Reaction of Woodward's reagent K with D-xylose isomerases

Modification of an active site carboxylate residue

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D-Xylose isomerases from *Streptomyces violaceoruber*, *Streptomyces* sp., *Lactobacillus xylosus*, *Lactobacillus brevis* and *Bacillus coagulans* were rapidly inactivated by Woodward's reagent K. Second-order rate constants in the absence of ligands, at pH 6.0 and 25 °C, were 41, 36, 22, 95 and 26 M⁻¹·min⁻¹ respectively. Spectral analysis at 340 nm revealed that inactivation was correlated with modification of five, six, two, three and six carboxylate residues per monomer respectively. In the presence of protecting ligands, modification of one carboxylate group was prevented. The results support the idea of an active site glutamate or aspartate group that may contribute to the catalytic activity of all these D-xylose isomerases.

INTRODUCTION

Aldo-keto isomerizations act via a *cis*-enediol open substrate intermediate (Schray & Rose, 1971). An essential carboxylate residue as well as a histidine and lysine group seem to be involved in the catalytic reaction mechanism of triose- and glucose-phosphate isomerase (Shaw & Muirhead, 1976; Raines *et al.*, 1986). For D-xylose isomerase activity (D-xylose ketol-isomerase, EC 5.3.1.5), the presence of bivalent metal cations is an absolute requirement. In addition, evidence has been provided for the presence of a histidine residue with potential active centre functions, although its distinct role has not been elucidated (Vangrýsperre *et al.*, 1988; Gaikwad *et al.*, 1988; Pawar *et al.*, 1988). Lysyl, sulphydryl, arginyl, tryptophanyl, tyrosyl and seryl side-chains are not involved in the reaction mechanism (W. Vangrýsperre, unpublished work).

The present paper considers the possibility of a catalytically important carboxylate group in D-xylose isomerase, and deals with the effects of Woodward's Reagent K (WRK), a reagent causing rapid and specific modification of aspartate or glutamate groups in proteins (Bodlaender *et al.*, 1969; Pétra, 1971). The first step in the reaction (Scheme 1) is the irreversible formation of a

ketoketenimine, which reacts with a carboxylate group to give an enol ester, showing an absorbance peak at 340 nm (Sinha & Brewer, 1985).

MATERIALS AND METHODS

Materials

D-Xylose and xylitol 'for biochemistry', CoCl₂·6H₂O, MgCl₂·6H₂O, MnCl₂·4H₂O and CaCl₂·2H₂O were products from E. Merck, Darmstadt, Germany. WRK was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. PD-10 Sephadex G-25 M columns were from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade.

Enzyme purification

Streptomyces violaceoruber (LMG 7183), *Streptomyces* sp. (ATCC 21132), *Lactobacillus xylosus* (NRRL B-4449), *Lactobacillus brevis* (ATCC 8287) and *Bacillus coagulans* [LMG 7386 = HN68 (Yoshimura *et al.*, 1966)] were cultivated as reported by Callens *et al.* (1985a), Sanchez & Smiley (1975), Yamanaka & Takahara (1977), Yamanaka (1968) and Danno *et al.* (1967) respectively.

Scheme 1. Reaction mechanism of WRK

Abbreviations used: WRK, Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3'-sulphonate); DEP, diethyl pyrocarbonate.

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The D-xylose isomerases were purified, using the procedure of Callens *et al.* (1985b), with slight modifications for each enzyme source as indicated. The isolation procedure involved ammonium sulphate fractionation (40–80, 40–80, 40–80, 45–80 and 55–80 % respectively), heat treatment (10 min at 70, 70, 60, 53 and 60 °C respectively), and chromatography on phenyl–Sephacryl CL-4B, DEAE–Sephacryl and Sephacryl S-200. A single buffer, i.e. 0.01 M-Tris/HCl buffer, pH 7.0, containing either Co^{2+} (D-xylose isomerase from Streptomyetaceae) or Mn^{2+} (D-xylose isomerase from *L. xylosus*, *L. brevis* and *B. coagulans*) was used throughout the purification process.

Protein concentrations of the purified enzymes (tetramers) were determined at 280 nm with a Uvikon 810 spectrophotometer (Kontron), using an average absorbance $A_{1\text{cm}}^{1\%}$ of 10 (W. Vangrysperre, unpublished work) and monomeric M_r s of 42 900, 43 300, 47 300, 52 300 and 49 500 for D-xylose isomerase from *S. violaceoruber*, *Streptomyces* sp., *L. xylosus*, *L. brevis* and *B. coagulans* respectively, as determined by SDS/polyacrylamide-gel electrophoresis (W. Vangrysperre, unpublished work). Metal-free enzyme was prepared by dialysis against 10 mM-EDTA (24 h) in 0.01 M-triethanolamine/HCl

buffer, pH 7.0. After EDTA treatment, the EDTA was removed by dialysis (34 h) against four batches of the same buffer.

Enzyme assays

Enzyme activities for D-xylose isomerase from *S. violaceoruber*, *Streptomyces* sp. *L. xylosus* and *L. brevis* were measured as described earlier (Vangrysperre *et al.*, 1988). For D-xylose isomerase from *B. coagulans*, reaction conditions were 1 mM- MnCl_2 and 0.01 M-triethanolamine/HCl buffer, pH 7.0.

Reaction of D-xylose isomerases with WRK

Freshly prepared stock solutions of 0.5 M-WRK in 1 mM-HCl were used. The modification reactions were carried out at pH 6.0 and 25 °C in 0.5 M-sodium/potassium phosphate buffer. The incubation mixtures (100 μl) contained 10–20 μM metal-free active sites and varying amounts of WRK. Aliquots (10 μl) were taken at different time intervals and assayed for enzyme activity. Ligands were added prior (5 min) to the addition of 30 mM-WRK.

The number of modified carboxylate residues was determined as follows. Active sites (10–19 μM) were

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

D-Xylose isomerases were from *S. violaceoruber* (a), *Streptomyces* sp. (b), *L. xylosus* (c), *L. brevis* (d) and *B. coagulans* (e). The experiments were carried out as described in the Materials and methods section. The numbers on the slopes indicate WRK concentrations (mM).

incubated with 20 mM-WRK for 10 min at 25 °C, after which a second portion of 20 mM-WRK was added. After 10 min of reaction, excess reagent was removed by gel filtration on PD-10 Sephadex G-25 M columns (equilibrated with 0.05 M-Tris/HCl buffer, pH 8.0) and the degree of modification was measured by the increase in absorbance at 340 nm (ϵ 7000 M⁻¹·cm⁻¹) (Sinha & Brewer, 1985), using Tris/HCl buffer as reference. All spectrophotometric experiments were carried out with a Uvikon 810 double-beam spectrophotometer (Kontron) equipped with thermostatted (25 °C) copper cuvette holders.

RESULTS AND DISCUSSION

Kinetics of inactivation

The investigated D-xylose isomerases were rapidly inactivated by excess WRK at 25 °C in 0.5 M-sodium/potassium phosphate buffer, pH 6.0. Plots of the logarithm of remaining activity versus time resulted in pseudo-first-order kinetics (Fig. 1) and the k_{obs} values seemed to be independent of initial enzyme concentrations as confirmed by the data obtained over a 6-fold range of enzyme concentration (results not shown). The k_{obs} versus [WRK] plots (Fig. 2) indicated that a

Fig. 2. Plots of pseudo-first-order rate constants versus WRK concentration

D-Xylose isomerases were from *S. violaceoruber* (○), *Streptomyces* sp. (●), *L. xylosus* (□), *L. brevis* (■) and *B. coagulans* (△). The plots were obtained from the experimental data of Fig. 1.

reversible enzyme-WRK complex is formed prior to covalent modification (Pétra, 1971).

Linearization of the data in a $[\text{WRK}] \cdot k_{\text{obs}}^{-1}$ versus [WRK] plot (Fig. 3), yielded (i) the apparent reagent-binding constants (K), (ii) the first-order rate constants at saturation of modifier (k_{max}) and (iii) the second-order rate constants (k) (Table 1). The latter constants were within the range of those reported for carboxylate modification of carboxypeptidase A (Pétra, 1971), staphylococcal nuclease (Dunn *et al.*, 1974) and phospholipase A₂ (Dinur *et al.*, 1981) (94, 35 and 26 M⁻¹·min⁻¹, respectively).

Effects of ligands on inactivation of D-xylose isomerases by WRK

The effects of the substrates D-xylose and D-glucose, the substrate analogue xylitol, the essential metal cofactors (Mg²⁺, Mn²⁺ and Co²⁺) and the nonactivating ligand Ca²⁺ on the rate of enzyme inactivation by WRK

Fig. 3. Plots of $[\text{WRK}] \cdot k_{\text{obs}}^{-1}$ versus [WRK]

D-Xylose isomerases were from *S. violaceoruber* (○), *Streptomyces* sp. (●), *L. xylosus* (□), *L. brevis* (■) and *B. coagulans* (△). The plots were linearizations of the plots presented in Fig. 2.

Table 1. Apparent binding constants (K), first-order rate constants at saturation (k_{max}) and second-order rate constants (k) for the reaction of D-xylose isomerase with WRK

The experiments were carried out as described in the Materials and methods section. Results are means, with s.e.m. values given in parentheses.

	<i>S. violaceoruber</i>	<i>Streptomyces</i> sp.	<i>L. xylosus</i>	<i>L. brevis</i>	<i>B. coagulans</i>
K (M ⁻¹)	89 (±4)	103 (±15)	27 (±5)	95 (±15)	107 (±36)
k_{max} (min ⁻¹)	0.46 (±0.01)	0.35 (±0.03)	0.8 (±0.1)	1.0 (±0.1)	0.24 (±0.04)
k (M ⁻¹ ·min ⁻¹)	41 (±2)	36 (±6)	22 (±5)	95 (±18)	26 (±10)

Table 2. Effect of ligands on the rate of inactivation of D-xylose isomerase by WRK

The experiments were carried out as described in the Materials and methods section. Protection (%) is expressed as $\{[k_{\text{obs. (unprotected)}} - k_{\text{obs. (protected)}}]/k_{\text{obs. (unprotected)}}\} \times 100$. The average variation found was 10 %.

Addition	Protection (%)				
	<i>S. violaceoruber</i>	<i>Streptomyces</i> sp.	<i>L. xylosus</i>	<i>L. brevis</i>	<i>B. coagulans</i>
None	0	0	0	0	0
0.2 M-D-Xylose	-8	-14	-19	-12	-2
0.5 M-D-Glucose	-13	-2	-13	-28	-6
50 mM-Xylitol	-19	34	27	64	52
1 mM-Mn ²⁺	84	73	-64	72	-43
10 mM-Mn ²⁺	80	86	-55	85	-42
50 mM-Xylitol + 1 mM-Mn ²⁺	-	69	-	-	-
50 mM-Xylitol + 10 mM-Mn ²⁺	100	95	90	100	76
0.5 M-D-Glucose + 10 mM-Mn ²⁺	91	81	-41	91	-39
1 mM-Co ²⁺	81	75	-	-	-
10 mM-Co ²⁺	97	95	45	91	37
50 mM-Xylitol + 1 mM-Co ²⁺	-	78	-	-	-
50 mM-Xylitol + 10 mM-Co ²⁺	83	91	98	100	36
0.5 M-D-Glucose + 10 mM-Co ²⁺	81	100	100	96	97
10 mM-Mg ²⁺	-16	-4	-10	-9	-98
50 mM-Xylitol + 10 mM-Mg ²⁺	100	93	53	86	52
0.5 M-D-Glucose + 10 mM-Mg ²⁺	7	9	-23	31	-228
1 mM-Ca ²⁺	40	27	14	0	25
50 mM-Xylitol + 1 mM-Ca ²⁺	62	64	5	76	52
0.5 M-D-Glucose + 1 mM-Ca ²⁺	31	48	12	26	-

are presented in Table 2. The effects of some combinations of ligands have also been studied.

The substrates D-xylose and D-glucose only slightly affect the inactivation rate. Their nonprotecting properties are easily understood if one considers the fact that metal ions are required to induce the proper conformation of the active site. The substrate analogue xylitol (open ring structure), however, gave more substantial protection.

The effect of the metal cofactor Mn²⁺ on the enzyme inactivation rate was different with the different isomerases. The metal ion seems to protect D-xylose isomerase from *S. violaceoruber*, *Streptomyces* sp. and *L. brevis*, while it enhances the inactivation of D-xylose isomerase from *L. xylosus* and *B. coagulans*. Analysis by $[WRK] \cdot k_{\text{obs.}}^{-1}$ versus $[WRK]$ plots (Fig. 4) for D-xylose isomerase from *L. xylosus* and *L. brevis* strongly suggests that the increase or decrease of the second-order rate constants in the presence of Mn²⁺ ions is mainly due to an increase or decrease in the apparent binding of WRK, yielding an overall effect of faster inactivation or apparent protection (Table 3). From the enhanced inactivation in the presence of Mn²⁺ (*L. xylosus* and *B. coagulans*), the direct co-ordination of Mn²⁺ to the essential carboxylate residue has to be excluded.

The Co²⁺-enzyme structure affords total protection against WRK inactivation (Tables 2 and 3), except for D-xylose isomerase from *L. xylosus* and *B. coagulans*. The active site carboxylate group of the latter enzymes, however, is fully protected in the Co²⁺-enzyme-D-glucose complex. If one considers the fact that Co²⁺ is the most effective metal cofactor for D-glucose isomerization

Fig. 4. Plots of $[WRK] \cdot k_{\text{obs.}}^{-1}$ versus $[WRK]$ in the presence or absence of Mn²⁺ or Co²⁺

The experimental conditions of inactivation were as described in the Materials and methods section; the concentrations of Mn²⁺ and Co²⁺ were as follows. (a) D-Xylose isomerase from *L. xylosus*: presence or absence of 1 mM-Mn²⁺ or 10 mM-Co²⁺ respectively; (b) D-xylose isomerase from *L. brevis*: presence or absence of 0.1 mM-Mn²⁺ or 0.05 mM-Co²⁺ respectively.

Table 3. Apparent binding constants (K), first-order rate constants at saturation ($k_{\max}$) and second-order rate constants (k) for D-xylose isomerases in the presence and absence of Mn^{2+} or Co^{2+} (*L. xylosus* and *L. brevis*)

The experiments were carried out as described in the Materials and methods section. Results are means, $\pm$ S.E.M. in parentheses.

Source of enzyme	Addition	$k_{\max}$ (min^{-1})	K (M^{-1})	k ($\text{min}^{-1} \cdot \text{M}^{-1}$)
<i>L. xylosus</i>	None	0.8 (± 0.1)	27 (± 5)	22 (± 5)
	1 mM- Mn^{2+}	0.8 (± 0.1)	46 (± 7)	36 (± 7)
	10 mM- Co^{2+}	0.9 (± 0.3)	13 (± 5)	12 (± 6)
<i>L. brevis</i>	None	1.0 (± 0.1)	96 (± 15)	95 (± 18)
	0.10 mM- Mn^{2+}	0.91 (± 0.04)	45 (± 2)	41 (± 3)
	0.05 mM- Co^{2+}	0.92 (± 0.04)	32 (± 1)	29 (± 2)

(except in Streptomycetaceae), the latter results may be easily understood. Unfortunately, Mn^{2+} -enzyme-D-xylose combinations could not be investigated due to interferences with the enzyme assay.

Formation of the binary Mg^{2+} -enzyme complex enhances the inactivation rate of D-xylose isomerase from *B. coagulans*. In contrast, only slight effects are observed on the inactivation rates of the other isomerases. At least formation of the tertiary Mg^{2+} -enzyme-xylitol complex is required to give substantial protection.

The protective effects of Ca^{2+} , possessing no activator capacities, seem to be of minor importance. Replacement of Mn^{2+} , Co^{2+} or Mg^{2+} by Ca^{2+} probably results in loss of the overall geometry of the active site and disturbance of the accessibility of the functional groups.

The overall results, together with previous observations concerning the structural role of the metal cofactors (W. Vangrýsperre, M. Callens & H. Kersters-Hilderson, unpublished work), tend to support the idea that, depending on the metal used, typical conformation changes are induced for each individual enzyme. As compared to the apoenzyme, the final M^{2+} -enzyme complex either enhances the accessibility to the essential groups or seems to block the access to it. In combination with substrates or substrate analogues, total protection may however be obtained.

Recently, similar effects of ligands have been reported

in our modification study with diethyl pyrocarbonate (DEP) (Vangrýsperre *et al.*, 1988), except for Mn^{2+} which unequivocally enhances the DEP inactivation, but slows down the WRK modification (*S. violaceoruber*, *Streptomyces* sp., *L. brevis*).

Quantification of modified carboxylate groups

Under the stipulated conditions as described by Sinha & Brewer (1985), protein modification by WRK enables quantification of carboxylate modification at 340 nm, provided sulphhydryl groups do not react. Reaction of WRK with sulphhydryl side-chains is very unlikely since thiol groups were not detected by Ellman's reagent in any of the native (either reduced or not) D-xylose isomerases (Callens *et al.*, 1985b; W. Vangrýsperre, unpublished work).

Spectral analysis at 340 nm of the protein samples revealed that complete inactivation of D-xylose isomerases from *S. violaceoruber*, *Streptomyces* sp., *L. xylosus*, *L. brevis* and *B. coagulans* was accompanied by the modification of five, six, two, three and six carboxylate residues per monomer respectively (Table 4).

Inactivation in the presence of protecting ligand further indicated that modification of one carboxylate residue was prevented, with maintenance of enzyme activity (Fig. 5, Table 4).

The fact that modification of one carboxylate group

Table 4. Numbers of carboxylate groups of D-xylose isomerases modified by WRK

The experiments were carried out as described in the Materials and methods section. Ligands were selected from the data presented in Table 2.

Source of enzyme	Addition	Modified residues (mol/mol of monomer)	Residual activity (%)
<i>S. violaceoruber</i>	None	4.8	21
	50 mM-Xylitol + 10 mM- Mg^{2+}	4.1	88
<i>Streptomyces</i> sp.	None	5.8	15
	50 mM-Xylitol + 10 mM- Mg^{2+}	5.0	90
<i>L. xylosus</i>	None	2.2	15
	50 mM-Xylitol + 10 mM- Mn^{2+}	1.2	78
<i>L. brevis</i>	None	2.9	5
	50 mM-Xylitol + 10 mM- Mn^{2+}	1.8	100
<i>B. coagulans</i>	0.5 M-D-Glucose + 10 mM- Mg^{2+}	5.8	21
	0.5 M-D-Glucose + 10 mM- Co^{2+}	5.3	70

Fig. 5. Difference spectra for modification of D-xylose isomerases by WRK

The experimental conditions of inactivation were as described in the Materials and methods section. (a), (b) D-Xylose isomerases from *S. violaceoruber* and *Streptomyces* sp. respectively: 1, absence of ligands; 2, presence of 50 mM-xylitol + 10 mM-Mg²⁺ (cf. Table 2); 3, unmodified D-xylose isomerase. (c), (d) D-Xylose isomerases from *L. xylosus* and *L. brevis* respectively: 1, absence of ligands; 2, presence of 50 mM-xylitol + 10 mM-Mn²⁺ (cf. Table 2); 3, unmodified D-xylose isomerase. (e) D-xylose isomerase from *B. coagulans*: 1, presence of 0.5 M-D-glucose + 10 mM-Mg²⁺ (cf. Table 2); 2, presence of 0.5 M-D-glucose + 10 mM-Co²⁺ (cf. Table 2); 3, unmodified D-xylose isomerase.

per active site abolishes the catalytic activity of D-xylose isomerases from Streptomycetaceae, Lactobacillaceae and *B. coagulans* offers support for our proposal that, besides the previously reported essential histidine residue (Vangrysperre *et al.*, 1988; Gaikwad *et al.*, 1988; Pawar *et al.*, 1988), one carboxylate group may contribute to the catalytic activity.

The distinct roles of the active site histidine and carboxylate group have not yet been elucidated. To

evaluate their specific roles, the use of site-specific mutants and extensive structural information will be required.

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