Single active-site histidine in D-xylose isomerase from *Streptomyces violaceoruber*

Identification by chemical derivatization and peptide mapping

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Group-specific chemical modifications of D-xylose isomerase from *Streptomyces violaceoruber* indicated that complete loss of activity is fully correlated with the acylation of a single histidine. Active-site protection, by the ligand combination of xylitol plus Mg^{2+} , completely blocked diethyl pyrocarbonate derivatization of this particular residue [Vangrýsperre, Callens, Kersters-Hilderson & De Bruyne (1988) *Biochem. J.* **250**, 153–160]. Differential peptide mapping between D-xylose isomerase, which has previously been treated with diethyl pyrocarbonate in the presence or absence of xylitol plus Mg^{2+} , allowed specific isolation and sequencing of a peptide containing this active-site histidine. For this purpose we used two essentially new techniques: first, a highly reproducible peptide cleavage protocol for protease-resistant, carbethoxylated proteins with guanidinium hydrochloride as denaturing agent and subtilisin for proteolysis; and second, reverse-phase liquid chromatography with dual-wavelength detection at 214 and 238 nm, and calculation of absorbance ratios. It allowed us to locate the single active-site histidine at position 54 in the primary structure of *Streptomyces violaceoruber* D-xylose isomerase. The sequence around this residue is conserved in D-xylose isomerases from a diversity of micro-organisms, suggesting that this is a structurally and/or functionally essential part of the molecule.

INTRODUCTION

D-Xylose isomerases (D-xylose ketol-isomerase, EC 5.3.1.5) are enzymes of prime economic importance because of their exclusive use in the industrial production of the high-fructose corn syrups that constitute a large share in the vast food and drink sweeteners market. D-Xylose isomerases are tetrameric enzymes, mostly of microbial origin, that catalyse the conversion of D-xylose and D-glucose to D-xylulose and D-fructose respectively. Their constituting monomers have an average chain length of about 370–470 amino acids.

Industrial interests in D-xylose isomerase with improved catalytic properties and thermostability have sparked many searches for mutant enzymes, traditionally through selection of numerous naturally occurring mutant strains. However, with the advent of DNA recombinant technology and protein engineering, strategies have now shifted towards rationally modified, improved enzymes.

As a first step in the structure–function studies, usually preceding subsequent gene manipulations, active site residues, and more importantly catalytic residues, need to be identified in the enzyme sequence. Some of us recently reported evidence for a single essential active-site histidine residue in D-xylose isomerase from *Streptomyces violaceoruber* (Vangrýsperre *et al.*, 1988). Modification of the enzyme (M_r 171 700; Callens *et al.*,

1985b) with diethyl pyrocarbonate (DEP) in the absence or presence of the protecting ligand combination of xylitol plus Mg^{2+} indicated that four histidines per monomer in the unprotected enzyme and three in the protected enzyme were acylated to form carbethoxy-histidines. The modification of the one differential histidine is totally correlated with complete loss of activity.

In this report, we describe experiments leading to the accurate localization of this histidine residue in the total sequence (J. Seurinck, unpublished work). The method consists of differential peptide mapping by reverse-phase h.p.l.c., based on the changed absorbance ratios and retention times of carbethoxyhistidine-containing peptides as compared with their underivatized counterparts, followed by isolation and sequencing of the single differential peptide.

EXPERIMENTAL

Materials

D-Xylose and xylitol were products ‘for Biochemistry’ from Merck (Darmstadt, Germany). DEP was obtained from Aldrich (Milwaukee, WI, U.S.A.). Hydroxylamine was purchased from Serva (Heidelberg, Germany). Guanidinium hydrochloride (GdnHCl, sequential grade), trifluoroacetic acid (TFA), CH_3CN and phenyl isothiocyanate (PITC) were from Pierce (Rockford, IL,

Abbreviations used: DEP, diethyl pyrocarbonate; TFA, trifluoroacetic acid; PITC, phenyl isothiocyanate; PTC, phenylthiocarbonyl; GdnHCl, guanidinium hydrochloride.

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U.S.A.). L-1-*p*-Tosylamino-2-phenyl ethyl chloromethyl ketone-treated trypsin (type XIII), and chymotrypsin and elastase (type VII) were from Sigma (St. Louis, MO, U.S.A.); subtilisin was obtained from Novo (Bagsvaerd, Denmark). The synthetic peptide CSF19 (a gift from Dr. Suzanna Horvath, Caltech, Pasadena, CA, U.S.A.) has the following sequence: TVERPWKHVEAIKEALNLA.

Enzyme purification and assay

Cultivation of *S. violaceoruber* (strain LMG 7183) and purification of D-xylose isomerase to a homogeneous state were as described (Callens *et al.*, 1985a,b). Enzymic activity was measured by the coupled D-xylose isomerase/D-sorbitol dehydrogenase assay (Callens *et al.*, 1986; Kersters-Hilderson *et al.*, 1987).

Reaction of D-xylose isomerase with DEP

Concentration assessment of commercial DEP solutions, reaction (and inactivation) of D-xylose isomerase with DEP, subsequent reactivations using hydroxylamine and differential absorption spectroscopy were exactly as described before (Vangrýsperre *et al.*, 1988). The amount of *N*-carbethoxyhistidine per monomer of D-xylose isomerase was calculated by using $3200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ as the molar absorption coefficient at 238 nm (Ovádi *et al.*, 1967).

Denaturation and proteolysis

DEP-modified D-xylose isomerase was denatured by adding 3 vol. of 8 M-GdnHCl in 0.05 M-sodium phosphate buffer, pH 7.0, and subsequent incubation for 10 min at 30 °C. The solution was then diluted with 0.05 M-sodium phosphate buffer, pH 7.0, to a final GdnHCl concentration of 2 M. Protease (5% of the mass of substrate) was added at this stage. All digests were performed at 30 °C for 24 h, except with subtilisin (2 h). Peptide mixtures were then immediately injected on to a reverse-phase h.p.l.c. column for separation.

Reverse-phase h.p.l.c. of peptides

The reverse-phase h.p.l.c. system consisted of a complete ABI 150A system and an additional ABI-Kratos 757 detector (Applied Biosystems Inc., Ramsey, NJ, U.S.A.). The two variable wavelength detectors were connected in series and set at 214 and 238 nm. A Vydac 214TP54 reverse-phase (C4) column (0.46 cm × 25 cm) from the Separations Group (Hesperia, CA, U.S.A.) was used with two different solvent systems: system I, or 12 mM-sodium phosphate buffer, pH 6.0 (solvent A)/12 mM-phosphate buffer, pH 6.0 in CH₃CN/water (70:30, v/v) (solvent B); and system II, or 0.1% TFA (solvent A)/0.1% TFA in CH₃CN/water (70:30, v/v) (solvent B). Flow rates were always 1 ml/min and gradient slopes were as indicated in the legends to the Tables and Figures. System II was always used to isolate peptides for amino acid analysis and sequencing. Collected fractions were then dried in a Speedvac concentrator (Savant, Hicksville, NY, U.S.A.).

Amino acid analysis

Peptides were hydrolysed under vacuum at 110 °C in 6 M-HCl containing 0.05% phenol for 24 h. Hydrolysates were derivatized with PITC and the phenylthiocarbamyl (PTC)-amino acids were analysed with a Waters h.p.l.c. system and a C18 reverse-phase column (Waters,

Millipore Corp., Milford, MA, U.S.A.) as described by Bidlingmeyer *et al.* (1984).

Automatic sequence determination

Peptide XI-1/NH₂OH (see the Results section) was sequenced using a 470A Applied Biosystems gas-phase sequencer (Hewick *et al.*, 1981) equipped with a 120 A on-line phenylthiohydantoin (PTH)-analysis system (Applied Biosystems Inc.). Yields of stepwise liberated PTH-amino acids were measured using an integrative recorder (Hitachi/Merck, Darmstadt, Germany).

RESULTS

DEP-derivatized D-xylose isomerase preparations, in the presence and absence of xylitol plus Mg²⁺, were freshly made before all experiments. Complete loss of activity was correlated with modification of a single active-site histidine residue. To locate this residue in the known amino acid sequence (J. Seurinck, unpublished work) we wanted to perform comparative peptide mapping between totally inactive and fully active forms of DEP-derivatized D-xylose isomerase, isolate the single differential peptide and sequence it. However, D-xylose isomerases are proteins with an eight-stranded parallel β -barrel architecture (Carrell *et al.*, 1984) and therefore have a tight and very stable structure. D-Xylose isomerase from *S. violaceoruber* is resistant to degradation by a wide variety of proteases (P. Tempst, E. Bossier & W. Vangrýsperre, unpublished work). Thus, in order to cleave the isomerase enzymically, it needed to be denatured first. Since we expected a rather complex peptide mixture, we decided to use a highly selective peptide separation technique such as reverse-phase h.p.l.c. (Tempst *et al.*, 1987).

Table 1. Reverse-phase h.p.l.c. retention times and A_{238}/A_{214} absorbance ratios of synthetic and proteolytic peptides

The absorbance ratios were calculated from the values obtained by dual-wavelength detection of h.p.l.c. effluents. Unmodified CSF19 (for sequence, see the Experimental section) were tested separately; a fraction of the resulting carboxy derivative was also treated with an equal volume of 1 M-NH₂OH (pH 7.0) prior to chromatography (CSF19-DEP/NH₂OH). Subtilisin peptides XI-1 and XI-2 are derived from D-xylose isomerase treated with a 20-fold molar excess (over monomer) of DEP in the absence or presence of xylitol plus Mg²⁺ respectively; an aliquot of XI-1, after chromatography, was treated with NH₂OH and rechromatographed (XI-1/NH₂OH). For chromatography of the first three peptides, solvent system II was used with a gradient slope of 2% solvent B/min; in the last three experiments we used solvent system I and a gradient slope of 1% solvent B/min (see Experimental section).

Peptide	Reverse-phase h.p.l.c. retention (min)	$10^2 \times A_{238}/A_{214}$
CSF19	27	6.7
CSF19-DEP	33	12.3
CSF19-DEP/NH ₂ OH	28	6.5
XI-1	35	11.8
XI-2	31	3.1
XI-1/NH ₂ OH	31	3.1

Reverse-phase h.p.l.c. of carboxyhistidyl peptides

A single histidine-containing synthetic peptide (CSF19) was used as a model system to test chromatographic behaviour and the use of two-wavelength detection (at 214 and 238 nm) of carboxyhistidine derivatives. The result is shown in Table 1 and indicates, as anticipated, that the addition of two carbon atoms to the single histidine of a 19-residue long peptide has a dramatic effect upon the retention on a reverse-phase (C4) support. Moreover, a reverse shift is observed following decarboxylation upon NH_2OH treatment. A similar observation has been reported by Biscoglio *et al.* (1986) for peptides varying in length from 3 to 13 residues. The fact that the retention time of decarboxylated CSF19 is slightly longer than that of native CSF19, although the A_{238}/A_{214} ratios are the same, can be explained by assuming that derivatization of other groups than histidyl (α - and ϵ -amino or arginyl) has occurred, resulting in irreversible derivatives with no apparent absorption increase at 238 nm (Miles, 1977). Most importantly, dual-wavelength detection at 238 and 214 nm, the former wavelength being the absorption maximum of carboxyhistidine (Ovadi *et al.*, 1967), indicates that the A_{238}/A_{214} ratio of the carboxylated peptide is twice that of its native form.

Stability of carboxyhistidine to pH changes

Since carboxyhistidine is only stable around pH 6.0 (Miles, 1977), we investigated the stability of carboxyhistidyl derivatives in sodium phosphate buffer, pH 6.0 to 8.5, for 5 h at 30 °C and 37 °C. We derivatized synthetic peptide CSF19 with a 20-fold molar excess of DEP and mapped the position on reverse-phase h.p.l.c. (solvent system II) before and after treatment with 0.5 M- NH_2OH . Peak heights at 214 nm of 1 nmol of the underivatized, derivatized, and derivatized/decarboxylated (DEP/ NH_2OH) peptides were identical and could consequently be used for quantification of each of these molecules. Freshly prepared aliquots of carboxyhistidyl peptide CSF19 were incubated for 5 h at different pH and temperature, immediately

Table 2. Carboxyhistidyl CSF19-peptide remaining after 5 h incubation at 30 °C or 37 °C and at different pH values

Amounts are calculated from the peptide peak heights during u.v. detection (214 nm) of reverse-phase h.p.l.c. effluents. The combined peak heights of the carboxyhistidyl CSF19-peptide and its decarboxylated form are constant and amount to 0.09 absorbance unit per mol at 214 nm under the chromatographic conditions as described in the Experimental section employing solvent system II. The % carboxyhistidyl CSF19 is expressed relative to the amount at pH 6.0 and at time zero. N.D., not determined.

pH	Temp. ...	Carboxyhistidyl peptide (%)	
		30 °C	37 °C
6.0		99	86
6.5		90	81
7.0		86	72
8.0		61	53
8.5		N.D.	25

analysed by reverse-phase h.p.l.c. and the destruction of the derivative quantified (Table 2).

Peptide mapping of DEP-modified D-xylose isomerase

According to the results summarized in Table 2, denaturation and digestion of carboxylated D-xylose isomerase needed to be done at a pH between 6 and 7, at 30 °C or below and in a total period of time not exceeding 5 h, preferably faster. Using the results of a systematic study (P. Tempst, unpublished work) of enzymic cleavage of protease-resistant proteins, we developed the optimized protocol described below. *S. violaceoruber* D-xylose isomerase, treated with a 20-fold molar excess (over monomer) of DEP for 40 min at 25 °C, in 0.05 M-sodium phosphate buffer, pH 6.0, was made 6 M with respect to GdnHCl in the same buffer at pH 7.0, and further treated as described in the Experimental section; 5% (w/w) subtilisin was then added and the digestion proceeded for 2 h at 30 °C. In order to obtain a complete digest under those conditions, substrate concentration

Fig. 1. Reverse-phase h.p.l.c. profiles of *S. violaceoruber* D-xylose isomerase digested with subtilisin

Isomerases (10 nmol) were treated with a 20-fold molar excess (over monomer) of DEP in the absence (a) or presence (b) of xylitol plus Mg^{2+} . Conditions for the digests and h.p.l.c. experiments are described in the Experimental section; solvent system I was used. Gradient slopes (—) were 0–50% solvent B in 50 min and 50–100% solvent B in 5 min. U.v. detection was done at 214 nm (—) and 238 nm (-----); A_{238} peaks are slightly off to the right of their A_{214} counterparts. Collected peaks XI-1 and XI-2 are indicated with the arrows on the profiles concerned as are their corresponding positions on the other chromatogram.

must exceed 0.4 mg/ml. Peptide mixtures were then injected on the C4 reverse-phase column equilibrated with buffer system I.

Isolation and sequencing of the 'active-site His'-peptide

D-Xylose isomerase (10 nmol of monomer) modified with DEP in the presence or absence of xylitol plus Mg^{2+} was digested with subtilisin and chromatographed as described above in two parallel experiments. The reverse-phase h.p.l.c. profiles are presented in Fig. 1. The patterns are nearly identical, with the exception of one major peak that has shifted considerably. Both peptide peaks, designated XI-1 and XI-2 in Fig. 1, were collected for further characterization. Peak XI-1, displaying the highest retention time of the two and derived from the unprotected carbethoxylated D-xylose isomerase and therefore inactive, was subsequently incubated with an equal volume of 1 M-NH₂OH for 30 min at 25 °C (designated XI-1/NH₂OH). An aliquot was then rechromatographed under conditions used for the total peptide mixtures (result not shown). The retention times and the A_{238}/A_{214} absorbance ratios for peptides XI-1, XI-2 and XI-1/NH₂OH are presented in Table 1. Peptide XI-1 has an almost 4-fold higher A_{238}/A_{214} ratio than XI-2, and also elutes later from the C4 support; but when treated with NH₂OH, peptide XI-1 is converted into a molecule with exactly the same A_{238}/A_{214} ratio and reverse-phase retention time as XI-2. This clearly indicates that XI-1 is a carbethoxyhistidyl peptide; moreover, XI-2 is most probably its uncarbethoxylated counterpart.

Final purification of peptides XI-1/NH₂OH and XI-2 was accomplished by preparative reverse-phase h.p.l.c. using the same chromatographic system but operated with solvent system II (see the Experimental section). The additional advantage of this final purification is the complete volatility of the solvent which allows optimal amino acid composition and sequence analysis of the peptides afterwards (Mahoney & Hermodson, 1980). The amino acid composition of peptides XI-1/NH₂OH and XI-2 proved to be the same: 1.6 Asx, 1.2 Thr, 1.6

Gly, 0.8 Val, 1.2 Ile, 1.0 Leu, 1.8 Phe, 0.7 His and 1.0 Pro. Automatic sequence determination of XI-1/NH₂OH yielded the following sequence: Gly-Val-Thr-Phe-His-Asp-Asp-Asp-Leu-Ile-Pro-Phe. Given the peptide composition and subtilisin specificity, this sequence is most likely to be full length. The peptide matches region 50–61 of the amino acid sequence deduced from the nucleotide sequence of the *S. violaceoruber* D-xylose isomerase gene (J. Seurinck, unpublished work). Consequently, His-54 is located in the active site of this enzyme.

DISCUSSION

To gain further information about the proposed single active-site histidine, a procedure has been developed for isolation and amino acid sequencing of the peptide containing the essential histidyl residue.

To this purpose, the protease-resistant enzyme was unfolded in 6 M-GdnHCl and subsequently digested with subtilisin in 2 M-GdnHCl at pH 7.0 for only 2 h and at 30 °C. The use of any other set of denaturing conditions and/or proteases was either too harsh, too lengthy, irreproducible or did not produce the right size fragments.

Reverse-phase h.p.l.c. effluents were monitored simultaneously at 238 nm (the absorbance maximum of the difference spectrum between carbethoxylated and native isomerase) and at 214 nm, a common wavelength for peptide detection. It allowed us to detect the shift of a single peak by comparison of subtilitic peptide profiles of active and inactive D-xylose isomerases, containing three and four carbethoxyhistidines respectively (Fig. 1). In addition to the retention differences, the peaks also differed sharply in absorbance properties (Table 1). Subsequent isolation of differential peptides was simple. The properties of the carbethoxylated one could be converted by NH₂OH treatment into exactly those of its unmodified counterpart.

U.v. detection at 238 nm of reverse-phase h.p.l.c. effluents during separation of carbethoxylated peptide mixtures, in combination with subsequent decarbethoxyl-

<i>Actinomycetales:</i>														
<i>S. violaceoruber</i>	50	Gly	Val	Thr	Phe	His	Asp	Asp	Asp	Leu	Ile	Pro	Phe	61
(peptide sequence)*														
<i>S. violaceoniger</i>	49	Gly	Val	Thr	Phe	His	Asp	Asp	Asp	Leu	Ile	Pro	Phe	60
(gene sequence)†														
<i>Ampullariella</i> sp.	50	Gly	Val	Thr	Phe	His	Asp	Asp	Asp	Leu	Val	Pro	Phe	61
(gene sequence)‡														
<i>Bacillus subtilis</i>	103	Phe	Phe	Ala	Phe	His	Asp	Arg	Asp	Ile	Ala	Pro	Glu	114
(gene sequence)§														
<i>Escherichia coli</i>	97	Phe	Tyr	Cys	Phe	His	Asp	Val	Asp	Val	Ser	Pro	Glu	108
(gene sequence)														

* This work.

† Drocourt *et al.* (1988).

‡ Saari *et al.* (1987).

§ Wilhelm & Hollenberg (1985).

|| Schellenberg *et al.* (1984).

Fig. 2. Sequence alignment of the active-site His peptide from *S. violaceoruber* with similar regions of D-xylose isomerases from other bacteria

The numberings are according to published reports or to the *S. violaceoruber* D-xylose isomerase gene sequence (J. Seurinck, unpublished work). Identical or chemically conserved amino acids are boxed with a solid and a dashed line respectively.

ation followed by rechromatography and detection at 220 nm, has already been employed for specific isolation of histidine peptides (Biscoglio *et al.*, 1986). In contrast to that report, our studies deal with DEP-derivatization prior to proteolysis and rely on peptide profile comparisons in which simultaneous detection of DEP-derivatized and underivatized peptides is necessary.

Following isolation, decarboxylation, rechromatography and sequencing of the active-site histidine-containing peptide, comparison with the complete primary structure of *S. violaceoruber* D-xylose isomerase allowed us to identify His-54 as an active-site residue. When the experimentally determined peptide sequence surrounding His-54 is compared with the best matching stretches of the known amino acid sequences of D-xylose isomerases from the two related species *Ampullariella* sp. (Saari *et al.*, 1987) and *S. violaceoniger* (Drocourt *et al.*, 1988) and from the two more divergent micro-organisms *E. coli* (Schellenberg *et al.*, 1984) and *B. subtilis* (Wilhelm & Hollenberg, 1985) significant sequence similarity was observed (Fig. 2). Five out of the twelve residues are identical in all sequences and two more positions are chemically conserved. Even greater similarity is observed among the sequences of the isomerases from the three species belonging to the *Actinomycetales* order; all residues are identical except for one conservative substitution.

These highly conserved regions, as compared with the overall similarities, allow one to suggest that they are located in a structurally essential part of the molecule, adding more value to our claim that this particular histidine is part of the active site in those enzymes of the micro-organisms concerned. This conclusion is substantiated by the recently published structural analysis of D-xylose isomerase from *Actinoplanes missouriensis* (Rey *et al.*, 1988) classified in the same order as *S. violaceoruber* (*Actinomycetales*). According to these authors, the active site is positioned at the carboxyl ends of the β -barrel strands and more specifically, His-54 is placed at the C-terminus of the β_2 -strand.

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