Acid–base catalysis in *Leuconostoc mesenteroides* sucrose phosphorylase probed by site-directed mutagenesis and detailed kinetic comparison of wild-type and Glu²³⁷ → Gln mutant enzymes

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The role of acid–base catalysis in the two-step enzymatic mechanism of α -retaining glucosyl transfer by *Leuconostoc mesenteroides* sucrose phosphorylase has been examined through site-directed replacement of the putative catalytic Glu²³⁷ and detailed comparison of purified wild-type and Glu²³⁷ → Gln mutant enzymes using steady-state kinetics. Reactions with substrates requiring Brønsted catalytic assistance for glucosylation or deglucosylation were selectively slowed at the respective step, about 10⁵-fold, in E237Q. Azide, acetate and formate but not halides restored catalytic activity up to 300-fold in E237Q under conditions in which the deglucosylation step was rate-determining, and promoted production of the corresponding α -glucosides. *In situ* proton NMR studies of the chemical rescue of E237Q by acetate and formate revealed that enzymatically formed α -glucose 1-esters decomposed spontaneously via acyl group migration and hydrolysis. Using pH profiles of k_{cat}/K_m , the pH dependences of kinetically isolated glucosylation and deglucosylation steps were analysed for wild-type and E237Q. Glucosylation of the wild-type proceeded optimally above and below apparent pK_a

values of about 5.6 and 7.2 respectively whereas deglucosylation was dependent on the apparent single ionization of a group of $pK_a \approx 5.8$ that must be deprotonated for reaction. Glucosylation of E237Q was slowed below apparent $pK_a \approx 6.0$ but had lost the high pH dependence of the wild-type. Deglucosylation of E237Q was pH-independent. The results allow unequivocal assignment of Glu²³⁷ as the catalytic acid–base of sucrose phosphorylase. They support a mechanism in which the pK_a of Glu²³⁷ cycles between ≈ 7.2 in free enzyme and ≈ 5.8 in glucosyl enzyme intermediate, ensuring optimal participation of the glutamate residue side chain at each step in catalysis. Enzyme deglucosylation to an anionic nucleophile took place with Glu²³⁷ protonated or unprotonated. The results delineate how conserved active-site groups of retaining glycoside hydrolases can accommodate enzymatic function of a phosphorylase.

Key words: Brønsted catalysis, chemical rescue, family GH-13, phosphorylase, retaining mechanism, pK_a modulation.

INTRODUCTION

Most glycoside hydrolases employ Brønsted catalysis to facilitate their reactions. Cleavage of the glycosidic bond between two sugar residues and attack of a nucleophilic water molecule are known to receive substantial catalytic assistance in enzyme active sites from groups serving as general acid and base respectively [1–3]. In the canonical two-step mechanism of glycoside hydrolases that retain the anomeric configuration of their substrate in the hydrolysis product, a single amino acid, typically a glutamate or aspartate residue, cycles between catalytic functions of a general acid and base in glycosylation and deglycosylation half-reactions respectively (Figure 1A) [3].

SPase (sucrose phosphorylase) catalyses conversion of sucrose (α -D-glucopyranosyl-1,2- β -D-fructofuranoside) and phosphate into D-fructose and α G1P (α -D-glucose 1-phosphate) (Figure 1B) [4]. Configurational retention during glucosyl transfer to phosphate is dictated by the molecular mechanism of SPase, featuring two successive nucleophilic displacements at the anomeric centre and a covalent β -glucosyl-enzyme intermediate formed by an active site aspartate residue [5,6]. In the systematic sequence-based categorization of carbohydrate-active enzymes [7], SPase was classified as a member of family GH-13 of the glycoside hydrolase families. The X-ray crystal structure of SPase from *Bifidobacterium adolescentis* (termed BaSPase in the present study)

revealed a highly conserved architecture of the active centre, implicating Glu²³² as the catalytic acid–base [6]. It seems therefore that SPase achieves its special transferase activity (EC 2.4.1.7), which is unique among retaining glycoside hydrolases for its involvement of an anionic nucleophile, through non-conventional exploitation of the common catalytic machinery. In the present study, we asked the question of how the putative acid–base participates in each step of catalysis by SPase, considering that breaking the glycosidic bond in sucrose will depend strongly on help provided by a Brønsted acid whereas nucleophilic attack of phosphate should proceed well without assistance from a general base but requires that the catalytic centre accommodates a negatively charged substrate. An observation that is often made with retaining glycoside hydrolases is, however, that anions are prevented access to the active site, presumably because of charge screening by the base catalyst.

In the present study we report on a detailed mechanistic comparison of wild-type SPase from *Leuconostoc mesenteroides* (termed LmSPase in the present study) and a site-directed mutant thereof in which the candidate acid–base catalyst (Glu²³⁷) was replaced by a glutamine residue. Results from steady-state kinetic analysis of half-reactions leading to and from the covalent intermediate, pH-dependence studies, and chemical rescue experiments pinpoint the proposed catalytic acid–base function of Glu²³⁷. They also delineate an enzymatic reaction co-ordinate in

Abbreviations used: α G1N₃, α -D-glucose 1-azide; α G1P, α -D-glucose 1-phosphate; SPase, sucrose phosphorylase; BaSPase, SPase from *Bifidobacterium adolescentis*; LmSPase, SPase from *Leuconostoc mesenteroides*.

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Figure 1 Reaction mechanisms of retaining (trans)glycosidases and catalytic mutants thereof

(A) Double-displacement mechanism of an α -retaining enzyme, which interconverts glycosides with the leaving groups R₁-OH and R₂-OH. In the case of SPase R₁-OH is D-fructose and R₂-OH is phosphate. (B) Two-step (Ping Pong Bi Bi) kinetic mechanism of SPase. (C) Functional complementation of an otherwise inactive mutant in which the acid–base catalyst (an aspartate or glutamate residue) was replaced by a non-functional residue (in the present study, a glutamine residue). Nu is an external nucleophile such as azide, formate, acetate and halides.

which the apparent pK_a of Glu²³⁷ changes to suit a catalytic function of the glutamate residue during glucosylation and deglycosylation. Recognition of fructose or phosphate as a leaving group/nucleophile may require changes in the active-site environment of Glu²³⁷. Although glutamate residues homologous to Glu²³⁷ in LmSPase have been replaced before in other members of family GH-13 [8–10], a full kinetic analysis of a point mutant lacking the proposed catalytic acid–base has so far only been described for the human pancreatic α -amylase [11].

EXPERIMENTAL

Materials

Materials for site-directed mutagenesis, protein purification and enzymatic assays have been described previously [5]. Oligonucleotide synthesis and DNA sequencing were performed at VBC Biotech Services GmbH. α G1N₃ (α -D-glucose 1-azide) was synthesized from 2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl azide (CMS Chemicals) by de-*O*-acetylation with sodium methoxide in methanol. All other chemicals were of the highest available purity and were obtained from Sigma.

Site-directed mutagenesis, protein expression and purification

The point mutation Glu²³⁷ → Gln was introduced via a two-stage PCR strategy that has been reported previously [5]. The following oligonucleotide primers were used: 5'-CCATTAAAGGCTGAA-ATTTTACCACA**AA**ATTCATG-3' (forward primer) and 5'-CA-TGAATTTGTGGTAA**AA**ATTCAGCCTTTAATGG-3' (reverse primer). Mutated codons are indicated in bold. The plasmid

pQE30-LmSPase containing the gene for wild-type LmSPase was used as the template. After digestion of the parental template DNA by DpnI, the mutagenized plasmid was transformed into *Escherichia coli* JM109 cells. Wild-type LmSPase and the E237Q mutant were produced and purified to apparent electrophoretic homogeneity using previously reported procedures [5]. Stock solutions of purified enzymes were concentrated to 5–10 mg/ml in 20 mM Mes buffer (pH 7.0) and stored at 4 °C. Protein concentrations were determined using the Bio-Rad dye binding assay. CD spectroscopic measurements were carried out on a JASCO J-715 spectropolarimeter using enzyme solutions (0.85 mg/ml) in 20 mM Mes buffer (pH 7.0).

Kinetic studies

Enzyme activity was routinely measured at 30 °C in the direction of phosphorolysis of sucrose [250 mM substrate, 50 mM potassium phosphate and 20 mM Mes buffer (pH 7.0)] using a continuous coupled assay with phosphoglucumutase and glucose 6-phosphate dehydrogenase as described previously [5]. Initial rates of glucosyl transfer to and from phosphate and arsenolysis of α G1P were determined in discontinuous assays in which formation of α G1P, phosphate or glucose respectively, was measured and rates (v) were obtained from linear plots of released product concentration against reaction time [5, 12]. Typical protein concentrations of wild-type and E237Q used in the reactions were 0.7 μ g/ml and 20 μ g/ml respectively. Assay times varied between 20 min (wild-type) to about 5 h (E237Q). Control reactions lacking the enzyme or one of the substrates were performed in all cases, and the values reported are corrected for the blank readings.

Apparent kinetic parameters were obtained from non-linear fits of eqn (1) to the data, where k_{cat} is the catalytic-centre activity, $[E]$ and $[S]$ are molar concentrations of the 55.7 kDa subunit of *LmSPase* and substrate respectively, and K_m is an apparent Michaelis constant for *S*. We are not aware of a suitable method with which to determine the enzyme active-site concentration for *LmSPase* and E237Q. Therefore $[E]$ was calculated from the concentration of purified protein.

$$v = k_{\text{cat}}[E][S]/(K_m + [S]) \quad (1)$$

pH-dependence studies were carried out in 20 mM Mes buffer (pH 5.2–8.0). All buffer pH values were adjusted at the temperature of measurement (30°C) and controlled before and after recording each enzyme-catalysed initial rate. No pH change was observed during the typical time-span (up to 30 min) of the enzymatic reactions. To confirm pH dependences seen in the ranges $\text{pH} \leq 5.2$ and $\text{pH} \geq 7.2$ where Mes alone ($\text{p}K = 6.2$) has limited buffering capacity, additional experiments were performed using a mixture of 20 mM Mes and 20 mM sodium acetate (pH 4.5–6.0), and 50 mM Tes (pH 6.5–8.0). Note, however, that the ionizable phosphorylase substrate (phosphate, arsenate or αG1P) provides an additional buffering effect under all the conditions used. Ionic strength effects due to pH-dependent ionization of the Mes buffer were not controlled. However, it was proven in separate experiments carried out at pH 5.0, 6.0 and 7.0, where NaCl (or NaN_3) was added to the initial-rate assays at concentrations of up to 150 mM, that none of the reported pH-dependences (determined in the absence of added salt) was significantly distorted by relevant changes in ionic strength of the buffer. Also note that determination of kinetic parameters for phosphate and αG1P necessarily involves a substantial change in ionic strength at each pH value caused by the variable substrate concentration. It is counterproductive to compensate these changes by working at very high concentrations of NaCl. Further control experiments showed that the enzyme used was fully stable over the time of the initial rate assay under each of the different pH conditions.

The appropriate equations were fitted to pH profiles of $\log(k_{\text{cat}})$ and $\log(k_{\text{cat}}/K_m)$. Eqn (2) describes a log Y versus pH curve that decreases with a slope of +1 below $\text{p}K_1$. Eqn (3) describes a curve that decreases with slopes of +1 and –1 below $\text{p}K_1$ and above $\text{p}K_2$ respectively.

$$\log Y = \log[C/(1 + [\text{H}^+]/K_1)] \quad (2)$$

$$\log Y = \log[C/(1 + [\text{H}^+]/K_1 + K_2/[\text{H}^+])] \quad (3)$$

where Y is k_{cat} or k_{cat}/K_m , C is the pH-independent value of Y at the optimal state of protonation, K_1 and K_2 are macroscopic dissociation constants and $[\text{H}^+]$ is the proton concentration.

Chemical rescue studies using exogenous nucleophiles

Initial rates of E237Q-catalysed conversion of αG1P (1 mM) were recorded in the absence and presence of various reagents which by functioning as external nucleophiles could ‘rescue’ activity that was lost in the mutant (for the general case, see Figure 1C). The sodium salts of chloride (10–250 mM), bromide (10–250 mM), cyanide (5–100 mM), azide (2–50 mM), acetate (10–250 mM) and formate (10–250 mM) were tested. The enzyme (0.4 μM subunits of E237Q) was incubated at 30°C in 20 mM Mes buffer (pH 7.0), and the release of phosphate was measured in dependence of time of incubation up to 24 h. The reported values are corrected for the amount of phosphate produced by spontaneous hydrolysis of αG1P which was significant but did not impair the determination

of the enzymatic rates. The effect of the nucleophiles on initial rates of the wild-type was measured in suitable controls.

Monitoring E237Q-catalysed reactions with external nucleophiles by *in situ* proton NMR

Measurements were carried out on a Bruker DRX 600 AVANCE spectrometer using the Topspin 1.3 software. The reactions were performed at 300 K directly in the NMR sample tube (0.70 ml), which is placed in the magnet. Reaction mixtures contained 9 μM subunits of E237Q, 10 mM αG1P and 250 mM sodium formate or sodium acetate respectively, in $^2\text{H}_2\text{O}$ (99.9% ^2H). Proton spectra were taken every 30 min over a time of 14–16 h. The measurements were recorded at 600.13 MHz irradiation frequency, a relaxation delay of 1.0 s with presaturation of the HDO frequency, acquisition of 32768 data points and accumulation of 64 scans. After zero filling to 65536 data points, the free induction decays were Fourier transformed leading to spectra with a range of 5400 Hz. All measurements were referenced on external acetone (δ_{H} : 2.225 p.p.m.).

Structural analysis of glycosidic product formed upon the reaction of E237Q with azide

A solution of E237Q (9 μM subunits) in 20 mM Mes buffer (pH 7.0), was incubated at 30°C in the presence of 10 mM αG1P and 50 mM sodium azide. The reaction time course was monitored by TLC of samples taken at certain times up to 24 h as well as by measuring the release of phosphate. When αG1P was depleted to about 80 %, the reaction products were further analysed by ^1H , ^{13}C and corresponding two-dimensional homo- and heteronuclear NMR spectroscopy. Sample preparation involved removal of the enzyme from the reaction mixture by centrifugal ultrafiltration, lyophilization of the filtrate and dissolution into $^2\text{H}_2\text{O}$. Proton chemical shifts and coupling constants were compared with those of independently synthesized αG1N_3 . The following spectra were obtained: $\delta_{\text{H}}[(^2\text{H})\text{water}]$ 5.62 (1 H, d, J 4.2 Hz, H -1), 3.97 (1 H, dd, J 12.6, 2.2 Hz, H -6a), 3.89 (1 H, ddd, J 9.0, 5.5, 2.2 Hz, H -5), 3.85 (1 H, dd, J 12.6, 5.5 Hz, H -6b), 3.75 (1 H, dd, J 9.6, 4.2 Hz, H -2), 3.69 (1 H, dd, J 9.6, 9.6 Hz, H -3), 3.50 (1 H, dd, J 9.6, 9.0 Hz, H -4). δ_{C} 89.6 (C-1), 74.2 (C-5), 73.1 (C-3), 71.2 (C-2), 69.6 (C-4), 60.8 (C-6). Chemical shifts are referenced to external acetone (δ_{H} : 2.225 p.p.m. and δ_{C} : 31.45 p.p.m.).

RESULTS

Preparation of the E237Q mutant

The X-ray crystal structure of *BaSPase* has provided strong evidence in support of a catalytic function of Glu²³² [6]. Figure 2(A) displays a partial multiple sequence alignment comparing regions that surround the tentative acid–base catalyst in SPases and two close structural neighbours of *BaSPase* inside family GH-13. Because sequence homology in the relevant parts of the primary structures is quite low (<15 %), we utilized an alignment based on predicted and experimentally determined secondary structures to eliminate uncertainty in the identification of the candidate glutamate residue in *LmSPase*. Glu²³⁷ was replaced by a glutamine residue, and the E237Q mutant was obtained as an apparently homogeneous protein preparation (Figure 2B). The specific activity of E237Q for phosphorolysis of sucrose (0.0017 ± 0.00004 units/mg) was ≤ 0.001 % that of the wild-type. CD spectra of isolated wild-type and E237Q were nearly superimposable in the recorded wavelength range 200–250 nm (results not shown), indicating that the relative proportions of

Figure 2 (A) Comparison of the region containing the catalytic acid–base of different members of family GH-13 and (B) SDS/PAGE of purified wild-type *LmSPase* and E237Q

(A) The sequences shown are *LmSPase* (D90314), *BaSPase* (AF543301), amylosucrase from *Neisseria polysaccharea* (*NpASase*, AJ011781) and 4- α -glucantransferase from *Thermotoga maritima* (*TmGTase*, AE000512). The alignment was performed with the Vector NTI program using the AlignX-module with the PAM250 scoring matrix. Conserved amino acids are shaded in black (100% identity), dark grey (80–100% identity) and light grey (60–80% identity). **(B)** Lane 1, wild-type; lane 2, E237Q; lane 3, molecular mass standard. The staining of protein bands was performed with Coomassie Blue.

secondary structural elements in both enzymes were virtually identical.

Kinetic comparison of wild-type SPase and the E237Q mutant

Kinetic consequences of the E237Q mutant were determined through steady-state kinetic analysis of reactions that are distinguished according to their requirement for acid–base catalytic assistance during glucosylation or deglucosylation steps. Sucrose differs widely from α G1P and α G1N₃ in leaving group ability, and phosphate and arsenate are much better nucleophiles than

fructose. Kinetic parameters of an enzyme mutant lacking the general acid–base catalyst should thus vary in response to altered substrate chemical reactivity. Initial rates were recorded under conditions in which the concentration of glucosyl donor or acceptor was varied at a constant saturating concentration of the respective other substrate. Eqn (1) was fitted to the data, and the results are summarized in Table 1. Catalytic steps involving a poor leaving group or nucleophile were slowed by five orders of magnitude in E237Q compared with wild-type. By contrast, steps expected to proceed without assistance from an acid–base were not strongly affected by the mutation. α G1N₃ was an even better substrate to be phosphorylated by E237Q than wild-type.

Chemical rescue of E237Q using external nucleophiles

E237Q catalysed the hydrolysis of α G1P at a very low rate that was at the limit of detection by analytical procedures ($k_{\text{cat}} \approx 10^{-5} \text{ s}^{-1}$). By way of comparison, the corresponding k_{cat} of the wild-type was 2 s^{-1} [5]. However, when E237Q was presented with an external nucleophile such as azide, the steady-state rate of enzymatic cleavage of α G1P, measured as release of phosphate, was increased significantly, up to about 330-fold in dependence of the azide concentration. Rate enhancement under the chosen conditions should reflect more rapid reaction of glucosylated E237Q with azide than water and thus lead to formation of a new glucosidic product (compare with Figure 1C). Consistent with this notion, chemical rescue of E237Q by azide was not accompanied by increased formation of glucose, indicating that hydrolysis did not occur. The glucoside produced by E237Q co-migrated in TLC with an authentic standard of α G1N₃ (results not shown). Its proposed chemical structure featuring the expected retained anomeric configuration was confirmed unequivocally by NMR analysis of the product mixture obtained after exhaustive E237Q-catalysed conversion of α G1P in the presence of azide.

Acetate and formate also enhanced the rate of α G1P conversion by E237Q although about 10-fold less efficiently than azide (Table 2). Cyanide, chloride and bromide were inactive. The enzymatic reaction of E237Q in the presence of acetate yielded no glucose whereas a significant concentration of hydrolysis product was detected at all times when formate was added. As the amount of released glucose was always smaller than that of phosphate, we considered the possibilities that hydrolysis had occurred (i) in competition with transglucosylation at the level of the β -glycosyl-enzyme, or (ii) because the new glucosidic product, likely α -glucose 1-formic acid ester decomposes non-enzymatically. *In situ* proton NMR studies were therefore performed to characterize

Table 1 Kinetic parameters for wild-type and E237Q mutant

Initial rates were determined at 30 °C in 20 mM Mes buffer (pH 7.0) in the presence of 250 mM sucrose, 50 mM phosphate, 100 mM α G1P, 75 mM (wild-type) and 150 mM (E237Q) arsenate respectively, and 100 mM D-fructose. Results are from non-linear fits of eqn (1) to the experimental data. k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ have S.E.M. values of $\leq 6\%$ and $\leq 13\%$ respectively; n.d., not determined. Values of k_{cat} were obtained from specific enzyme activities (units/mg) using the factor 0.93 which includes a subunit molecular mass of 55 700 g/mole.

Reaction type	Varied substrate	In the presence of	Wild-type		E237Q mutant		$k_{\text{cat}} \text{ wild-type} / k_{\text{cat}} \text{ E237Q}$ ($k_{\text{cat}}/K_{\text{m}} \text{ wild-type} / k_{\text{cat}}/K_{\text{m}} \text{ E237Q}$)
			k_{cat} (s^{-1})	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \text{ mM}^{-1}$)	k_{cat} (s^{-1})	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1} \text{ mM}^{-1}$)	
Phosphorolysis	Phosphate	250 mM sucrose	195	20.5	n.d.	n.d.	n.d.
	Sucrose	50 mM phosphate	165	28.7	1.6×10^{-3}	2.9×10^{-4}	1×10^5 (1×10^5)
	α G1N ₃	50 mM phosphate	4.3×10^{-3}	4.9×10^{-5}	5.2×10^{-3}	2.5×10^{-3}	0.8 (0.02)
Arenolysis	Arsenate	100 mM α G1P	170	26.5	24	0.39	7 (70)
	α G1P	75 mM (wild-type)/150 mM (E237Q) arsenate	261	5.5	21	0.61	12 (9)
Synthesis	α G1P	100 mM D-fructose	91	5.3	6.3×10^{-4}	0.23	1×10^5 (25)
	D-Fructose	100 mM α G1P	72	3.4	n.d.	n.d.	n.d.

Table 2 Kinetic parameters for the reaction of E237Q with α G1P and exogenous nucleophiles

Kinetic parameters were determined as described in the Experimental section. Results are from non-linear fits of eqn (1) to the experimental data. k_{cat} and k_{cat}/K_m have S.E.M. values of $\leq 5\%$ and $\leq 4\%$ respectively.

Nucleophile	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)
Azide	0.21	42	5.0×10^{-3}
Acetate	0.067	173	3.9×10^{-4}
Formate	0.051	137	3.7×10^{-4}

the course of α G1P conversion by E237Q in the presence of acetate or formate. The results depicted in Figures 3 and 4 provide a time-resolved analysis of the composition of the respective reaction mixture. Note that when incubated under exactly identical conditions as E237Q, the wild-type hydrolysed α G1P but did not catalyse glucosyl transfer to azide, acetate or formate.

Figures 3 and 4 confirm that chemical rescue of catalytic activity in E237Q by acetate and formate goes along with formation of the corresponding α -glucose 1-esters. However, it also shows that both glucosidic products underwent spontaneous transformation due to acetyl- or formyl-group migration as well as mutarotation.

α -Glucose 1-acetic acid ester was converted primarily into the α - and β -anomers of 2-*O*-acetyl D-glucose [13] whereas only traces of consecutive products of acetyl group migration to positions three and six were detectable (Figure 3). By contrast, formyl group migration took place preferentially up to position six [14] (Figure 4). All products of formyl group migration were unstable and hydrolysed with time. Such migration of acetyl and formyl groups on monosaccharides are well known as undesired (side) reactions in organic and biocatalysed synthesis. However, only recently positional, kinetic and thermodynamic aspects of acetyl group migration have been investigated in more detail for the first time [15]. The regio- and stereoselective formation of α -glucose 1-acetic acid ester by E237Q can be helpful to improve these studies because alternative ways to synthesize glycosyl monoacetates are very cumbersome.

pH-dependence studies of wild-type and E237Q enzymes

pH effects on initial rates of phosphorolysis and synthesis of sucrose as well as arsenolysis of α G1P were measured under conditions in which the concentration of glucosyl donor or acceptor was varied at a constant saturating concentration of the respective other substrate. Kinetic parameters were determined at six to eight pH values in the pH range 5.0–8.0, and the results are shown in Figure 5. The appropriate equations were fitted to pH profiles of $\log(k_{\text{cat}})$ and $\log(k_{\text{cat}}/K_m)$, and apparent pK values thus obtained

Figure 3 ¹H NMR monitoring of product formation during E237Q-catalysed α G1P conversion in the presence of sodium acetate (A) and the corresponding reaction scheme (B)

The primary product is α -glucose 1-acetic acid ester [δ_{H} 6.05 (1 H, d, J 3.6 Hz, H-1), 2.14 (3 H, s, CO-CH₃)]. Migration of the acetyl group and mutarotation lead to accumulation of 2-*O*-acetyl- α -D-glucose [δ_{H} 5.32 (1 H, d, J 3.8 Hz, H-1), 2.118 (3 H, s, CO-CH₃)] and 2-*O*-acetyl- β -D-glucose [δ_{H} 4.76 (1 H, d, J 8.1 Hz, H-1), 2.121 (3 H, s, CO-CH₃) not shown]. Consecutively, the α - and β -anomers of 3-*O*-acetyl-D-glucose and 6-*O*-acetyl-D-glucose are also formed in smaller amounts. Signals of their acetate groups appear in the range of 2.14 p.p.m. to 2.05 p.p.m. and the α -anomeric protons cause signals between 5.25 p.p.m. and 5.20 p.p.m.. All β -anomeric proton signals are partly overlapped by the overwhelming H₂O signal and not shown. 4-*O*-Acetyl-D-glucose has not been formed in detectable amounts after 16 h.

Figure 4 ^1H NMR monitoring of product formation during E237Q-catalysed αG1P conversion in the presence of sodium formate (A) and the corresponding reaction scheme (B)

αG1P [δ_{H} 5.41 (1 H, dd, J 7.2, 3.4 Hz, $\text{H}-1$)]. The primary product is α -glucose 1-formic acid ester [δ_{H} 8.21 (1 H, s, CHO), 5.36 (1H, d, J 3.8 Hz, $\text{H}-1$)]. Migration of the formyl group and mutarotation consequently lead to formation of small amounts of α - and β -anomers of 2-,3- or 4- O -acetyl- D -glucose. The signals of the corresponding formyl groups are in the range of 8.31 p.p.m. to 8.25 p.p.m., and one α -anomeric proton signal appears at 5.25 p.p.m. (indicated by asterisks). The further α -anomeric and all β -anomeric proton signals are overlapped by other signals. 6- O -Formyl α - D -glucose [δ_{H} 8.15 (1 H, s, CHO), 5.18 (1 H, d, J 3.8 Hz, $\text{H}-1$), 4.42 (dd, 12.7, 2.4 Hz, 1H, $\text{H}-6\text{a}$), 4.38 (dd, 12.7, 4.7 Hz, 1H, $\text{H}-6\text{b}$)] and 6- O -formyl β - D -glucose [δ_{H} 8.15 (1 H, s, CHO), 4.63 (1 H, d, J 8.0 Hz, $\text{H}-1$), 4.47 (1 H, dd, J 12.4, 1.9 Hz, $\text{H}-6\text{a}$), 4.32 (1 H, dd, J 12.7, 5.3 Hz, $\text{H}-6\text{b}$)] have been accumulated in detectable amounts prior to hydrolysis to formate and free glucose. The signal at 8.24 p.p.m. is a ^{13}C -satellite of the formate signal at 8.41 p.p.m..

are summarized in Table 3. The pH profiles of $\log(k_{\text{cat}}/K_{\text{m}})$ for arsenolysis of αG1P decreased at high pH with slopes different from -1 . Eqn (3) could not be fitted to the data unless suitable correction of the data was made for the pH-dependent ionization of arsenate ($\text{p}K_{\text{a}} = 6.94$) or αG1P ($\text{p}K_{\text{a}} = 6.51$) respectively, assuming that the protonated form of the oxyanion is required for binding or catalysis. Figures 6(B) and 6(C) show both measured and corrected values. The pH-rate profiles of E237Q featured marked changes, in particular complete removal of apparent ionizations, in comparison with corresponding pH-rate profiles of the wild-type.

DISCUSSION

Analysis of kinetic consequences in E237Q and chemical rescue studies

Substitution of Glu²³⁷ with a glutamine residue caused a 10^5 -fold decrease in wild-type activity, expressed as k_{cat} or $k_{\text{cat}}/K_{\text{m}}$ (for sucrose) in the phosphorolysis direction, which is consistent with the expected effect of complete loss of protonic assistance to the departure of a poor leaving group such as fructose during

formation of the glucosyl-enzyme intermediate. More detailed information about the role of Glu²³⁷ was obtained by examining glucosylation and deglucosylation as kinetically isolated steps. In a Ping Pong Bi Bi kinetic mechanism such as that of *LmSPase* (Figure 1B; [5]), $k_{\text{cat}}/K_{\text{m}}$ for the glucosyl donor and acceptor measure formation and breakdown of the covalent intermediate respectively. k_{cat} includes all steps of the catalytic cycle and reflects the rate-determining step(s) under conditions of enzyme saturation with both substrates.

Glucosyl transfer from phosphate to the enzyme was only moderately affected in E237Q, reflecting the good leaving group ability of αG1P . However, due to removal of general base catalytic assistance deglucosylation of E237Q was slowed about 10^5 -fold in comparison with wild-type when fructose was used as the nucleophile. Under these conditions, an unusually small apparent K_{m} for αG1P ($= 2.7 \mu\text{M}$) was observed which was four orders of magnitude lower than the wild-type value, probably indicating accumulation of the covalent glucosyl-enzyme intermediate. Although k_{cat} values of E237Q for phosphorolysis and synthesis of sucrose were decreased by about the same factor of $\approx 10^5$ in comparison with the corresponding wild-type values, rate limitation occurred at different steps in enzymatic glucosyl transfer to and

Figure 5 pH profiles for k_{cat} (A) and k_{cat}/K_m (B) of the phosphorolysis (●) and synthesis (○) reaction catalysed by wild-type LmSPase

Initial rates were obtained in 20 mM Mes buffer at 30°C. Control experiments for the phosphorolysis reaction were performed in 20 mM Mes/sodium acetate (pH 4.5–6.0) and 50 mM Tes (pH 6.5–8.0) buffer (■). Solid lines are fits of eqn (2) or eqn (3) to the data as described in the Experimental section.

Table 3 pK -values from pH-profiles of k_{cat} and k_{cat}/K_m for wild-type

pK -values were obtained from non-linear fits of the indicated eqn to $\log(k_{\text{cat}})$ and $\log(k_{\text{cat}}/K_m)$. The fits of the data are illustrated in Figures 5 and 6.

	eqn	pK_1	pK_2
Phosphorolysis			
$\log(k_{\text{cat}})$ (sucrose)	3	5.31 ± 0.06	7.90 ± 0.09
$\log(k_{\text{cat}}/K_m)$ (sucrose)	3	5.75 ± 0.10	7.24 ± 0.11
Synthesis			
$\log(k_{\text{cat}})$ (D-fructose)	3	5.33 ± 0.17	6.99 ± 0.11
$\log(k_{\text{cat}}/K_m)$ (D-fructose)	2	5.84 ± 0.06	—
Arsenolysis			
$\log(k_{\text{cat}})$ (arsenate)	3	5.49 ± 0.14	7.11 ± 0.13
$\log(k_{\text{cat}}/K_m)$ (arsenate)	3	4.97 ± 0.10	8.30 ± 0.25
$\log(k_{\text{cat}})$ (α G1P)	3	5.65 ± 0.13	7.60 ± 0.15
$\log(k_{\text{cat}}/K_m)$ (α G1P)	3	5.51 ± 0.36	7.17 ± 0.12

from phosphate catalysed by the mutant. In the arsenolysis of α G1P and the phosphorolysis of α G1N₃, k_{cat} and k_{cat}/K_m were only weakly changed as a result of the mutation, consistent with the expectation that both glucosylation and deglucosylation in these conversions should proceed well in the absence of acid-base catalysis. The energetic contribution of Brønsted catalytic assistance to a stabilization of the transition state of glucosylation and deglucosylation can be calculated from a comparison of k_{cat}/K_m values for wild-type and E237Q. It is similar, about 29 kJ mol⁻¹ ($= -RT \ln 10^5$), for catalytic steps involving fructose

Figure 6 pH profiles for k_{cat} (A) and k_{cat}/K_m (B and C) of the arsenolysis of α G1P catalysed by the wild-type (●) and the E237Q mutant (○)

Initial rates were obtained in 20 mM Mes buffer at 30°C. Control experiments for wild-type LmSPase were performed in 20 mM Mes/sodium acetate (pH 4.5–6.0) and 50 mM Tes (pH 6.5–8.0) buffer (■). k_{cat}/K_m values were corrected for the fraction of protonated α G1P (B) or arsenate (C) respectively. Solid lines are fits of eqn (2) or eqn (3) to the data as described in the Experimental section. Dotted lines show the trend of the uncorrected data of k_{cat}/K_m for the wild-type (▼) and the E237Q mutant (▽).

as the leaving group and nucleophile. Making and breaking of a glucosidic bond to phosphate requires little stabilization from Glu²³⁷ (< 10.7 kJ mol⁻¹), reflected by the mere < 70 -fold rate difference for reactions catalysed by wild-type and E237Q.

Enhancement of catalytic activity of E237Q was observed when conversion of α G1P, a substrate with good leaving group ability, was assayed in the presence of anionic nucleophiles. This kinetic behaviour of E237Q under conditions in which deglucosylation was rate-determining is diagnostic of a catalytic acid-base function of Glu²³⁷ in the wild-type enzyme. Chemical rescue of activity showed saturation-like behaviour on the concentration of the nucleophile (Table 2). The observed saturation might indicate

true binding of the anionic nucleophile at the active site of E237Q or instead, reflect a change in the rate-limiting step as the nucleophile concentration was increased, such that eventually another step, likely glucosylation, became rate-determining. At apparent saturation with the nucleophile, the k_{cat} values of E237Q measured with azide, acetate and formate were significantly (≈ 4 -fold) different and not the same, as would be expected if enzyme glucosylation was the common rate-determining step in reactions of the mutant with these nucleophiles. The catalytic centre activity of E237Q in the presence of azide was about 10 % and 0.1 % of wild-type k_{cat} values for hydrolysis and arsenolysis of α G1P respectively. The proposed mechanism of functional complementation of E237Q whereby external nucleophiles intercept the glucosyl-enzyme intermediate and thus facilitate catalytic turnover was confirmed by NMR analysis of the respective chemical rescue products, each of which featured the expected α -anomeric configuration.

Comparison of k_{cat}/K_m values for E237Q-catalysed conversion of α G1P and α G1N₃ can be used to determine that interactions with a phosphate leaving group promoted glucosylation of the mutant about 244-fold more efficiently than interactions with an azide leaving group. Another interesting observation was that glucosyl transfer from α G1N₃ to wild-type and E237Q enzymes took place with identical efficiencies, suggesting that general acid catalysis from the carboxylic side chain of Glu²³⁷ to the cleavage of the C–N bond was not important.

pH-rate dependences for wild-type and E237Q

The pH-rate profiles of some retaining glycoside hydrolases were shown to be determined by ionizations of the major active-site residues, the nucleophile and the acid–base catalyst (for review, see [3]). Therefore site-directed replacement of the acid–base catalyst should, in an ideal situation, be accompanied by complete removal of the corresponding ionization from the pH profile.

The pH profiles of $\log(k_{\text{cat}})$ and $\log(k_{\text{cat}}/K_m)$ for glucosyl donor substrates (α G1P, sucrose) decreased at high and low pH, suggesting the involvement of two ionizable groups in the variation of activity of the wild-type enzyme with pH. Apparent pK values ($pK_1 \approx 5.3$ – 5.8 , $pK_2 \approx 7.2$ – 7.9) were comparable in these pH profiles and sufficiently far apart (≥ 1.7 pH units) to treat them as separate ionizations. The pH-dependence of k_{cat}/K_m on the glucosyl donor reflects ionizations in free enzyme and substrate whereas that of k_{cat} mirrors variation of the rate-determining step with pH when the enzyme is fully saturated with substrate. Unlike α G1P, sucrose does not ionize in the relevant pH range and therefore, pK values seen in pH profiles of $\log(k_{\text{cat}}/K_m)$ for the glucosyl donor are due to protonation/deprotonation of groups on the enzyme. The pH profiles of $\log(k_{\text{cat}})$ for phosphorolysis and synthesis of sucrose as well as arsenolysis of α G1P were similar, indicating that the pH-dependence of the rate-determining step(s) was not altered in response to (i) variation of the glucosyl donor substrate, and (ii) replacement of fructose by arsenate as the nucleophile of the reaction. The pH profile of $\log(k_{\text{cat}}/K_m)$ for fructose followed an apparent single ionization of a group of $pK = 5.8$ that must be unprotonated for activity. Because the k_{cat}/K_m for fructose measures breakdown of the β -glucosyl-enzyme intermediate, it is clear that ionization due to the catalytic nucleophile Asp¹⁹⁶, which is trapped in the covalent intermediate, must be eliminated from this pH profile. It is therefore reasonable to assign the observed pK of 5.8 to the side chain of the acid–base catalyst in the glucosylated enzyme. The pH profile of $\log(k_{\text{cat}}/K_m)$ for arsenate decreased at high pH above an apparent pK of 8.3, suggesting that the deglycosylation rate in the presence of the anionic nucleophile is independent of the ionization of the acid–

base catalyst and deprotonation of another enzyme group dominates the pH dependence of this step.

k_{cat} for the arsenolysis of α G1P by E237Q varied with pH according to a single apparent pK of 6.0–6.2 below which the activity was lost. The high-pH ionization seen in the corresponding pH-rate profile of the wild-type was completely eliminated from the profile of the mutant. $\log(k_{\text{cat}}/K_m)$ for arsenate was not dependent on pH, revealing removal of the group of $pK = 8.3$ observed in the analogous pH dependence of the wild-type. These results are fully consistent with mutation of the acid–base catalyst in E237Q and allow pK -to-group assignments for wild-type phosphorylase.

Asp¹⁹⁶ and Glu²³⁷ are proposed to ionize in the free enzyme with pK values of about 5.6 and 7.2 respectively. Asp¹⁹⁶ must be unprotonated and Glu²³⁷ protonated for catalysis to formation of the covalent intermediate. Considering the good leaving group ability of α G1P and also the fact that mutation of Glu²³⁷ did not cause a large decrease in k_{cat}/K_m for α G1P, it is interesting that deprotonation of Glu²³⁷ appears to dominate the high pH-dependence of the reaction with this glucosyl donor. Productive binding of α G1P may require Glu²³⁷ to be protonated. Enzyme glucosylation is suggested to control k_{cat} , implying that the binary complex with glucosyl donor is the main form of the enzyme at the steady-state under conditions of saturating substrate concentrations. The clear pH-dependence of k_{cat} supports a mechanism in which the donor substrate binds to the enzyme in its reactive or unreactive protonation state, and protonation/deprotonation can take place in free enzyme and in the enzyme–substrate complex. The significant upshift in the apparent pK of Glu²³⁷ by approximately 0.4–0.7 pH units upon moving along the reaction co-ordinate from free enzyme to enzyme–glucosyl donor complex is most reasonably explained by an effect of hydrogen bonding on apparent pK , perhaps involving the glycosidic oxygen of the substrate. By contrast, the apparent pK of Asp¹⁹⁶ seems not to be affected by substrate binding, fully consistent with the ionized side chain of the aspartate residue functioning as a catalytic nucleophile. Once the β -glucosyl-enzyme has been formed and the leaving group departed, the pK of Glu²³⁷ is depressed by ≈ 2 pH units to a value of 5.8, compared with the pK of enzyme–substrate. It is probable that changes in active-site environment resulting from the absence of negative charge on Asp¹⁹⁶ in the covalent intermediate are responsible for the observed drop in pK . Modulation of the pK of the acid–base is consistent with optimum participation of the catalytic side chain at each step in the reaction of a true glycoside hydrolase, requiring protonic assistance to leaving group departure and facilitation to the attack of water during deglycosylation [16]. But is there a need for a similar ‘ pK cycling’ of Glu²³⁷ during phosphorolysis of sucrose, which is the physiological reaction of *Lm*SPase?

The pH dependence of k_{cat}/K_m for arsenate suggests that reaction of the glucosyl-enzyme with the anionic nucleophile takes place independently of the protonation state of Glu²³⁷ but requires that another enzyme group with an apparent pK of 8.3 be protonated. The structure of unliganded *Ba*SPase [6] has revealed His²³⁴ (amino acid numbering of *Ba*SPase) as a candidate interaction partner with phosphate. Lys¹⁹⁹ is also a contributor to subsite +1 of the phosphorylase and might play a role in binding and positioning of the nucleophile. Recent structural studies of glucosylated *Ba*SPase also implicate Arg¹³⁵, Asp³⁴² and Tyr³⁴⁴ [17]. Some structural rearrangements of the active site at the level of the covalent intermediate may thus be necessary to accommodate fructose or arsenate (and by analogy, phosphate) as the nucleophile. While the ionized side chain of Glu²³⁷ is clearly needed to provide general base catalytic assistance to the reverse reaction with fructose, it appears to be passive in catalytic terms during nucleophilic attack by the oxyanion.

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