Wild-type and mutant D-xylose isomerase from *Actinoplanes missouriensis*: metal-ion dissociation constants, kinetic parameters of deuterated and non-deuterated substrates and solvent-isotope effects

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The metal-ion dissociation constants (Mg^{2+} , Mn^{2+}) of wild-type and mutant D-xylose isomerases from *Actinoplanes missouriensis* have been determined by titrating the metal-ion-free enzymes with Mg^{2+} and Mn^{2+} respectively. Substitution of amino acids co-ordinated to metal-ion 1 (E181D, D245N) dramatically affects the dissociation constants, pH-activity profiles and apparent substrate binding. Mutagenesis of groups ligated to metal-ion 2 is less drastic except for that of Asp-255: a decrease in metal-ion affinity, a change in metal-ion preference and an improved apparent substrate binding (at pH values above the optimum), especially in the presence of Mn^{2+} , are observed for the D255N

enzyme. Similar effects, except for a slightly increased metal-ion affinity, are obtained by mutagenesis of the adjacent Glu-186 to Gln and the unconserved Ala-25 to Lys. Moreover, the striking acidic-pH shifts observed for the D255N and E186Q enzymes support the crucial role of the water molecule, Wa-690, Asp-255 and the adjacent Glu-186 in proton transfer from 2-OH to O-1 of the open and extended aldose substrate. Mutations of other important groups scarcely affect the metal-ion dissociation constants and pH-activity profiles, although pronounced effects on the kinetic parameters may be observed.

INTRODUCTION

D-Xylose isomerase (XylI) (EC 5.3.1.5), an intracellular bacterial enzyme, catalyses the conversion of D-xylose into D-xylulose and D-glucose into D-fructose. The latter reaction is of considerable importance for the production of sweeteners (high-fructose corn syrup). The enzyme is tetrameric and requires Mg^{2+} , Co^{2+} or Mn^{2+} for catalytic activity. Crystal structures of a number of XylIs from Actinomycetaceae have been determined [*Actinoplanes missouriensis* (Rey et al., 1988; Jenkins et al., 1992); *Streptomyces rubiginosus* (Carrell et al., 1989; Whitlow et al., 1991); *Arthrobacter* strain B3728 (Henrick et al., 1989; Collyer et al., 1990); *Streptomyces olivochromogenes* (Farber et al., 1989; Allen et al., 1994b)]. The existence of two metal-ion sites per active site have been reported from these studies, in accordance with binding studies by Callens et al. (1988) and Van Bastelaere et al. (1992). On the basis of the structural data (Farber et al., 1989; Collyer et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992), site-directed mutagenesis (Lee et al., 1990; Jenkins et al., 1992; Lambeir et al., 1992; van Tilbeurgh et al., 1992), isotopic-exchange and kinetic experiments (Allen et al., 1994a), a reaction mechanism in which both metal ions play a crucial role has been proposed. It involves the binding of the cyclic sugar via co-ordination of O-3 and O-4 to the structural metal ion (M_1) and, after ring-opening, of the open-chain substrate via co-ordination of O-2 and O-4 to M_1 . The isomerization proceeds with proton transfer from 2-OH to O-1 of the extended aldose substrate and a rate-limiting hydride shift from C-2 to C-1 (Lee et al., 1990; Lambeir et al., 1992; Smart et al., 1992). The metal ion, M_2 , and a water molecule ligated to it, would play a direct role in the catalytic steps.

On the basis of the kinetic parameters for α - and β -D-glucose of the wild-type, the H101N and the D104N enzymes from *Thermoanaerobacterium thermosulfurigenes* (in the enzymes from Actinomycetaceae these residues correspond to His-53 or -54 and Asp-56 or -57 respectively), Meng et al. (1993) proposed another reaction model in which the hydride shift would occur on the cyclic form of the substrate rather than on the extended form. In this mechanism only one metal ion (M_1) would play an important role, in contrast with the conclusions from the crystallographic and metal-ion binding studies.

In order to obtain more information on the mechanism involved, we determined the metal-ion dissociation constants for the structural and catalytic M_1 and M_2 sites of wild-type and mutant XylIs from *A. missouriensis* and studied how the metal-ion affinities affect the catalytic properties of wild-type and mutant enzymes.

EXPERIMENTAL

Materials

D-Xylose, D-glucose, $MgCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$ and $MnCl_2 \cdot 4H_2O$ were from Merck, Darmstadt, Germany. EGTA and D-glucose deuterated (97% 2H) at the C-2 position were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Deuterium oxide (99.8% 2H) was purchased from Acros Chimica, Beerse, Belgium. Sorbitol dehydrogenase and NADH grade I were from Boehringer, Mannheim, Germany, and Bio-Rex ion-exchange membranes from Bio-Rad Laboratories, Richmond, CA, U.S.A. Buffers were prepared in Millipore Milli-Q water and stored in acid-washed plastic containers.

Wild-type and mutant enzymes

Wild-type and mutant enzymes from *A. missouriensis* were generously given by Gist-Brocades (Delft, Nederland) and Plant Genetic Systems (Gent, Belgium).

Metal-ion-free enzymes were prepared by dialysis for more than 24 h of 1–3 ml samples against 1 litre of 0.01 M triethanolamine/HCl buffer, containing 10 mM EGTA, pH 7, followed by dialysis (again for more than 24 h) against two 1-litre batches of metal-ion-free 0.01 M triethanolamine/HCl buffer, pH 7, to remove excess EGTA.

Metal-ion-free buffer was prepared by filtration of the 1 M stock buffer through a Bio-Rex ion-exchange membrane, followed by dilution to the required buffer molarity with Millipore Milli-Q water. The enzyme preparations were analysed for residual metal contamination by atomic-absorption spectrometry (equiv./monomer ≤ 0.005 for Mn^{2+} , Co^{2+} , Ca^{2+} and ≤ 0.03 for Mg^{2+}). Residual activities were about 0.1% or less of those observed at optimal concentration of the most effective metal ion.

The enzyme concentrations were routinely estimated at 280 nm with a Uvikon absorption 810 spectrophotometer (Kontron) by using the specific absorption coefficient ($A_{1\text{cm}}^{0.1\%} = 1.0$; M_r of monomer = 43300). The concentrations are expressed as μM active sites.

Enzyme assays

Isomerization of D-xylose or D-glucose was monitored by the two-step Xyl/sorbitol dehydrogenase method (100 μl assays; reaction times 15 min) at 60 °C (Callens et al., 1986; Kersters-Hilderson et al., 1987).

To avoid chemical isomerization of the substrate solutions, concentrated stock solutions (2.5 M) were made up in Millipore Milli-Q water and stored at -18°C until used. Appropriate dilutions were made up in a Hamilton Microlab 1000 apparatus.

Determination of the metal-ion dissociation constants

The metal-ion dissociation constants were determined at 60 °C by typical titrations (Van Bastelaere et al., 1992) carried out at fixed enzyme concentrations [at optimum pH 0.04 μM (Mg^{2+}) or 0.1 μM (Mn^{2+}) for the wild-type enzyme and 0.06–1.0 μM (Mg^{2+}) or 0.1–5.0 μM (Mn^{2+}) for the mutant enzymes] and various metal-ion equivalents [at optimum pH 50–1000 equiv. of Mg^{2+} or 1–50 equiv. of Mn^{2+} for the wild-type enzyme and 100–1000 (or 1000–10000) equiv. of Mg^{2+} or 1–10 (or 50–500) equiv. of Mn^{2+} for the mutant enzymes with considerable (respectively low) remaining activity]. The protein–metal-ion solutions (90 μl) were allowed to equilibrate (60 min, 60 °C) in 0.028 M Veronal/acetate buffers (containing 0.12 M NaCl for constant ionic strength) before the enzyme reaction was started by addition of 10 μl of 4 M D-xylose, and the enzyme activity (indicator of active complex or $[\text{M}_1\text{EM}_2]$) was measured (15 min at 60 °C). This minor dilution of the preincubated enzyme–metal-ion mixture did not affect the established equilibrium (Van Bastelaere et al., 1992). The dissociation constants were evaluated by an approximation procedure using an NCR Tower 32/800 computer (Dayton, OH, U.S.A.) (Van Bastelaere et al., 1992).

Concentrated stock solutions of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (100 mM), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (50 mM) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (500 mM) were made up in Millipore Milli-Q water and stored at -18°C until used. Appropriate dilutions in Millipore Milli-Q water were made up in a Hamilton Microlab 1000 apparatus. Metal-ion-free buffers were prepared in Millipore Milli-Q water, filtered through Bio-

Rex exchange membranes and stored in acid-washed plastic containers.

pH-activity profiles

pH-activity profiles were determined at 60 °C. Michaelis's sodium diethylbarbiturate (0.028 M)/sodium acetate (0.028 M)/HCl buffers of constant ionic strength (0.12 M NaCl) (Michaelis 1931) were used (except for solvent-isotope studies). Taking into account the rather high temperature coefficient (Dawson et al., 1969), the pH values were always carefully measured at 60 °C.

Activities were monitored in the presence of 1 M D-xylose, 10 mM Mg^{2+} or 1 mM Mn^{2+} , the required buffer of specific pH (at 60 °C) and the following Xyl concentrations: 0.04 μM (Mg^{2+}) or 0.1 μM (Mn^{2+}) for the wild-type enzyme and 0.05–1.0 (13 for D255A) μM (Mg^{2+}) or (0.05–4.7) μM (Mn^{2+}) for the mutant enzymes. Standards without Xyl were included at pH values of 7 or higher since chemical isomerization at these pH values is considerable. At the concentration of D-xylose used (1 M or greater than substrate saturation), the curves mainly reflect pH effects on k_{cat} , except for the E181D and D245N mutant enzymes where effects on the K_m are not excluded.

Kinetic parameters

The kinetic parameters K_m (mM) and k_{cat} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) were derived from plots of $[S]/v$ versus $[S]$. The plots were fitted by least-squares analysis. The parameters were determined at different pH values at 60 °C in the presence of 10 mM Mg^{2+} or 1 mM Mn^{2+} (Co^{2+}) (metal-ion saturation). Preincubation of metal ions and enzyme in Veronal/acetate buffer was not performed as no incubation effects occur at saturated metal-ion concentrations (Van Bastelaere et al., 1992).

In the presence of D-xylose which varied from 6 to 250 mM, wild-type enzyme concentrations were 0.04 μM . With D-glucose which varied from 100 to 1000 mM in the presence of Mg^{2+} , and from 400 to 2000 mM in the presence of Co^{2+} or Mn^{2+} , wild-type concentrations were 0.76 μM (Mg^{2+}), 7.6 μM (Co^{2+}) or 15 μM (Mn^{2+}). As compared with the wild-type enzyme, mutant concentrations were mostly higher in the presence of D-xylose (Mg^{2+} , Mn^{2+}) but often lower in the presence of D-glucose (Mn^{2+}). The Xyl and substrate concentrations were adjusted to obtain measurable initial velocities and valid $[S]/v$ versus $[S]$ plots.

Deuterium isotope effects

Deuterium isotope effects on k_{cat} and K_m of wild-type and mutant enzymes were studied with D-glucose deuterated (97% ^2H) at the C-2 position at different pH values. The Xyl and substrate concentrations were adjusted to obtain measurable initial rates and valid $[S]/v$ versus $[S]$ plots. Parallel experiments using non-deuterated D-glucose were included as standards.

Solvent-isotope effects

Solvent-isotope effects on the catalytic rate constants were determined using $^2\text{H}_2\text{O}$ as solvent in conditions in which the enzyme was saturated with metal ions (1 mM Mn^{2+} or 10 mM Mg^{2+}) and substrate (250 mM D-xylose). The reaction medium contained 0.15 M NaCl and 10 mM triethanolamine (free base) adjusted to the desired pH with HCl in presence of water and

with ^2HCl when the reaction was carried out in $^2\text{H}_2\text{O}$. The pH was adjusted at room temperature and the pH at 60°C was calculated from the temperature coefficient of triethanolamine (Dawson et al., 1969). The pD was calculated as follows: $\text{pD} = \text{pH} + 0.4$, where pH is the value measured with a pH-meter calibrated with standard buffers in water.

Two series of buffer solutions were made spanning the useful range for triethanolamine to obtain the following pH or pD values at 60°C : 8.1, 7.7, 7.3, 6.9, 6.5 (H_2O) or 6.4 ($^2\text{H}_2\text{O}$), and 6.1. Ten times concentrated enzyme solutions (typically around $1\ \mu\text{M}$) were made up by diluting a concentrated stock solution in either water or deuterium oxide immediately before the start of the experiment. The amount of water originating from the stock solution never exceeded 0.15% of the total volume in the reaction mixture. The enzyme was incubated with the metal ions for at least 30 min before the reaction (60°C) was started by the addition of D-xylose. After 15 min the reaction was stopped by a fivefold dilution in 50 mM triethanolamine buffer, pH 7, containing 0.25 mM EDTA (Mn^{2+}) or 2.5 mM EDTA (Mg^{2+}). The samples were kept on ice until analysed. The amount of D-xylulose was determined (30°C) from the total absorbance change (after 15 min) at 340 nm after a 20-fold dilution in triethanolamine buffer, pH 7, containing 0.2 mM NADH and $10\ \mu\text{g}/\text{ml}$ sorbitol dehydrogenase. The amount of NADH consumed never exceeded 40%. The amount of D-xylose converted into D-xylulose during the isomerase reaction was around 1%.

RESULTS AND DISCUSSION

Determination of the dissociation constants for Mg^{2+} and Mn^{2+}

From the reported crystallographic data and binding studies, two metal-ion-binding sites (1 and 2)/monomer should be considered. The first metal-ion site, M_1 , is co-ordinated to four carboxylates (Glu-181, Glu-217, Asp-245, Asp-292). The second, M_2 , is liganded by three carboxylates (Glu-217, Asp-255, Asp-257) and further by His-220 and a water molecule (Wa-690).

According to Kasumi et al. (1982), Lambeir et al. (1992) and the present paper (Figure 1), a random equilibrium system, in which binding of the metal ions increases the affinity for the substrate, is proposed. In contrast, Yamanaka (1968) and Rangarajan and Hartley (1992) suggest a compulsory order of binding. Yamanaka (1968), Danno (1971), Sanchez and Smiley (1975), Kasumi et al. (1982), Lambeir et al. (1992) and

Figure 1 $[S]/v$ versus $[S]$ plots for D-xylose (wild-type enzyme) in the presence of 0.02 and 1 mM Mg^{2+}

The solutions containing Xyl ($0.04\ \mu\text{M}$) and Mg^{2+} [0.02 and 1 mM] were allowed to equilibrate (1 h, 60°C) in $90\ \mu\text{l}$ of 0.028 M Veronal/acetate buffer, pH 7.4, containing 0.12 M NaCl, before the D-xylose isomerization was started by the addition of various D-xylose concentrations ($10\ \mu\text{l}$ aliquots). Activities were monitored as described in the Experimental section.

Rangarajan and Hartley (1992) estimated the metal-ion dissociation constants from the substrate- and metal-ion-dependence of steady-state kinetic measurements. The interpretation of their data, however, may be incorrect since they [except for Rangarajan and Hartley (1992)] reckoned with only one metal-ion site. Moreover, steady-state kinetic analysis is not adequate in the case of tight metal-ion binding (Mn^{2+} , Co^{2+} , K_1 and $K_2 < 0.5\ \mu\text{M}$).

In the present paper, a simple and accurate titration method is used as described by Van Bastelaere et al. (1992). The method is based on the titration of metal-ion-free enzymes with the respective metal ions, using the enzyme activity as indicator of the active-complex concentration or $[\text{M}_1\text{EM}_2]$, and taking into account the required preincubation of the enzyme and metal ions to attain equilibrium (60°C , 1 h). The dissociation constants were determined at different pH values for the wild-type enzyme and near the pH optimum for the mutant enzymes (Table 1).

From the results one may make several conclusions. (i) The data fit the equations of the random and independent-sites model according to the results obtained by Van Bastelaere et al. (1992). (ii) The binding constants K_1 and K_2 for Mn^{2+} are nearly identical, in contrast with the binding of Mg^{2+} where K_1 and K_2 differ by at least a factor of 50. Calculation of K_1 for Mg^{2+} was not possible except at higher pH where both dissociation constants attain nearly the same value. (iii) The dissociation constants decrease with increasing pH. (iv) Substitution of M_1 ligands leads to a dramatic decrease in metal-ion binding on both sites (D245N, E181D): a conformational change occurring at the M_1 site thus seems to disturb the conformation and metal-ion binding at the M_2 site. As both sites are located less than 0.5 nm (5 Å) from each other and share a common glutamate group (Glu-217), this is not surprising. Furthermore, as the enzyme activity is used as an indicator of active-complex-formation $[\text{M}_1\text{EM}_2]$, less productive substrate binding, caused by substitution of the M_1 ligands (see Table 2), may also lead to a low estimate for metal-ion binding at the M_2 site. (v) Substitution of the M_2 ligands is less dramatic except for the D255N mutant enzyme. Asp-255, however, has been reported to be a bidentate ligand in the absence of substrate (Collyer et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992). (vi) Mutagenesis of other groups such as Glu-186 into Gln (van Tilbeurgh et al., 1992) and Ala-25 into Lys (A.-M. Lambeir, unpublished work), creating a local positive charge in the vicinity of metal-ion 2, slightly improves metal-ion affinity. (vii) Finally, mutations not directed toward the metal-ion ligands but to other important groups such as His-54, Lys-183 or Trp-137 (Lambeir et al., 1992) scarcely affect the metal-ion dissociation constants.

Kinetic parameters of deuterated and non-deuterated substrates, pH-activity profiles and solvent-isotope effects

The kinetic parameters for D-xylose and deuterated (D-[2- ^2H]glucose) and non-deuterated D-glucose were determined at metal-ion saturation (Mg^{2+} , Mn^{2+}) at different pH values. They are summarized in Tables 2 and 3. The deuterium isotope effects on the kinetic parameters of D-glucose are expressed as $k_{\text{cat}}(^1\text{H})/k_{\text{cat}}(^2\text{H})$ and $K_{\text{m}}(^1\text{H})/K_{\text{m}}(^2\text{H})$. As deuterium effects around 3 or 4 were reported for the wild-type and H54N(H101N) enzymes by Lee et al. (1990), Lambeir et al. (1992) and Smart et al. (1992), the hydride shift (from C-2 to C-1 of the extended aldose substrate) was assumed to be rate-determining. However, in the presence of Mg^{2+} and pH 7.5 (above the pH optimum), the E186Q enzyme has an isotope effect near unity, implying another rate-limiting step (van Tilbeurgh et al., 1992). In order to obtain

Table 1 Numerical values of the metal-ion dissociation constants for Xyl from *A. missouriensis* (wild-type and mutant enzymes)

The experiments were carried out as described in the Experimental section. Maximum standard error was 2% except at the start of the titration curves (10%).

Xyl	Mg ²⁺			Mn ²⁺		
	pH	K ₁ (μM)	K ₂ (μM)	pH	K ₁ (μM)	K ₂ (μM)
Wild-type	6.3	K ₁ ≪ K ₂ *	300	6.0	0.44	0.50
	7.1	K ₁ ≪ K ₂	35	6.8	0.13	0.13
	7.5	K ₁ ≪ K ₂	18	7.5	0.040	0.040
	8.0	2.0	5.0			
	8.4	2.0	2.0			
A25K	7.0	K ₁ ≪ K ₂	22	6.8	0.060	0.080
	8.0	2.0	2.5			
D245N	8.4	900	900	6.8	75	75
D255N	7.1	K ₁ ≪ K ₂	650	6.0	5.0	6.0
D257N	7.5	K ₁ ≪ K ₂	250	6.8	0.50	0.50
E181D	8.0	200	400	6.8	6.0	6.0
E186Q	6.2	K ₁ ≪ K ₂	250	6.8	0.060	0.080
H54N	7.1	K ₁ ≪ K ₂	50	6.8	0.10	0.10
H220Q	7.5	K ₁ ≪ K ₂	300	6.8	0.20	0.20
K183Q	7.0	K ₁ ≪ K ₂	40	6.8	0.060	0.10
W137F	7.0	46	46	6.8	0.10	0.10

* At least a factor 50.

more evidence on a possible change in rate-determining step, substrate-isotope effects were studied at different pH values.

pH-activity profiles of wild-type and mutant enzymes with D-xylose as substrate, in the presence of either Mg²⁺ or Mn²⁺, are shown in Figures 2–4. On the acidic side they are supposed to be governed by the protonation of the metal-ion ligands and (or) of His-54 (fixed in position via Asp-57) which, according to the crystallographic studies, stabilizes the transition state and thus may affect k_{cat} . A water molecule (Wa-690), tightly bound to the cation at site 2 [thus having a lower pK_a (near 8–9) than free water], is proposed to play a crucial role in the proton transfer from 2-OH to O-1 of the aldose substrate (Collyer et al., 1990; Whitlow et al., 1991; van Tilbeurgh et al., 1992; Allen et al., 1994b). If this hypothesis is correct, the decrease in enzyme activity at the alkaline flank of the pH profile may be due to a less efficient proton shuttle which may become rate-limiting at pH values above the pK_a of the metal-ion-bound water molecule. This would also explain the disappearance of the substrate-isotope effects observed with the E186Q enzyme (van Tilbeurgh et al., 1992). As the proton shuttle involves water molecules and carboxylate groups which readily exchange protons with the solvent, more information about the proton transfer as rate-limiting step at pH values above the pK_a of Wa-690 may be gained by a study of kinetic solvent-isotope effects at different pH values. The substrate and solvent-isotope effects are summarized in Tables 3 respectively.

Wild-type enzyme

The pK_a of Wa-690, which is decreased as a result of the co-ordination with M₂, is lower in the presence of Mn²⁺ (stronger co-ordination) than in the presence of Mg²⁺, as exemplified by the difference in pH optima (7.2–7.8 and 6.7–7.4 respectively in Figures 2–4). In order to avoid precipitation of Mn(OH)₂ or Mg(OH)₂ above pH 8 and pH 8.5 respectively, the alkaline flank of the pH-activity curves could not be studied at higher pH values.

The substrate-isotope effects are larger in the presence of Mn²⁺

(3.4) than in the presence of Mg²⁺ (2.7) and suggest that the hydride shift is more rate-determining with Mn²⁺ than with Mg²⁺ (Table 3). This correlates well with the solvent-isotope effects which are larger in the presence of Mg²⁺ (Table 4). The magnitude of the kinetic solvent deuterium effect, however, is rather small. It suggests an essential step in the mechanism, which is not rate-limiting at optimal pH values, involving proton transfer at exchangeable sites, e.g. the substrate's hydroxy groups, the co-ordinated water molecule or the carboxylates of the proton shuttle.

A considerable decrease in K_m for D-glucose, which has an extra -CH₂OH group compared with D-xylose, is further observed with decreasing pH in the presence of Mn²⁺ (Table 2). This may be ascribed to the protonation and thus changed position of the interacting His-54–Asp-57 residues resulting in better accommodation of the open and extended D-glucose (see below).

H54N, D57N (His-54 fixed in its position by interaction with Asp-57)

Both mutant enzymes exhibit normal wild-type pH-activity profiles (not shown). According to Collyer et al. (1990) and Whitlow et al. (1991) but not to other authors (Lee et al., 1990; Lambeir et al., 1992), His-54 catalyses the ring opening. If correct, this step cannot be rate-limiting because the substrate-isotope effects are identical with those obtained for the wild-type enzyme (Table 3). The same conclusion has been reported previously by Lee et al. (1990), Lambeir et al. (1992) and Smart et al. (1992). However, as mutagenesis of His-54 or Asp-57 affects k_{cat} in particular (Table 2), a role in the isomerization process (i.e. the stabilization of the extended enzyme–substrate intermediate) is suggested rather than in the process of ring opening (Lee et al., 1990; Lambeir et al., 1992).

In addition, further evidence is provided that His-54 sterically hinders the binding of D-glucose in the presence of Mn²⁺ (see the section on the wild-type enzyme above): the K_m of the H54N mutant enzyme for D-glucose is lower (by a factor 4) than the K_m of the wild-type enzyme at the pH optimum and approaches the wild-type value obtained at low pH (Table 2).

Table 2 Kinetic parameters for D-xylose and D-glucose

The experiments were carried out as described in the Experimental section. The average standard error on k_{cat} was 2% and that on K_m was 4% except where indicated (*). The values for k_{cat} are expressed as $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and those for K_m as mM. The pH values underlined are optima.

Xyl	D-Xylose						D-Glucose					
	Mg ²⁺			Mn ²⁺			Mg ²⁺			Mn ²⁺		
	pH	k_{cat}	K_m	pH	k_{cat}	K_m	pH	k_{cat}	K_m	pH	k_{cat}	K_m
Wild-type	5.3	34	73	5.0	24	69	5.3	7.4	300	5.0	1.1	300
	6.0	62	24	5.8	48	69	6.0	15	250	6.0	3.7	740
	<u>7.4</u>	120	23	<u>6.9</u>	65	69	<u>7.4</u>	42	230	<u>6.9</u>	6.6*	1300*
	8.0	110	21	8.0	53	38	8.0	45	250	8.0	12*	2300*
A25K	5.3	38	46	5.0	37	93	5.3	6.4	490	6.0	11*	1500*
	6.0	71	41	6.0	54	52	<u>6.0</u>	27	340	<u>6.9</u>	22*	1200*
	6.6	71	26	<u>6.9</u>	54	36	6.4	34	260	8.0	23	300
	8.0	31	18	8.0	49	18	6.9	33	180			
							8.0	28	130			
D57N	<u>7.2</u>	70	36	<u>6.9</u>	29	64	<u>7.3</u>	12	250	5.0	1.4	660
							7.6	12	210	<u>6.9</u>	1.6	480
										7.1	1.7	480
D245N	<u>8.4</u>	2.4	170	<u>6.7</u>	1.4	670						
D255A	<u>6.4</u>	0.30	35	<u>5.5</u>	4.8	58						
				6.1	3.8	38						
D255N	5.8	2.7	56	5.1	14	80	5.8	0.36	340	5.0	5.7	350
	<u>7.0</u>	7.7	56	<u>5.7</u>	16	35	<u>7.0</u>	2.7	290	<u>5.6</u>	12	400
	7.7	4.1	26	6.0	15	35	7.6	2.2	250	6.4	11	220
				7.2	6.1	15	8.0	1.9	240	7.2	11	200
				8.0	2.1	13				8.0	5.0	120
D257N	<u>7.2</u>	22	42	<u>6.7</u>	25	51	<u>7.2</u>	11	260			
E181D	6.1	5.4	270	6.0	3.7	490						
	7.1	15	200	<u>6.9</u>	3.9	450						
	<u>7.8</u>	16	180	8.0	3.6	440						
E186Q	5.3	18	90	5.0	19	100	5.3	8.2	310	<u>6.3</u>	40*	3100*
	5.7	27	22	5.7	37	84	5.7	26	320	6.9	51	1000
	<u>6.1</u>	35	15	<u>6.3</u>	50	32	<u>6.3</u>	32	330	7.5	40	430
	6.3	28	18	7.4	43	12	7.4	8.0	110	8.0	39	290
	7.2	5.9	10				8.0	2.8	110			
H54N	7.4	18	26	5.0	3.7	120	5.3	0.65	300	5.0	0.16	480
	<u>7.6</u>	24	30	5.2	4.6	80	<u>7.2</u>	3.0	290	<u>6.9</u>	0.18	430
				6.0	5.4	77						
				<u>6.9</u>	6.2	81						
				8.0	4.5	61						
H54Q	<u>7.4</u>	12	47	<u>6.9</u>	2.6	100						
H220Q	<u>7.4</u>	7.8	53	<u>6.8</u>	0.74	88						
K183Q	6.1	35	37	6.1	23	73	<u>7.4</u>	22	240	<u>6.9</u>	4.5*	1300*
	7.2	51	26	<u>7.3</u>	29	71						
	<u>7.4</u>	53	27	8.0	25	49						
	8.0	52	17									
W137F	<u>7.3</u>	12	64	<u>6.7</u>	1.8	160						

* Limited accuracy because K_m is too high and solubility of the substrate is limited (4 M).

E181D, D245N (site 1)

The pronounced decrease in metal-ion binding especially for Mg²⁺ (Table 1) is compensated for, at least partially, at higher pH. This leads to an alkaline shift of the acidic side and of the pH optimum (pH–activity curve not shown). Besides the pronounced decrease in metal-ion-binding properties, the unfavourable k_{cat} and K_m values (Table 2) reflect a distorted positioning of the substrate.

D255N (site 2)

The pK_a of Asp-255 is influenced most strongly by the co-ordination with the second cation and also by the charge distribution in its immediate neighbourhood. Moreover, its

proton-accepting or -donating properties probably change during the catalytic cycle when the second cation moves from one site to another and loses Asp-255 as a ligand. Asp-255 has been reported to adopt different conformations in several crystal structures (Collyer et al., 1990; Whitlow et al., 1991; Jenkins et al., 1992; van Tilbeurgh et al., 1992). When a mutation stabilizes the 'wrong' conformation, the proton shuttle will be impaired. Van Tilbeurgh et al. (1992) describe how Mn²⁺ is able to correct the effect of the E186Q mutation by co-ordination of the Asp-255 side chain which is turned away from the cation in the presence of Mg²⁺.

Removal of the negative charge in D255N (thus introducing an extra positive charge in the vicinity of Wa-690) causes an extra decrease in the pK_a of the co-ordinated water which results in a dramatic acidic shift of the pH optimum in the presence of Mn²⁺

Table 3 Deuterium isotope effects

The experiments were carried out as described in the Experimental section. The average standard error on k_{cat} was 2% and that on K_m was 4% except where indicated (*). The pH values underlined are optima.

Xyl	[2- ² H]-D-glucose					
	Mg ²⁺			Mn ²⁺		
	pH	k_{cat} (¹ H)/ k_{cat} (² H)	K_m (¹ H)/ K_m (² H)	pH	k_{cat} (¹ H)/ k_{cat} (² H)	K_m (¹ H)/ K_m (² H)
Wild-type	6.1	2.7	1.0	5.1	3.4	1.2
	7.4	2.6	1.1	6.9	3.4*	0.8*
	8.3	2.7	1.0	8.0	3.9*	1.0*
A25K	5.3	2.7	1.0	7.0	3.4*	1.0*
	6.9	2.2	0.9	8.0	3.4	1.0
	8.0	1.9	1.0			
D255N	5.4	1.7	1.0	5.6	3.0	1.0
	5.7	1.5	1.0	8.0	1.5	1.0
	7.0	1.2	1.0			
	8.0	1.1	1.0			
D257N	5.3	2.4	1.0			
	7.2	3.4	1.2			
	8.0	3.0	1.0			
E186Q	5.3	1.8	1.0	5.6	3.4*	1.0*
	6.2	1.8	0.9	7.0	2.6	1.0
	7.4	1.3	1.0	8.0	2.1	1.0
H54N	6.1	2.4	1.0			
	7.2	2.2	0.7			
	8.4	2.7	1.0			
K183Q	7.4	2.6	1.0			

* Limited accuracy because K_m is too high and solubility of the substrate is limited (4 M).

Figure 2 pH-activity profiles of the wild-type (●) and D255N mutant (○) enzymes in the presence of (a) Mg²⁺ or (b) Mn²⁺

The experiments were carried out as described in the Experimental section.

(Figure 2). The neutral pH optimum in the presence of Mg²⁺ may be explained by the reduced affinity for Mg²⁺, becoming less unfavourable at higher pH.

The fact that the deuterium substrate-isotope effect decreases with D255N (Table 3) suggests that the hydride transfer is not rate-determining. At the same time, the low specific activity and pH-activity shifts point to a less-efficient proton shuttle. Average kinetic solvent-effects (pH range 6.4–7.7) of 1.9 were obtained in the presence of Mg²⁺ (Table 4). The size of the solvent-isotope effect does not support the suggestion that the proton transfer is

rate-limiting in this mutant enzyme any more than in the wild-type enzyme. 'Inverse' solvent-isotope effects are shown in the presence of Mn²⁺ (1.0 → 0.68 in the pH range 6.5–8.1) (Table 4). The inverse effect is not easily explained. Schowen and Schowen (1982) argue that it may signify a tightening at contributing hydrogenic sites on formation of the transition state. However, the same authors warn that, for correct interpretation of the solvent-isotope effects, the rate constants need to be compared at the pH optimum or at equivalent positions in the titration curve. This is necessary because pK_a values of ionizable groups often

Figure 3 pH-activity profiles of the wild-type (●) and E186Q mutant (○) enzymes in the presence of (a) Mg^{2+} or (b) Mn^{2+}

The experiments were carried out as described in the Experimental section.

Figure 4 pH-activity profiles of the wild-type (●) and A25K mutant (○) enzymes in the presence of (a) Mg^{2+} or (b) Mn^{2+}

The experiments were carried out as described in the Experimental section.

Table 4 Solvent-isotope effects

The experiments were carried out as described in the Experimental section.

Xyl	Mg^{2+}		Mn^{2+}	
	pH(D) range	$k(^1H)/k(^2H)$	pH(D) range	$k(^1H)/k(^2H)$
Wild-type	6.1–8.1	2.1	6.1–8.1	1.5
A25K	6.1–7.3	1.8	6.1–8.1	1.5
D255N	6.5 (4)–7.7	1.9	6.5 (4)–8.1	1.0 → 0.68
E186Q	6.1–6.5 (4)	1.0	6.1–8.1	2.0 → 1.3
	6.9–8.1	0.68		

shift (typically an increase of 0.4–0.5 unit) in deuterium oxide. This may well be the case with D255N in the presence of Mn^{2+} as indicated by the decreasing trend in solvent-isotope effect with increasing pH(D). Even so, the isotope effect would not be much larger than unity.

Furthermore, Table 2 shows that, at the alkaline flanks of the pH-activity profiles, the apparent substrate binding increases, especially in the presence of Mn^{2+} , the decrease in K_m being more pronounced for D-glucose than for D-xylose. This suggests that the pK_a of the substrate 2-OH group is lowered (resulting in

improved or lower K_m values) by replacement of the negative carboxyl group with a neutral amide group (positive charge effect in the vicinity of M_2). In addition, from the comparable K_m values of the least-active mutant enzyme, D255A, and the wild-type enzyme (Table 2), evidence exists that the M_2 site is not directly involved in the process of substrate binding.

D257N (site 2)

Similar pH shifts to those obtained for the D255N enzyme were expected for this mutant enzyme, as Asp-257 is proposed by Whitlow et al. (1991) and van Tilbeurgh et al. (1992) to be the point of initiation of the proton transfer from 2-OH to O-1. However, from the rather small pH shifts (not shown) and from the analogue deuterium effects (Table 3) of the wild-type and the D257N enzymes, Asp-257 is probably not the compulsory initiation point of the proton shuttle. Nevertheless, the same conclusions as for the D255N enzyme may be drawn about the low specific activity, especially for Mg^{2+} (Table 2).

E186Q (positive charge in the vicinity of site 2)

In contrast with Asp-255, Glu-186 is not a metal-ion ligand and the positive charge effect even slightly improves the metal-ion affinity (Table 1), as suggested for the weakly bound Mg^{2+} from the acidic shift on the acidic side of the pH-activity curve (Figure 3). The striking acidic shift on the alkaline side in the presence of Mg^{2+} can be explained by movements of Asp-255 turning toward Gln-186 which competes with the cation for the negative Asp-255 (van Tilbeurgh et al., 1992). This leads to an extra positive charge effect in the vicinity of M_2 , to a lowering of the pK_a of Wa-690 and to a less favourable proton transfer. Once more, the dramatic shift in the pH-activity profile seems to be correlated with 'inverse' isotope effects from 1.0 to 0.68 in the pH range 6.1 to 8.1 (see also D255N in the presence of Mn^{2+}) and decreased substrate-isotope effects (Tables 3 and 4). As with D255N, even allowing for a pK_a shift of 0.4 unit, the solvent kinetic deuterium effect does not rise above one and certainly does not increase at alkaline pH(D) where the substrate deuterium effect disappears and the hydride shift is no longer rate-determining. Therefore the rate of the step that governs the catalytic rate constant at alkaline pH is not crucially dependent on exchangeable proton sites. It could be, for example, the conformational change leading to repositioning of the Asp-255 side chain in order to exchange a proton with Wa-690. In the presence of Mn^{2+} , pH-activity profiles, solvent- and substrate-isotope effects comparable with those for the wild-type enzyme were observed. Furthermore, a change in metal-ion preference is obvious and K_m values decrease for the same reason as suggested for the D255N enzyme, especially at higher pH (Table 2).

A25K (positive charge in the vicinity of site 2)

Ala-25 is neither a metal-ion ligand nor a conserved residue. The substitution by a positive group slightly improves the cation affinity (Table 1) as suggested for the weaker Mg^{2+} cation from the acidic shift of the acidic side of the pH curve (Figure 4). The positive charge also affects the pK_a of Wa-690, especially in the presence of Mg^{2+} , as shown by the acidic shift of the alkaline side of the curve (Figure 4). The deuterium substrate-isotope effects rather decrease (Table 3) whereas solvent-isotope effects [1.8 (Mg^{2+}) and 1.5 (Mn^{2+})] comparable with those for the wild-type enzyme (Table 4) are observed.

Conclusions

In summary, the pH-activity profiles of the wild-type and mutant enzymes emphasize the crucial role of Wa-690 (co-ordinated to M_2), Asp-255 and Glu-186 in proton transfer from 2-OH to O-1 of the aldose substrate.

The hydride shift (from C-2 to C-1) seems to be rate-limiting. Mutagenesis of Asp-255 or Glu-186 leads to a change in rate-determining step at pH values above the pK_a of Wa-690. As asparagine at position 255 is not able to accept or donate a proton, the change in rate-limiting step is due to impaired proton transfer. The absence of a sizeable kinetic solvent-isotope effect suggests that it is not the proton transfer *per se* that is rate-limiting. Perhaps it is due to a disturbance of the structural rearrangement that, according to crystallographic observations, may accompany the proton transfer.

As Wa-690, Asp-255 and Glu-186 are located in the vicinity of metal-ion 2, the proton shuttle and hydride shift obviously occur on the open substrate form and not on the cyclic form as suggested by Meng et al. (1993). In addition, the effects on the alkaline flank (Figures 2–4) cannot be attributed to local changes at position 292 (corresponding to Asp-339 in Xyl from *Thermoanaerobacterium thermosulfurigenes*).

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