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**Acid-base catalysis in *Leuconostoc mesenteroides* sucrose phosphorylase probed by site-directed mutagenesis and detailed kinetic comparison of wild-type and Glu-237→Gln mutant enzymes**

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## ABBREVIATIONS AND TEXTUAL FOOTNOTES

$\alpha$ G1N<sub>3</sub>,  $\alpha$ -D-glucose 1-azide;  $\alpha$ G1P,  $\alpha$ -D-glucose 1-phosphate; SPase, sucrose phosphorylase; *Ba*SPase, SPase from *Bifidobacterium adolescentis*; *Lm*SPase, SPase from *Leuconostoc mesenteroides*

## SYNOPSIS

The role of acid-base catalysis in the two-step enzymatic mechanism of  $\alpha$ -retaining glucosyl transfer by *Leuconostoc mesenteroides* sucrose phosphorylase has been examined through site-directed replacement of the putative catalytic Glu-237 and detailed comparison of purified wild-type and Glu-237→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  $10^5$ -fold, in E237Q. Azide, acetate and formate but not halides restored catalytic activity, up to 300-fold, in E237Q under conditions in which the deglycosylation step was rate-determining, and promoted production of the corresponding  $\alpha$ -glucosides. *In situ* proton NMR studies of the chemical rescue of E237Q by acetate and formate revealed that enzymatically formed  $\alpha$ -glucose 1-esters decomposed spontaneously via acyl group migration and hydrolysis. Using pH profiles of  $k_{\text{cat}}/K_{\text{m}}$ , the pH dependences of kinetically isolated glucosylation and deglucosylation steps were analyzed for wild-type and E237Q. Glucosylation of the wild-type proceeded optimally above and below apparent  $\text{p}K_{\text{a}}$  values of about 5.6 and 7.2 respectively whereas deglucosylation was dependent on the apparent single ionization of a group of  $\text{p}K_{\text{a}} \approx 5.8$  that must be deprotonated for reaction. Glucosylation of E237Q was slowed below apparent  $\text{p}K_{\text{a}} \approx 6.0$  but had lost the high pH dependence of the wild-type. Deglucosylation of E237Q was pH-independent. The results allow unequivocal assignment of Glu-237 as the catalytic acid/base of sucrose phosphorylase. They support a mechanism in which the  $\text{p}K_{\text{a}}$  of Glu-237 cycles between  $\approx 7.2$  in free enzyme to  $\approx 5.8$  in glucosyl enzyme intermediate, ensuring optimal participation of the Glu side chain at each step in catalysis. Enzyme deglucosylation to an anionic nucleophile took place with Glu-237 protonated or unprotonated. The results delineate how conserved active-site groups of retaining glycoside hydrolases can accommodate enzymatic function of a phosphorylase.

**Keywords:** phosphorylase; retaining mechanism; family GH-13; Brønsted catalysis; chemical rescue;  $pK_a$  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 Glu or Asp residue, cycles between catalytic functions of a general acid and base in glycosylation and deglycosylation half-reactions respectively (Figure 1A) [3].

SPase (sucrose phosphorylase) catalyzes conversion of sucrose ( $\alpha$ -D-glucopyranosyl-1,2- $\beta$ -D-fructofuranoside) and phosphate into D-fructose and  $\alpha$ -D-glucose 1-phosphate ( $\alpha$ G1P) (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  $\beta$ -glucosyl enzyme intermediate formed by an active site Asp [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* (*Ba*SPase) revealed a highly conserved architecture of the active centre, implicating Glu-232 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. 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 often made observation with retaining glycoside hydrolases is however, that anions are prevented access to the active site, presumably because of charge screening by the base catalyst.

We report here on a detailed mechanistic comparison of wild-type SPase from *Leuconostoc mesenteroides* (*LmSPase*) and a site-directed mutant thereof in which the candidate acid/base catalyst (Glu-237) was replaced by a glutamine. 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 Glu-237. They also delineate an enzymatic reaction coordinate in which the apparent  $pK_a$  of Glu-237 changes to suit a catalytic function of the Glu during glucosylation and deglucosylation. Recognition of fructose or phosphate as leaving group/nucleophile may require changes in the active-site environment of Glu-237. Although Glu residues homologous to Glu-237 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 been described only for the human pancreatic  $\alpha$ -amylase [11] so far.

## EXPERIMENTAL

### *Materials*

Materials for site-directed mutagenesis, protein purification and enzymatic assays were described elsewhere [5]. Oligonucleotide synthesis and DNA sequencing were performed at

VBC Biotech Services GmbH (Vienna, A).  $\alpha$ -D-glucose 1-azide ( $\beta$ G1N<sub>3</sub>) was synthesized from 2,3,4,6-tetra-*O*-acetyl- $\alpha$ -D-glucopyranosyl azide (CMS Chemicals Ltd., Oxfordshire, UK) by de-*O*-acetylation with MeONa in methanol. All other chemicals were of the highest available purity and obtained from Sigma.

#### *Site-directed mutagenesis, protein expression and purification*

The point mutation Glu-237→Gln was introduced via a Two-Stage PCR strategy reported elsewhere [5]. The following oligonucleotide primers were used:

5'-CCATTAAAGGCTGAAATTTTACCACAAATTCATG-3' (forward primer)

5'-CATGAATTTGTGGTAAAATTTTCAGCCTTTAATGG-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 *E. coli* JM109 cells. Wild-type *LmSPase* and E237Q mutant were produced and purified to apparent electrophoretic homogeneity using 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 was 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; 20 mM MES buffer, pH 7.0) using a continuous coupled assay with phosphoglucomutase and glucose 6-phosphate dehydrogenase as described elsewhere [5]. Initial rates of glucosyl transfer to and from phosphate and arsenolysis of  $\alpha$ G1P were determined in discontinuous assays in which

formation of, respectively,  $\alpha$ G1P, phosphate or glucose 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  $\mu$ g/mL and 20  $\mu$ 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 carried out 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  $k_{\text{cat}}$  is the catalytic-centre activity,  $[E]$  and  $[S]$  are molar concentrations of the 55.7-kDa subunit of *Lm*SPase 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 *Lm*SPase 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-catalyzed 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 ionisable phosphorylase substrate (phosphate, arsenate, or  $\alpha$ G1P) provides an additional buffering effect under all the conditions used. Ionic strength effects due to pH-dependent ionisation 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  $\text{NaN}_3$ ) was added to the initial-rate assays in concentration 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  $\alpha$ G1P 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  $\log(k_{\text{cat}}/K_m)$ . Equation (2) describes a  $\log Y$  versus pH curve that decreases with a slope of +1 below  $\text{p}K_1$ . Equation (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  $k_{\text{cat}}$  or  $k_{\text{cat}}/K_m$ ,  $C$  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-catalyzed conversion of  $\alpha$ G1P (1 mM) were recorded in the absence and presence of a 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  $\mu$ M 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  $\alpha$ G1P 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-catalyzed 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  $\mu$ M subunits of E237Q, 10 mM  $\alpha$ G1P, and 250 mM sodium formate or sodium acetate, respectively, in D<sub>2</sub>O (99.9% D). Proton spectra were taken every 30 min over a time of 14 h to 16 h. The measurements were recorded at 600.13 MHz irradiation frequency, relaxation delay of 1.0 s with presaturation of the HDO frequency, acquisition of 32 768 data points, and accumulation of 64 scans. After zero filling to 65 536 data points the free induction decays are Fourier transformed leading to spectra with a range of 5400 Hz. All measurements were referenced on external acetone ( $\delta_{\text{H}}$ : 2.225 ppm).

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

A solution of E237Q (9  $\mu$ M subunits) in 20 mM MES buffer, pH 7.0, was incubated at 30 °C in the presence of 10 mM  $\alpha$ G1P 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  $\alpha$ G1P was depleted to about 80%, the reaction products were further analyzed by <sup>1</sup>H, <sup>13</sup>C, and corresponding 2D 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 D<sub>2</sub>O. Proton chemical shifts and coupling constants were compared with those of independently synthesized  $\alpha$ G1N<sub>3</sub>. 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 (1H, dd, *J* 9.6, 9.6 Hz, *H*-3), 3.50 (1 H, dd, *J* 9.6, 9.0 Hz, *H*-4).  $\delta_C$  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 ( $\delta_H$ : 2.225 ppm and  $\delta_C$ : 31.45 ppm).

## RESULTS

### *Preparation of E237Q mutant*

The X-ray crystal structure of *BaSPase* has provided strong evidence in support of a catalytic function of Glu-232 [6]. Figure 2A 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 Glu in *LmSPase*. Glu-237 was replaced by Gln, and the E237Q mutant was obtained as an apparently homogeneous protein preparation (Figure 2B). The specific activity of E237Q for phosphorolysis of sucrose ( $0.0017 \pm 0.00004$  U/mg) was  $\leq 0.001\%$  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 secondary structural elements in both enzymes were virtually identical.

### *Kinetic comparison of wild-type SPase and E237Q mutant thereof*

Kinetic consequences in E237Q were determined through steady-state kinetic analysis of reactions that are distinguished according their requirement for acid/base catalytic assistance

during glycosylation or deglycosylation steps. Sucrose differs widely from  $\alpha$ G1P and  $\alpha$ G1N<sub>3</sub> 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 results are summarized in Table 1. Catalytic steps involving a poor leaving group or nucleophile were slowed by 5 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.  $\alpha$ G1N<sub>3</sub> was even a better substrate to be phosphorylated by E237Q than wild-type.

#### *Chemical rescue of E237Q using external nucleophiles*

E237Q catalyzed the hydrolysis of  $\alpha$ G1P at a very low rate that was at the limit of detection by the analytical procedures ( $k_{\text{cat}} \approx 10^{-5} \text{ s}^{-1}$ ). By way of comparison, the corresponding  $k_{\text{cat}}$  of the wild-type was  $2 \text{ s}^{-1}$  [5]. However, when E237Q was presented with an external nucleophile such as azide, the steady-state rate of enzymatic cleavage of  $\alpha$ 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 (cf. 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  $\alpha$ G1N<sub>3</sub> (data 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-catalyzed conversion of  $\alpha$ G1P in the presence of azide.

Acetate and formate also enhanced the rate of  $\alpha$ G1P conversion by E237Q although about 10-fold less efficiently than azide (Table 2). Cyanide, chloride, and bromide were inactive. 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  $\beta$ -glycosyl-enzyme, or (ii) because the new glucosidic product, likely  $\alpha$ -glucose 1-formic acid ester decomposes non-enzymatically. *In situ* proton NMR studies were therefore carried out to characterize the course of  $\alpha$ G1P conversion by E237Q in the presence of acetate or formate. Results are depicted in Figures 3 and 4, which provide a time-resolved analysis of the composition of the respective reaction mixture. Note that when incubated under the exactly identical conditions as E237Q, the wild-type hydrolyzed  $\alpha$ G1P but did not catalyze 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  $\alpha$ -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.  $\alpha$ -Glucose 1-acetic acid ester was converted primarily into the  $\alpha$ - and  $\beta$ -anomers of 2-*O*-acetyl D-glucose [13] whereas only traces of consecutive products of acetyl group migration to positions 3 and 6 were detectable (Figure 3). By contrast, formyl group migration took place preferentially up to position 6 [14] (Figure 4). All products of formyl group migration were unstable and hydrolyzed with time. Such migration of acetyl and formyl groups on monosaccharides are well known as undesired

(side) reactions in organic and biocatalyzed 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  $\alpha$ -glucose 1-acetic acid ester by E237Q can be helpful to improve these studies because alternative ways to synthesize glycosyl mono-acetates 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  $\alpha$ 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 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 are summarized in Table 3. The pH profiles of  $\log(k_{\text{cat}}/K_m)$  for arsenolysis of  $\alpha$ G1P decreased a 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 ( $pK_a = 6.94$ ) or  $\alpha$ G1P ( $pK_a = 6.51$ ), respectively, assuming that the protonated form of the oxyanion is required for binding or catalysis. Panels B and C in Figure 6 show both measured and corrected values. The pH-rate profiles of E237Q featured marked changes, in particular complete removals of apparent ionizations, in comparison to corresponding pH-rate profiles of the wild-type.

## DISCUSSION

#### *Analysis of kinetic consequences in E237Q and chemical rescue studies*

Substitution of Glu-237 with Gln caused a  $10^5$ -fold decrease in wild-type activity, expressed as  $k_{\text{cat}}$  or  $k_{\text{cat}}/K_m$  (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 Glu-237 was obtained by examining glucosylation and deglucosylation as kinetically isolated steps. In a Ping-Pong BiBi kinetic mechanism such as that of *LmSPase* (Figure 1B; [5]),  $k_{\text{cat}}/K_m$  for glucosyl donor and acceptor measure formation and breakdown of the covalent intermediate respectively.  $k_{\text{cat}}$  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  $\alpha$ G1P. 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  $\alpha$ G1P ( $= 2.7 \mu\text{M}$ ) was observed which was 4 orders of magnitude lower than the wild-type value, probably indicating accumulation of the covalent glucosyl-enzyme intermediate. Although  $k_{\text{cat}}$  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 from phosphate catalyzed by the mutant. In the arsenolysis of  $\alpha$ G1P and the phosphorolysis of  $\alpha$ G1N<sub>3</sub>,  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_m$  were only weakly changed as 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  $k_{\text{cat}}/K_m$  values for wild-type and E237Q. It is similar, about  $29.0 \text{ kJ mol}^{-1}$  ( $= -RT \ln 10^5$ ), for catalytic steps

involving fructose as leaving group and nucleophile. Making and breaking of a glucosidic bond to phosphate requires little stabilization from Glu-237 ( $< 10.7 \text{ kJ mol}^{-1}$ ), reflected by the mere  $< 70$ -fold rate difference for reactions catalyzed by wild-type and E237Q.

Enhancement of catalytic activity of E237Q was observed when conversion of  $\alpha$ 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 Glu-237 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 nucleophile, the  $k_{\text{cat}}$  values of E237Q measured with azide, acetate, and formate were significantly ( $\approx 4$ -fold) different and not the same, as 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  $k_{\text{cat}}$  values for hydrolysis and arsenolysis of  $\alpha$ 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  $\alpha$ -anomeric configuration.

Comparison of  $k_{\text{cat}}/K_{\text{m}}$  values for E237Q-catalyzed conversion of  $\alpha$ G1P and  $\alpha$ G1N<sub>3</sub> 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  $\alpha$ G1N<sub>3</sub> to wild-type and E237Q enzymes took place with identical efficiencies, suggesting that general acid catalysis from the carboxylic side chain of Glu-237 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  $\log(k_{\text{cat}}/K_{\text{m}})$  for glucosyl donor substrates ( $\alpha$ 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 ( $\text{p}K_1 \approx 5.3 - 5.8$ ;  $\text{p}K_2 \approx 7.2 - 7.9$ ) were comparable in these pH profiles and sufficiently far apart ( $\geq 1.7$  pH units) to treat them as separate ionizations. The pH dependence of  $k_{\text{cat}}/K_{\text{m}}$  for glucosyl donor reflects ionizations in free enzyme and substrate whereas that of  $k_{\text{cat}}$  mirrors variation of the rate-determining step with pH when the enzyme is fully saturated with substrate. Unlike  $\alpha$ G1P, sucrose does not ionize in the relevant pH range and therefore, pK values seen in pH profiles of  $\log(k_{\text{cat}}/K_{\text{m}})$  for 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  $\alpha$ G1P were similar, indicating that the pH dependence of the rate-determining step(s) was not altered in response to (1) variation of the glucosyl donor substrate, and (2) replacement of fructose by arsenate as nucleophile of the reaction. The pH profile of  $\log(k_{\text{cat}}/K_{\text{m}})$  for fructose followed an apparent single ionization of a group of  $\text{p}K = 5.8$  that

must be unprotonated for activity. Because the  $k_{\text{cat}}/K_{\text{m}}$  for fructose measures breakdown of the  $\beta$ -glucosyl-enzyme intermediate, it is clear that ionization due to the catalytic nucleophile Asp-196, 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  $\log(k_{\text{cat}}/K_{\text{m}})$  for arsenate decreased at high pH above an apparent  $pK$  of 8.3, suggesting that the deglucosylation 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.

$k_{\text{cat}}$  for the arsenolysis of  $\alpha$ 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.  $\log(k_{\text{cat}}/K_{\text{m}})$  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.

Asp-196 and Glu-237 are proposed to ionize in the free enzyme with  $pK$  values of about 5.6 and 7.2 respectively. Asp-196 must be unprotonated and Glu-237 protonated for catalysis to formation of the covalent intermediate. Considering the good leaving group ability of  $\alpha$ G1P and also the fact that mutation of Glu-237 did not cause a large decrease in  $k_{\text{cat}}/K_{\text{m}}$  for  $\alpha$ G1P, it is interesting that deprotonation of Glu-237 appears to dominate the high-pH dependence of the reaction with this glucosyl donor. Productive binding of  $\alpha$ G1P may require Glu-237 to be protonated. Enzyme glucosylation is suggested to control  $k_{\text{cat}}$ , 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  $k_{\text{cat}}$  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 enzyme-substrate complex. The significant upshift in the apparent  $\text{p}K$  of Glu-237 by approximately 0.4 – 0.7 pH units upon moving along the reaction coordinate from free enzyme to enzyme-glucosyl donor complex is most reasonably explained by an effect of hydrogen bonding, perhaps involving the glycosidic oxygen of the substrate, on apparent  $\text{p}K$ . By contrast, the apparent  $\text{p}K$  of Asp-196 seems to be not affected by substrate binding, fully consistent with the ionized side chain of the Asp functioning as catalytic nucleophile. Once the  $\beta$ -glucosyl-enzyme has been formed and the leaving group departed, the  $\text{p}K$  of Glu-237 is depressed by  $\approx 2$  pH units to a value of 5.8, compared with the  $\text{p}K$  of enzyme-substrate. It is probable that changes in active-site environment resulting from the absence of negative charge on Asp-196 in the covalent intermediate are responsible for the observed drop in  $\text{p}K$ . Modulation of the  $\text{p}K$  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 Glu-237 during phosphorolysis of sucrose, which the physiological reaction of *LmSPase*?

The pH dependence of  $k_{\text{cat}}/K_{\text{m}}$  for arsenate suggests that reaction of the glucosyl-enzyme with the anionic nucleophile takes place independently of the protonation state of Glu-237 but requires that another enzyme group with an apparent  $\text{p}K$  of 8.3 be protonated. The structure of unliganded *BaSPase* [6] has revealed His-234 (numbering of *BaSPase*) as candidate interaction partner with phosphate. Lys-199 is also contributed to sub-site +1 of the phosphorylase and might play a role in binding and positioning of the nucleophile. Very

recent structural studies of glucosylated *BaSPase* also implicate Arg-135, Asp-342, and Tyr-344 [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 Glu-237 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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**TABLES**
**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 <sup>\*</sup>250 mM sucrose, <sup>†</sup>50 mM phosphate, <sup>‡</sup>100 mM  $\alpha$ G1P, <sup>§</sup>75 mM (wild-type) and 150 mM (E237Q) arsenate, respectively, and <sup>||</sup>100 mM D-fructose. Results are from non-linear fits of eqn (1) to the experimental data.  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_{\text{m}}$  have S.E. values of  $\leq 6\%$  and  $\leq 13\%$ , respectively; n.d., not determined. Values of  $k_{\text{cat}}$  were obtained from specific enzyme activities (U/mg) using the factor 0.93 which includes a subunit molecular mass of 55,700 g/mole.

reaction type	varied substrate	wild-type		E237Q		$k_{\text{cat}} \text{ wild-type}/k_{\text{cat}} \text{ E237Q}$
		$k_{\text{cat}} \text{ (s}^{-1}\text{)}$	$k_{\text{cat}}/K_{\text{m}} \text{ (s}^{-1} \text{ mM}^{-1}\text{)}$	$k_{\text{cat}} \text{ (s}^{-1}\text{)}$	$k_{\text{cat}}/K_{\text{m}} \text{ (s}^{-1} \text{ mM}^{-1}\text{)}$	$(k_{\text{cat}}/K_{\text{m}} \text{ wild-type}/k_{\text{cat}}/K_{\text{m}} \text{ E237Q})$
phosphorolysis	phosphate <sup>*</sup>	195	20.5	n.d.	n.d.	n.d.
	sucrose <sup>†</sup>	165	28.7	$1.6 \cdot 10^{-3}$	$2.9 \cdot 10^{-4}$	$1 \cdot 10^5 \text{ (} 1 \cdot 10^5\text{)}$
	$\alpha$ G1N <sub>3</sub> <sup>†</sup>	$4.3 \cdot 10^{-3}$	$4.9 \cdot 10^{-5}$	$5.2 \cdot 10^{-3}$	$2.5 \cdot 10^{-3}$	0.8 (0.02)
arsenolysis	arsenate <sup>‡</sup>	170	26.5	24	0.39	7 (70)
	$\alpha$ G1P <sup>§</sup>	261	5.5	21	0.61	12 (9)
synthesis	$\alpha$ G1P <sup>  </sup>	91	5.3	$6.3 \cdot 10^{-4}$	0.23	$1 \cdot 10^5 \text{ (} 25\text{)}$
	D-fructose <sup>‡</sup>	72	3.4	n.d.	n.d.	n.d.

**Table 2****Kinetic parameters for the reaction of E237Q with  $\alpha$ 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.  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_{\text{m}}$  have S.E. values of  $\leq 5\%$  and  $\leq 4\%$ , respectively.

nucleophile	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_{\text{m}}$ (mM)	$k_{\text{cat}}/K_{\text{m}}$ ( $\text{mM}^{-1} \text{s}^{-1}$ )
azide	0.21	42	$5.0 \cdot 10^{-3}$
acetate	0.067	173	$3.9 \cdot 10^{-4}$
formate	0.051	137	$3.7 \cdot 10^{-4}$

**Table 3****pK-values from pH-profiles of  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_{\text{m}}$  for wild-type.**

pK-values were obtained from non-linear fits of the indicated eqn to  $\log(k_{\text{cat}})$  and  $\log(k_{\text{cat}}/K_{\text{m}})$ .

The fits of the data are illustrated in Figures 5 and 6.

	eqn	pK <sub>1</sub>	pK <sub>2</sub>
<b>phosphorolysis</b>			
$\log(k_{\text{cat}})$ (sucrose)	3	5.31 ± 0.06	7.90 ± 0.09
$\log(k_{\text{cat}}/K_{\text{m}})$ (sucrose)	3	5.75 ± 0.10	7.24 ± 0.11
<b>synthesis</b>			
$\log(k_{\text{cat}})$ (D-fructose)	3	5.33 ± 0.17	6.99 ± 0.11
$\log(k_{\text{cat}}/K_{\text{m}})$ (D-fructose)	2	5.84 ± 0.06	—
<b>arsenolysis</b>			
$\log(k_{\text{cat}})$ (arsenate)	3	5.49 ± 0.14	7.11 ± 0.13
$\log(k_{\text{cat}}/K_{\text{m}})$ (arsenate)	3	4.97 ± 0.10	8.30 ± 0.25
$\log(k_{\text{cat}})$ ( $\alpha$ G1P)	3	5.65 ± 0.13	7.60 ± 0.15
$\log(k_{\text{cat}}/K_{\text{m}})$ ( $\alpha$ G1P)	3	5.51 ± 0.36	7.17 ± 0.12

## FIGURE LEGENDS

**Figure 1: Reaction mechanisms of retaining (trans)glycosidases and catalytic mutants thereof.** (A) Double-displacement mechanism of an  $\alpha$ -retaining enzyme, which interconverts glycosides with the leaving groups  $R_1$ -OH and  $R_2$ -OH. In the case of sucrose phosphorylase (SPase)  $R_1$ -OH is D-fructose and  $R_2$ -OH is phosphate. (B) Two-step (Ping Pong BiBi) kinetic mechanism of SPase. (C) Functional complementation of an otherwise inactive mutant in which the acid/base catalyst (Asp or Glu) was replaced by a non-functional residue (here, Gln). Nu is an external nucleophiles such as azide, formate, acetate and halides.

**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 *Lm*SPase and E237Q.** (A) The sequences shown are *Lm*SPase (D90314), *Ba*SPase (AF543301), amylosucrase from *Neisseria polysaccharea* (*Np*ASase, AJ011781) and 4- $\alpha$ -glucantransferase from *Thermotoga maritima* (*Tm*GTase, AE000512). The alignment was performed with the Vector NTI program using the AlignX-modul with the PAM250 scoring matrix. Conserved amino acids are shaded in black (100% identity), grey (80-100% identity) and light grey (60-80% identity). (B) Lane 1, wild-type; lane 2, E237Q; lane 3, molecular mass standard. Staining of protein bands was done with Coomassie Blue.

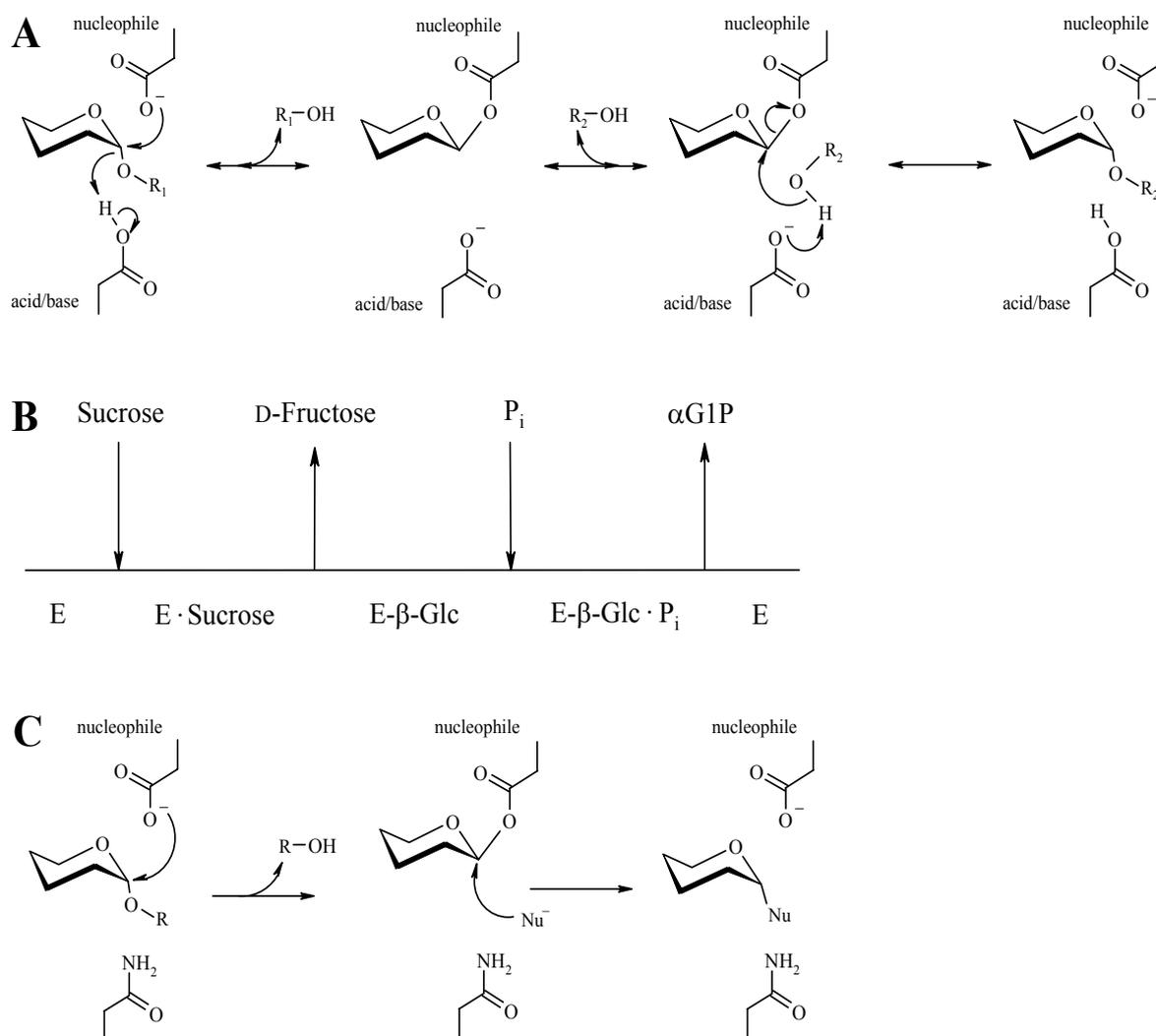
**Figure 3:  $^1\text{H}$  NMR monitoring of product formation during E237Q catalyzed  $\alpha$ G1P conversion in the presence of sodium acetate (A) and the corresponding reaction scheme (B).** The primary product is  $\alpha$ -glucose 1-acetic acid ester [ $\delta_{\text{H}}$  6.05 (1 H, d,  $J$  3.6 Hz,  $H$ -1), 2.14 (3 H, s, CO- $\text{CH}_3$ )]. Migration of the acetyl group and mutarotation lead to accumulation of 2- $O$ -acetyl- $\alpha$ -D-glucose [ $\delta_{\text{H}}$  5.32 (1 H, d,  $J$  3.8 Hz,  $H$ -1), 2.118 (3 H, s, CO- $\text{CH}_3$ )] and 2- $O$ -acetyl- $\beta$ -D-glucose [ $\delta_{\text{H}}$  4.76 (1 H, d,  $J$  8.1 Hz,  $H$ -1), 2.121 (3 H, s, CO- $\text{CH}_3$ ) not shown]. Consecutively, the  $\alpha$ - and  $\beta$ -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  $\alpha$ -anomeric protons cause signals between 5.25 p.p.m. and 5.20 p.p.m.. All  $\beta$ -anomeric proton signals are partly overlapped by the overwhelming HDO signal and not shown. 4- $O$ -Acetyl-D-glucose has not been formed in detectable amounts after 16 h.

**Figure 4:  $^1\text{H}$  NMR monitoring of product formation during E237Q catalyzed  $\alpha\text{G1P}$  conversion in the presence of sodium formate (A) and the corresponding reaction scheme (B).**  $\alpha\text{G1P}$  [ $\delta_{\text{H}}$  5.41 (1 H, dd,  $J$  7.2, 3.4 Hz,  $H-1$ )]. The primary product is  $\alpha$ -glucose 1-formic acid ester [ $\delta_{\text{H}}$  8.21 (1 H, s, CHO), 5.36 (1H, d,  $J$  3.8 Hz,  $H-1$ )]. Migration of the formyl group and mutarotation consequently lead to formation of small amounts of  $\alpha$ - and  $\beta$ -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  $\alpha$ -anomeric proton signal appears at 5.25 p.p.m. (indicated by asterisks). The further  $\alpha$ -anomeric and all  $\beta$ -anomeric proton signals are overlapped by other signals. 6-*O*-Formyl  $\alpha$ -D-glucose [ $\delta_{\text{H}}$  8.15 (1 H, s, CHO), 5.18 (1 H, d,  $J$  3.8 Hz,  $H-1$ ), 4.42 (dd, 12.7, 2.4 Hz, 1H, H 6a), 4.38 (dd, 12.7, 4.7 Hz, 1H, H 6b)] and 6-*O*-formyl  $\beta$ -D-glucose [ $\delta_{\text{H}}$  8.15 (1 H, s, CHO), 4.63 (1 H, d,  $J$  8.0 Hz,  $H-1$ ), 4.47 (1 H, dd,  $J$  12.4, 1.9 Hz,  $H-6a$ ), 4.32 (1 H, dd,  $J$  12.7, 5.3 Hz,  $H-6b$ )] have been accumulated in detectable amounts prior to hydrolysis to formate and free glucose. The signal at 8.24 p.p.m. is a  $^{13}\text{C}$ -satellite of the formate signal at 8.41 p.p.m..

**Figure 5: pH profiles for  $k_{\text{cat}}$  (A) and  $k_{\text{cat}}/K_{\text{m}}$  (B) of the phosphorolysis (●) and synthesis (○) reaction catalyzed 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 (3) to the data as described in the Experimental section.

**Figure 6: pH profiles for  $k_{\text{cat}}$  (A) and  $k_{\text{cat}}/K_{\text{m}}$  (B,C) of the arsenolysis of  $\alpha\text{G1P}$  catalyzed 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 (■).  $k_{\text{cat}}/K_{\text{m}}$  values were corrected for the fraction of protonated  $\alpha\text{G1P}$  (B) or arsenate (C), respectively. Solid lines are fits of eqn (2) or (3) to the data as described in the Experimental section. Dotted lines show the trend of the uncorrected data of  $k_{\text{cat}}/K_{\text{m}}$  for the wild-type (▼) and the E237Q mutant (▽).

Figure 1



**Figure 2**

**A**

*Lm*SPase : LKAEIILPE<sup>237</sup>IHEHYSI

*Ba*SPase : RGLEIILIE<sup>232</sup>VHSYYKK

*Np*ASase : PAVFFKSE<sup>336</sup>AIVHPDQ

*Tm*GTase : LKGIFLAE<sup>216</sup>IWAEARM

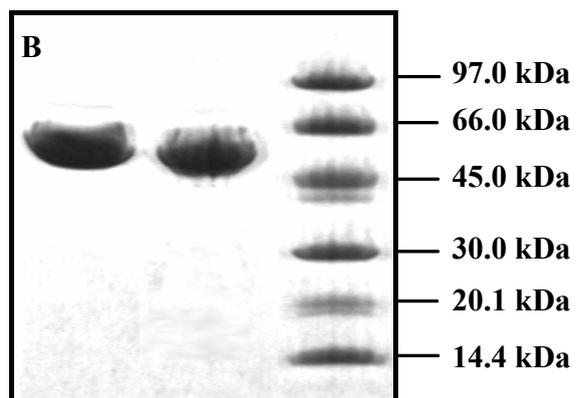




Figure 4

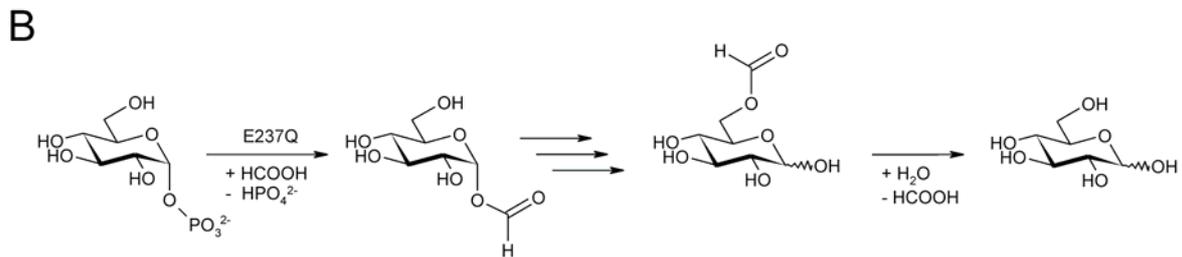
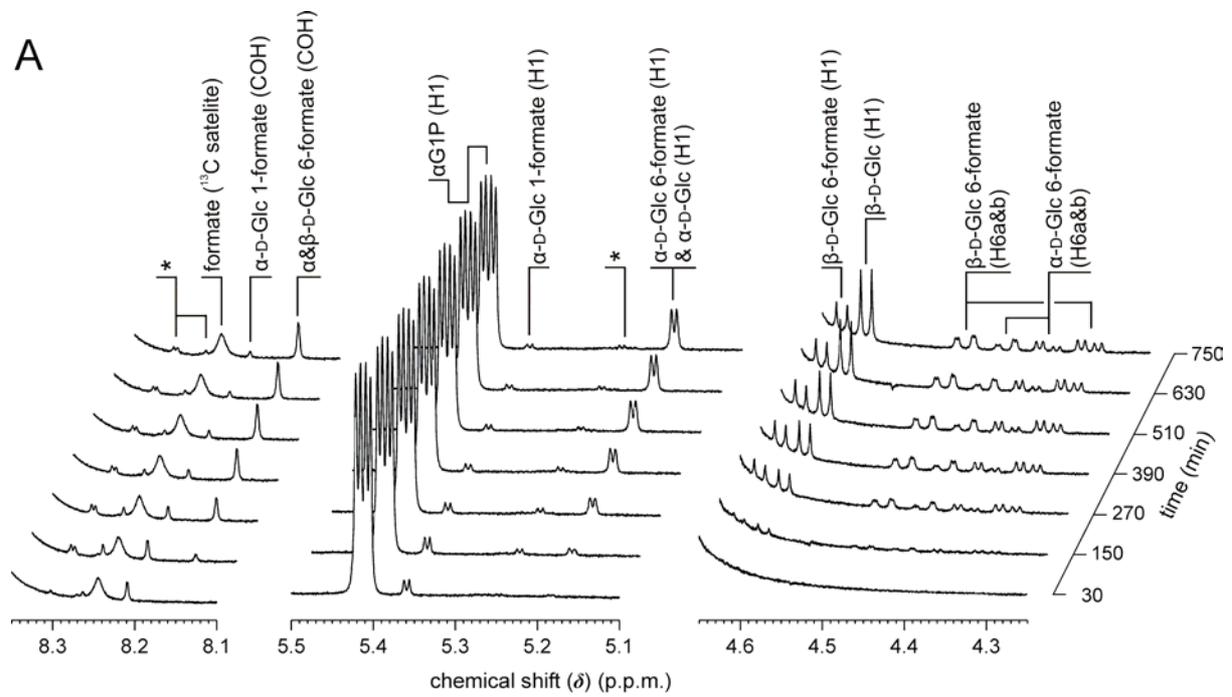


Figure 5

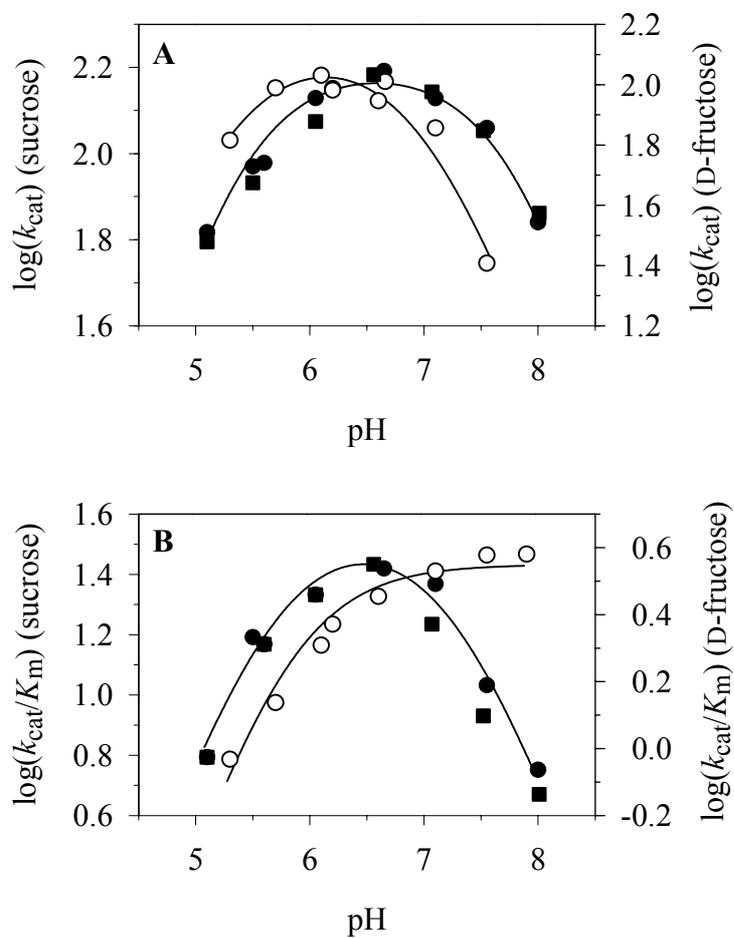


Figure 6

