Group A streptococcus (Streptococcus pyogenes) is the causative agent of severe invasive infections such as necrotizing fasciitis (the so-called ‘flesh eating disease’) and toxic-shock syndrome. Spy1600, a glycoside hydrolase from family 84 of the superfamily of glycoside hydrolases, has been proposed to be a virulence factor. In the present study we show that Spy1600 has no activity toward galactosaminides or hyaluronan, but does remove β-O-linked N-acetylglucosamine from mammalian glycoproteins—an observation consistent with the inclusion of eukaryotic O-glycoprotein 2-acetamido-2-deoxy-β-D-glucopyranosidases within glycoside hydrolase family 84. Proton NMR studies, structure-reactivity studies for a series of fluorinated analogues and analysis of 1,2-dideoxy-2-methyl-α-D-glucopyranosyl-[2,1-d]-Δ2-thiazoline as a competitive inhibitor reveals that Spy1600 uses a double-displacement mechanism involving substrate-assisted catalysis. Family 84 glycoside hydrolases are therefore comprised of both prokaryotic and eukaryotic β-N-acetylglucosaminidases using a conserved catalytic mechanism involving substrate-assisted catalysis. Since these enzymes do not have detectable hyaluronidase activity, many family 84 glycoside hydrolases are most likely incorrectly annotated as hyaluronidases.

Key words: β-N-acetylglucosaminidase (GlcNAcase), mammalian glycoproteins, 1,2-dideoxy-2-methyl-α-D-glucopyranosyl-[2,1-d]-Δ2-thiazoline (NAG-thiazoline), O-glycoprotein 2-acetamido-2-deoxy-β-D-glucopyranosidase (O-GlcNAcase), O-glycoprotein 2-acetamido-2-deoxy-β-D-glucopyranosidase (O-GlcNAc), Spy1600, substrate-assisted catalysis.

INTRODUCTION

Family 84 glycoside hydrolases [1] are a group of enzymes found in higher eukaryotes and pathogenic or symbiotic bacteria. The first family 84 glycoside hydrolase cloned was identified as a tumour-associated antigen termed MGEA5 (meningioma expressed antigen 5) [2]. This enzyme had most likely been previously isolated as hexosaminidase C [3] and shown to have exo-β-N-acetylglucosaminidase activity. Further studies [4] using protein purified from rat brain defined this enzyme as O-GlcNAcase (O-glycoprotein 2-acetamido-2-deoxy-β-D-glucopyranosidase), a key regulatory enzyme responsible for mediating the removal of O-GlcNAc [O-linked GlcNAc (N-acetylglucosamine, 2-acetamido-2-deoxy-D-glucopyranose)] from serine and threonine residues of post-translationally modified proteins [5]. The rat brain O-GlcNAcase was later cloned and found to be identical with MGEA5 in sequence, and it has been proposed that this enzyme has both O-GlcNAcase and hyaluronidase activity [6]. Preliminary studies of the family 84 NagH enzyme from Clostridium perfringens have also suggested that this enzyme is a hyaluronidase [7]. On the basis of these studies, several members of this family of enzymes have been annotated as hyaluronidases. Human family 84 O-GlcNAcase has been shown to use a substrate-assisted catalytic mechanism to effect removal of O-GlcNAc residues from proteins [8], a mechanism in which the 2-acetamido group of the substrate acts as a nucleophile to form a bicyclic oxazoline intermediate (Scheme 1). This is in contrast with some other hexosaminidases, such as the bacterial GlcNAcases (β-N-acetylglucosaminidases) from glycoside hydrolase family 3 that, instead, use an enzymatic nucleophile [9]. Recently, X-ray structural studies of two bacterial family 84 enzymes have given strong support to the substrate-assisted catalytic mechanism for family 84 enzymes [10,11] yet, despite genome annotations, no unambiguous studies have addressed whether family 84 glycosidases do genuinely process hyaluronan [6].

As part of an ongoing study into virulence factors from the human pathogen group A streptococcus, we are interested in Spy1600, a family 84 glycoside hydrolase from this organism. Group A streptococcus causes severe invasive infections such as necrotizing fasciitis and toxic-shock syndrome [12], and it has recently been estimated that these organisms cause half a million deaths each year [13]. The streptococcal family 84 enzyme has been shown to be up-regulated during phagocytosis and has, accordingly, been implicated as a virulence factor [14]. To better understand this putative virulence factor as well as to facilitate the design of potential inhibitors of this enzyme that could eventually prove useful in a clinical setting, we have undertaken a series of studies of this enzyme. In the present study we describe the cloning, recombinant overexpression and purification, studies on

Abbreviations used: GlcNAc, N-acetylglucosamine (2-acetamido-2-deoxy-D-glucopyranose); GlcNAcase, β-N-acetylglucosaminidase; MGEA5, meningioma expressed antigen 5; MU, 4-methylumbelliferon; MU-GlcNAc, Mu-2-acetamido-2-deoxy-β-D-glucopyranoside; MU-GlcNAcF, Mu-2-deoxy-2-fluoroacetamido-β-D-glucopyranoside; O-Mu-GlcNAc, O-methylacetamido-2-deoxy-β-D-glucopyranoside; O-GlcNAcase, O-glycoprotein 2-acetamido-2-deoxy-β-D-glucopyranosidase; ORF, open reading frame.

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the stereocchemical outcome of the enzyme catalysed reaction, substrate structure–function studies and inhibition studies. Collectively, these studies show that Spy1600, in common with eukaryotic O-GlcNAcases, uses substrate-assisted catalysis. This enzyme is also capable of cleaving O-GlcNAc from post-translationally modified proteins, opening up the possibility that it may encounter this modification during the life cycle of the bacterium. Furthermore, despite annotation as a putative hyaluronidase, we show that Spy1600 displays no detectable hyaluronidase activity, suggesting that glycoside hydrolase family 84 comprises GlcNAcases and not, as implied by genomic annotations, hyaluronidases.

EXPERIMENTAL

Materials

All materials were purchased from Sigma–Aldrich, unless stated otherwise. All methods followed the manufacturer’s instructions, where given, or were performed as described by Sambrook et al. [15].

Methods

Amplification and cloning of the ORF (open reading frame) encoding Spy1600, a family 84 glycoside hydrolase from Streptococcus pyogenes M1 GAS SF370

The amino acid sequence of Spy1600 was checked for the presence of a signal peptide using the SignalP 3.0 server [16]. The amino acid sequence, spy1600, was amplified from genomic DNA of S. pyogenes SF370 (A.T.C.C. 700294; [18]) using the following primers: 5′- CAT ATG ACC ATT TAC GGC TTA AAG -3′ and 5′- GGA TCC GAT TCG TAT CAG AAG GCC AGA AC -3′. The amplified product was cloned into pCR-Blunt (Invitrogen), then subcloned into pET-28a (Novagen) on an Nde I–Bam HI fragment, and the resulting plasmid designated pSpy1600. The conditions used for amplification were as specified for use with Platinum Pfx DNA polymerase (Invitrogen).

Production and purification of Spy1600

N-terminally hexahistidine-tagged Spy1600 was produced via induction of Escherichia coli BL21 (DE3; Novagen) cultures carrying pSpy1600 following a protocol described in Brown et al. [19] for expression of a different protein, except cultures were grown at 20°C post-induction. The recombinant protein product was purified via affinity chromatography using nickel-charged SepharoseTM chelating fast flow resin (GE Healthcare) as described in the manufacturer’s instructions. The fractions containing pure protein were concentrated, exchanged into 18.2 MΩ/cm water using a 30 kDa-cut-off concentrator unit (Viva Science), and the protein concentration determined according to the method of Bradford [20], using BSA as a standard. The purity of Spy1600 was judged by SDS/PAGE [21] and static nanospray ion-trap MS (ThermoFinnigan LCQ Advantage) of a tryptic digest was used to confirm the identity of the protein.

Spy1600 assays

Assays using MU (4-methylumbelliferyl) glycosides, i.e. 2-MU-α-D-N-acetylneuraminic acid, MU-α-D-galactoside, MU-α-D-mannopyranoside, MU-β-D-galactoside, MU-β-D-glucuronide, MU-β-D-mannopyranoside, MU-β-acetyl-β-D-glucosaminide, (MU-2-acetamido-2-deoxy-β-D-glucopyranoside or MU-GlcNAc), MU-β-acetyl-β-D-galactosaminide and the three N-fluoroacetyl derivatives of MU GlcNAc (Figure 1A; MU-GlcNAcF, (MU-2-deoxy-2-fluoroacetamido-β-D-glucopyranoside), MU-GlcNAcF2 (MU-2-deoxy-2-difluoroacetamido-β-D-glucopyranoside) and MU-GlcNAcF3 (MU-2-deoxy-2-trifluoroacetamido-β-D-glucopyranoside)) were performed in triplicate and liberating 4-methylumbelliferone was measured, in real time, using a BioTek FL600 fluorescence microplate reader. Fluorinated substrates were prepared as previously described [8]. Excitation and emission wavelengths of 360 and 460 nm respectively were used. Standard assays were performed at 37°C in a total volume of 100 µl of 20 mM Hepes buffer, pH 7.6, containing 1.0 mg/ml BSA and 0.25 µg of Spy1600. The ranges of substrate concentrations used for determining kcat and Km values were 0.5–7.0 mM for MU-GlcNAc, 0.7–3.7 mM for MU-GlcNAcF, 0.7–3.7 mM for MU-GlcNAcF2 and 0.3–1.3 mM for MU-GlcNAcF3. For the latter three substrates, 0.5 µg, 1 µg and 100 µg of enzyme was added per 100 µl of reaction mixture respectively. Goodness-of-fit statistical analyses of linear trendlines of the resulting Lineweaver–Burk plots produced r2 values of 0.9744. For inhibition assays the range of concentrations of MU-GlcNAc and NAG-thiazoline (1,2-dideoxy-2-fluoroacetamido-β-D-galactosyl-α-D-mannopyranoside, MU-2-deoxy-2-fluoroacetamido-β-D-glucopyranosyl-α-D-acetylated neuraminic acid, MU-acetyl-2-D-mannopyranosyl-β-D-acetylneuraminic acid, MU-acetyl-2-D-galactosyl-β-D-acetylneuraminic acid, and MU-acetyl-2-D-glucuronide) were added per 100 µl of reaction mixture respectively. Goodness-of-fit statistical analyses of linear trendlines of the resulting Lineweaver–Burk plots produced r2 values of 0.9744. For inhibition assays the range of concentrations of MU-GlcNAc and NAG-thiazoline (1,2-dideoxy-2-fluoroacetamido-β-D-galactosyl-α-D-mannopyranoside, MU-2-deoxy-2-fluoroacetamido-β-D-glucopyranosyl-α-D-acetylated neuraminic acid, MU-acetyl-2-D-mannopyranosyl-β-D-acetylneuraminic acid, MU-acetyl-2-D-galactosyl-β-D-acetylneuraminic acid, and MU-acetyl-2-D-glucuronide) were added per 100 µl of reaction mixture respectively. Goodness-of-fit statistical analyses of linear trendlines of the resulting Lineweaver–Burk plots produced r2 values of 0.9744.

1H-NMR studies of hydrolysis of MU-GlcNAc

1H-NMR spectra were collected at ambient probe temperature with a JEOL Eclipse spectrometer operating at 270 MHz. All reactions
were performed in $\text{H}_2\text{O}$ using 5-mm-long NMR tubes of total volume 1 ml. Both Spy1600 and MU-GlcNAc were freeze-dried and resuspended in $\text{H}_2\text{O}$ prior to monitoring the reactions. MU-GlcNAc (3.3 mg/ml) was hydrolysed using 2.5 mg/ml Spy1600.

Western-blot analyses of human proteins treated with Spy1600 COS-7 cells were cultured to 75% confluence and a cell extract was obtained as previously described [8]. The cell extract was incubated at 37°C for 36 h in the presence of 1 mM PMSF, 1 mM 2-mercaptoethanol, 10 mM imidazole (to reduce non-specific binding to Ni–agarose) and 2 mg/ml purified Spy1600 enzyme where appropriate. Spy1600 was removed prior to Western blotting by two consecutive affinity precipitations using Ni–agarose beads (Qiagen). The samples were electrophoresed through a 10% (w/v) polyacrylamide gel, blotted on to nitrocellulose and probed as previously described [8].

RESULTS AND DISCUSSION

Spy1600 is produced in large quantities in E. coli and shares a high degree of sequence similarity with the N-terminal domain of the human O-GlcNACase

Spy1600 is predicted to be a non-secretory protein by the SignalP server [16] and contains a single PfamA domain, annotated as Hyaluronidase.2 [17]. We recombiantly expressed the full-length ORF, spy1600, in E. coli, and its product, Spy1600, was obtained in large quantities (∼200 mg/l) as a N-terminally hexahistidine-tagged protein. The enzyme was readily purified to homogeneity as judged by SDS/PAGE (see the Supplementary Figure at http://www.BiochemJ.org/bj/399/bj3990241add.htm). Spy1600 is significantly smaller than the human family 84 O-GlcNACase; however, they are very similar over a ∼300-residue stretch that coincides with the location of the Hyaluronidase.2 PfamA domain, and this level of identity includes two aspartate residues that have been shown to be the catalytic residues in human O-GlcNACase [24] as well as within the structurally characterized bacterial family 84 enzymes [10,11] (Figure 2). This observation suggests that the Hyaluronidase.2 PfamA domain, which these enzymes share in common, likely performs related functions in nature, as was first proposed by Hanover [25] and more recently demonstrated by kinetic studies [24,26,27].

Spy1600 shows no hyaluronidase activity and is only active against β-N-acetylglucosaminides

Spy1600 and many other family 84 glycoside hydrolases are annotated in GenBank®, GenPept and Swiss-Prot as putative hyaluronidases. Indeed, eukaryotic O-GlcNACases have been proposed to have hyaluronidase activity on the basis of zymogram-like assays [2]. We, however, were unable to detect any hyaluronidase activity when Spy1600 was incubated with hyaluronan using a reducing-sugar assay [22] or using high-performance anion-exchange chromatography [23] to look for expected oligosaccharide breakdown products (Figure 3). Given the detection limits of these assays, the upper limit of hyaluronidase activity that could be present without being observed here is ∼1 pmol·min$^{-1}$·mg$^{-1}$. We also find that Spy1600 shows activity only against β-N-acetylglucosaminides, with no detectable activity against β-N-acetylgalactosaminides. This difference in epimeric specificity contrasts with the family 20 β-hexosaminidases, which have nearly equivalent activity towards these substrates [28], and is consistent with the behaviour of other family 84 glycoside hydrolases [4]. We also assayed Spy1600 with several other β- and α-glycosides of methylumbelliferone (see the list in the Experimental section) and found that none of these are substrates. Owing to the marked sequence similarity between Spy1600 and the N-terminal domain of human family 84 O-GlcNACase, we expected that Spy1600 might have the ability to remove O-GlcNACe from eukaryotic glycoproteins. Using a COS-7 cell extract and incubating Spy1600 with these glycoproteins we
were able to show, using an anti-\(O\)-GlcNAc antibody in a Western-blot analysis, that this enzyme effectively removed \(O\)-GlcNAc from eukaryotic \(O\)-GlcNAc modified glycoproteins (Figure 4). This \(O\)-GlcNAcase activity may be of importance, since Spy1600 may, at some point in the life cycle of \(S.\) pyogenes, encounter \(O\)-GlcNAc and process this modification either for purposes of foraging or for interfering with the host cellular machinery.

Biochemical and biophysical parameters of Spy1600 are similar to those of other GlcNAcases

The pH–activity optimum for Spy1600 towards MU-GlcNAc is 7.4. When subjected to a 20 min exposure to various temperatures, Spy1600 showed no significant decrease in activity after pre-incubations at 37.6, 46.9, 49.1, 51.4 and 53.1 \(^\circ\)C, but was only 33\% active after a pre-incubation at 57 \(^\circ\)C, and was completely inactive after a pre-incubation at 68 \(^\circ\)C. The percentage inhibition of enzyme activity resulting from the inclusion of 1 mM bivalent metal ions in the assays was as follows: 28\% (Ba\(^{2+}\)), 3\% (Ca\(^{2+}\)), 28\% (Co\(^{2+}\)), 3\% (Mg\(^{2+}\)), 20\% (Mn\(^{2+}\)) and 46\% (Ni\(^{2+}\)). The kinetic parameters, \(k_{cat}\) and \(K_m\), for Spy1600 against MU-GlcNAc, were 32 \(\pm\) 3 s\(^{-1}\) and 3.6 \(\pm\) 0.3 mM respectively, with a \(k_{cat}/K_m\) value of 9.0 s\(^{-1}\)·mM\(^{-1}\). The \(k_{cat}\) and \(K_m\) values for Spy1600 against MU-GlcNAc are in the same order of magnitude as those for several other GlcNAcases [8,9,29].

Spy1600 uses substrate-assisted catalysis

Stereochemical outcome

To ascertain the stereochemical outcome of the Spy1600-catalysed hydrolysis of \(\beta\)-\(D\)-glucosaminides, the progress of the early stages of the enzyme-catalysed reaction was monitored using \(^1\)H NMR. Because \(N\)-acetyl-\(D\)-glucosamine undergoes mutarotation only slowly in water [30], it is possible to monitor the stereochemistry of the anomeric centre of the first-formed product of the reaction. The equilibrium state in water at 20 \(^\circ\)C is a 2:1 ratio of \(\alpha\)- to \(\beta\)-anomers for \(N\)-acetyl-\(D\)-glucosamine [30].

The anomeric region of the \(^1\)H NMR spectrum of a reaction mixture containing MU-GlcNAc at several time points after adding Spy1600 is shown in Figure 5. The data shows that the first product formed is, unambiguously, the \(\beta\)-anomer and only over time does this species slowly mutarotate to form the \(\alpha\)-anomer. The assignment of the resonance at the higher chemical shift as the \(\alpha\)-anomer and the lower chemical shift resonance as the \(\beta\)-anomer is consistent with literature values [31]. The \(\alpha\)- and \(\beta\)-anomers

Figure 4 Western blot of proteins from COS-7 cells incubated in the presence or absence of Spy1600

Top panel: probed with anti-\(O\)-GlcNAc monoclonal IgM antibody; bottom panel: probed with anti-\(\beta\)-actin IgG; lane 1, COS-7 cell extract + Spy1600; lane 2, COS-7 cell extract only; lane 3, Spy1600 only; lane 4, COS-7 cell extract + boiled Spy1600. The molecular masses (in kDa) of the marker proteins are indicated on the extreme left.
Family 84 glycosidases are β-N-acetylglucosaminidases lacking hyaluronidase activity

Figure 5 1H NMR spectra of the hydrolysis of MU-GlcNAc by Spy1600

The anomic resonances of MU-GlcNAc, α-GlcNAc and β-GlcNAc are centred (relative to water at δ 4.78 p.p.m.) on δ 5.21 p.p.m. (J 10 Hz), δ 5.14 p.p.m. (J 4.5 Hz) and δ 4.65 p.p.m. (J 11 Hz) respectively. Trace A, MU-GlcNAc only; trace B, MU-GlcNAc + Spy1600 at t = 10 min; trace C, MU-GlcNAc + Spy1600 at t = 1 h; trace D, MU-GlcNAc + Spy1600 at t = 2 h; trace E, MU-GlcNAc + Spy1600 at t = 16 h.

Table 1 Michaelis–Menten parameters for Spy1600 a series of 4-MU 2-N-acyl-2-deoxy-β-D-glucopyranosides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>σ*</th>
<th>K_m (mM)</th>
<th>V_max · [E]_0/[(µmol · min⁻¹ · mg⁻¹)]</th>
<th>V_max · [E]_0/K_m (µmol · mM⁻¹ · min⁻¹ · mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MU-GlcNAc</td>
<td>0</td>
<td>3.58 ± 0.34</td>
<td>28.78 ± 2.70</td>
<td>8.04</td>
</tr>
<tr>
<td>MU-GlcNAcF_1</td>
<td>0.8</td>
<td>5.21 ± 0.17</td>
<td>4.96 ± 0.16</td>
<td>0.95</td>
</tr>
<tr>
<td>MU-GlcNAcF_2</td>
<td>2.0</td>
<td>14.07 ± 0.70</td>
<td>0.60 ± 0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>MU-GlcNAcF_3</td>
<td>2.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

are also identifiable from the magnitudes of the vicinal coupling constant, which is larger for the trans-diaxial arrangement of the H-1 and H-2 protons of the β-anomer (11 Hz) than that observed for the α-anomer (4.5 Hz). Further, comparison with a standard of N-acetyl-D-glucosamine in solution in which the known equilibrium position is obtained is consistent with the proposed nomenclature. Spy1600 therefore catalyses the reaction using a retaining catalytic mechanism, consistent with the mechanism proposed for the human family 84 enzyme [8,24], as well as with that for β-hexosaminidases from family 20 of glycoside hydrolases [32].

Substrate-assisted catalysis from the 2-acetamido group

To provide direct support for a role of the 2-acetamido group in catalysis, a series of fluorinated substrate analogues were studied. Four substrates with different levels of fluorine on the N-acetyl group were used (Figure 1A). Since increasing the number of fluorine-withdrawing groups decreases the nucleophilicity of the carbonyl oxygen atom, it is expected that a commensurate decrease in the rate constant governing the enzyme-catalysed reaction should result only if substrate-assisted catalysis is operative. Assays using these compounds as substrates for Spy1600 revealed Michaelian saturation kinetics for MU-GlcNAc, MU-GlcNAcF_1 and MU-GlcNAcF_2; however, the enzymatic activity towards the trifluorinated derivative was too low to be accurately measured using the assay conditions described here (Table 1; Figure 6A).

A plot of the log (V_max · [E]_0/K_m) against the Taft electronic parameter (σ*) of the N-acyl substituent shows a steep negative linear correlation on increasing fluorine substitution (Figure 6B). Such negative correlations have been previously observed for glycoside hydrolases from families 20 and 84 that are known to use substrate-assisted catalysis [8,9,33], yet remarkably absent for family 3 glycoside hydrolases that use an enzymic nucleophile [8]. Accordingly, these data strongly implicate the 2-acetamido group of the substrate as an intramolecular nucleophile.

Inhibition of Spy1600 using NAG-thiazoline

NAG-thiazoline (Figure 1B) is a potent inhibitor of human O-GlcNAcase [8], as well as of β-hexosaminidases from family 20 of glycoside hydrolases [8,34].
substrates as shown in (σ the Taft parameter (H17033 against log (V max[E]0/K m)[ l o g( catalytic mechanism in which the N- towards hyaluronan. Catalysis occurs via a substrate-assisted with activity towards Spy1600 has been shown to be a family 84 glycoside hydrolase Conclusions analyses reveal a pattern of competitive inhibition (Figure 7). Non-linear regression was assayed as an inhibitor of Spy1600, we observed a clear as a good competitive inhibitor. Indeed, when the compound involving substrate-assisted catalysis, NAG-thiazoline should act expected that if, as we propose, Spy1600 uses a mechanism to the intermediate or a structurally related transition state. We for these enzymes is attributed to the similarity of this molecule to the intermediate or a structurally related transition state. We expected that if, as we propose, Spy1600 uses a mechanism involving substrate-assisted catalysis, NAG-thiazoline should act as a good competitive inhibitor. Indeed, when the compound was assayed as an inhibitor of Spy1600, we observed a clear pattern of competitive inhibition (Figure 7). Non-linear regression analyses reveal a K_i value for NAG-thiazoline of 5.82 ± 0.01 μM. Although the inhibitor binds approx. 80-fold less tightly to the streptococcal enzyme than it does to human O-GlcNAcase (K_i 0.070 μM), it remains an effective inhibitor of Spy1600. Nevertheless, the observation that NAG-thiazoline is a good inhibitor of Spy1600 is consistent with the substrate-structure studies described above as well as with the stereochemical outcome experiment and strongly suggests that all members of glycoside hydrase family 84, both eu karyotic and prokaryotic, use a catalytic mechanism involving anchimeric assistance.

Figure 6  Activity of Spy1600 with N-fluoroacetyl derivatives of MU-GlcNAc (A) Initial velocity of Spy1600-catalysed hydrolysis of N-fluoroacetyl derivatives of MU-GlcNAc: ○ MU-GlcNAc; ■ MU-GlcNAcF1; ▲ MU-GlcNAcF2. (B) Linear-free-energy analysis plotting the Taft parameter (σ*) of the N-fluoracetyl substituent of MU-GlcNAc substrate analogues against log (Vmax[E]/Km) (log [μmol·min⁻¹·mg⁻¹·Km⁻¹]) values measured from the substrates as shown in (A) with Spy1600.

Figure 7 Lineweaver–Burk plot of Spy1600 hydrolysis of MU-GlcNAc in the presence of NAG-thiazoline

The concentrations of NAG-thiazoline were as follows: ♦ 0.00; ■ 0.01; ▲ 0.0037; □ 0.00123; and ● 0.0 mM.

for these enzymes is attributed to the similarity of this molecule to the intermediate or a structurally related transition state. We expected that if, as we propose, Spy1600 uses a mechanism involving substrate-assisted catalysis, NAG-thiazoline should act as a good competitive inhibitor. Indeed, when the compound was assayed as an inhibitor of Spy1600, we observed a clear pattern of competitive inhibition (Figure 7). Non-linear regression analyses reveal a K_i value for NAG-thiazoline of 5.82 ± 0.01 μM. Although the inhibitor binds approx. 80-fold less tightly to the streptococcal enzyme than it does to human O-GlcNAcase (K_i 0.070 μM), it remains an effective inhibitor of Spy1600. Nevertheless, the observation that NAG-thiazoline is a good inhibitor of Spy1600 is consistent with the substrate-structure studies described above as well as with the stereochemical outcome experiment and strongly suggests that all members of glycoside hydrase family 84, both eu karyotic and prokaryotic, use a catalytic mechanism involving anchimeric assistance.

Conclusions
Spy1600 has been shown to be a family 84 glycoside hydrolase with activity towards N-acetylglucosaminides and no activity towards hyaluronan. Catalysis occurs via a substrate-assisted catalytic mechanism in which the N-acetamido group of the sub-


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Family 84 glycosidases are β-N-acetylglucosaminidases lacking hyaluronidase activity 247