Aspartate 313 in the *Streptomyces plicatus* Hexosaminidase Plays a Critical Role in Substrate-assisted Catalysis by Orienting the 2-Acetamido Group and Stabilizing the Transition State*

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SpIHex, a retaining family 20 glycosidase from *Streptomyces plicatus*, catalyzes the hydrolysis of N-acetyl-β-hexosaminides. Accumulating evidence suggests that the hydrolytic mechanism involves substrate-assisted catalysis wherein the 2-acetamido substituent acts as a nucleophile to form an oxazolinium ion intermediate. The role of a conserved aspartate residue (D313) in the active site of SpIHex was investigated through kinetic and structural analyses of two variant enzymes, D313A and D313N. Three-dimensional structures of the wild-type and variant enzymes in product complexes with N-acetyl-D-glucosamine revealed substantial differences. In the D313A variant the 2-acetamido group was found in two conformations of which only one is able to aid in catalysis through anchimeric assistance. The mutation D313N results in a steric clash in the active site between Asn-313 and the 2-acetamido group preventing the 2-acetamido group from providing anchimeric assistance, consistent with the large reduction in catalytic efficiency and the insensitivity of this variant to chemical rescue. By comparison, the D313A mutation results in a shift in a shift in the pH optimum and a modest decrease in activity that can be rescued by using azide as an exogenous nucleophile. These structural and kinetic data provide evidence that Asp-313 stabilizes the transition states flanking the oxazoline intermediate and also assists to correctly orient the 2-acetamido group for catalysis. Based on analogous conserved residues in the family 18 chinatinases and family 56 haluronidases, the roles played by the Asp-313 residue is likely general for all hexosaminidases using a mechanism involving substrate-assisted catalysis.

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S. *plicatus* N-acetyl-β-hexosaminidase (SpIHex) is a glycosidase that catalyzes the hydrolysis of N-acetyl-β-hexosaminides (NAG and GalNAc) from the non-reducing end of glycoconjugates, oligosaccharides, and polysaccharides (1, 2). N-Acetyl-β-hexosaminidases are common in nature and are found in molecules such as chitin, which forms the exoskeleton of crustaceans and insects, in glycosaminoglycans that populate the extracellular matrix, and as an essential component of the carbohydrate structure of glycoproteins and glycolipids. There is considerable interest in the mechanism of N-acetyl-β-hexosaminidases because of their importance in human disease (3), biotechnology, and the control of fungal and insect pests (4).

Glycosidases can catalyze the cleavage of the glycosidic bond with one of two possible stereochemical outcomes, retention or inversion of stereochemistry at the anomeric center (5, 6). Henrissat *et al.* (7–9) have classified the glycosidases into more general for all hexosaminidases using a mechanism involving substrate-assisted catalysis. Fig. 1. In this reaction, a strategically located carboxylic acid group provides general acid catalysis to the glycosidic oxygen of the bound, distorted substrate in order to facilitate the cleavage of the glycosidic linkage. As the glycosidic bond is breaking, the anomic carbon migrates toward the oxygen atom of the 2-acetamido group of the substrate, which acts as a waiting nucleophile to accept

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1 The abbreviations used are: SpIHex, *S. plicatus* β-hexosaminidase; SmChB, *S. marcescens* chitobiase; 3,5-DNPGlcNAc, 3,5-dinitrophenyl N-acetyl-β-D-glucosamine; pNPGlCNac, 4-nitrophenyl N-acetyl-β-D-glucosamine; PhGlcNAc, phenyl N-acetyl-β-D-glucosamine; NAG, N-acetyl-β-D-glucosamine; NTA, nitrolotriacetic acid.
the electrophilic anomic center, thereby forming an oxazolinium ion intermediate. This intermediate is then hydrolyzed in a process that is the near microscopic reverse of the first step. Ring opening of the high energy oxazolinium ion intermediate results in the electrophilic migration of the anomic carbon to a water molecule. The nucleophilicity of this water molecule is enhanced by the now deprotonated carboxylate residue that acts as a general base. The two transition states flanking the intermediate (Fig. 1, I and II) are believed to have considerable oxacarbenium ion character.

Convincing structural evidence in support of this proposed mechanism has accumulated through the elucidation of a number of crystal structures of family 20 β-hexosaminidases including those of the native enzyme (16, 17), the Michaelis complex (17, 18), and that of a non-hydrolysable analog of the proposed intermediate (16, 19). In the case of the Michaelis complex formed between chitobiose and the Serratia marcescens chitosamidase (SmChB), the substrate was seen bound in the −1 and +1 subsites with the −1 saccharide modeled in a 1S2 conformation with distinct electron density noted for its 2-acetamido moiety. This density was interpreted as showing the amide carbonyl to be poised for nucleophilic attack on the anomic carbon. The crystal structure of SpHex in complex with a stable analog of the reaction intermediate, NAG-thiazoline, provides convincing evidence for a mechanism involving anameric assistance (16). In this data set unambiguous electron density was seen for the NAG-thiazoline and revealed the saccharide moiety in a 1C1 conformation with an aspartate residue (Asp-313) forming a short hydrogen bond (2.5 Å) to the nitrogen of the thiazoline ring. Most recently, a structure of SpHex in complex with GalNAc-isofagomine was determined to 1.75 Å resolution (20). This inhibitor has a nitrogen in place of carbon at the anomeric center. In the structure of the complex, the carbonyl of the 2-acetamido moiety was directed at the piperidine nitrogen. The piperidine ring of the inhibitor adopted a conformation intermediate between a 2E envelope and a 1C1 chair, with a proton shared between the piperidine nitrogen and the acid/base in a 2.8 Å hydrogen bond. Collectively, these structural data reveal that the family 20 enzymes carefully control the orientation of the 2-acetamido group of the substrate through a number of interactions. They are also consistent with an electrophilic migration mechanism that has been proposed to operate for all retaining hexopyranosidases (21).

Interestingly, recent evidence has accumulated that another set of retaining β-hexosaminidases, those from family 3, proceed through a somewhat different mechanism. These enzymes are also believed to proceed by an electrophilic migration mechanism like most retaining glycosidases, but one which involves the participation of an enzymic nucleophile as originally postulated by Koshland (22). This mechanism involves two suitably positioned carboxyl-containing residues, with one acting as a general acid/base catalyst in the same manner as the acid/base residue of the family 20 β-hexosaminidases. The second carboxyl group acts as the catalytic nucleophile to form a covalent glycosyl enzyme. It appears that the identity of the nucleophile is the major distinction between the mechanisms of the family 20 enzymes, where the nucleophile is the 2-acetamido group of the substrate, and the family 3 enzymes, where the nucleophile is an enzymic carboxylate. Evidence for the covalency of the glycosyl enzyme intermediate in the mechanism of the family 3 β-hexosaminidases was obtained with the Vibrio furnissii N-acetyl-β-N-acetylglucosaminidase through studies with the slow substrate, N-acetyl-5-fluoro-α-L-idopyranosaminyl fluoride. Vocadlo et al. (23) showed that incubation of this substrate with the enzyme afforded a steady-state amount of the glycosyl enzyme that could be detected by electrospray ionization mass spectrometry. Proteolysis of the covalently labeled enzyme and tandem mass spectrometric analysis of the labeled peptide enabled identification of the nucleophilic residue. Very recently, Vocadlo et al. have provided strong evidence that hen egg-white lysozyme, a member of family 22 of the glycoside hydrolases that catalyzes the hydrolysis of the glycosidic linkage of the NAG-NAM peptide-glycan repeat, also proceeds through a mechanism involving a glycosyl enzyme intermediate (21). This has allowed the generalization of a catalytic mechanism involving covalent participation of a nucleophile and electrophilic migration for all retaining β-hexopyranosidases (21).

In the case of the family 20 SpHex, there remain significant questions as to how this enzyme achieves its catalytic prowess. While convincing evidence for the identity and role of the acid/base residue has been supplied through crystallographic studies and analysis of variants, the role of other active site residues remains indistinct. In particular, a highly conserved aspartate residue immediately preceding the acid/base residue has been found to be of varying importance in catalysis (Fig. 2) (24, 25). In the three-dimensional structure of SpHex with NAG-thiazoline described above, this residue (Asp-313) was shown to be in close proximity to the thiazoline nitrogen atom (2.5 Å) and a Coulombic interaction was proposed to provide stabilization of the positively charged oxazolium ring during the enzyme-catalyzed reaction (16). The environment surrounding Asp-313 ensures that the carboxyl group of Asp-313 bears a negative charge and remains properly positioned throughout catalysis. In particular, O5 of Asp-313 shares a proton with the carbonyl group of Asp-246 through a short 2.5 Å hydrogen bond so that O5 of Asp-313 remains well positioned to stabilize the positive charge that develops upon cyclization of the substrate. Additionally, a role for this residue in distortion of the substrate to direct the 2-acetamido group toward the anomic carbon has also been suggested.

The obvious importance of Asp-313 for the activity of family 20 hexosaminidases prompted us to describe our own studies into the role played by this residue in a structurally defined system. The D313A and D313N variants of SpHex were constructed and expressed, and it is shown here that these substitutions have substantially different effects on the activities of the two variant enzymes compared with each other and to the wild-type enzyme. We have examined the activity of one of these variants in the presence of external nucleophiles and...
have observed significant chemical rescue of enzymatic activity. In addition, we have determined x-ray crystal structures of the wild-type enzyme and the two variants in complex with the reaction product, N-acetyl-D-glucosamine (NAG). The complex with wild-type SpHex is the first example of a product complex for a family 20 enzyme and, with other studies, permits a structural description of every stable species along the reaction coordinate throughout the course of the enzyme-catalyzed reaction. Moreover, the structures of the D13N and D13A variant enzymes in complex with NAG reveal the importance of Asp-313 in controlling both the conformation of the saccharide residue bound in the −1 subsite and the orientation of the 2-acetamido group.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized by the Nucleic Acid and Peptide Service Unit at the University of British Columbia (Vancouver, Canada). Restriction enzymes were obtained from New England Biolabs or Invitrogen. T4 DNA ligase and DNA polymerase (Roche Molecular Biochemicals) were used for all extension and ligation reactions. Plasmid minipreps were performed using the Promega Wizard kit according to the manufacturer’s instructions.

The binding of His7-tagged proteins were performed using affinity chromatography on NiNTA agarose resin (Qiagen) as described by Mark et al. (16). Mutagenesis—The construction of the plasmid pAHX-1.8, which was created from insertion of the SpHex gene into a pET3a vector with an N-terminal His6-tag, has been described previously (2). pAHX-1.8 was modified by site-directed mutagenesis using a four primer cassette strategy to introduce the requisite substitution and a neighboring silent KpnI restriction site. PCR amplifications were performed using pAHX-1.8 as template and the following pairs of oligonucleotides (restriction sites indicated in bold, mutated residues are underlined): reaction 1: 5′-GGTGGCCGCCCTGTTGACCCG-3′ and 5′-GGCTGGAGGTGCCCCTTCTGCGCCCGAGTGGAGTACCCGCGGGGT-AGACC-3′; reaction 2: 5′-GGTGGCCGCCCTGTTGACCCG-3′ and 5′-GGCTGGAGGTGCCCCTTCTGCGCCCGAGTGGAGTACCCGCGGGGT-AGACC-3′; reaction 3: 5′-GAGGCGGCCCCTGCGGACCCGGCT- TCC-3′ and 5′-GGCTGGACCCCGCGTACCCATCACATCGCG- GCCAGAGGGCGACCTCCAGC-3′; reaction 4: 5′-GAGGCGGCCCCTG- GACCCGGCT-3′ and 5′-GCGCTGACCCCGCGTGACCCATCACATCGCG- GCCAGAGGGCGACCTCCAGC-3′; reaction 5: 5′-GAGGCGGCCCCTGCGGACCCGGCT-TCC-3′ and 5′-GGCTGGACCCCGCGTACCCATCACATCGCG- GCCAGAGGGCGACCTCCAGC-3′; reaction 6: 5′-GAGGCGGCCCCTGCGGACCCGGCT-TCC-3′ and 5′-GGCTGGACCCCGCGTACCCATCACATCGCG- GCCAGAGGGCGACCTCCAGC-3′.

The product from each PCR was purified using QiaexII beads (Qiagen). The purified products from a pair of reactions (1 and 3 and reactions 2 and 4) were combined and subjected to five thermal cycles in a reaction mixture containing dNTPs with Pwo polymerase. A portion of each reaction mixture containing the PCR product was used as the template for a PCR amplification using the flanking primers 5′-GGTGGCCGCCCTGTTGACCCG-3′ and 5′-GGCTGGAGGTGCCCCTTCTGCGCCCGAGTGGAGTACCCGCGGGGT-AGACC-3′. The 300- bp products were separated by electrophoresis in 3% agarose gel, excised from gel, and then purified using QiaexII. The purified blunt-ended PCR product was ligated into pCR-Blunt, according to the manufacturer’s protocol using the Zero Blunt PCR cloning kit (Invitrogen) and the insert confirmed by sequencing. The resulting product was digested with AgeI and BsrGI and ligated into a similarly digested sample of p3AHEX-1.8. After transformation into Escherichia coli XL1-Blue, plasmid minipreps were screened for the correct insert using KpnI to confirm the presence of the unique silent mutation to afford the two desired variants, p3AHEX-1.8D313A and p3AHEX-1.8D313N.

Expression and Purification—E. coli BL21DE3 cells containing the variant plasmids were grown to log-phase (OD600 0.5–0.6) at 37 °C in 2YT media containing 100 mg liter−1 ampicillin with vigorous shaking. The culture was allowed to grow overnight at 25 °C and precipitated by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM. The culture was allowed to grow overnight at 25 °C. The cells were collected by centrifugation and subjected to mechanical lysis using a French press. The lysate was centrifuged and the supernatant applied to a column of NiNTA agarose resin and eluted with 20 mM Tris buffer (pH 7.5) containing 100 mM NaCl and a gradient of imidazole from 0 to 250 mM. The fractions containing protein were concentrated and stored at 4 °C in elution buffer (20 mM Tris-Cl, pH 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol, 250 mM imidazole, pH 8.0) and were stable for several months. ES1-MS was used to confirm the mass of the protein: m/z: wild-type, 56063 (calculated, 56053); D313A, 56013 (calculated, 56009); D313N, 56063 (calculated, 56052). Protein concentrations were measured using the Pierce Micro BCA analysis kit.

Cryocrystallization and Data Collection—Purified enzyme was diazylated against pH 5.0 buffer containing 50 mM trisodium citrate and 300 mM NaCl and concentrated to ∼10 mg/ml with a Millipore concentrator. Wild-type and the two variants of SpHex were co-crystallized with NAG using the vapor diffusion method at room temperature. Concentrated wild-type SpHex was incubated with 30 mM NAG for 30 min and then mixed in a 1:1 ratio with mother liquor (2.1 M ammonium sulfate, 100 mM trisodium citrate, pH 6.0 and 20% glycerol) just prior to data collection.

Structure Determination and Refinement—Prior to structure determination, refinement, 10% of the diffraction data from the wild-type SpHex-D313A-NAG and SpHex-D313N-NAG complexes were collected with an R-axis IV27 image plate detector mounted on a Rigaku rotating anode x-ray generator. All crystals were flash-cooled to 100 K within a N2(g) stream during data collection, and all diffraction data were processed using DENZO and SCALEPACK (26).

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Figure 2. Multiple sequence alignment of a region surrounding the acid/base residue of selected family 20 hexosaminidases. Consensus is shown below with uppercase letters indicating entirely conserved residues and lowercase letters indicating similar residues. Dark shading indicates highly conserved residues and light shading indicates conserved similar residues. The sequences were aligned with ClustalW, and shading was performed with BoxShade Version 3.21. The abbreviations used and data bank accession numbers are as follows: SpHex, hexosaminidase from S. plicatus (GenBank™ identifier O85568). Canahl hex, hexosaminidase from Candida albicans (GenBank™ identifier P43077). Aratha_hex, hexosaminidase from Arabidopsis thaliana (GenBank™ identifier Q9SYK0). Homosa_hex, HexA hexosaminidase from Homo sapiens (GenBank™ identifier P06865). HeXb, HeXb from Homo sapiens (GenBank™ identifier P07686). SmCHB, chitobiase from S. marcescens (GenBank™ identifier Q54468). Burcep_chi, chitinase from Burkholderia cepacia (GenBank™ identifier BAB20043). The glutamate residue corresponding to the general acid/base is indicated with (○) and the conserved aspartate studied in this work is indicated with (○).
The change in absorbance with time was fitted to a first-order rate reaction. The mixture was then checked, and it was thus established that no time it was apparent that 5 the release of phenolate monitored at 360 nm for 5 position molecular models of WT

using a maximum likelihood target function, rigid body refinement and tallographic complex. Initial

solution of PhGlcNAc (100 units, were relocated using the CNS program and manually inspected from all three models prior to positioning into the new asymmetric unit. Solvent molecules, which had been removed using the program O, and topology and parameter files of the sugar were gener-

Coordinates—Coordinates and structure factors have been deposited into the Protein Data Bank (accession code: WT

using a maximum likelihood target function, rigid body refinement and several rounds of conjugate gradient minimization were sufficient to position molecular models of WT

The pH of the —initiated by the addition of

TABLE I

Crystallographic statistics

|                | WT   | NAG  | D313A NAG | D313N NAG |
|----------------|------|------|-----------|-----------|
| Crystal Information | P6,22 | P6,22 | P6,22     | P6,22     |
| Space group      |      |      |           |           |
| Unit cell dimensions | a = b = 132.3 | a = b = 132.9 | a = b = 133.7 |
| (Å)              | c = 176.2 | c = 176.9 | c = 176.1 |
| Data Collection (values in parentheses refer to the high-resolution shell) | | | | |
| Detector         | MAR345 image plate | RaxisIV-+ image plate | RaxisIV-+ image plate |
| Wavelength (Å)   | 0.979 | 1.54 | 1.54       |           |
| Resolution (Å)   | 70.0–2.10 | 40.0–1.90 | 40.0–1.95 |
| High-resolution (Å) | 2.14–2.10 | 1.96–1.90 | 2.02–1.95 |
| Total observations | 747643 | 1251130 | 1300022 |
| Unique reflections | 54264 (2604) | 70431 (5572) | 65848 (6556) |
| Completeness (%) | 99.8 (97.4) | 96.8 (93.3) | 98.9 (98.5) |
| R<sub>free</sub> (%) | 0.038 (0.100) | 0.060 (0.189) | 0.056 (0.256) |
| Refinement | | | | |
| Resolution (Å)   | 70.0–2.1 | 38.4–1.90 | 39.2–1.95 |
| R<sub>work</sub> | 0.195 | 0.194 | 0.198 |
| R<sub>free</sub> | 0.216 | 0.214 | 0.218 |
| Number of atoms  | protein | 3864 | 3861 | 3864 |
|                  | heterogen | 41 | 50 | 29 |
|                  | water | 280 | 295 | 287 |
| Average B (Å<sup>2</sup>) | 22.0 | 17.9 | 24.6 |
| RMSD ideal geometry | bond lengths (Å) | 0.005 | 0.005 | 0.005 |
|                  | bond angles (°) | 1.3 | 1.3 | 1.3 |
| Ramachandran plot<sup>a</sup> | %—most favoured | 89.9 | 90.9 | 90.1 |
|                  | %—additionally allowed | 10.1 | 9.1 | 9.9 |

<sup>a</sup> Calculated by treating Bijvoet pairs as equivalent.

<sup>b</sup> R<sub>free</sub> = Σ<sub>i</sub> |I<sub>i</sub>|<sub>obs</sub> – |I<sub>i</sub>|<sub>calc</sub> / Σ<sub>i</sub> |I<sub>i</sub>|<sub>obs</sub> = |I<sub>i</sub>|<sub>calc</sub> for reflections in the working and test sets (10% of all data) respectively.

<sup>c</sup> Regions defined by PROCHECK (31).

<sup>d</sup> Regions defined by PROCHECK (31).

using the program Grafit 4.0, yielding values for the pseudo-

first-order rate constant at each pH value. Because at low substrate concentrations ([S] < Km) the reaction rates are given by Equation 1,

\[ V = \frac{k_{cat}[E][S]}{K_m} \]  

(Eq 1)

the k<sub>cat</sub>, values correspond to [E]k<sub>cat</sub>/K<sub>m</sub>. Thus, k<sub>cat</sub>/K<sub>m</sub> values can be extracted by division of these obtained rate constants by the enzyme concentration. By analyzing the bell-shaped plots of k<sub>cat</sub>/K<sub>m</sub> versus pH using the program Grafit 4.0 (33) two apparent pK<sub>a</sub> values of ionizable groups were assigned. Enzyme stability over the pH range, assay time, and at the temperature of the study was examined by adding enzyme at the same concentration as examined in the pH study to a preincubated cell containing 0.02% bovine serum albumin and the appropriate buffer at 37 °C. After 10 min an aliquot of the mixture was removed and injected into another preincubated solution containing PhGlcNAc in 25 mM NaH<sub>2</sub>PO<sub>4</sub>/25 mM sodium citrate buffer (pH 5.0) containing 100 mM NaCl. Data were retained for those pH values at which enzyme was stable during the assay period. Data were discarded if more than 10% enzyme death had occurred over a 10-min period. V<sub>max</sub> values at each pH value were determined by measuring the rate of reaction using a concentration of PhGlcNAc of 4.75 mM (10 x Km) over a period in which less than 10% substrate was consumed. The kinetic parameters for PhGlcNAc at pH 5.5 are k<sub>cat</sub> = 172 s<sup>-1</sup>, K<sub>m</sub> = 0.48 mM, and k<sub>cat</sub>/K<sub>m</sub> = 360 s<sup>-1</sup> mM<sup>-1</sup>.

**Kinetic Analysis**—The SpHex-catalyzed rates of hydrolysis of 3,5-DNP-GlcNAc were measured at 25 °C using a 25 mM NaH<sub>2</sub>PO<sub>4</sub>/25 mM sodium citrate buffer (pH 6.0) containing 100 mM NaCl. Reactions were initiated by the addition of SpHex. Using a Pye Unicam spectrophotometer the increase in absorption at 400 nm was monitored continuously. In all cases the rates were linear over the assay period. Michaelis-Menten parameters (V<sub>max</sub> and K<sub>m</sub>) were extracted from these data by non-linear fitting to the Michaelis-Menten equation using the program Grafit 4.0 (33). K<sub>m</sub> and k<sub>cat</sub>/K<sub>m</sub> values were obtained by measuring rates in a series of cells at a range of substrate concentrations (6–10 concentrations), which encompassed the K<sub>m</sub> value ultimately determined from 0.2 x K<sub>m</sub> to 5 x K<sub>m</sub>. Because of the extremely low activity of the SpHex D313N variant, kinetic runs were performed over a period of up to 12 h in parallel using an automated cell-changer. For chemical rescue ex-
Two variants (D313A and D313N) of the conserved Asp-313 constant to that of an authentic sample of 3,4,6-tri-... 

The pH profile of the wild-type enzyme reveals an approximately bell-shaped profile with a pH optimum of 5.0, which in the simplest interpretation results from the ionization of two residues (with pH 6.0). TLC analysis of a sample of the reaction mixture containing the wild-type enzyme appeared as a single spot in the above phosphate/citrate/NaCl buffer (pH 6.0). The kinetic parameters of the wild-type, the D313A, and the D313N variants were determined using 3,5-DNPGlcNAc as substrate (Table II). The reductions in the $k_{cat}/K_m$ values for the two variants compared with the wild-type enzyme largely reflect the decrease in the $k_{cat}$ values. The low values of $K_m$ relative to the $k_{cat}$ determined for NAG (3.0 mM) (34) suggest the accumulation of a relatively high steady-state concentration of a reaction intermediate, most likely the oxazolinium ion intermediate, in all three cases. A convenient way to consider this is that the $K_m$ value represents an equilibrium between free enzyme and substrate and all forms of enzyme substrate and intermediate complexes according to Equation 2.

$$K_m = \frac{[E][S]}{[\text{enzyme bound species}]} \quad \text{(Eq. 2)}$$

Thus, in cases where the second step, ring-opening, is rate determining the intermediate accumulates to a relatively high steady-state concentration and the $K_m$ value will be considerably lower than the $K_m$ value of the substrate. This in turn suggests that the rate-determining step is the hydrolysis of the oxazolinium ion intermediate. Consequently, the reduction in the value of $k_{cat}$ appears to reflect a decrease in the rate of hydrolysis of the oxazolinium ion intermediate in the variants as compared with the wild-type enzyme. Therefore, at least one effect of the Asp-313 mutation appears to be the destabilization of the second transition state of the enzyme-catalyzed reaction (Fig. 1, II).

Insight into the degree of transition state stabilization afforded in the first transition state (Fig. 1, I) by the Asp-313 residue may be gained by an analysis of the change in the $k_{cat}/K_m$ values for the variants versus the wild-type enzyme. For the reaction catalyzed by SpHex the apparent second order rate constant, $k_{cat}/K_m$, reflects the energy difference between the free enzyme and substrate and the transition state of the first irreversible chemical step: cleavage of the glycosidic bond and formation of the oxazoline intermediate. The large reductions in the values of $k_{cat}/K_m$ observed with the two variants relative to the wild-type enzyme indicate that replacement of Asp-313 with either Ala or Asn has marked effects on transition state stabilization. As the $K_m$ values of the variant and wild-type enzymes are similar, changes in the values of $k_{cat}/K_m$ necessarily parallel changes in the values of $k_{cat}$. This suggests that Asp-313 has similar importance in both steps of the reaction and is consistent with an electrophilic migration mechanism in which the second step is the near microscopic reverse of the first. However, the destabilizing effects of each of the two variants differ considerably and correspond to a loss of transition state stabilization of 2.6 kcal mol$^{-1}$ for the D313A variant and 7.8 kcal mol$^{-1}$ for the D313N variant (Table II).

The degrees of residual activity with the D313A and D313N variants may be a consequence of the size of the two substituting amino acids. While the conversion of aspartate to asparagine appears at first glance a conservative mutation, close examination of the x-ray structure of SpHex shows that the Asp-313 residue makes a close contact of 2.5 Å with N2 of the thiazoline. Thus, in the D313N variant, in addition to the loss of charge stabilization seen with the D313A variant, there may be additional steric congestion resulting from the substitution of a carboxyl group by a CONH$_2$ moiety, which results in further destabilization of the intermediate and its flanking transition states, or perhaps may even induce an alternative mechanism. These possibilities prompted the investigation of the consequences of these substitutions on the structures of the product complexes of the wild-type and variant enzymes.
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Table II

| Enzyme       | $k_{\text{cat}}$ | $K_m$  | $k_{\text{cat}}/K_m$ | Fold decrease |
|--------------|------------------|--------|----------------------|---------------|
|              | s$^{-1}$         | $\mu$M | s$^{-1}$µM$^{-1}$    |               |
| SpHex$^a$    |                  |        |                      |               |
| Wild-type    | 222              | 48     | 4.6                  | —             |
| D313A        | 3.30             | 56     | 0.059                | 78            |
| D313N        | 0.00028          | $\sim$34 | 0.0000082         | 560,000       |
| SmCHB$^b$    |                  |        |                      |               |
| Wild-type    | 110$^e$          | 56,700$^d$ | 0.00195$^f$        | —             |
| D539A        | 827$^e$          | 0.063$^c$ | 13,000$^e$         | —             |
| E540A        | 17               | 1.991  | 0.8                  | 16,000$^e$    |
| D354N        | 6                | 0.014  | 420                  | 31$^e$        |
| D355Q        | 530              | 690    | 1.08                 | —             |
|              | 0.31             | 680    | 0.00044              | 2,500         |
|              | 0.14             | 450    | 0.00033              | 3,300         |

$^a$ Using 3,5-DNPGlcNAc.  
$^b$ Using pNPGlcNAc.  
$^c$ Using MUGlcNAc.  
$^d$ Data from Ref. 12.  
$^e$ Data from Ref. 25; interestingly, the $k_{\text{cat}}/K_m$ value reported by Prag et al. in this report was greater than the diffusion limit.  
$^f$ Ratios calculated using data from Ref. 25.

Structural Analysis of Product Complexes with Wild-type, D313A, and D313N SpHex—The structure of a product complex between NAG and SpHex was solved and yielded well-defined electron density for a sugar bound in the −1 subsite (Fig. 4). The 2-acetamido group is positioned such that the carbonyl oxygen is under the mean plane of the sugar ring and within 2.5 Å of the anomeric carbon, C1 (Fig. 5A). A hydrogen-bonding contact (2.5 Å) is observed between the anomeric hydroxyl and the carboxyl group of the general acid/base residue Glu-314. Although the sugar ring is not in a completely relaxed 4C1 conformation, it is also not distorted to the same extent as seen for the skew-boat conformation of the analogous sugar found in the Michaelis complex between chitobiose and SmCHB (17). Rather, the conformation of the NAG ring is remarkably similar to the conformation of the piperidine ring in the complex of NAG-isofagomine with SpHex (16). The difference in conformation of the sugar ring bound in the −1 subsite of the Michaelis complex as compared with the product complex determined here suggests that enzyme-substrate interactions at the +1 subsite contribute significantly to the distortion of the pyranose ring in the −1 subsite, positioning the scissile bond and leaving group in a pseudo-axial orientation prior to bond cleavage.

Complexes of NAG bound to the D313A and D313N SpHex variants also yielded well-defined electron density for the product bound in the −1 subsite (Fig. 5, B and C). When Asp-313 is substituted by Ala in SpHex, a sizeable pocket is created in the active site (Fig. 5B). Interestingly, unusual electron density for the 2-acetamido moiety of the NAG residue was observed in the complex and was understood to arise from the 2-acetamido side-chain adopting two alternate conformations in the crystal structure. One conformation, with an occupancy of 0.4, appears to be compatible with catalysis and is similar to that of NAG bound to the wild-type enzyme (Fig. 5A). For the alternate conformation, with an occupancy of 0.6, the 2-acetamido group is swung out from under the sugar ring and into the position usually occupied by the carboxyl group of Asp-313. Curiously, the sugar ring in this conformer is bound in a 1,4B conformation. This second conformer is similar to that reported by Prag et al. (25) where the analogous Asp variant of SmCHB in complex with chitobiose revealed that the 2-acetamido group occupies the pocket created by the deleted Asp carboxyl. Together, the structural and kinetic studies with the SpHex and SmCHB enzymes indicate that the Asp-313 residue plays a critical role in controlling the orientation of the 2-acetamido group in addition to stabilizing the two transition states of the enzyme-catalyzed reaction.

In the case of the complex of the SpHex D313N variant with NAG, the sugar ring is in a 4C1 conformation and is tilted forward with C1 dropped by 1.1 Å from its position in the wild-type complex (Fig. 5C). The Asn-313 residue occupies an almost identical position to Asp-313 in the wild-type complex, however, the 2-acetamido moiety is rotated about the C2-N bond 117° from that of the complex of NAG with the wild-type enzyme. The substitution of a slightly bulkier CONH2 for the carboxyl moiety of Asp-313 has likely introduced sufficient steric conflicts that the 2-acetamido moiety is unable to adopt its optimal conformation. Difference electron density of an omit map for the Asn-313 side-chain indicates that the CONH2 group is oriented so that Nδ2 points toward the 2-acetamido moiety; whereas, the carbonyl oxygen shares a proton with the
FIG. 5. Stereographic representations of NAG bound in the active site of wild-type SpHex (A), SpHex D313A (B), and SpHex D313N (C). The ball-and-stick models shown in each panel represent the final refined coordinates of each complex. The initial $\alpha$-A weighted $F_o - F_c$, difference electron density, calculated prior to modeling the NAG residue and solvent of each complex, is shown in blue (see “Experimental Procedures”). A, wild-type SpHex in complex with NAG. SpHex carbon atoms shown in gray, whereas the carbon atoms of NAG are shown in green. For both the enzyme and sugar, nitrogen atoms are shown in blue and oxygen atoms in red. The enzymic residue, Asp-313, is labeled in red. Notable features of this complex include the conformation of the pyranose ring of the NAG sugar, which approaches that of a 4$C_1$ chair and a strong hydrogen bonding interaction between the hydroxyl group of the anomeric center and the carboxyl group of the general acid/base residue Glu-314. Furthermore, the 2-acetamido group is positioned underneath the $\alpha$-face of the pyranose ring and partially held in position by hydrogen-bonding interactions with Asp-313 and Tyr-393. B, SpHex D313A in complex with NAG. SpHex carbon atoms shown in gray, nitrogen atoms shown in blue and oxygen atoms in red. The substituted residue Ala-313 is labeled in red. This mutation creates a pocket within the active site that can be occupied by the 2-acetamido group and a water. The NAG residue with its 2-acetamido group in this pocket is the dominant sugar conformation in the complex (overall occupancy of 0.6) and is drawn in with green carbon, blue nitrogen, and red oxygen atoms. The alternate conformation, which is more catalytically relevant, has an overall occupancy of 0.4 and is drawn with brown carbon, blue nitrogens, and magenta oxygen atoms. Electron density could not be seen for the anomeric hydroxyl group of either NAG conformation in this complex and may reflect large motions at this center. The refined positions of the anomeric hydroxyl groups in the models of the alternate NAG conformations resulted from an optimization of the geometry of all the remaining atoms for which there was clear electron density. For the dominant conformation, the pyranose ring refined into a 4$C_1$B boat, whereas in the alternate conformation, the pyranose ring is close to a $C_1$ chair and is similar in conformation to the NAG molecule bound to wild-type SpHex. C, SpHex D313N in complex with NAG. SpHex carbon atoms shown in gray, nitrogen atoms of NAG are shown in green. Nitrogen atoms shown in blue and oxygen atoms in red for both the enzyme and sugar. The mutated residue, Asn-313, is labeled in red. There is only one conformation for the sugar in this complex. The pyranose ring is close to a $C_1$ chair, while the 2-acetamido group is positioned with the carbonyl oxygen pointing up toward the $\beta$-face of the sugar in a conformation similar to that of the non-reducing sugar of the unbound crystal structure of chitobiose (42).
The D313N variant complex. The positive $F_o - F_c$ density indicates that the oxygen atom of the CONH$_2$ group lies to the left and is involved in a short 2.5 Å hydrogen bond with Asp-246 as shown in panel A. Panel B, $2F_o - F_c$ density (red) and positive $F_o - F_c$ (blue) of an omit map calculated after refinement of a D313A variant model of SpHex using structure factor amplitudes from the D313N variant complex. The positive $F_o - F_c$ density indicates that the oxygen atom of the CONH$_2$ group lies to the left and is involved in a short 2.5 Å hydrogen bond with Asp-246 as shown in panel A. Panel C, $F_o - F_c$ density map (blue, 2.7 σ; red, −2.0 σ) and side-chain B-factors calculated after refinement of a model of the D313N variant where the CONH$_2$ group was rotated 180°. The difference density peaks and B-factors suggest the original orientation of the amide to be correct when compared with panel D. Panel D $F_o - F_c$ density map (blue, 2.7 σ; red, −2.0 σ) and B-factors of the original and final D313N variant model of SpHex. The lack of difference peaks when contoured to the same σ level as in panel C confirms that this orientation of the amide is correct.

carboxyl group of Asp-246 via a short 2.5 Å hydrogen bond. Furthermore, inspection of the difference density and B-factors calculated for a model of the D313N variant refined with the amide of Asn-313 flipped 180° provides further support that N$\delta^2$ is pointing toward the 2-acetamido group of the bound product (Fig. 6). This orientation allows N$\delta^2$ to donate a hydrogen bond (3.0 Å) to the carbonyl oxygen atom of the 2-acetamido group. Indeed, this hydrogen bond results in a 24° rotation of the carbonyl oxygen atom out of the plane of the 2-acetamido group and towards atom N$\delta^2$ of Asn-313. This reorganisation prevents the 2-acetamido moiety from providing efficient anchimeric assistance, resulting in the very large reductions in both the values of $k_{cat}$ and $k_{cat}/K_m$ observed for this variant as described above. The kinetic and structural data suggest that the D313A variant is able to catalyze substrate hydrolysis through a mechanism involving anchimeric assistance by the 2-acetamido moiety, albeit with significantly compromised efficiency. In contrast, the D313N mutation restricts the orientation of the 2-acetamido moiety, effectively prohibiting anchimeric assistance, suggesting that the hydrolytic mechanism of this variant may be fundamentally changed. An alternative explanation for the dramatic reduction in catalytic efficiency of the D313N variant could be that the mechanism of enzyme-catalyzed hydrolysis remains the same but is compromised to an even greater extent than that of the D313A variant.

**Chemical Rescue of D313A SpHex**—To provide further evidence of the ability of the D313A variant to proceed through a mechanism involving an oxazolinium ion intermediate and to examine the possibility that the D313N variant does not, the ability of these variants to undergo chemical rescue upon addition of small exogenous nucleophiles was investigated. In the case of the D313A variant, addition of azide causes a marked increase in the rate of reaction (Fig. 7), as can be seen in the plot of the normalized parameters. For increases in the concentration of azide up to 1.8 M, the $K_m$ value remains largely constant (only increasing 2-fold; Fig. 8), however, over the same range the $k_{cat}$ value increases substantially (to 16-fold its original value). Interestingly, no increase in the rate of hydrolysis catalyzed by the D313A variant was observed with concentrations of sodium formate up to 1.8 M, nor was any effect seen with the D313N variant using either azide or formate as exogenous nucleophiles. In addition, essentially no chemical rescue was seen with the wild-type enzyme when assayed in the presence of 1 M sodium azide.

Such small molecule chemical rescue of enzyme activity of glycosidase variants is not unique. Chemical rescue has been seen upon the addition of small, charged nucleophiles such as azide and formate to acid/base and nucleophile variants of retaining glycosidases (35). In the case of acid/base variants, chemical rescue has been observed with substrates bearing good leaving groups. In these cases, the good anomic leaving group allows the first step of the reaction, the formation of the glycosyl enzyme intermediate, to occur efficiently in the absence of general acid catalysis. However, the second step, the breakdown of the intermediate, is slowed substantially as there is no residue available to facilitate the attack of water through general base catalysis. In such cases, the intermediates often partition preferentially to small exogenous nucleophiles instead of water, and the resulting substitution products have the same anomic configuration as the substrate. Glycosidases in which the nucleophilic carboxyl group has been deleted have greatly reduced catalytic activity with up to 10$^2$-fold reduction in the catalytic efficiency. However, when such variant enzymes are presented with a substrate bearing an excellent leaving group and a small exogenous nucleophile such as azide or formate, the reaction proceeds relatively efficiently to afford a substitution product with inverted configuration at the anomic center. These two scenarios differ from that seen here with SpHex D313A, as this variant enzyme possesses both a nucleophile (the 2-acetamido moiety) and an acid/base residue (Glu-314). There are (at least) two possible explanations for the chemical rescue observed. Azide could be acting to provide...
charge stabilization of transition states that flank the oxazolinium ion, taking the place of the deleted carboxylate of the Asp-313 residue. Alternatively, azide could be acting as a nucleophile, attacking the anomeric carbon of the oxazolinium ion intermediate to form an azide substitution product (Fig. 9).

In support of this second proposal, analysis of the products of the SpHex D313A-catalyzed reaction allowed the identification of N-acetyl-β-D-glucosaminyl azide as confirmed by TLC and 1H NMR. Further, the increase in the value of $k_{cat}$ is consistent with an acceleration of the rate-determining step, which, as discussed above, is most likely the second step, the hydrolysis of the oxazolinium ion intermediate. Interestingly, there is a modest increase (5-fold) in the value of $k_{cat}/K_m$ with increasing azide concentration. The reason for this increase is ambiguous but may be a result of charge stabilization of the first transition state by azide ion. The absence of chemical rescue with formate may stem from the greater size of this anion making access to the enzyme active site difficult.

The absence of chemical rescue with the D313N variant would also be consistent with this variant enzyme using an altogether different mechanism. This possibility is supported by the structural data, which reveals that the acetalamido group is oriented so as to make its participation most improbable. The 560,000-fold reduction in the value of $k_{cat}/K_m$ observed for this variant is similar to that observed upon deletion of the carboxyl group of the enzyme nucleophiles of retaining glycosidases, which necessarily must then proceed by a different mechanism (35). Thus, misdirection of the 2-acetamido group effectively prevents it from acting as a nucleophile, resulting in an outcome similar to that of deletion of the enzyme nucleophile of a retaining glycosidase. Indeed, the $K_m$ value measured here for the D313N variant enzyme is suspiciously similar to that measured for the wild-type enzyme, which is suggestive of wild-type contamination. Quite possibly, spontaneous deamination and/or translational misincorporation may have resulted in contamination of the variant enzyme preparation with a very small quantity (at most 1 part in 560,000) of the wild-type enzyme. The kinetic parameters observed here therefore likely represent an upper limit for the values of $k_{cat}$ and $k_{cat}/K_m$.

Relevance of SpHex Data for other Family 20 Hexosaminidases—Prag et al. (25) have investigated the role of the equivalent residue in SmCHB (Asp-539) by mutagenesis and x-ray crystallography. These authors found that substitution of alanine for aspartate in SmCHB resulted in a greater than 1,000-fold reduction in the value of $k_{cat}/K_m$ when using pNPGlCNac as substrate. However, in that case there seem to be some ambiguities in the values of the kinetic parameters reported for SmCHB. In the original characterization of SmCHB by Douillard et al. (12) the $K_m$ value reported for the wild-type enzyme differed by nearly 1 million-fold from those reported in the study of Prag et al. (25). For the SpHex D313A variant a moderate reduction in the value of $k_{cat}/K_m$ of 78-fold was observed, somewhat less than the 1,625-fold reduction seen for the corresponding SmCHB D539A variant by Prag et al. These workers also determined the x-ray structure of the SmCHB D539A variant in complex with the substrate, chitobiose, and in this structure the 2-acetamido group was seen to be rotated almost 180° from its position in the product complex of the wild-type enzyme, with the carbonyl group directed away from the anomeric carbon. In the cases of the substrate complex with SmCHB and the product complex with SpHex reported here, the D313A substitution results in the formation of a large pocket into which the 2-acetamido moiety can fit, resulting in the acetamido group adopting a conformation in which it is unable to provide anchimeric assistance (Fig. 10). While in the case of the SmCHB D539A substrate complex this conformation is the only one observed in the structure, in the case of the SpHex D313A product complex, a second conformation with an occupancy of 0.4 was also observed. This second conformation closely resembles that seen for wild-type SmCHB wherein the carbonyl group of the 2-acetamido is directed toward the anomeric carbon. It is most likely that this species reflects the
mechanistically relevant conformation of the 2-acetamido group. The kinetic results described above indicate that the D313A substitution has a very similar destabilizing effect on both the first and second steps of the reaction. As a consequence the intermediate is kinetically more stable, with hydrolysis occurring at a lower rate. In accord with the reactivity/selectivity principle, the increased stability of the intermediate accounts for the significant chemical rescue observed as this intermediate now partitions favorably to a better nucleophile such as azide. A further contributing factor could be that electrostatic repulsion between Asp-313 and an incoming charged group. The kinetic results described above indicate that the D313A substitution has a very similar destabilizing effect on both the first and second steps of the reaction. As a consequence the intermediate is kinetically more stable, with hydrolysis occurring at a lower rate. In accord with the reactivity/selectivity principle, the increased stability of the intermediate accounts for the significant chemical rescue observed as this intermediate now partitions favorably to a better nucleophile such as azide. A further contributing factor could be that electrostatic repulsion between Asp-313 and an incoming charged group.

Hou et al. (24) have described the preparation of the D354N variant enzyme of human HexB, which corresponds to the D313N variant of SpHex described here. When assayed against methylumbelliferyl N-acetyl-β-D-glucosaminide, the D354N variant was shown to have a 2,500-fold lower value of \( k_{\text{cat}}/K_m \) than does the wild-type. This rate reduction is considerably less than the 560,000-fold reduction observed for the SpHex D313N variant studied here. These investigators, studying this difficult system, indicate that the enzyme preparation is ~99.99% free of wild-type contamination, a marked improvement over earlier studies with the same enzyme (36). However, assuming 0.01% contamination and a completely inactive variant enzyme the greatest possible rate reduction that can be measured is 10,000-fold and is very close to the rate reduction that these workers report. Additionally, the \( K_m \) value is suspiciously close to that of the wild-type enzyme, and so the reduction in rate reported by Hou et al. likely does not fully reflect the consequences of the D354N mutation.

Family 18, 20, and 56 hexosaminidases All Utilize Substrate-assisted Catalysis—The chitinases of family 18, the hexosaminidases of family 20, and the hyaluronidases of family 56 all lack an enzymic nucleophile residue. In the case of the family 18 enzymes a mechanism identical to that of the family 20 enzymes has been proposed wherein the 2-acetamido moiety of the substrate acts as the nucleophile (15). Additionally, the tight binding of the naturally occurring inhibitor, allosamidin, which contains an oxazoline ring, to family 18 chitinases has been invoked as circumstantial but relevant evidence for an oxazoline intermediate (15). In a complex of the family 18 chitinase, ChiB from S. marcescens, with the natural product allosamidin, a close contact of 2.7 Å was observed between Asp-142 and the oxazoline nitrogen (37), and this is very similar to the contact observed between Asp-313 and the nitrogen of NAG-thiazoline (2.5 Å) (16). There is considerable equivalence in the active site architecture of family 18 and family 20 enzymes (and also family 56 enzymes, Ref. 14) with the acid/base acting as the nucleophile when the carboxyl directed either towards or away from the anomeric carbon. These workers opted to interpret their results in terms of the conformer of the D354N mutation.

**Fig. 10.** Stereographic superposition of NAG bound in the active sites of wild-type SpHex (gray), SpHex D313A (green), and SpHex D313N (yellow). Of the two NAG conformations refined in the SpHex D313A:NAG complex, only the catalytically incompetent conformer is shown. Panels A and B are oriented ~90° about the y-axis with respect to each other.
Role of Asp-313 in Streptomyces plicatus Hexosaminidase

Introduction

Kinetic analysis of the two SpHex Asp-313 variants, the structures of product complexes of the two variants and wild-type SpHex, and the observation of chemical rescue with azide ion provide a detailed description of the role of the conserved Asp-313 residue. The large effects observed on the rate of the enzyme-catalyzed reaction for both the D313A and D313N variants are consistent with an important role for this residue in carefully positioning the 2-acetamido group in a favorable geometry to act as a nucleophile. For the D313A variant enzyme, destabilization of the transition state bracketing the oxazoline intermediate results in the increased kinetic stability of this intermediate. This intermediate partitions to azide preferentially over water, rescuing enzymatic activity as a result of azide attacking the anomic center of the oxazoline intermediate to generate the ring-opened β-azide product. X-ray crystallographic analysis of the product complexes with the wild-type, D313A, and D313N variants provides strong evidence for the Asp-313 residue controlling the orientation of the 2-acetamido moiety and the pyranose ring of the sugar bound in the −1 subsite. The conformation of the product complex observed here is likely not that of the first formed product. In accordance with an electrophilic migration mechanism it is expected that the anomeric carbon of the first formed product adopts a position above the plane of the sugar ring where it has been intercepted by a water molecule hydrogen bonded to the basic enzymic carboxyl group of E314. Because of the absence of interactions at the +1 subsite, this unstable conformation relaxes to a lower energy conformation, in this case a distorted C1. This observation emphasizes the importance of interactions of the substrate in the +1 subsite in distorting the substrate within the Michaelis complex. A possible conformational itinerary of the sugar ring upon binding and during the catalytic cycle of SpHex can be formulated as 4C1 → [1S0 → 4H3 → 4C1 → 4H3 → 1S0 → 4C1]bound → 4C1 (where the conformation marked with an asterisk represents that of the first formed product). Throughout this process the anomeric carbon can be considered as scribing an arc where it starts above the plane of the sugar ring in the Michaelis complex, migrates to the nucleophile, the carbonyl group of the 2-acetamido group, that waits below the ring plane, and then moves back to an awaiting water molecule that lies above the plane.

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CONCLUSIONS

REFERENCES