Mechanistic Consequences of Mutation of the Active Site Nucleophile Glu 358 in *Agrobacterium* β-Glucosidase†

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ABSTRACT: The replacement of the active site nucleophile Glu 358 in *Agrobacterium* β-glucosidase by Asn and Gln by site-directed mutagenesis results in essentially complete inactivation of the enzyme, while replacement by Asp generates a mutant with a rate constant for the first step, formation of the glycosyl–enzyme, some 2500 times lower than that of the native enzyme. This low activity is shown to be a true property of the mutant and not due to contaminating wild-type enzyme by active site titration studies and also through studies of its thermal denaturation and of the pH dependence of the reaction catalyzed. Binding of ground-state inhibitors is affected relatively little by the mutation, while binding of transition-state analogues is greatly impaired, consistent with a principal role for Glu 358 being in transition-state stabilization, not substrate binding. Determination of kinetic parameters for a series of aryl glucosides revealed that the glycosylation step is rate determining for all these substrates in contrast to the native enzyme, where a switch from rate-limiting glycosylation to rate-limiting deglycosylation was observed as substrate reactivity was increased. These results coupled with secondary deuterium kinetic isotope effects of k_H/k_D = 1.17 and 1.12 measured for the 2,4-dinitrophenyl and *p*-nitrophenyl glucosides point to a principal role of the nucleophile in stabilizing the cationic transition states and in formation of the covalent intermediate. Indeed, these results constitute the first case in any enzyme in which a residue which functions as an active site nucleophile has been replaced by a shorter homologue and the kinetic consequences have been examined in detail, thus providing new insight into the consequences of mispositioning of enzymic nucleophiles.

As described in the previous two papers (Kempton & Withers, 1992; Street et al., 1992), the cleavage of glycosides by "retaining" β-glycosidases involves the formation and hydrolysis of a covalent α-D-glycopyranosyl–enzyme intermediate via oxocarbonium ion-like transition states. In all cases investigated to date [see Legler (1990) and Sinnott (1991) for recent reviews], the amino acid side chain involved as the nucleophile in this process has been shown to be an aspartic or glutamic acid, though other residues such as histidine have been suggested previously as potential nucleophiles (Paice & Jurasek, 1979).

Replacement of this nucleophilic residue using site-directed mutagenesis provides an attractive way of further probing its role and of exploring the consequences of its modification. Such studies have already been performed on several glycosidases, but all such studies to date have encountered problems which either invalidate the conclusions drawn initially or limit the experiments which can be performed. The earliest such studies (apart from ingenious chemical modification studies which unfortunately are unreliable when only low residual activities are observed) involved the conversion of the proposed nucleophile in human lysozyme, Asp 53, to glutamic acid (Muraki et al., 1987), yielding a mutant with activities around 1–5% of wild type. Unfortunately, the choice of a larger side chain replacement, with its potential attendant steric problems, limits the interpretation of this result, even though the three-dimensional structure of this mutant has recently been determined (Muraki et al., 1991). Other early studies involved replacement of the putative catalytic nucleophile of T4 lysozyme (Asp 20) by Asn and Gln, yielding mutants with 1.2% and 0.9% of wild-type activity, respectively (Anand et al., 1988). However, the meaning of these results, and even the assignment of Asp 20 as the catalytic nucleophile, is cast into serious doubt by the finding that the Asp20Cys mutant has 80% of wild-type activity (Hardy & Poteete, 1991) and particularly by the recent revelation (Rennell et al., 1991) that the Asp20Ala mutant has very similar levels of activity.

Similar, but somewhat more detailed, studies have been performed on chicken egg white lysozyme (Malcolm et al., 1989) in which the putative nucleophile, Asp 52, was replaced by asparagine. Assays with *Micrococcus luteus* cell walls revealed an activity corresponding to 5% of wild type initially, which dropped to 0.5% after 15 min, while assay with synthetic substrates yielded from 0.025% to 2% of native enzyme activity. No further results have been reported, undoubtedly due to the considerable difficulties of performing detailed kinetic studies with this enzyme and even more so with the T4 lysozyme which has stricter substrate requirements. Mutagenesis has also been used to help identify candidates for the catalytic nucleophile in amylases (Holm et al., 1990) and cellulases (Py et al., 1991), as well as in a β-glucosidase (Grace et al., 1990), but in none of these cases has there been good independent evidence for the identity of the nucleophilic residue.

The most detailed kinetic characterization of a mutant glycosidase in which the presumed nucleophile has been replaced is that on the *Escherichia coli* (*lac z*) β-galactosidase (Cupples et al., 1990). In this case the presumed nucleophile, Glu 461, identified by affinity labeling studies with conduritol C *cis*-epoxide (Herrchen & Legler, 1984) was replaced by a series of amino acid residues, and the catalytic consequences were examined. Replacement by aspartic acid reduced the

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activity almost 10 000-fold, though replacement by glycine, glutamine, and even lysine reduced the activity by smaller amounts. Most surprising was the fact that replacement by histidine resulted in a mutant with a full 6% of the activity of wild-type enzyme. These results are hard to reconcile with a role for glutamic acid 461 as a nucleophile *and*, in the subsequent step, as a good leaving group. This lead to the reinvestigation of the identity of the catalytic nucleophile (Gebler et al., 1992) by trapping the 2-deoxy-2-fluoro-α-D-galactopyranosyl–enzyme intermediate and identifying the amino acid involved using techniques analogous to those employed on the *Agrobacterium* β-glucosidase (Withers et al., 1990). This resulted in the identification of glutamic acid 537 as the catalytic nucleophile and the suggestion that glutamic acid 461 functions instead as the general acid/base catalyst.

In no case, therefore, has a detailed investigation been performed on mutants of a "retaining" β-glucosidase in which the catalytic nucleophile has been replaced by other amino acid residues. This paper describes such a study in which Glu 358 of *Agrobacterium* β-glucosidase, identified previously as the catalytic nucleophile (Withers et al., 1990), has been replaced by the amino acids Asn, Gln, and Asp and detailed kinetic studies have been performed.

MATERIALS AND METHODS

Reagents

Growth media components were obtained from Difco. Restriction enzymes, polymerases, and nucleotides were from BRL and Pharmacia. Radionucleotides were from New England Nuclear Corp. XGlu[1] and PNPGlu were obtained from Sigma. All other reagents, substrates, and buffer materials were obtained as described previously (Kempton et al., 1992; Street et al., 1992).

Mutagenesis

Strains and Culture Conditions. Escherichia coli strains JM101 (Viera & Messing, 1988) and RZ1032 (Kunkel, 1987) have been described. Plasmid pTZ18R::abg was constructed by taking the coding sequence of the β-glucosidase gene (abg) from pABG5 (Wakarchuk et al., 1986) and inserting it into pTZ18R (Mead et al., 1986). JM101 was maintained on M9 minimal medium (Viera & Messing, 1988). Plasmid-containing strains were grown in Luria broth (Miller, 1972) containing 100 μg/mL ampillicin.

DNA Techniques and in Vitro Mutagenesis. Single-stranded DNA was isolated by the following method. Cultures were grown on TYP (16 g/L tryptone, 16 g/L yeast extract, 5 g/L NaCl, 2.5 g/L K_2HPO_4) medium containing 100 μg/mL ampillicin and 10^9 PFU/mL helper phage M13K07 (Viera & Messing, 1987). Kanamycin (50 μg/mL) was added 1 h after inoculation, and the culture was grown 6–10 h at 37 °C. Phagemid were precipitated with 1.7 M ammonium acetate and 12% (w/v) PEG-6000. Single-stranded DNA was isolated from the phagemid by the method of Kristensen et al. (1987). Uracil-containing template was generated by growing the plasmid in strain RZ1032 (*dut⁻ ung⁻*). Site-directed mutants were generated by the method of Kunkel (1987) with modifications for phagemid vectors (McClary et al., 1989). Specific mutations of the active site nucleophile (E358) were carried out with oligonucleotide primers: pTACATCACCXXXAACGGCGCCTGC (The underline shows the location of mismatches, and the X indicates positions where different nucleotides were used in the various primers [GAC-Asp, CAA-Gln, AAT-Asn].) T7 DNA polymerase was used instead of the Klenow fragment of DNA pol I for the extension reactions. After in vitro mutagenesis, the plasmid DNA was transformed into JM101. Transformants were selected on LB agar containing 2% XGlu, 1 mM IPTG, and 100 μg/mL ampillicin. Possible mutants were screened by single-track sequencing and confirmed by complete sequencing reactions. The entire coding region of *Agrobacterium* β-glucosidase was then sequenced to confirm that only the desired mutations were present. DNA sequencing was performed by the procedure of Tabor and Richardson (1987) with the following modifications: the reaction temperature was increased to 43 °C and deaza-7-dGTP replaced dGTP. Expression levels of mutant proteins were monitored by SDS–PAGE (Laemmli, 1970) followed by Western blot analysis (Gilkes et al., 1988) with wild-type enzyme as a control.

Purification

The mutant polypeptides were purified essentially according to the protocol used for the native *Agrobacterium* β-glucosidase (Kempton & Withers, 1992) except that the presence and quantity of the Asp mutant was determined by assay with 2,4-DNPGlu, while the presence of the Asn and Gln mutants was determined by means of Western blots using antibody raised against native *Agrobacterium* β-glucosidase (Gilkes et al., 1988). Essentially inactive proteins were also followed during the purification by SDS–PAGE using 20% homogenous PHAST gels from Pharmacia. Total protein was detected by silver stain (Pharmacia). CD spectra were run on each of the mutants at 37 °C. Thermal denaturation of each mutant was also monitored by circular dichroism at 50 °C in 50 mM sodium phosphate buffer, pH 7.00, by monitoring changes at 222 nm.

Kinetic Characterization

All assays, determinations of kinetic parameters, isotope effect measurements, pH-dependence studies, and burst measurements on the Asp mutant were performed exactly as described in the preceding two papers in this issue (Kempton & Withers, 1992; Street et al., 1992) except that approximately 100-fold more enzyme was used in each case. Kinetic studies with the Asn and Gln mutants were performed using a protein concentration of approximately 1 mg mL^{-1}. Determination of the reaction stereochemistry by ^1H NMR directly followed the procedures described previously (Withers et al., 1986) and involved monitoring the reaction of 2,4-DNPGlu (23 mM) with β-glucosidase (7 mg mL^{-1}) in a 5-mm NMR tube. Spectra were taken at time = 0, 4.5, 10, 22.5, and 41 min. The thermal stability of the native enzyme and the Asp mutant was determined by incubating the enzyme at the defined temperature, removing aliquots at time intervals, and assaying these for residual activity at 37 °C in the usual manner.

RESULTS AND DISCUSSION

Mutagenesis and Isolation. Site-directed mutagenesis was used to change the active site nucleophile Glu 358 to Asp, Asn, and Gln. Possible mutants were first screened on X-Glu plates, and white colonies, indicating loss of activity, were

[1] Abbreviations: PNPGlu, *p*-nitrophenyl β-D-glucopyranoside; PGlu, phenyl β-D-glucopyranoside; 2,4-DNPGlu, 2′,4′-dinitrophenyl β-D-glucopyranoside; 2,4-DNP2FGlu, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside; XGlu, 5-bromo-4-chloro-3-indolyl β-D-glucopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside.

Table I: Apparent Activities of Mutants against 2,4-DNPGlu

enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
wild type	88	0.031	2839
Glu358Asp	0.72	0.054	13.3
Glu358Asn[a]	9.5×10^{-5}	0.08	0.0012
Glu358Gln[a]	2.4×10^{-4}	0.07	0.0034

[a] At least part of these activities appears to be due to contaminating native enzyme or Asp mutant.

picked and sequenced to confirm the presence of the desired mutations. All white colonies screened contained the correct mutation, and sequencing of the entire coding region of the mutant *Agrobacterium* β-glucosidase clones showed that only the single mutation was present. Western blots of small scale cultures showed that the mutant proteins were expressed at levels equivalent to wild-type protein. No significant differences in behavior during purification could be detected, with the proteins eluting at approximately the same point as wild-type enzyme in the ion exchange columns used. Each purified protein ran as a single band on SDS–PAGE (>95% purity by inspection), at the same position (50 kDa) as native enzyme. CD spectra of all the mutants were essentially indistinguishable from that of the native enzyme, indicating that no gross conformational changes had resulted from the substitutions.

Activities of the Purified Proteins against 2,4-DNPGlu. Each of the purified polypeptides was assayed with 2,4-DNPGlu, and the (apparent) k_{cat} and K_m values so determined are presented in Table I. As can be seen, the (apparent) k_{cat} values for the Asn and Gln mutants are *extremely* low (approximately 10^6 times lower than wild type) while the k_{cat} value for the Asp mutant is reduced much less, only some 10^2 fold. Values of K_m in each case are fairly similar to those of the wild-type enzyme. Interestingly, the Asp mutant undergoes slow inactivation in the presence of 2,4-DNPGlu but not in its absence. Fortunately, this inactivation is slow enough not to affect the initial rate kinetics significantly, and this interesting phenomenon is currently the subject of further investigation.

Since these low levels of activity could arise from contamination of the mutant with a minute quantity of wild-type enzyme, either by physical contamination (unlikely as new column packings were used for the isolation of all mutants), from spontaneous deamidation,[2] or from translational misreading (Schimmel, 1989), especially given the similarity of the K_m values measured to those of the wild-type enzymes, it was necessary to further investigate the source of the observed activity. One of the best ways to do this is by means of an active site titration, a method which is particularly well-suited to the Asp mutant.

Active Site Titration of the Asp Mutant: Reaction with 2-Deoxy-2-fluoroglucosides. The Asp mutant was subjected to an active site titration experiment using 2,4-DNP2FGlu as the titrant (Street et al., 1992). The release of 2,4-dinitrophenolate after the addition of a known quantity of mutant to an excess of 2,4-DNP2FGlu was monitored at 400 nm, and the results are shown in Figure 1. As can be seen, a *slow* release of *one full equivalent* of dinitrophenolate was observed, according to pseudo-first-order kinetics, in an initial relatively rapid phase. This was followed by the slow steady-state release of dinitrophenolate. Assay of the residual enzyme activity in a parallel experiment revealed that time-dependent inactivation of the enzyme accompanied the release of dinitrophenolate. This indicates that the enzyme is being

[2] We thank the referees for pointing out this possibility.

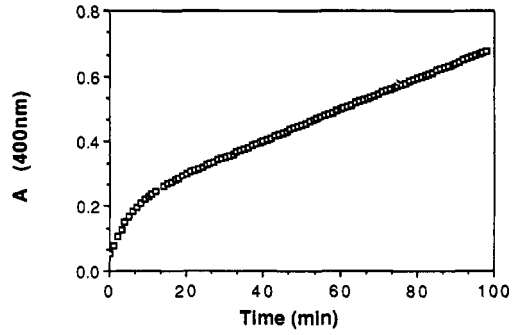

FIGURE 1: Reaction of Glu358Asp β-glucosidase with 2,4-DNP2FGlu. Time-dependent changes were monitored at 400 nm. The reaction cell contains 9.3 μM mutant enzyme and excess (263 μM) 2,4-DNP2FGlu.

inactivated in a manner similar to that found for the native enzyme, presumably via the formation of a relatively stable 2-deoxy-2-fluoro-α-D-glucopyranosyl–enzyme intermediate, but *very* slowly.

This was investigated in more detail by monitoring the rate of release of dinitrophenolate at several different concentrations of 2,4-DNP2FGlu (data not shown). The reaction rate was found to be dependent upon the inactivator concentration, in a saturable fashion, and analysis of the early phases of the reaction at each concentration allowed determination of pseudo-first-order rate constants for the inactivation process. Nonlinear regression analysis of these data yielded values for the inactivation rate constant, k_i = 0.05 min^{-1}, and the dissociation constant, K_i = 0.2 mM. Comparison of these with values for the native enzyme (k_i = 297 min^{-1}, K_i = 0.26 mM) reveals that, once again, the dissociation constant is not significantly altered, but the rate constant for inactivation is decreased some 5940-fold.

The turnover of this inactivated enzyme was investigated by rapidly purifying some of the material by gel filtration at low temperatures and then incubating it at 37 °C and assaying for activity regain. Return of activity was relatively rapid, with a rate constant of k_{react} = 0.017 min^{-1}, which compares with a value of k_{react} = 1×10^{-5} min^{-1} for the native enzyme. As with the native enzyme, the reactivation rate was considerably increased by the presence of a reactivating ligand. Investigation of the reactivation provided by the hydrolytically inert glycosyl benzene revealed a saturable dependence of the rate upon reactivator concentration, and analysis of these data according to the method used previously (Street et al., 1992) allowed determination of reactivation parameters of k_{react} = 0.185 min^{-1} and K_{react} = 34 mM. The equivalent parameters for the native enzyme are k_{react} = 0.0053 min^{-1} and K_{react} = 59 mM. Thus reactivation by hydrolysis and by transglycosylation are some 1700- and 35-fold, respectively, more rapid with the mutant than with the native enzyme, the reactivating ligand binding with a comparable affinity in the two cases.

This observation of a full-sized burst of dinitrophenolate released from 2,4-DNP2FGlu at much lower rates than with the native enzyme, and concomitant inactivation, clearly indicates that the activity observed is associated with the pure mutant and does not arise from a small contaminant of native enzyme in a completely dead mutant. Were the activity to be due to contaminating native enzyme in a dead mutant, only a very small "burst" of dinitrophenolate should have been seen, and this would have appeared very rapidly, accompanied by rapid inactivation of the enzyme. The time-dependent reactivation which is accelerated by glucosyl benzene, with dissociation constants for the reactivator very similar to that seen with native enzyme, provides further evidence for the

nature of the inactivation process occurring here and suggests that there has been no change in mechanism as a consequence of mutation, at least with regard to the 2-deoxy-2-fluoroglucoside inactivators.

Investigation of the Gln and Asn Mutants. The extremely low activities of the Asn and Gln mutants (approximately 10^6 times lower than native enzyme) raise serious concerns as to the source of the activity. Experiments with the 2-deoxy-2-fluoroglucosides were therefore performed to probe this. Reaction of the Asn mutant (1 mg mL^{-1}, 5.4 nmol) with 1.8 mM 2FβGlu-F (400 nmol) for 20 min resulted in complete inactivation of the enzyme, while reaction with 0.18 mM 2FβGlu-F (40 nmol) and 0.01 mM 2FβGlu-F (2.4 nmol) resulted in 80% and 75% activity loss, respectively. This latter number is particularly important since in this case only 0.44 equiv of inactivator per equivalent of enzyme has been used, yet this has resulted in 75% inactivation. Clearly this is not possible if the mutant is the active species which is being inactivated. Similar experiments were performed on the Gln mutant (1.0 mg mL^{-1}, 4 nmol) using 2,4-DNP2FGlu as inactivator. Incubation for 20 min with 0.056 mM 2,4-DNP2FGlu (11.3 nmol) resulted in 100% inactivation, while incubation at a concentration of 0.009 mM (1.9 mmol) resulted in 80% inactivation. Again such a result is clearly inconsistent with the mutant being the active species. Further, the inactivation mixtures were monitored by UV/vis spectroscopy at 400 nm, and no increase in absorbance was observed indicating that quantities of dinitrophenol released were beneath the detectable limit, as would be the case if the active species were a contaminant present at low levels. The very low residual activity is therefore almost certainly due to contaminating native enzyme or Asp mutant, probably arising from either translational misreading or spontaneous deamidation.

Stereochemistry. Replacement of the nucleophilic glutamic acid by the smaller aspartic acid must leave some space in the active site cavity which may be filled by some reshuffling of the protein structure or by a water molecule. If the latter is the case, it may be possible for the water molecule to directly attack the anomeric center of this activated sugar moiety resulting in hydrolysis of the glycosidic linkage with overall inversion of anomeric configuration. The fact that the 2-deoxy-2-fluoroglucosyl–enzyme intermediate has been shown to accumulate on this mutant renders this unlikely; nonetheless, reaction with normal substrates was investigated by following the enzyme-catalyzed reaction by ^1H NMR and determining the stereochemistry of the product so formed. The limitations of this experiment imposed by the low activity of the mutant and the need for the formation of an observable quantity of the product before significant mutarotation could occur dictated that a high concentration of enzyme was required and that 2,4-DNPGlu be used as the substrate at high concentrations, despite the problems associated with the time-dependent inactivation caused by this substrate. Under these conditions, the only sugar product formed was clearly 2,4-dinitrophenyl β-D-cellobioside, the product of transglycosylation, demonstrating that reaction is indeed occurring according to the normal mechanism, via a glycosyl–enzyme intermediate.

Thermal Stability. The thermal stability of each mutant and of the native enzyme at 50 °C was monitored by changes in circular dichroism at 222 nm upon incubation at 50 °C, and results are presented, along with those for the native enzyme, in Figure 2. As can be seen, the Asp mutant is somewhat less stable than the wild-type enzyme, but the other two mutants are of very similar stability to wild type or slightly more stable.

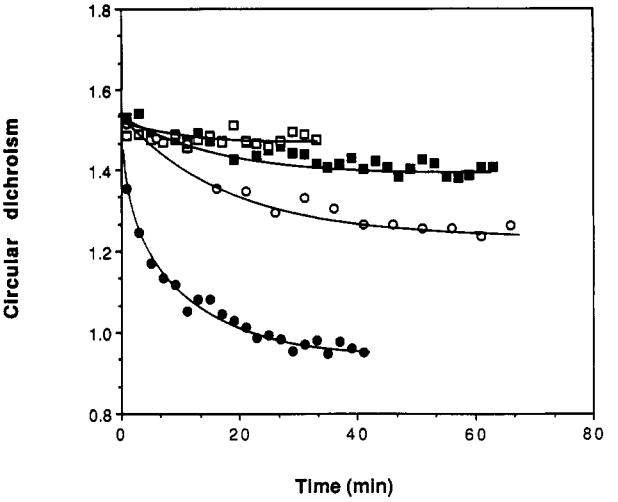

FIGURE 2: Thermal stability of native enzyme and mutants. Change in circular dichroism at 222 nm vs time at 50 °C for (○) native enzyme; (●) Asp mutant, (■) Asn mutant, and (□) Gln mutant. Values plotted are $-\theta \times 10^4$.

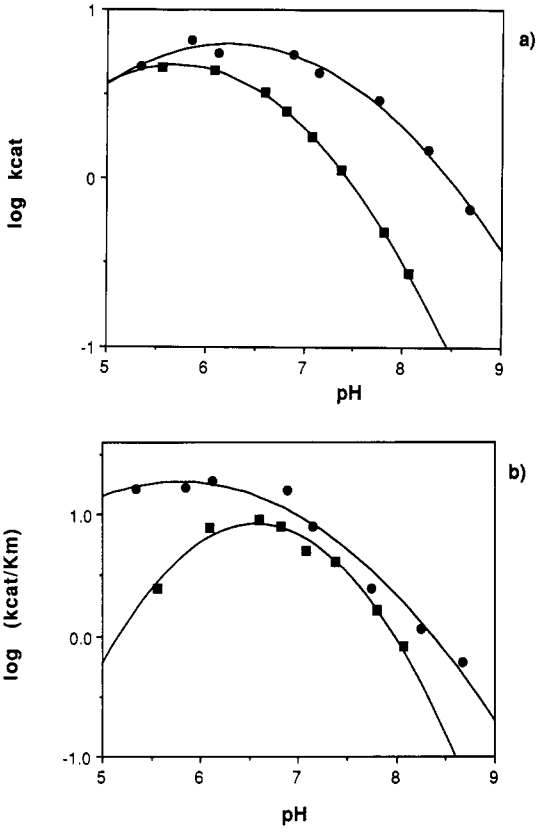

FIGURE 3: pH dependence of catalysis by the Asp mutant. (a) Plot of log k_{cat} vs pH for hydrolysis of 2,4-DNPGlu by Asp mutant (■) and hydrolysis of PGlu by native enzyme (●). (b) Plot of log $k_{cat}/_{Km}$ vs pH for hydrolysis of 2,4-DNPGlu by Asp mutant (■) and hydrolysis of PGlu by native enzyme (●). Lines drawn through the points are simply illustrative of the shape of the profile.

pH Dependence. Values of k_{cat} and K_m for the hydrolysis of 2,4-DNPGlu by the Asp mutant at a series of different pH values within the pH stability range (5.5–9) of the enzyme were determined, and the results are presented graphically in Figure 3a,b, along with the comparable profiles for the substrate phenyl glucoside with the native enzyme, for which the glycosylation step is rate limiting (Kempton & Withers, 1992). Relatively little difference is observed between the

Table II: Kinetic Parameters for Aryl Glucosides with the Asp358 Mutant

substrate (aryl β-glucoside)	aglycon pK_a	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
2′,4′-dinitrophenyl-	3.96	0.72	0.054	13.3
2′,5′-dinitrophenyl-	5.15	1.22	0.20	0.58
3′,4′-dinitrophenyl-	5.36	0.040	0.068	0.60
		0.090	0.08	1.12
2′-nitro-4′-chlorophenyl-	6.45	0.054	0.07	0.77
4′-nitrophenyl-	7.18	0.021	0.093	0.22
3′-nitrophenyl-	8.39	0.043	1.9	0.022

basic limbs of the profiles of k_{cat}/K_m for the native and mutant enzymes, indicating that ionizations in the free enzymes have very similar pK_a values (pK_2 = 6.8 for the mutant; pK_2 = 7.0 for native enzyme). There is, however, a significant difference between the profiles of k_{cat} for the two enzymes, that for the mutant having a basic limb with an ionization (pK_2 = 6.7) depressed approximately 1 pH unit relative to that seen for wild type (pK_2 = 7.6), suggesting that an ionization in the enzyme–substrate complex is perturbed as a result of the change in geometry. The ionization in the basic limb is unlikely to be due to the nucleophilic carboxylate since deprotonation results in a *decrease* in activity and thus is more likely to be associated with the acid catalytic residue. Such a depression of the pK_a of the acid catalytic group in the enzyme–substrate complex is quite reasonable since the negatively charged carboxylate nucleophile has been pulled approximately 1 Å farther away from the acid catalytic group. The pK_a depression may not show up to the same degree in the free enzyme (thus in the k_{cat}/K_m plot) either because the two residues may not be held so close together or because of the insulating effect of solvent present at the active site of the unliganded enzyme. The absence of any significant data for the acid limb in most cases and the instability of the mutant below pH 5.5 preclude any speculations on the effects of mutation on this part of the profile.

Structure/Reactivity Studies. Values of k_{cat} and K_m were determined for a series of aryl glycoside substrates, exactly as with the native enzyme (Kempton & Withers, 1992), except that only the more reactive substrates (aglycon pK_a < 8.4) were investigated because the very low inherent reactivity of the mutant enzyme (and smaller $\Delta\epsilon$ values of the other substrates) precluded the reliable study of others. Results are presented in Table II and plotted in the form of Hammett relationships in Figure 4a,b. A quite reasonable Hammett relationship for log k_{cat}/K_m is observed, with a slope of β_{lg} = –0.6 and a correlation coefficient of 0.9. This suggests that the chemical step of initial glycosidic bond cleavage is indeed the rate-limiting first irreversible step. The slope observed is similar to that of β_{lg} = –0.7 found for the native enzyme in the leaving group-dependent part of the equivalent plot, suggesting that bond cleavage at the transition state proceeds to approximately the same extent in the two cases. The plot of log k_{cat} vs pK_a appears to be very scattered (however, the number of points is relatively low, and it is possible that other effects may be superimposed); thus it is somewhat hard to interpret. If all the data are correlated with a single line, a plot of slope β_{lg} = –0.3 is obtained, with a correlation coefficient of 0.5. While this is clearly not a satisfactory correlation, it does constitute a trend and likely indicates that the glycosylation step is rate determining for these substrates. This interpretation was reinforced by nucleophilic competition experiments (Kempton & Withers, 1992) performed by adding DTT to reaction mixtures containing the Asp mutant and several different concentrations of either 2,4-DNPGlu or PNPGlu. In no case was there a significant rate increase observed, indicating that deglycosylation was not rate determining for these substrates.

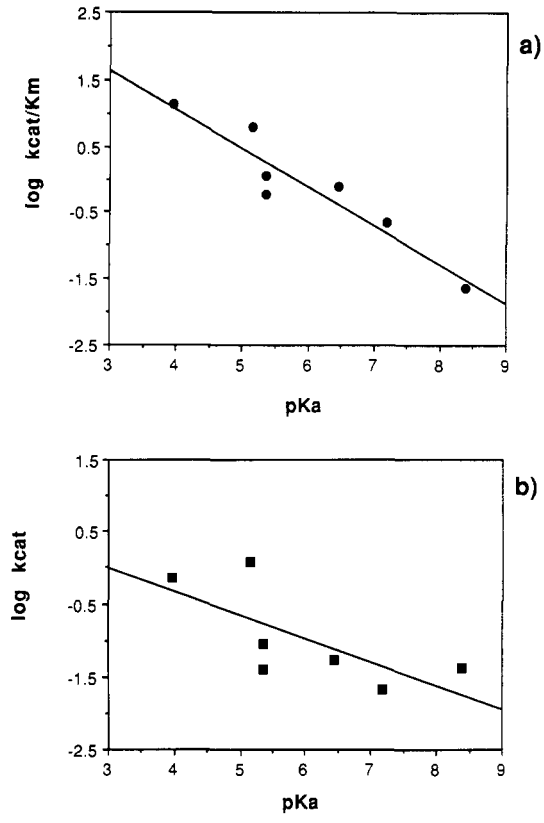

FIGURE 4: Hammett relationships for the Asp mutant. (a) Plot of the log k_{cat}/K_m vs aglycon pK_a for aryl glycosides. (b) Plot of log k_{cat} vs aglycon pK_a for aryl glycosides.

Table III: Inhibition Constants for Native and Glu358Asp β-Glucosidase

inhibitor	category	K_i native[a] (mM)	K_i mutant (mM)	K_i ratio (mutant/native)
β-glucosyl benzene	ground state	3.4	17	5
β-glucosylamine	ground state/transition state	0.16	8.2	51
gluconolactone	transition state	0.0014	0.53	380
glucono-phenylurethane	transition state	0.0012	0.70	580
glucono-hydroximolactone	transition state	0.03	3.6	120

[a] Data from Kempton and Withers (1992).

Inhibitor Binding. In order to further probe to what extent the structure of the active site pocket has been conserved upon mutation, and also as a means of further probing the role of Glu 358, the inhibition constants for a series of inhibitors of the enzyme which mimic both ground-state and transition-state binding were determined. All were found to inhibit competitively with respect to substrate, and the K_i values determined are listed in Table III. Also listed in this table are the K_i values determined previously (Kempton & Withers, 1992) for the native enzyme, the ratio of these two values, and a designation of the generally perceived character of these inhibitors (ground state or transition state) [see, for example, Legler (1990)]. A fairly clear progression can be noted in the ratio of the K_i values, with the ground-state analogue glucosyl benzene binding only slightly more weakly to the mutant than to the native enzyme, while the best transition-state analogues

bind over 500-fold more weakly. This is consistent with a principal role of Glu 358 being in the stabilization of the transition state, presumably largely through electrostatic stabilization of the developing positive charge. Moving the negatively charged counterion approximately 1 Å farther away, upon changing the residue from Glu to Asp, must result in less effective stabilization of the transition state and therefore also weakened transition-state analogue binding. The very small effect of the mutation on the binding of the ground-state inhibitor, and also, but less reliably, on K_m values for substrates, suggests that Glu358 has very little role in ground-state binding.

Secondary Deuterium Kinetic Isotope Effects. Secondary deuterium kinetic isotope effects of k_H/k_D = 1.17 (±0.03) and 1.12 (±0.02) were measured for the hydrolysis of 2,4-DNPGlu and PNPGlu, respectively, by the Asp mutant. These are larger than that of k_H/k_D = 1.05 determined for the glycosylation step with the native enzyme (Kempton & Withers, 1992). However, this is probably largely due to the greater leaving group ability of these phenolates (pK_a = 3.96 and 7.16) than of those used for this determination with the native enzyme (pK_a > 8.4), making the reaction inherently more dissociative in character. Indeed, the significantly greater isotope effect measured for 2,4-DNPGlu than PNP-Glu would appear to support this notion. Nonetheless, the presence of this large degree of oxocarbonium ion character, despite the fact that the stabilizing counterion (Asp 358 carboxylate) is nominally farther away, is of considerable interest. Presumably this is a reflection of the fact that preassociation by the carboxylate is even more severely compromised by the greater distance than is the electrostatic stabilization.

Conclusions. It is useful to consider the results obtained here in light of expectations concerning the role of the nucleophile, Glu 358, based upon the mechanism proposed (Kempton & Withers, 1992). The glutamate side chain might be expected to have very little role in substrate binding, since any such interactions would likely have to be broken in proceeding to the transition state, thus decreasing the amount of "differential binding energy" (Fersht, 1985) available for selective stabilization of the transition state. Its principal roles would therefore be in electrostatic stabilization of the two positively charged transition states and in the formation of a covalent linkage with the sugar in the glycosyl–enzyme intermediate. In addition to this it must serve as a good leaving group for the second step of catalysis, hydrolysis of the glycosyl–enzyme. Thus replacement of Glu 358 by Asp would not be expected to affect the free energy of the ground state much but should raise that of the transition states considerably. In addition, since the covalent intermediate formed would necessarily be strained, the free energy of the intermediate would also be raised considerably. This situation is depicted in the hypothetical reaction coordinate diagrams shown in Figure 5 for the native enzyme and the Asp mutant. The extents to which the two transition states and the intermediate have been destabilized are arbitrarily shown as being equal, while ground state destabilization is shown as being small. Results obtained are quite consistent with this profile as follows.

The finding of similar K_m values for the substrates and similar K_i values for ground-state inhibitors with the native enzyme and Asp mutant is consistent with the minimal role of the residue in ground-state binding, with the largest differences in energy being no greater than 1.3 kcal mol^{-1}. A major role in transition-state stabilization is clearly indicated by the severely decreased activities (k_{cat}/K_m) of the Asn, Gln,

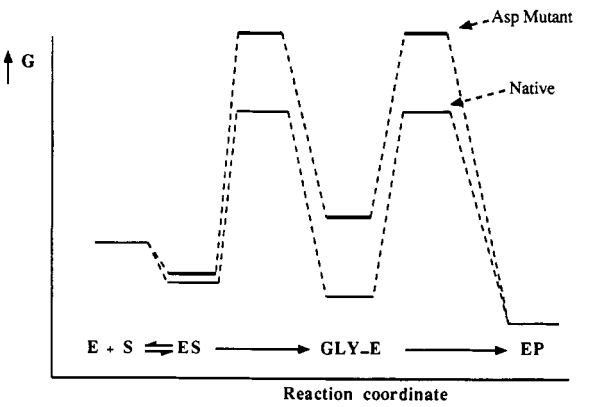

FIGURE 5: Hypothetical reaction coordinate diagrams for native enzyme and Asp mutant.

and Asp mutants and by the observations of *much* weaker binding of the transition-state analogue inhibitors to the Asp mutant than to the native enzyme. Some estimate of the extent by which each transition state has been destabilized can be obtained by calculating the rate reductions for each step. Removal of the charge results in a transition-state destabilization of *at least* 8.5 kcal mol^{-1} (based upon the Gln data) and almost certainly considerably more. The effect of displacement of the charge is more subtle. The best estimate of the rate reduction in the glycosylation step resulting from the change of Glu to Asp is obtained by comparing values for *m*-nitrophenyl glucoside, a substrate for which the glycosylation step is rate determining in *both* enzymes. In this case a 2500-fold reduction in k_{cat} is observed, corresponding to an increase in $\Delta G^{\ddagger}$ of approximately 4.5 kcal mol^{-1}. The actual increase in transition state energy is larger since the above value is attenuated by ground-state binding effects. Relative values of k_{cat}/K_m indicate an increase in transition-state energy of approximately 6 kcal mol^{-1}. A similar estimate is obtained by comparing the rates of formation of the 2-deoxy-2-fluoro-α-D-glucopyranosyl–enzyme intermediates from 2,4-DNP2FGlu, the mutant reacting approximately 5940-fold more slowly than the native enzyme. Comparison of deglycosylation rate constants is more difficult since no substrate has been identified for which the deglycosylation step is rate determining with the Asp mutant. (This result is itself consistent with the prediction from Figure 5 that the glycosylation step will tend to be rate limiting). However, the k_{cat} value of 1.22 s^{-1} for 2′,5′-dinitrophenyl glucoside puts a *lower* limit on this rate constant, and thus comparison with the value of approximately 150 s^{-1} for the native enzyme (Kempton & Withers, 1992) reveals that this step is slowed *less than* 125-fold. Moreover, the hydrolysis of the 2-deoxy-2-fluoro-α-D-glucopyranosyl–enzyme intermediate was found to be some 1400-fold *faster* for the mutant than the native enzyme; thus it may well be the case that the deglycosylation step with normal substrates is faster for the mutant than the native enzyme. These results suggest very substantial destabilization of the glycosyl–enzyme intermediate upon mutation.

On the basis of the large secondary deuterium kinetic isotope effects measured for the mutant, it is tempting to suggest that the forming bond is more stretched at the transition state for the mutant than the native enzyme. However, as noted this may simply be a reflection of the different leaving groups employed in these two sets of measurements. Unfortunately, the fact that enzyme–ligand recombination events are probably rate determining in k_{cat}/K_m for the native enzyme (Kempton & Withers, 1992) precludes any reliable measurement of

isotope effects on the glycosylation step for the "fast" substrates, and thus a direct comparison cannot be made.

The apparent inactivity of the Asn and Gln mutants is of particular interest and is quite reasonable on chemical grounds, being a consequence of some combination of the reduced ability to stabilize a cationic transition state, the difficulty of forming a covalent linkage with the weakly nucleophilic amide moiety, and possibly also the poor leaving group ability of the amide, should an intermediate ever form. However, there is no evidence for the formation of even a stable intermediate which cannot turn over, since upon reaction of large quantities of these mutants with 2,4-DNPGlu there was no sign of a "burst" of dinitrophenolate release. It is hard to rationalize these rates with results for the chicken egg white lysozyme (Malcolm et al., 1989), where substantial (0.5–5%) enzyme activity was observed upon replacement of the active site Asp 52 with Asn. The reason for this difference may lie in slightly different mechanisms or in the somewhat more complex assay system employed for lysozyme. In the case of the Asp mutant, catalysis is severely compromised due to the relatively poor placement of the catalytic groups. Nonetheless, sufficient flexibility is present in the active site for the carboxylate to still function in stabilizing the positive charge and to form the covalent intermediate, as was shown by stereochemical experiments, and particularly by the trapping and subsequent turnover of the 2-deoxy-2-fluoroglucopyranosyl–enzyme intermediate.

This work represents the first case in which a glutamate which functions as a nucleophile in an enzymic reaction mechanism has been replaced by the shorter aspartate. Indeed, it likely represents the first case in which *any* amino acid which functions as a nucleophile has been replaced by a smaller congener *of the same chemical type* and therefore provides useful insight into the steric requirements of such processes. However, since such interpretations are made in the absence of structural information, the possibility of significant structural change in the protein must always be considered. There are several other enzymes in which a key glutamate residue which serves as an acid/base catalyst has been replaced by an aspartic acid, these being triose phosphate isomerase (Raines et al., 1986), staphylococcal nuclease (Hibler et al., 1987), and β-lactamase I (Gibson et al., 1990). In all these cases, the shortening of the side chain reduced rate constants approximately 1000–2000-fold, a rate reduction very similar to that of 2500-fold found for the glycosylation step in β-glucosidase. This perhaps reflects the somewhat similar chemical nature of these two steps, reaction of a carboxylate anion with a "hard" electrophile (the oxocarbonium ion or the proton), accompanied by displacement of an anionic leaving group.

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