Mechanistic consequences of replacing the active-site nucleophile Glu-358 in *Agrobacterium* sp. β -glucosidase with a cysteine residue

Sherry L. LAWSON, R. Antony J. WARREN and Stephen G. WITHERS¹

Protein Engineering Network of Centres of Excellence and the Departments of Chemistry and Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z1

Retaining glycosidases achieve the hydrolysis of glycosidic bonds through the assistance of two key active-site carboxyls. One carboxyl functions as a nucleophile/leaving group, and the other acts as the acid–base catalyst. It has been suggested that a cysteine residue could fulfil the role of the active site nucleophile [Hardy and Poteete (1991) *Biochemistry* 30, 9457–9463]. To test the validity of this proposal, a kinetic evaluation was conducted on the active-site nucleophile cysteine mutant (Glu-358 $\rightarrow$ Cys) of the retaining β -glucosidase from *Agrobacterium* sp. The Glu-358 $\rightarrow$ Cys mutant was able to complete the first step (glyco-

sylation) of the enzymic mechanism, forming a covalent glycosyl-enzyme intermediate, but the rate constant for this step was decreased to $1/10^6$ of that of the native enzyme. The subsequent hydrolysis (deglycosylation) step was also severely affected by the replacement of Glu-358 with a cysteine residue, with the rate constant being depressed to $1/10^7$ or less. Thus Cys-358 functions inefficiently in both the capacity of catalytic nucleophile and leaving group. On the basis of these results it seems unlikely that the role of the active-site nucleophile in retaining glycosidases could successfully be filled by a cysteine residue.

INTRODUCTION

Glycosidases hydrolyse glycosidic bonds, either with retention or inversion of anomeric configuration. Inverting glycosidases are believed to employ a direct displacement mechanism, whereas retaining glycosidases are thought to act via the double displacement mechanism illustrated in Scheme 1 [1,2]. First proposed by Koshland, a key feature of the double displacement mechanism is the covalent glycosyl-enzyme intermediate. The formation and hydrolysis of this covalent intermediate occur via oxocarbenium ion-like transition states, with the assistance of two key active site carboxyls. In the glycosylation step, one carboxyl acts as the active-site nucleophile, attacking the anomeric carbon of the substrate to displace the aglycone (leaving group) and form the covalent intermediate. This same residue acts as a leaving group in the subsequent hydrolysis (deglycosylation) step. The other carboxyl has the dual role of acid–base catalyst.

Hardy and Poteete [3] proposed that cysteine could fulfil the role of the active-site nucleophile in retaining glycosidases. This proposal was based on site-directed mutagenesis work done on T4 lysozyme, a glycosidase that was assumed to act via a retaining mechanism. Nearly wild-type activity was observed on mutating Asp-20, the putative catalytic nucleophile, to a cysteine residue, suggesting that cysteine could function effectively as the catalytic nucleophile. However, recent work done by Kuroki et al. [4] revealed that T4 lysozyme is actually an inverting glycosidase, not a retaining enzyme as originally believed. Therefore, Asp-20 cannot act as the active-site nucleophile, and its role in catalysis is unclear.

Despite this new finding, the possibility still remains that a cysteine residue could function as the active-site nucleophile in retaining glycosidases, given its inherent strong nucleophilicity. Indeed, Nature has used the cysteine residue in this capacity in several other enzyme systems: the cysteine proteases [5], tyrosine

phosphatases [6], thiolase [7] and glyceraldehyde-3-phosphate dehydrogenase [5,8] are some examples. Consequently we were interested in testing the feasibility of a cysteine residue functioning as the catalytic nucleophile in the well-characterized family 1 [9] β -glucosidase from *Agrobacterium* sp. [10,11]. (In the rest of the paper the enzyme will be referred to as *Agrobacterium* sp. β -glucosidase. The bacterium was originally classified as *Alcaligenes faecalis* [12].)

Agrobacterium sp. β -glucosidase catalyses the hydrolysis of cellobiose and β -glucosides with net retention of anomeric configuration [10]. The enzyme is believed to employ the double displacement mechanism illustrated in Scheme 1. As stated above, the key catalytic residues in this mechanism are two active-site carboxyls. The active-site nucleophile has been identified as Glu-358 by using the mechanism-based inactivator 2,4-dinitrophenyl 2-deoxy-2-fluoro- β -D-glucopyranoside (2F-DNPG) [13]. Results obtained from mutagenesis studies were consistent with this assignment. Replacement of Glu-358 with residues incapable of functioning as nucleophiles, such as asparagine, glutamine, glycine or alanine, resulted in marked decreases in activity (to $1/10^4$ or less) [14,15]. In contrast, replacing Glu-358 with its shortened analogue, an aspartic residue, caused only a $1/2500$ decrease in the rate constant for the glycosylation step, emphasizing the importance of a correctly positioned carboxyl at this position [15]. The acid–base catalyst of *Agrobacterium* sp. β -glucosidase has been identified as Glu-170, an assignment based on a detailed kinetic analysis of the Glu-170 $\rightarrow$ Gly mutant [16].

Whereas the three-dimensional structure of *Agrobacterium* sp. β -glucosidase is still undetermined, those of two related family 1 β -glucosidases, white clover β -glucosidase [17] and *Lactococcus lactis* 6-phospho- β -galactosidase [18], have been solved. Inspection of these two structures revealed that the residues equivalent to Glu-358 and Glu-170 are suitably positioned within the enzymes' active sites to fulfil the respective roles of catalytic nucleophile and acid–base catalyst. Thus these results provide

Abbreviations used: ES–MS, electrospray MS; 2F-DNPG, 2,4-dinitrophenyl 2-deoxy-2-fluoro- β -D-glucopyranoside; DNP, 2,4-dinitrophenolate; 2,4-DNPG, 2,4-dinitrophenyl β -D-glucopyranoside; PITC 311, 4-(3-pyridinylmethylaminocarboxypropyl)phenyl isothiocyanate.

¹ To whom correspondence should be addressed.

Scheme 1 Proposed double displacement mechanism for a retaining glycosidase (shown for a β -glucosidase, where R can be either a glucose residue or an alkyl or aryl group)

additional support for the assignment of Glu-358 and Glu-170 as the active-site nucleophile and the acid–base catalyst respectively of *Agrobacterium* sp. β -glucosidase.

This study describes the detailed kinetic evaluation of the active-site nucleophile cysteine mutant (denoted Glu-358 $\rightarrow$ Cys) of *Agrobacterium* sp. β -glucosidase. The results of this investigation should provide insight into the possibility of a cysteine residue acting as the catalytic nucleophile in retaining glycosidases.

EXPERIMENTAL

General

The compounds 2,4-DNPG and 2,4-dinitrophenyl 2-deoxy-2-fluoro- β -D-glucopyranoside (2F-DNPG) were kindly synthesized by Dr. Mark Namchuk by published procedures [11,19]. Pepsin was purchased from Boehringer Mannheim; all other chemicals and buffer materials were obtained from the Sigma Chemical Company.

All enzyme assays and kinetic studies, unless stated otherwise, were done with 1 cm pathlength micro black quartz cuvettes, in either a Unicam 8700 or a Pye Unicam PU 8800 UV/Vis

spectrophotometer, each of which was equipped with a circulating-water bath and thermostatically controlled cuvette holders. Thiol titrations were performed with plastic cuvettes (1 cm path length) in a Pye Unicam PU 8800 UV/Vis spectrophotometer. The CD spectra of native β -glucosidase and the Glu-358 $\rightarrow$ Cys mutant were obtained in a Jasco J-710 spectropolarimeter with a protein concentration of 0.1 mg/ml. The CD spectra, typically acquired from 260 to 200 nm, were corrected for the buffer background before normalization for the protein concentration and the optical pathlength.

All electrospray MS (ES–MS) experiments were done with an HPLC–ES–MS setup consisting of a microbore HPLC (Michrom UMA) connected on-line to a Sciex API III MS as described by Hess et al. [20]. Intact β -glucosidase samples (10–20 μ g) were injected onto a microbore PLRP column (1 mm $\times$ 50 mm) and eluted with a gradient of 20–100% solvent B over 10 min followed by 100% solvent B over 2 min [solvent A was 0.05% trifluoroacetic acid/2% (v/v) acetonitrile in water; solvent B was 0.045% trifluoroacetic acid/80% (v/v) acetonitrile in water]. For the proteolytic digests, a Reliasil C₁₈ column (1 mm $\times$ 150 mm) was used with a gradient of 0–60% solvent B over 20 min followed by 100% solvent B for 2 min. A post-column flow splitter was used to introduce 15% of the HPLC eluate into the MS, via an ion spray ion source, whereas the remaining 85% was recovered.

Enzyme production and purification

Native *Agrobacterium* sp. β -glucosidase and the Glu-358 mutants used for this work were cloned and expressed in *Escherichia coli* [14,21]. The mutagenesis, the detection of β -glucosidase mutants and the production of the mutant enzymes and native β -glucosidase were kindly performed by Dr. Don Trimbur (Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada) as described previously [14]. The oligonucleotide primers used in the mutagenesis were: for Glu-358 $\rightarrow$ Cys, 5'-TAC ATC ACC **TGC** AAC GGC GCC TGC-3', and for Glu-358Gly, 5'-TAC ATC ACC **GGC** AAC GGC GCC TGC-3' (the bold letters indicate the mutated codon).

The isolation of native β -glucosidase and the Glu-358 mutants was performed as described previously [11,15]. During purification the presence of the mutant proteins was determined by SDS/PAGE in addition to Western blot analysis [22].

Thiol titrations

The number of free thiol groups was determined with the method of Ellman [23] as follows. To a solution of 0.2 mM 5,5'-dithiobis-(2-nitrobenzoic acid)/6 M guanidine hydrochloride/20 mM Hepes/1 mM EDTA (pH 7.4), pre-equilibrated at 25 $^{\circ}$ C, was added enzyme (2.5–5 μ M). The absorbance at 412 nm, due to the released 2-nitro-5-thiobenzoate, was monitored over a 30 min period. The concentration of free thiol groups was calculated from the net ΔA_{412} , corrected for a buffer blank, with the absorption coefficient determined from a cysteine standard curve generated under the same conditions (ϵ_{412} values typically ranged from 13 to 15 mM⁻¹·cm⁻¹). Division of this obtained value by the protein concentration yielded the number of free thiol groups.

Glu-358 $\rightarrow$ Cys + 2,4-DNPG: determination of kinetic parameters

Various concentrations of 2,4-DNPG in 50 mM sodium phosphate buffer, pH 6.8, were warmed to 37 $^{\circ}$ C in 1 mm path-length quartz cells. The reaction was initiated by the addition of enzyme (final concentration 1.9 mg/ml; 0.039 mM) or buffer and sub-

strate hydrolysis was monitored by measuring the release of 2,4-dinitrophenol (DNP) at 400 nm ($\Delta\epsilon$ 10.91 mM⁻¹·cm⁻¹) [11]. Reaction rates were measured at six different substrate concentrations ranging from 0.4-fold to 8-fold the estimated K_d value.

The data for A_{400} versus time obtained were corrected for the absorbance due to enzyme, 2,4-DNPG and the spontaneous hydrolysis of 2,4-DNPG. The corrected data were then fitted to an equation describing a first-order reaction followed by a steady state, thus yielding the pre-steady-state pseudo first-order rate constant (k_{obs}), the steady-state rate and the y intercept (net ΔA_{400}) at each substrate concentration. The pre-steady-state kinetic parameters, K_d and k_2 , were determined from these k_{obs} values by direct fitting to eqn. (1):

$$k_{obs} = k_2[S]/(K_d + [S]) \quad (1)$$

Direct fitting of the results for steady-state rate versus substrate concentration to the Michaelis–Menten equation yielded the steady-state kinetic parameters K_m and k_{cat} . The program GraFit [24] was used for the fitting of all data and the determination of standard error values.

Glu-358 → Cys + 2F-DNPG: contamination check

To determine whether the observed steady-state activity for the Glu-358 → Cys mutant was real or due to a contaminant the following experiment was performed. The enzyme (15 mg/ml; 0.3 mM) was incubated at 37 °C with either 0 or 0.02 mM 2F-DNPG (0.07 equiv.) in 50 mM sodium phosphate, pH 6.8. At intervals, enzyme activity was determined by assaying a 30 μ l aliquot of the inactivation mixture with 0.3 mM 2,4-DNPG (170 μ l, pH 6.8, 37 °C) and following the release of DNP at 400 nm.

Treatment of Glu-358 → Cys and Glu-358 → Gly with excess 2,4-DNPG: ES–MS analysis and thiol titrations

The Glu-358 → Cys mutant (3.6 mg/ml; 0.07 mM) was incubated with 5.2 mM 2,4-DNPG (74-fold molar excess)/1 mM EDTA/50 mM sodium phosphate buffer (pH 7.0) at 37 °C for 24 h. An aliquot was removed for ES–MS analysis and the remaining reaction mixture was loaded on a Sephadex G-25 column (to remove any unreacted 2,4-DNPG and the DNP released). After elution of the column with 50 mM sodium phosphate buffer, pH 7.0, the appropriate fractions were pooled and the protein concentration was determined from A_{280} measurements (ϵ_{280} 2.184 mg·ml⁻¹·cm⁻¹) [11]. A thiol titration was then performed on the column-purified colourless Glu-358 → Cys mutant.

A control experiment was run simultaneously in which the inactive Glu-358 → Gly mutant was also treated with a 74-fold molar excess of 2,4-DNPG under the above conditions. The treated enzyme was subjected to ES–MS analysis.

ES–MS analysis of digested glucosylated Glu-358 → Cys

Glucosylated Glu-358 → Cys was prepared by incubating the mutant with a 74-fold molar excess of 2,4-DNPG (pH 7.0, 37 °C, 24 h) as described in the previous procedure. Samples (0.86 mg/ml) of the glucosylated Glu-358 → Cys and a control, exposed to the same conditions in the absence of 2,4-DNPG, were digested by incubation with pepsin (0.016 mg/ml) in 50 mM sodium phosphate buffer, pH 2, at room temperature for 1 h.

The resulting peptide mixtures were separated by reverse-phase HPLC, with ES–MS as a detector. ES–MS analysis was performed in the single-quadrupole mode with the quadrupole

mass analyser scanning over an m/z range 300–2400. A comparative analysis of the HPLC–ES–MS data obtained for the glucosylated sample and its control resulted in the identification of the HPLC peak containing the glucosylated peptide. The labelled peptide was subsequently collected via the post-column flow splitter with the mass spectrometer, operating in the single-quadrupole mode, as a detector. This peptide was further purified on the Reliasil C₁₈ column by using a shallower gradient (0–20 % solvent B over 50 min).

The amino acid sequence of the isolated glucosylated peptide was determined with a modified Edman degradation procedure as described previously [25]. The peptide was degraded, in a stepwise manner, with the novel Edman sequencing reagent 4-(3-pyridinylmethylaminocarboxypropyl)phenyl isothiocyanate (denoted PITC 311). The 311 phenylthiohydantoin-derivatized amino acids were separated by reverse-phase HPLC and then analysed by ES–MS. The mass spectrometer was operated in the single-quadrupole mode with the quadrupole mass analyser scanning over a m/z range 300–850.

Denaturation of glucosylated Glu-358 → Cys with urea

Glucosylated Glu-358 → Cys (prepared as described above) and a control sample (0.12 mg/ml) were incubated at 40 °C with various concentrations of freshly prepared urea (0–8 M) in 35 mM Hepes buffer, pH 7.5. At intervals (1, 4 and 8 h) CD spectra were acquired from 260 to 200 nm. The data were corrected for the buffer background and normalized for the protein concentration and the optical path-length.

RESULTS AND DISCUSSION

Preliminary characterization of native β -glucosidase and the Glu-358 → Cys mutant

Native β -glucosidase and the Glu-358 → Cys mutant were subjected to ES–MS analysis, thiol titrations and CD analysis. For native enzyme and Glu-358 → Cys, the molecular masses were determined as 51192 ± 2 (S.D.) Da and 51171 ± 6 (S.D.) Da respectively. The observed mass decrease of 21 ± 8 Da, for the Glu-358 → Cys mutant relative to the native enzyme, is as expected for the replacement of a glutamic residue by cysteine (the theoretical mass decrease is 26 Da). The thiol titration results were also consistent with the introduction of a cysteine residue at position 358 in Glu-358 → Cys. The Cys mutant contained 7.18 ± 0.25 free cysteine residues, as opposed to the native enzyme, which contained only 6.07 ± 0.15 cysteine residues. Very similar CD spectra were obtained for the two enzymes, suggesting that the mutation had not grossly altered the conformation of the enzyme (results not shown).

Kinetic evaluation of the Glu-358 → Cys mutant

The replacement of Glu-358 with a cysteine residue changes the length, nucleophilicity and leaving-group ability of the side chain at the active-site nucleophile position of *Agrobacterium* sp. β -glucosidase. Because a thiol group is a reactive nucleophile, one might expect the shortened side chain of cysteine still to function effectively as a nucleophile, as long as it can reach the anomeric carbon of the substrate. This would seem likely because the Glu-358 → Asp mutant was catalytically active. However, the thiolate side chain should serve as a relatively poor leaving group when compared with the carboxyl side chain of a glutamic residue.

Enzymic activity could therefore be affected in several possible ways as a result of this substitution. If the shortened thiol side chain were too far removed or sterically hindered to make the nucleophilic attack on the substrate, the Cys mutant would be

Scheme 2 Hydrolysis of 2,4-DNPG (1) by the Glu-358 → Cys mutant

The proposed reaction scheme is based on the double displacement mechanism illustrated in Scheme 1. The rate constants k_2 and k_3 are for the glycosylation and deglycosylation steps respectively.

inactive. Alternatively, if the cysteine side chain could reach the anomeric carbon of the substrate, one might expect the Cys mutant to hydrolyse substrate via the mechanism shown in Scheme 2. The strong nucleophilicity of the cysteine residue's side chain should result in the formation of the covalent glycosyl-enzyme intermediate. However, the subsequent hydrolysis of this intermediate to yield free enzyme should be hindered by the poor leaving-group ability of the cysteine residue's side chain, resulting in the accumulation of the intermediate and a burst of release of phenolate.

The Glu-358 → Cys mutant (0.039 mM) was incubated with various concentrations (0.10–2.1 mM) of the substrate 2,4-DNPG (shown as structure 1 in Scheme 2) and the release of DNP (structure 2 in Scheme 2) monitored at 400 nm. Shown in Figure 1 are the absorbance–time data obtained for the reaction of 0.26 mM 2,4-DNPG with 0.039 mM enzyme. As can be seen, the release of DNP occurs as an initial burst followed by a steady state. From the magnitude of the burst (observed ΔA_{400} , 0.044 ± 0.0016 ; expected ΔA_{400} , 0.043), it was determined that 1 mol of DNP is released per mol of enzyme. A full-sized DNP burst was observed at all substrate concentrations tested, with the exception of 0.10 mM 2,4-DNPG. At this decreased substrate concentration, only approx. 0.6 equiv. of DNP was released.

The fact that a full-sized burst of DNP is released when Glu-358 → Cys is treated with 0.26 mM 2,4-DNPG clearly indicates that the observed pre-steady-state activity is associated with the Cys mutant and is not the result of a contaminating enzyme. Such a conclusion is reached because one full equivalent of DNP could be released only if Glu-358 → Cys itself is undergoing this reaction. The rate measured must therefore be that of this mutant reacting with 2,4-DNPG. To determine whether the observed steady-state activity is real or due to a contaminant, an inactivation study was performed with 2F-DNPG. Treatment of the mutant (0.3 mM) with 2F-DNPG (0.02 mM) resulted in a 70% decrease in the steady-state rate in 26 h. Because only 0.07 equiv. of inactivator was used, a 7% decrease in the steady-

Figure 1 Hydrolysis of 2,4-DNPG (0.26 mM) by the Glu-358 → Cys mutant (0.039 mM)

The results shown have been corrected for the absorbance due to enzyme, 2,4-DNPG and the spontaneous hydrolysis of 2,4-DNPG.

state rate would be expected if this activity were due solely to Glu-358 → Cys. Thus the observation of a 70% decrease in activity indicates that 63% ($70\% - 7\%$) of the observed steady-state activity for Glu-358 → Cys is due to a contaminant (possibly either low levels of native β -glucosidase or another active mutant). A parallel experiment was run in which native enzyme (72 pM, the estimated concentration that would produce the steady-state activity shown in Figure 1) was also incubated with 2F-DNPG (0.02 mM). Complete inactivation of native β -glucosidase was observed in 1 min. This suggests that the contaminant is most probably not native enzyme but some other mutant because a much longer incubation period (26 h) was

required to achieve the observed inactivation in the Glu-358 $\rightarrow$ Cys mutant.

Analysis of the absorbance–time data obtained for 2,4-DNPG with Glu-358 $\rightarrow$ Cys yielded values for the pre-steady-state pseudo-first-order rate constants (k_{obs}) and the steady-state rates at each concentration of 2,4-DNPG. From these results, values for the pre-steady-state kinetic parameters of $K_d = 0.3 \pm 0.07$ mM and $k_2 = (55 \pm 4.5) \times 10^{-5} \text{ s}^{-1}$ were determined. The corresponding values of K_d and k_2 for the hydrolysis of 2,4-DNPG by native β -glucosidase (at 5 °C) had previously been determined as 0.65 ± 0.022 mM and $1300 \pm 21 \text{ s}^{-1}$ respectively [26]. Replacing the active-site nucleophile Glu-358 with a cysteine residue therefore had a marked effect on the glycosylation step of the double displacement mechanism (see Scheme 2), the rate constant for the glycosylation step (k_2) being decreased to $1/(2 \times 10^6)$ or less. These experiments were conducted at pH 7.0, conditions under which Glu-358 in the native enzyme is expected to be deprotonated, yet the Cys residue at this position in the mutant might well remain protonated. The rate difference at a higher pH value therefore might well be considerably less. Because the cysteine residue's side chain should function effectively as a nucleophile, this decrease in k_2 is most probably due to the poor positioning of the side chain for attack on the substrate.

The insertion of a cysteine residue at the active-site nucleophile position also had a drastic impact on the deglycosylation step of the double displacement mechanism. The apparent k_{cat} and K_m values for the Glu-358 $\rightarrow$ Cys mutant with 2,4-DNPG were determined as $(35 \pm 1.8) \times 10^{-6} \text{ s}^{-1}$ and less than 0.10 mM respectively. The results of the 2F-DNPG inactivation study indicated that at least 63 % of the steady-state activity (and therefore k_{cat}) is due to a contaminant, whereas only approx. 37 % is attributed to the Glu-358 $\rightarrow$ Cys mutant, yielding a corrected k_{cat} for Glu-358 $\rightarrow$ Cys of $(130 \pm 6.7) \times 10^{-7} \text{ s}^{-1}$. This value represents a maximum because it is always possible that an active low-level contaminating enzyme might not be inactivated by 2F-DNPG. For native β -glucosidase the corresponding k_{cat} and K_m for 2,4-DNPG are $130 \pm 3 \text{ s}^{-1}$ and 0.022 ± 0.0012 mM respectively, and the deglycosylation step was shown to be rate-limiting [26]. Therefore substitution of a cysteine for a glutamic residue at the active-site nucleophile position results in a decrease in the k_{cat} (k_3) value to $1/10^7$ or less. Thus, as expected on the basis of chemical reactivity alone, the cysteine residue's side chain functions as a relatively poor leaving group when compared with the carboxyl side chain of a glutamic residue.

Treatment of the Glu-358 $\rightarrow$ Cys and Glu-358 $\rightarrow$ Gly mutants with excess 2,4-DNPG: ES–MS analysis and thiol titrations

Glu-358 $\rightarrow$ Cys, and the inactive Glu-358 $\rightarrow$ Gly mutant, were incubated with a 74-fold molar excess of 2,4-DNPG (37 °C, 24 h) and then subjected to ES–MS analysis. After treatment with 2,4-DNPG, the Glu-358 $\rightarrow$ Cys mutant (molecular mass 51176 Da) was completely converted to a new species with a molecular mass of 51340 Da (results not shown). This mass increase of 164 Da is as expected for the covalent attachment of a single glucosyl moiety (molecular mass 162 Da) and confirms the trapping of the covalent glycosyl-enzyme intermediate. No such mass increase was seen for Glu-358 $\rightarrow$ Gly exposed to the same treatment, suggesting that the glucosyl moiety is indeed attached to Cys-358 in the treated Glu-358 $\rightarrow$ Cys mutant. The thiol titration results were consistent with this proposal, with Glu-358 $\rightarrow$ Cys possessing approximately one less free cysteine residue after treatment with 2,4-DNPG (treated and untreated Glu-358 $\rightarrow$ Cys contained 5.64 ± 0.06 and 7.11 ± 0.02 free cysteine residues respectively).

Identification of the glucosylated residue in 2,4-DNPG-treated Glu-358 $\rightarrow$ Cys

To provide additional proof that Cys-358 was indeed the site of glucosylation in 2,4-DNPG-treated Glu-358 $\rightarrow$ Cys, the treated mutant and a control were digested with pepsin and the glucosylated peptide was identified by HPLC–ES–MS. Shown in Figures 2(a) and 2(b) are the HPLC traces of the digested, treated mutant and its control. A comparative analysis of the mass spectra obtained for the two HPLC traces showed that the only significant differences between the treated Glu-358 $\rightarrow$ Cys digest and its control were in HPLC peak areas 1 and 4. Examination of the mass spectra of HPLC peak area 1 [see Figures 2(c) and 2(d); for Figure 2(d) no peaks were detected at $m/z > 900$] revealed the presence of a unique peak (m/z 1006.5) in the digest of treated Glu-358 $\rightarrow$ Cys. Similarly, a comparison of the mass spectra of HPLC peak 4 (see Figures 2e and 2f) revealed a peak at m/z 844.5 unique to the digested control sample. These two unique peaks, differing in mass by 162 Da ($z = +1$; the mass of a glucosyl moiety is 162 Da), are attributed to the glucosylated peptide and its non-glucosylated counterpart. The glucosylated peptide (m/z 1006.5), being more polar, is retained less by the reverse-phase HPLC column (retention time 21–24 min) than the corresponding non-glucosylated peptide (m/z 844.5) (retention time approx. 27 min).

A computer search of the sequence of the Glu-358 $\rightarrow$ Cys mutant for peptides with a mass of 844.5 ± 1 Da (the mass of the non-glucosylated peptide) yielded 17 candidates, five of which contained Cys-358. To identify the glucosylated peptide unambiguously and confirm that the glucosyl moiety was attached to Cys-358, the labelled peptide (m/z 1006.5) was purified and sequenced with a modified Edman degradation procedure in which the novel sequencing reagent PITC 311 [25] replaced the normal phenyl isothiocyanate and the released 311 phenylthiohydantoin-derivatized amino acids were identified by HPLC–ES–MS. The isolated glucosylated peptide was subjected to seven rounds of the modified Edman degradation procedure. The results, summarized in Table 1, identified the peptide sequence as YITXNGA (where X corresponds to an unknown amino acid). Examination of the mass spectrum obtained for cycle 4 revealed a unique peak at m/z 577.0 that was absent from cycle 3 (results not shown). This mass does not correspond to that expected for the 311 phenylthiohydantoin derivative of any known amino acid, but is the exact mass predicted for a derivatized glucosylated cysteine residue (see Figure 3).

The determined sequence of the glucosylated peptide in 2,4-DNPG-treated Glu-358 $\rightarrow$ Cys, YITXNGA, aligns with residues 355–361 of the published sequence of *Agrobacterium* β -glucosidase [27]. Therefore the unknown amino acid (X), which exhibited a molecular mass consistent with a glucosylated cysteine residue, corresponds to position 358, the active-site nucleophile position, demonstrating that Cys-358 forms a stabilized covalent glycosyl-enzyme intermediate with this substrate.

Denaturation of glucosylated Glu-358 $\rightarrow$ Cys with urea

Previous work had indicated that the presence of a covalently bound sugar in the active site of a glycosidase enhances its stability [19]. In native β -glucosidase the presence of a 2-fluoroglucosyl moiety covalently bound to Glu-358 resulted in considerable stabilization (at least 12.5 kJ/mol) to the chemical denaturant urea [28]. Whereas exposure to 8 M urea (at 40 °C) caused essentially complete unfolding of native β -glucosidase, no significant unfolding (less than 10 %) was observed for the glucosylated enzyme. This increased stability is attributed to the

Figure 2 ES-MS analysis of digested, 2,4-DNPG-treated Glu-358 → Cys and its control

(a) HPLC trace of digested, treated Glu-358 → Cys; (b) HPLC trace of the digested Glu-358 → Cys control; (c) mass spectrum of HPLC peak area 1 in (a) (treated Glu-358 → Cys); (d) mass spectrum of HPLC peak area 1 in (b) (control; note that no peaks were detected at $m/z > 900$); (e) mass spectrum of HPLC peak 4 in (a) (treated Glu-358 → Cys); (f) mass spectrum of HPLC peak 4 in (b) (control).

Table 1 Results of the modified Edman degradation of the glucosylated peptide

The identification of each amino acid was made by comparing the mass and chromatographic retention time of the released 311 phenylthiohydantoin (PTH) derivative with the values obtained for authentic 311 PTH standards. Xaa is an unknown amino acid.

Cycle	m/z of 311 PTH derivative	Identified amino acid
1	475.0	Tyr
2	425.2	Ile
3	413.2	Thr
4	577.0	Xaa
5	426.2	Asn
6	369.0	Gly
7	383.2	Ala

hydrogen-bonding interactions that occur between the bound sugar moiety and the active-site residues.

To test whether the presence of a sugar moiety covalently bound to Cys-358 also confers stability, a urea denaturation study was conducted on Glu-358 → Cys and the glucosylated

Figure 3 Structure of the 311 phenylthiohydantoin derivative of glucosylated cysteine

mutant. The two enzymes were incubated with various concentrations of urea (at 40 °C) and the extent of protein unfolding was monitored by CD. As illustrated in Figure 4, the glucosylated mutant was more resistant to denaturation by urea. For Glu-358 → Cys and glucosylated Glu-358 → Cys, respective urea concentrations of approx. 5 and approx. 6 M were required to achieve 50 % unfolding ($[\theta]/[\theta]_0 = 0.5$). Thus for both the native enzyme and Glu-358 → Cys, the presence of a sugar moiety covalently bound to the side chain at the active-site nucleophile position

Figure 4 Urea denaturation curves for the Glu-358 $\rightarrow$ Cys mutant (▼) and the glucosylated mutant (△)

The $[\theta]$ values shown were determined at 222 nm after an incubation time of 1 h (no further unfolding was observed at incubation times longer than 1 h); $[\theta]_0$ is the mean residue ellipticity at 0 M urea.

results in enhanced stability to denaturation by urea. This suggests that in the glucosylated Glu-358 $\rightarrow$ Cys mutant, the sugar is binding in the active site in a manner similar to that of the normal glycosyl-enzyme intermediate.

Conclusion

Replacement of the catalytic nucleophile Glu-358 in *Agrobacterium* β -glucosidase by a cysteine residue yields an enzyme of very low activity that nevertheless follows the same double displacement mechanism. Rate constants for the formation and hydrolysis of the glycosyl-enzyme intermediate are decreased to $1/10^6$ and $1/10^7$ or less respectively, compared with those of the wild-type enzyme. Undoubtedly, much of this rate decrease is a consequence of the shorter side chain of the cysteine residue, resulting in poor positioning of this key residue. In addition, the relatively poor leaving-group ability of the thiol must result in considerable rate decrease in the deglycosylation step as revealed through the greater effect on that step. On the basis of these results it seems unlikely that a cysteine residue could effectively function as the active-site nucleophile in a retaining glycosidase.

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REFERENCES

- Koshland, D. E. (1953) *Biol. Rev.* **28**, 416–436
- Sinnott, M. L. (1990) *Chem. Rev.* **90**, 1171–1202
- Hardy, L. W. and Poteete, A. R. (1991) *Biochemistry* **30**, 9457–9463
- Kuroki, R., Weaver, L. H. and Matthews, B. W. (1995) *Nature Struct. Biol.* **2**, 1007–1011
- Walsh, C. T. (1979) *Enzymatic Reaction Mechanisms*, W. H. Freeman, New York
- Cho, H., Krishnaraj, R., Kitas, E., Bannwarth, W., Walsh, C. T. and Anderson, K. S. (1992) *J. Am. Chem. Soc.* **114**, 7296–7298
- Thompson, S., Mayerl, F., Peoples, O. P., Masamune, S., Sinskey, A. J. and Walsh, C. T. (1989) *Biochemistry* **28**, 5735–5742
- Stryer, L. (1988) *Biochemistry*, 3rd edn., W. H. Freeman, New York
- Henrissat, B. and Bairoch, A. H. (1993) *Biochem. J.* **293**, 781–788
- Day, A. G. and Withers, S. G. (1986) *Biochem. Cell Biol.* **64**, 914–922
- Kempton, J. B. and Withers, S. G. (1992) *Biochemistry* **31**, 9961–9969
- Han, Y. W. and Srinivasan, V. R. (1969) *J. Bacteriol.* **100**, 1355–1363
- Withers, S. G., Warren, R. A. J., Street, I. P., Rupitz, K., Kempton, J. B. and Aebersold, R. (1990) *J. Am. Chem. Soc.* **112**, 5887–5889
- Trimbur, D. E., Warren, R. A. J. and Withers, S. G. (1992) *J. Biol. Chem.* **267**, 10248–10251
- Withers, S. G., Rupitz, K., Trimbur, D. and Warren, R. A. J. (1992) *Biochemistry* **31**, 9979–9985
- Wang, Q., Trimbur, D., Graham, R., Warren, R. A. J. and Withers, S. G. (1995) *Biochemistry* **34**, 14554–14562
- Barrett, T., Suresh, C. G., Tolley, S. P., Dodson, E. J. and Hughes, M. A. (1995) *Structure* **3**, 951–960
- Wiesmann, C., Beste, G., Hengstenberg, W. and Schulz, G. E. (1995) *Structure* **3**, 961–968
- Street, I. P., Kempton, J. B. and Withers, S. G. (1992) *Biochemistry* **31**, 9970–9978
- Hess, D., Covey, T. C., Winz, R., Brownsey, R. W. and Aebersold, R. (1993) *Protein Sci.* **2**, 1342–1351
- Wakarchuk, W. W., Kilburn, D. G., Miller, R. C. and Warren, R. A. J. (1986) *Mol. Genet.* **205**, 146–152
- Gilkes, N. R., Warren, R. A. J., Miller, R. C. J. and Kilburn, D. G. (1988) *J. Biol. Chem.* **263**, 10401–10407
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Leatherbarrow, R. J. (1992) *GraFit Version 3.0*, Erithacus Software, Staines, U.K.
- Hess, D., Nika, H., Chow, D. T., Bures, E. J., Morrison, H. D. and Aebersold, R. (1995) *Anal. Biochem.* **224**, 373–381
- Namchuk, M. N. and Withers, S. G. (1995) *Biochemistry* **34**, 16194–16202
- Wakarchuk, W. W., Greenberg, N. M., Kilburn, D. G., Miller, R. C. and Warren, R. A. J. (1988) *J. Bacteriol.* **170**, 301–307
- Stevenson, A. D. (1994) M.Sc. Thesis, Department of Chemistry, University of British Columbia, Vancouver