine group. In accordance with the recently published data for D-xylose isomerase from *S. rubiginosus* (Carrell et al., 1989) the histidine group is positioned on the conserved Phe-His-Asp-Xaa-Asp-Xaa-Xaa-Pro-Xaa-Gly sequence.

**DISCUSSION**

The N-terminal sequences of D-xylose isomerase from *S. violaceoruber*, *Streptomyces* sp., *B. coagulans*, *L. brevis* and *L. xylosus*, as determined in the present study (Fig. 1), have been extensively compared with the available total sequences of the *E. coli*, *B. subtilis*, *Ampullariella* sp. and *S. violaceoniger* isomerases. A striking similarity, including conservative substitutions, is noticed in the N-terminal sequences of D-xylose isomerases from Actinomycetaceae (Group I). Moreover, alignment with D-xylose isomerases from more divergent micro-organisms (Group II) points at the less extended N-terminal region of the first group of enzymes. These observations, to-

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**Fig. 6. Reverse-phase h.p.l.c. profiles of the contents of peak XI-1 (35.5 min) from Fig. 5**

The collected fraction XI-1 (35.5 min) was diluted with 0.1% TFA and immediately chromatographed with solvent system II.

**Fig. 7.** \(k_{cat}\), \(K\) and \(k_{cat}/K\) versus pH profiles for D-xylose isomerase from *B. coagulans*

Assays (200 \(\mu l\)) were run in 0.029 m-barbital/sodium acetate buffers containing 0.12 mM NaCl, at 1 mM-Co\(^{3+}\), with the substrate D-fructose varied from 0.025 to 1 mM, and with 0.047 unit of D-xylose isomerase. The curves through the data are hand-drawn and fit to \(pK_a\) values of 5.72 and 5.65 respectively of the free enzyme.

According to these authors, this active-site histidine is thought to be the base catalyst involved in the proton abstraction process, in contrast to the proposed participation in substrate binding as suggested in the present pH study.
together with previously reported differences in catalytic or physicochemical properties (see the Introduction) corroborate the existence of two distinct groups of D-xylose isomerases. This also follows from the presented reverse-phase h.p.l.c. patterns of the digested D-xylose isomerases, the essential histidine-containing peptides from the first group of isomerases being easily positioned compared with those from the second group of isomerases. Although both classes of D-xylose isomerases show distinct properties, they probably all act by the same catalytic mechanism. This follows from our studies identifying ligand-protected histidine and carboxylate groups in close proximity with each other and in a highly conserved region with consensus sequence Phe-His-Asp-Xaa-Asp-Xaa-Pro-Xaa-Gly. Interestingly, all isomerases belonging to Group I display an Asp-Asp-Asp sequence following the essential histidine. In contrast, those belonging to the high-molecular-mass isomerases show an Asp-Xaa-Asp stretch in the corresponding region (with Xaa either being arginine or valine). This may be one of the reasons for the poor stability of the DEP-modified active-site histidine residues in this class of enzymes.

The presence of the essential carboxylate group within a very short distance of the active-site histidine residue further suggests that both groups co-operate in either the binding or the isomerization process. In order to obtain more information on the possible role of these residues in the enzymatic process, we have studied the pH-dependence of the kinetic parameters. The results are consistent with a single histidine group implicated in the process of substrate binding. In contrast with the suggestions of Carrell et al. (1989), this group seems not to be involved in the isomerization process since our studies did not reveal variations in the $k_{\text{cat}}$ values in the pH range 5–8.

The participation of the carboxylate group is less clear. Probably one of the conserved aspartates serves as a nucleophile for the neighbouring histidine, locking the latter in a tautomeric form. The opposite N atom may then form a hydrogen bond with the substrate. It is structurally difficult to envisage a direct interaction between the histidine and the next aspartate. However, several secondary structures (β-turn, α-helix and some coiled structures) (Rey et al., 1988) will allow direct interactions of the type described between the histidine and the aspartate residues away from the histidine. Our hypothesis has been confirmed in a very recent paper which was published during the preparation of this manuscript and which describes the atomic structure of the active centre of D-xylose isomerase from S. rubiginosus (Carrell et al., 1989). Indeed, the presented diagram proposes hydrogen-bond formation between Asp-57 (one of the two conserved aspartates) and His-54. Following the authors the interaction between His-54 and Asp-57 is necessary for proper hydrogen dislocation in His-54. The role of the other conserved residues in the consensus sequence Phe-His-Asp-Xaa-Asp-Xaa-Xaa-Pro-Xaa-Gly will become apparent when more information is available on the three-dimensional structure of this enzyme.

**Note added in proof (received 17 November 1989)**

Since this work was submitted two papers have been published (Farber et al., 1989; Henrick et al., 1989) that support our hypothesis that the Phe-His-Asp-Xaa-Asp region is essential for substrate binding.

We are indebted to C. Capiu, K. Conrath and O. Van Opstal (Smith–Kline R.I.T., Belgium) for sequence analysis of some peptides and encouragement, to Professor Danno for the gift of *B. coagulans* HN-68, to Dr. M. Claeyssens for h.p.l.c. facilities made possible by a Research Grant from the National Fund for Scientific Research, Belgium, to G. Wallays for determining the kinetic parameters, to M. Cambier and L. Mees for technical assistance and to W. Van de Wiele for artwork. J.V. and R.C. are Research Associate and Research Director respectively of the National Fund for Scientific Research, Belgium. W.V. is a bursar of I.W.O.N.L., Belgium.

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Received 7 July 1989/14 August 1989; accepted 18 August 1989

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