Subsite structure of the endo-type chitin deacetylase from a Deuteromycete, *Colletotrichum lindemuthianum*: an investigation using steady-state kinetic analysis and MS

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The endo-type chitin deacetylase (EC 3.5.1.41) from a Deuteromycete, *Colletotrichum lindemuthianum* (ATCC 56676), catalyses the hydrolysis of the acetamido group of GlcNAc (2-acetamido-2-deoxy-D-glucose) residues in chitin or chito-oligosaccharides with a degree of polymerization (n) equal to or greater than 2. The steady-state kinetic parameters for the initial deacetylation reactions of (GlcNAc)_{2–6} were determined using a direct, continuous spectrophotometric assay in combination with ESI-MS (electrospray ionization MS) analysis of the products. The dependence of the observed K_m and k_{cat}/K_m on n suggests the presence of four enzyme subsites (–2, –1, 0 and +1) that interact with GlcNAc residues from the non-reducing end to the reducing end of the substrate. The turnover number (k_{cat} , 7 s^{–1}) is independent of n and represents the intrinsic rate constant (k_{int}) for the hydrolysis of the acetamido group in subsite 0. The subsite affinities for the GlcNAc residues were calculated

from the observed k_{cat}/K_m values (A_{-2} , –11.0; A_{-1} , –1.5; A_0 , –7.7; A_{+1} , –12.5 kJ·mol^{–1}). The increments in the subsite affinities due to the recognition of the acetamido groups were calculated [$\Delta\Delta G_{(N-acetyl)} = 3.3, 0, 4.0$ and 0 kJ·mol^{–1} for subsites –2, –1, 0 and +1 respectively]. The steady-state kinetic parameters for the second deacetylation reaction of (GlcNAc)₄ were also determined using (GlcNAcGlcNAcGlcNAcGlcNAc) as the substrate. The comparison of the experimental and theoretical values (calculated using the subsite affinities) suggests that the mono-deacetylated substrate binds strongly in a non-productive mode occupying all four subsites, thereby inhibiting the second deacetylation reaction.

Key words: amino sugar N-deacylase, continuous assay, endo-type enzyme, mass spectrometry (MS), subsite affinity.

INTRODUCTION

Amino sugar N-deacylases play important roles in the modification of N-substituted saccharides that are found in a variety of organisms as components of both cell walls (chitin, peptidoglycans, etc.) and extracellular matrices (heparan sulphate, hyaluronic acid, etc.) [1]. Hence these enzymes could serve as crucial targets for future drug development. Carbohydrate esterases and N-deacylases, which comprise 13 families based on sequence similarities (<http://afmb.cnrs-mrs.fr/~cazy/CAZY>), have been subjected to very little study. Family 4 of carbohydrate esterases includes five major members that share a conserved region in the primary structure assigned as the NodB homology domain [2]. Rhizobial NodB chito-oligosaccharide deacetylases (EC 3.5.1.–) catalyse the removal of the acetyl group from the non-reducing-end GlcNAc (2-acetamido-2-deoxy-D-glucose) of chito-oligosaccharides [3]. NodB deacetylases together with other Nod proteins are involved in the biosynthesis of Nod factors, chito-oligosaccharides that carry a fatty acyl chain at the non-reducing-end GlcNAc unit, which act as morphogenic signal molecules to leguminous roots during development of nitrogen-fixing nodules [4,5]. Peptidoglycan GlcNAc deacetylases (EC 3.1.1.–) play a role in the modification of the bacterial cell wall, providing resistance against host lysozyme [6]. Acetylxylose esterases (EC 3.1.1.72) and modular xylanases A, C, D and E (EC 3.2.1.8) catalyse the removal of the acetyl groups from acetylated xylan [7,8]. Many xylan-degrading bacteria and fungi

produce acetyl xylan esterases and modular xylanases with both glycosidase and esterase activities in order to overcome the inhibitory effects of naturally occurring acetylated xylan in plant cell walls [7,8]. Chitin deacetylases (EC 3.5.1.41) catalyse the hydrolysis of the acetamido group of GlcNAc units in chitin or chito-oligosaccharides (Scheme 1). Chitin, a linear homopolymer of β -(1–4)-linked GlcNAc units, is one of the most abundant biopolymers on Earth, next to cellulose, and is found in the cell walls of most fungi and some algae [9] and in arthropod integuments [10]. Two types of chitin deacetylase have been investigated to date: chitin deacetylases from Zygomycetes and those from Deuteromycetes. Chitin deacetylase from *Mucor rouxii*, a Zygomycete, has been purified, characterized [11] and shown to be a monomeric glycoprotein [12] with an exo-type mode of action in which multiple attacks by the enzyme remove N-acetyl groups from the non-reducing GlcNAc units of the substrate [13,14]. This enzyme is involved in the biosynthesis of chitosan [15], a linear homopolymer of β -(1–4)-linked GlcN units with much higher water solubility and broader applications than chitin [16–18]. In contrast, the extracellular chitin deacetylase from *Colletotrichum lindemuthianum*, a Deuteromycete (ATCC 56676), was shown to have an endo-type pattern of action in which the chito-oligosaccharide substrates with a degree of polymerization (n) equal to or greater than 4 are eventually fully deacetylated but through a specific deacetylation pathway [19]. Chitotriose is also fully deacetylated, but through a random deacetylation process in which either of three GlcNAc units can

Abbreviations used: CDAH, recombinant chitin deacetylase from *Colletotrichum lindemuthianum* containing a C-terminal hexahistidine tag; ESI-MS, electrospray ionization MS; GlcN, 2-amino-2-deoxy-D-glucose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; β -GlcNAcase, β -N-acetylhexosaminidase.

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Scheme 1 Reaction catalysed by the chitin deacetylase from *C. lindemuthianum*

The smallest substrate, chitobiose, is deacetylated only at the non-reducing-end sugar residue whereas larger substrates are fully deacetylated.

be deacetylated first, while the smallest substrate, chitobiose, is only deacetylated at the non-reducing GlcNAc residue [19,20]. This enzyme, also a monomeric glycoprotein [21], has been shown to catalyse the reverse reaction at very high (3.0 M) acetate concentrations [22]. CDAH (the recombinant non-glycosylated chitin deacetylase from *C. lindemuthianum* containing a C-terminal hexahistidine tag) can be produced easily in the culture medium of *Escherichia coli* cells with the aid of a signal sequence from *Streptomyces lividans* chitinase C [23]. Deuteromycetes that produce chitin deacetylase are all plant pathogens and secrete the enzyme during penetration into the plant cells [24–26], but the role of the enzyme in this process is unclear.

Recently, Tokuyasu et al. [19] carried out a structural analysis of the partially deacetylated products of $(\text{GlcNAc})_{2-6}$ [β -(1–4)-linked n -mers of GlcNAc] formed by CDAH and proposed that the enzyme has four subsites –2, –1, 0 and +1, where subsite 0 is the catalytic site. These authors also probed the recognition of N-acetyl groups of GlcNAc units of the substrate $(\text{GlcNAc})_4$ by the subsites –2, –1 and +1 and showed that subsite –2 strongly recognizes the N-acetyl group. Prompted by the unique endo-action pattern of CDAH and in order to fully investigate the subsite structure of the enzyme, we carried out a full steady-state kinetic characterization of CDAH for the first time using a direct, continuous spectrophotometric assay that relies on the extinction coefficient difference at 216 nm between the carbonyl functional group in the chito-oligosaccharide substrates and the acetate product released. Although this assay method has been used for other deacetylase enzymes [27], it has not been used for chitin deacetylase to date. So far, three indirect methods have been used to assay for chitin deacetylase hydrolytic activity: a stopped assay based on the detection of the glucosamine residues produced by the deacetylation reaction [28,29]; a stopped radioactive assay employing glycol chitin, radiolabelled in the N-acetyl group, as the substrate [30]; and a coupled assay based on the detection of released acetates using acetyl-CoA

synthetase, citrate synthase and malate dehydrogenase in a coupled system [31,32]. In combination with the kinetic analysis, we have used ESI-MS (electrospray ionization MS) in order to be able to distinguish between the first deacetylation reactions of the substrate chito-oligosaccharides and their subsequent deacetylations. This information was required for the accurate determination of subsite affinities in the catalytic domain of CDAH.

Calculation of binding contributions at each subsite to the overall affinity of the enzyme for the substrate relies on the assumption of the additivity of the individual subsite affinities [33], as is discussed in this report. This assumption breaks down if there is co-operativity among subsites. In the absence of co-operative behaviour, recent work has demonstrated that the energetic effects of multiple hydrogen bonds are nearly additive in systems modelling enzyme active sites [34].

EXPERIMENTAL

General materials and methods

Chito-oligosaccharides $(\text{GlcNAc})_{2-6}$ were purchased from Seikagaku Corporation (Tokyo, Japan) and Toronto Research Chemicals (North York, ON, Canada). All other chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada) and were of reagent grade. Recombinant β -GlcNAcase (β -N-acetylhexosaminidase) (which is β -GlcNAcase with an N-terminal heptahistidine tag) from *Streptomyces plicatus* was purified and quantified as described previously [35].

Enzyme purification

E. coli strain BL21(DE3, pLysS) harbouring the plasmid pS1CH was grown in Luria–Bertani medium at 27 °C for 3 h (D_{600} , 0.4), induced with isopropyl β -D-thiogalactoside (1.0 mM) and incubated overnight as described previously [23]. Plasmid pS1CH carried a 783-bp sequence that consisted of three parts: a signal sequence from *S. lividans* chitinase C (90 bp), part of the chitin deacetylase gene from *C. lindemuthianum* encoding a mature chitin deacetylase (663 bp) and a sequence encoding six histidines. Recombinant chitin deacetylase, secreted into the culture medium, was purified according to the method of Tokuyasu et al. [23] with a slight modification: after metal-ion-affinity chromatography (10 ml; His.Bind resin; Novagen, Madison, WI, U.S.A.), the CDAH sample was dialysed against assay buffer (20 mM $\text{Na}_2\text{B}_4\text{O}_7/\text{HCl}$ buffer, pH 8.5) and further purified via hydrophobic-interaction column chromatography (5 ml; Butyl-Toyopearl 650 M; Tosoh Co., Tokuyama, Japan) using a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ (25–0% saturation) in the assay buffer. Fractions with CDAH activity were pooled, dialysed against assay buffer and concentrated using an Amicon Centriprep-10 concentrator. Enzyme purity was checked using both SDS/PAGE and ESI-MS analyses. Protein concentration was determined in triplicate using the Micro BCA™ Protein Assay Reagent Kit with BSA standards (Pierce, Rockford, IL, U.S.A.).

Molar extinction coefficient difference determinations

The UV absorption spectra of solutions of 1.0 mM GlcNAc and 1.0 mM [GlcN (2-amino-2-deoxy-D-glucose)/HCl + sodium acetate] in the assay buffer at pH 8.5 and 30 °C were examined over a wavelength range of 190–300 nm, and a wavelength of 216 nm ($n \rightarrow \pi^*$ transition of carbonyl chromophore, where n indicates the occupied non-bonding orbital and π^*

indicates the unoccupied anti-bonding orbital) was chosen for all spectrophotometric assays. The values of $\Delta\varepsilon_{216}$ for the deacetylation of the substrates (GlcNAc)_{2–4} were determined by measuring the total change in absorbance at 216 nm upon complete turnover of each substrate in the presence of CDAH under assay conditions at pH 8.5 and 30 °C. The substrate and enzyme concentrations were as follows: 2.0–16 mM (GlcNAc)₂ in the presence of 5.50 $\mu\text{g} \cdot \text{ml}^{-1}$ CDAH, 0.2–4.0 mM (GlcNAc)₃ in the presence of 49.3 $\mu\text{g} \cdot \text{ml}^{-1}$ CDAH, and 0.01–0.17 mM (GlcNAc)₄ in the presence of 21.6 $\mu\text{g} \cdot \text{ml}^{-1}$ CDAH. Absorbance measurements were carried out using a UNICAM 8700 UV-Vis spectrophotometer equipped with a circulating-water bath that maintained the cells at 30 °C. Quartz cells of 0.1 and 1.0 cm pathlength were used for (GlcNAc)_{2,3} and (GlcNAc)₄ respectively. The completion of reactions was confirmed by ESI-MS. The plots of ΔA_{216} versus concentration were linear and the values of $\Delta\varepsilon_{216}$ were determined by linear regression using the program GraFit [36].

Steady-state initial velocity kinetics

Initial rates of hydrolysis by CDAH were determined at 30 °C with a continuous spectrophotometric assay by following the rate of decrease in absorbance at 216 nm resulting from the conversion of amide to amine functionalities in the chito-oligosaccharide with concurrent release of acetate groups. All kinetic studies were carried out in the quartz cells of 0.1 cm pathlength for (GlcNAc)_{2,3} and (GlcNAcGlcNAcGlcNAc), and 1.0 cm pathlength for (GlcNAc)_{4–6}. The standard assay was carried out in 20 mM sodium tetraborate/HCl buffer, pH 8.5. The substrate and enzyme concentrations in the assays were as follows: 2.0–30 mM (GlcNAc)₂ in the presence of 5.50 $\mu\text{g} \cdot \text{ml}^{-1}$ CDAH, 0.5–13 mM (GlcNAc)₃ in the presence of 55.0 $\mu\text{g} \cdot \text{ml}^{-1}$ CDAH, 0.01–0.5 mM (GlcNAc)_{4–6} in the presence of 0.880 $\mu\text{g} \cdot \text{ml}^{-1}$ CDAH, and 0.05–1.5 mM GlcNAcGlcNAcGlcNAc in the presence of 67.5 $\mu\text{g} \cdot \text{ml}^{-1}$ CDAH. Substrate concentrations ranged from 0.1–0.2 to 2–10 times the estimated K_m value for each substrate. The substrate in the assay buffer was preincubated for 10 min at 30 °C and reaction was initiated by the addition of enzyme. The plots of initial rates versus substrate concentrations were hyperbolic and the values of K_m and $V_{\max}$ were determined from fitting the initial velocity curves to the standard Michaelis–Menten equation using non-linear regression with the program GraFit. The k_{cat} values were calculated using a $\Delta\varepsilon_{216}$ of 330 $\text{M}^{-1} \cdot \text{cm}^{-1}$ and a CDAH molecular mass of 25 748 Da. The K_m and k_{cat} values were determined in triplicate and mean values are reported. The reported errors are S.D.

Substrate-depletion kinetics for k_{cat}/K_m determinations

The time course of substrate depletion was followed for two or more half-lives at a fixed concentration of both enzyme and substrate at 30 °C by monitoring the decrease in A_{216} with time. The standard assay conditions and procedures were as mentioned earlier. The substrate and enzyme concentrations in the assays were as follows: 2.0 mM (GlcNAc)₂ in the presence of 11.0 $\mu\text{g} \cdot \text{ml}^{-1}$ CDAH, 0.5 mM (GlcNAc)₃ in the presence of 55.0 $\mu\text{g} \cdot \text{ml}^{-1}$ CDAH and 0.01 mM (GlcNAc)_{4–6} in the presence of 0.880 $\mu\text{g} \cdot \text{ml}^{-1}$ CDAH. The substrate concentrations used were much lower than their respective K_m values for the pseudo-first-order kinetics to be valid. Where possible, the depletion curve was fitted to an exponential decay equation, $A(t) = \Delta A[\exp(-k_{\text{obs}}t)] + A_{\text{final}}$, using the program GraFit and

the value of k_{cat}/K_m was calculated from the equation $k_{\text{obs}} = (k_{\text{cat}}/K_m)[E_0]$.

ESI-MS

ESI-MS spectra were recorded on a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada) equipped with an ionspray ion source. The ion-source voltage was 5 kV and the orifice voltage was 50 V. The protein sample was introduced by reversed-phase HPLC on an Ultrafast Microprotein Analyser (Michrom BioResources, Pleasanton, CA, U.S.A.) directly interfaced with the mass spectrometer. The sample was loaded on to a PLRP-S column (1.0 mm $\times$ 50 mm; Michrom BioResources, Auburn, CA, U.S.A.) and then eluted with a linear gradient of 5–90% solvent B at a flow rate of 50 $\mu\text{l} \cdot \text{min}^{-1}$ (where solvent A is 0.05% trifluoroacetic acid/2% acetonitrile in water and solvent B is 0.045% trifluoroacetic acid/80% acetonitrile in water). Carbohydrate samples were introduced directly on to the mass spectrometer by injection. Samples of the assay mixture containing the highest and lowest substrate concentrations were run on the ESI-MS within 10 min after the spectrophotometric assay time period in order to confirm that the substrates were deacetylated at only one centre during the reaction time used for the measurement of initial rates. Aliquots from the assay mixture were also run on ESI-MS at time points hours and days after initiation of the reaction in order to follow the course of the full deacetylation of the substrates and to check complete substrate turnover for the determination of $\Delta\varepsilon_{216}$ values. The enzyme retained its original specific activity for several weeks at both room temperature (23 ± 1 °C) and 30 °C. All ESI-MS experiments were carried out at room temperature and all spectra were obtained in the positive-ion, single quadrupole scan (LC-MS) mode. GlcN/HCl and GlcNAc at equal concentrations were added to all carbohydrate samples as internal standards before injection.

Structural analysis of the mono-deacetylated products

Three assay mixtures containing CDAH at concentrations used in the steady-state kinetic experiments and substrates (GlcNAc)_{2–4} at concentrations lower than their respective K_m values were incubated at 30 °C and stopped by boiling for 2 min before any di-deacetylation occurred (checked by ESI-MS). Each mixture was directly analysed by normal-phase HPLC (Waters Chromatography System; Millipore Co., Mississauga, ON, Canada). The sample was loaded on to an HPLC column (2.15 cm $\times$ 30.0 cm; TSK-GEL® Amide-80; Tosoh Co.) and eluted with a linear gradient of 100–50% acetonitrile in water at a flow rate of 5 $\text{ml} \cdot \text{min}^{-1}$. Fractions (5 ml) containing the mono-deacetylated products were analysed by ESI-MS, pooled and lyophilized. Samples were then re-dissolved in the assay buffer and subjected to β -GlcNAcase hydrolysis for 5 days at 30 °C. The reactions were terminated by boiling for 2 min and samples were run on ESI-MS.

RESULTS

Purification of the recombinant chitin deacetylase

The enzyme sample resulting from metal-ion-affinity chromatography showed minor protein contaminants as judged by SDS/PAGE and was further purified to electrophoretic homogeneity via hydrophobic interaction column chromatography.

SDS/PAGE analysis of the final sample of recombinant chitin deacetylase (bearing a C-terminal hexahistidine tag) revealed the presence of a single protein band with the expected mobility [23]. The purity of the enzyme sample was also tested via ESI-MS over a mass range of 1–100 kDa. The reconstruct of the original mass spectrum revealed a molecular mass of $25\,756 \pm 5$ Da, in reasonable agreement with the calculated value of 25 748 Da. The specific activity of the recombinant chitin deacetylase was 19 ± 2 units $\cdot$ mg $^{-1}$ using the substrate (GlcNAc) $_4$ (1 unit of activity/unit volume was defined as the amount causing the release of 1 μ mol of acetate/min per unit volume). This specific activity is essentially identical to that of the wild-type enzyme purified directly from the fungal source [29]. The recombinant enzyme had been shown previously to have kinetic properties comparable with those of the wild-type enzyme [23] and therefore was used in all kinetic studies.

Determination of a molar extinction coefficient difference for the release of acetate

The values of $\Delta\epsilon_{216}$ for the deacetylation of the substrates (GlcNAc) $_{2-4}$ to the products GlcNGlcNAc and (GlcN) $_{3,4}$ [β -(1–4)-linked n -mers of GlcN] were 338, 899 and 1412 M $^{-1}$ $\cdot$ cm $^{-1}$ respectively. These values correlate well in a linear fashion with the number of hydrolysed amide functional groups (number of deacetylation reactions) per substrate molecule and an average $\Delta\epsilon_{216}$ value of 330 ± 27 M $^{-1}$ $\cdot$ cm $^{-1}$ was determined and used in the determination of k_{cat} values. This $\Delta\epsilon_{216}$ value corresponds to the absorbance change resulting from the conversion of 1 mol $\cdot$ l $^{-1}$ of amide to amine functionalities in the chito-oligosaccharide substrate with the release of 1 mol $\cdot$ l $^{-1}$ of free acetate in solution.

Evaluation of the steady-state kinetic parameters for the first deacetylation of chito-oligosaccharide substrates catalysed by chitin deacetylase

The CDAH-catalysed mono-deacetylation of five chito-oligosaccharides (degrees of polymerization $n = 2-6$) was studied under steady-state conditions by continuous monitoring of the decrease in A_{216} over time. Comparison of the absorbance change over the course of the assay period with the expected absorbance change upon complete mono-deacetylation of the substrate (based on the $\Delta\epsilon_{216}$ value) confirmed that only 10% or less of the substrate was mono-deacetylated during the assay period. Initial rates for (GlcNAc) $_{2-6}$ followed Michaelis–Menten kinetics (Figure 1) and the average values of the kinetic parameters k_{cat} , K_m and k_{cat}/K_m were determined (Table 1). The observed K_m values decreased with increasing n while the observed k_{cat} values remained approximately constant. No activity was detected with GlcNAc as the substrate at concentrations up to 100 mM as judged by both UV/visible spectroscopy and ESI-MS, consistent with previous reports using a stopped-assay method [19,29]. When the time course of the deacetylation of (GlcNAc) $_2$ was followed for several half lives, the depletion of (GlcNAc) $_2$ was found to follow a pseudo-first-order exponential decay and a k_{cat}/K_m value was determined that was identical, within the limits of experimental error, to the value determined from steady-state initial-velocity kinetic experiments (Figure 2A and Table 1).

Confirmation of mono-deacetylation of substrates during the assay period using ESI-MS

In order to calculate and interpret meaningful kinetic data, it was necessary to first ascertain the extent of reaction during the

Table 1 Steady-state kinetic parameters for the recombinant chitin deacetylase from *C. lindemuthianum* with respect to chito-oligosaccharides (GlcNAc) $_n$

No activity was observed with GlcNAc as the substrate at concentrations up to 100 mM. All values are means $\pm$ S.D. calculated from triplicate determinations. The steady-state kinetic parameters were obtained from initial-velocity kinetic experiments and represent only the first deacetylation reactions as described in the text. The second-order rate constant shown in parentheses was obtained from substrate-depletion experiments.

n	K_m (mM)	k_{cat} (s $^{-1}$)	k_{cat}/K_m (mM $^{-1}$ $\cdot$ s $^{-1}$)
1	–	–	–
2	18 ± 2	11 ± 2	0.6 ± 0.2 (0.5 ± 0.1)
3	4.3 ± 0.1	6 ± 1	1.4 ± 0.4
4	0.125 ± 0.007	7 ± 2	56 ± 13
5	0.079 ± 0.004	7 ± 2	89 ± 20
6	0.048 ± 0.003	5.4 ± 0.8	113 ± 21

initial velocity assay and whether mono-deacetylated products accumulated to levels high enough for the rates of subsequent deacetylations to become significant. To check this, a sample of the assay mixture was analysed by ESI-MS within 10 min after the assay period. As illustrated in Figure 1 (insets), the only products observed were the mono-deacetylated oligomers [a mass difference of 42.0 (CH $_3$ CO–H) from the substrates]. Upon prolonged incubation, (GlcNAc) $_2$ was not further deacetylated, even after several weeks (Figure 1, inset). Tokuyasu et al. [20] had demonstrated previously that the final product was GlcNGlcNAc, resulting from deacetylation of the non-reducing-end GlcNAc residue in chitobiose. No multiply charged species were observed in ESI mass spectra and all oligosaccharides were observed as $[M + \text{Na}]^+$ species.

Structure of the mono-deacetylated products

The position of deacetylation in the mono-deacetylated products reveals the binding mode(s) of the substrates at each step and shows which subsites are occupied during the deacetylation step. This information is necessary for the accurate determination of the enzyme's subsite affinities. The structures of the mono-deacetylated products of (GlcNAc) $_{2-4}$ were determined via ESI-MS analysis (results not shown) of the β -GlcNAcase hydrolysates of the mono-deacetylated products, taking advantage of the fact that β -GlcNAcase is an exo-acting glycosidase that cleaves only N-acetylated GlcNAc residues from the non-reducing terminus of the oligosaccharides. The sequences of the partially deacetylated chito-oligosaccharides are shown using abbreviations GlcN and GlcNAc in the right sequence; for example, GlcNGlcNAc is the β -(1–4)-linked disaccharide with GlcN at the non-reducing end and GlcNAc at the reducing end. The mono-deacetylated product of (GlcNAc) $_2$ was not hydrolysed by β -GlcNAcase, indicating that the non-reducing-end sugar was deacetylated, producing GlcNGlcNAc as the only product. The hydrolysate of the mono-deacetylated product of (GlcNAc) $_4$ contained only GlcNAc and GlcNGlcNAc as the final products, which indicates that the only molecular species present was GlcNAcGlcNAcGlcNGlcNAc. The products of β -GlcNAcase hydrolysis of the mono-deacetylated (GlcNAc) $_3$ were GlcN, GlcNAc, GlcNGlcNAc and GlcNGlcNAcGlcNAc, which indicate that a mixture of three products was present: GlcNGlcNAcGlcNAc, GlcNAcGlcNGlcNAc and GlcNAcGlcNAcGlcN (in the proportions 22:50:28). The presence of three positional isomers

Figure 1 First deacetylation reactions of (GlcNAc)₂₋₆ catalysed by CDAH

Plots of initial velocity versus substrate concentration for CDAH using chito-oligosaccharides (GlcNAc)₂₋₆ as the substrates. The lines shown are the non-linear fits of the experimental data (●) to the Michaelis–Menten equation. The insets show the ESI mass spectra recorded within 10 min of the initial velocity assay period for (GlcNAc)₃₋₆ and a few hours after the assay period for (GlcNAc)₂. All peaks represent $[M + \text{Na}]^+$ species unless otherwise stated on the spectrum. The mass difference between the two peaks in each spectrum is 42.0 (CH₃CO–H). (GlcNAc)₂, 424.2 Da; (GlcNAc)₃, 627.3 Da; (GlcNAc)₄, 830.3 Da; (GlcNAc)₅, 1033.4 Da; (GlcNAc)₆, 1236.5 Da.

Figure 2 CDAH-catalysed mono-deacetylation of (GlcNAc)₂ and multiple deacetylations of (GlcNAc)₄

(A) Substrate-depletion patterns for (GlcNAc)₂ and (GlcNAc)₄. The curve for (GlcNAc)₂ is the non-linear fit of the experimental data (●) to a single exponential decay equation. The curve for (GlcNAc)₄ is the theoretical exponential decay curve obtained using the equation $A(t) = \Delta A[\exp(-k_{\text{obs}}t)] + A_{\text{final}}$, where $\Delta A = 0.0132$, $A_{\text{initial}} = 0.166$ and $k_{\text{obs}} = (k_{\text{cat}}/K_m)[E_0] = 0.00188 \text{ s}^{-1}$ using the k_{cat}/K_m value determined by steady-state kinetics. **(B)** ESI mass spectra showing the time scale of the consecutive deacetylation reactions of (GlcNAc)₄ catalysed by CDAH. The mass difference between successive peaks is 42.0 (CH₃CO-H). All peaks represent $[M + \text{Na}]^+$ species unless otherwise stated on the spectrum and no multiply charged species were observed. The substrate concentration was 0.5 mM in the presence of $0.880 \mu\text{g} \cdot \text{mL}^{-1}$ CDAH. On the right is the actual pathway for the full deacetylation of (GlcNAc)₄ reported in [19].

of mono-deacetylated (GlcNAc)₃ indicates that three parallel deacetylation reactions occurred simultaneously as a result of three different productive binding modes of (GlcNAc)₃ to the enzyme (Scheme 2). These findings are consistent with the results reported previously by Tokuyasu et al. [19,20], who used the wild-type enzyme purified directly from the fungal source in their study.

Investigation of the subsequent deacetylation reactions of mono-deacetylated chito-oligosaccharide substrates catalysed by chitin deacetylase

Time courses for the hydrolysis of the N-acetyl groups in (GlcNAc)₃₋₆ were followed spectrophotometrically for several half-lives. The depletion of the substrates (GlcNAc)₃₋₆ obeyed

Scheme 2 Schematic representations of the productive binding modes of (GlcNAc)₂₋₄ to CDAH

The N-acetyl group of the sugar residue at subsite 0 is removed by the enzyme. The equations used to calculate the subsite affinities are: eqn 3, $(k_{\text{cat}}/K_m)_2 = 0.018 \cdot k_{\text{int}} \cdot \exp[-(A_0 + A_{+1})/RT] = 500 \text{ M}^{-1} \cdot \text{s}^{-1}$; eqn 4, $0.22 \cdot (k_{\text{cat}}/K_m)_3 = 0.018 \cdot k_{\text{int}} \cdot \exp[-(A_0 + A_{+1})/RT] = 308 \text{ M}^{-1} \cdot \text{s}^{-1}$; eqn 5, $0.5 \cdot (k_{\text{cat}}/K_m)_3 = 0.018 \cdot k_{\text{int}} \cdot \exp[-(A_{-1} + A_0 + A_{+1})/RT] = 700 \text{ M}^{-1} \cdot \text{s}^{-1}$; eqn 6, $0.28 \cdot (k_{\text{cat}}/K_m)_3 = 0.018 \cdot k_{\text{int}} \cdot \exp[-(A_{-2} + A_{-1} + A_0)/RT] = 392 \text{ M}^{-1} \cdot \text{s}^{-1}$; eqn 7, $(k_{\text{cat}}/K_m)_4 = 0.018 \cdot k_{\text{int}} \cdot \exp[-(A_{-2} + A_{-1} + A_0 + A_{+1})/RT] = 56\,000 \text{ M}^{-1} \cdot \text{s}^{-1}$.

a multi-phasic decay due to the multiple deacetylation reactions occurring, thus the depletion curves could not be fitted to a simple first-order exponential decay equation. With these substrates, it was observed that full substrate depletion occurred much more slowly than would be expected if the subsequent deacetylation reactions had the same second-order rate constants (k_{cat}/K_m) as the first deacetylation. Figure 2(A) demonstrates this for the substrate (GlcNAc)₄. ESI-MS analysis also showed that, with these substrates, the first deacetylation occurred within a time scale of minutes whereas further deacetylations occurred within a time scale of hours to days; thus it was quite simple to measure the kinetic parameters for the first deacetylation reaction in each case. As an example, Figure 2(B) demonstrates the time course of the CDAH-catalysed consecutive deacetylation reactions of the substrate (GlcNAc)₄ followed by ESI-MS. Tokuyasu et al. [19] had previously illustrated that the di- and tri-deacetylated products were each mixtures of two positional isomers. The substrates (GlcNAc)₃₋₆ were eventually fully deacetylated as reported previously [19].

The CDAH-catalysed second deacetylation reaction of (GlcNAc)₄ was also studied under steady-state conditions using GlcNAcGlcNAcGlcNGlcNAc as the substrate directly. Initial rates determined for the deacetylation of GlcNAcGlcNAcGlcNGlcNAc followed Michaelis–Menten kinetics and the average values of the kinetic parameters k_{cat} , K_m and k_{cat}/K_m were determined (Figure 3 and Table 2). The observed K_m value was similar to that for (GlcNAc)₄ but the observed k_{cat} value was one order of magnitude smaller than the k_{cat} values for the first deacetylation reactions (Table 1). ESI-MS analysis showed that the substrate was deacetylated only once during the assay period, giving rise to di-deacetylated products (Figure 3, inset). As

Figure 3 The second deacetylation reaction of (GlcNAc)₄ catalysed by CDAH

Plot of initial velocity versus substrate concentration for CDAH using GlcNAcGlcNAcGlcNGlcNAc as the substrate. The curve shown is the non-linear fit of the experimental data (●) to the Michaelis–Menten equation. The inset shows the ESI mass spectrum recorded within 5 min of the initial velocity assay period. All peaks represent $[M + \text{Na}]^+$ species unless otherwise stated on the spectrum. The mass difference between the two peaks is 42.0 ($\text{CH}_3\text{CO}-\text{H}$). No multiply charged species were observed.

Table 2 Comparison of the experimental and theoretical steady-state kinetic parameters for CDAH with respect to GlcNAcGlcNAcGlcNGlcNAc as the substrate

The theoretical values were calculated using the subsite affinities (see Table 3) and appropriate equations (see the text). The binding modes p1, p2 and q1 are shown in Scheme 3.

Steady-state parameter	Experimental value	Theoretical value	
		p1, p2 and q1 binding modes	Only p1 and p2 binding modes
k_{cat} (s^{-1})	0.38 ± 0.03	0.15 ± 0.08	7 ± 2
K_m (mM)	0.6 ± 0.1	0.15 ± 0.08	7 ± 2
k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	0.6 ± 0.1	1.0 ± 0.4	1.0 ± 0.4

demonstrated in Figure 2(B), Tokuyasu et al. [19] had previously determined the structures of the di-deacetylated products to be GlcNAcGlcNGlcNGlcNAc and GlcNGlcNAcGlcNGlcNAc, indicating that two parallel reactions occurred yielding two positional isomers.

DISCUSSION

The number of subsites in the catalytic domain of CDAH

Recently, Tokuyasu et al. [19] proposed that CDAH has four subsites based on the first deacetylation patterns of (GlcNAc)₅ and (GlcNAc)₆ catalysed by the enzyme. Here, we have obtained steady-state kinetic data for the first deacetylation reactions of (GlcNAc)₂₋₆ that clearly demonstrates the presence of four subsites in the catalytic domain of the enzyme that are involved in recognition and binding of GlcNAc moieties in the substrate. As depicted in Figure 4, the k_{cat}/K_m values increase by two orders

Figure 4 Dependence of the steady-state kinetic parameters for CDAH on the degree of polymerization (n) of the chito-oligosaccharide substrates

of magnitude from (GlcNAc)₂ to (GlcNAc)₄ but remain the same, within experimental error, with substrates that have degrees of polymerization (n) higher than 4. Since the catalytic constant, k_{cat} , of this enzyme is independent of the degree of polymerization of the substrate, the increase in the second-order rate constants (k_{cat}/K_m) from the disaccharide to the tetrasaccharide can be attributed purely to the increase in the apparent affinity (or decrease in K_m). Thus the K_m values also undergo a decrease of two orders of magnitude from (GlcNAc)₂ to (GlcNAc)₄ and then remain similar from (GlcNAc)₄ to (GlcNAc)₆ (Figure 4). These data indicate clearly that a total of four GlcNAc moieties in the substrate are necessary for the optimal overall binding of the substrate chito-oligosaccharides to the enzyme. The four subsites are designated -2 , -1 , 0 and $+1$ based on the modification by Tokuyasu et al. [19] of the unified nomenclature for sugar-binding subsites in glycosylhydrolases originally proposed by Davies et al. [37] in which subsite 0 is the catalytic site of the enzyme where deacetylation takes place. Interestingly, the K_m values for (GlcNAc)₅ and (GlcNAc)₆ are roughly one-half and one-third of the K_m value for (GlcNAc)₄. These values are in good statistical agreement with the existence of only four subsites in CDAH because (GlcNAc)₄ binds to the enzyme in only one mode that occupies all four subsites whereas (GlcNAc)₅ and (GlcNAc)₆ have two and three such binding modes respectively (see also eqn 1, below), as was demonstrated previously via product analysis [19]. Finally, as shown in Figure 4, the observation that the k_{cat}/K_m values are quite similar for both (GlcNAc)₂ and (GlcNAc)₃ substrates, in spite of the higher subsite occupancy by the trisaccharide, is explained below based on the subsite affinity distribution in CDAH.

The subsite affinity distribution amongst the four subsites of CDAH

The combination of the steady-state kinetic data for (GlcNAc)_{2–4} presented in Table 1 and the product-analysis data for the first deacetylation reactions has been used in this study to determine the subsite affinity distribution for CDAH according to the subsite theory independently developed by Hiromi et al. [33,38–40] and Allen, Thoma and co-workers [41–44]. The subsite theory was first applied to exo- α -(1,4)-D-glucan glucohydrolase [38] by these workers and has since been tentatively applied to polysaccharide hydrolases of both the exo- and endo-type. The subsite theory has been widely applied to exo-type enzymes because of the simplicity of the system due to the fact that there is only one

productive binding mode between the substrate and the enzyme and the site of hydrolytic activity is known. In the case of exo-type enzymes, kinetic data are sufficient for the calculation of subsite affinities and no product analysis is required; the subsite affinities are calculated from the ratios of k_{cat}/K_m values for the successively larger substrates.

The subsite theory has also been used for some endo-acting polysaccharide hydrolases [45,46] but here we attempt to apply the theory for the first time to an endo-type enzyme, CDAH, which does not depolymerize the substrate oligosaccharides but rather modifies them. In applying the theory, we assume that the individual subsite affinities in CDAH are additive and that there is no co-operativity among subsites. At a first glance, the significant increase in k_{cat}/K_m that is observed when all four subsites are occupied ($n \geq 4$) could be viewed as a co-operative effect among subsites. However, a co-operative behaviour that would improve the positioning of the N-acetyl group in the active site for catalytic turnover might be expected to improve the catalytic constant k_{cat} as well as the substrate-binding process, but in the case of CDAH, the k_{cat} values are independent of the degrees of polymerization of the substrates (Figure 4). The initial rate (V_0) equation for a system in which the polymeric or oligomeric substrate binds to the multi-subsite enzyme in both productive and non-productive modes has a hyperbolic form as follows [40,47]:

$$V_0 = \frac{\{\Sigma K_p / (\Sigma K_p + \Sigma K_q)\} \cdot k_{\text{int}} \cdot [E]_t \cdot [S]}{\{1 / (\Sigma K_p + \Sigma K_q)\} + [S]} \quad (1)$$

where ΣK_p and ΣK_q are sums of all association constants for all productive (p) and non-productive (q) binding modes respectively [if eqn (1) is derived using the steady-state assumption instead of the rapid-equilibrium assumption, then $K_p = (k_1)/(k_{-1} + k_{\text{int}})$, where k_1 and k_{-1} are the on and off rate constants for each productive binding mode; in this study, we take K_p as the association constant], k_{int} is the intrinsic rate constant for the hydrolytic reaction and is assumed to be independent of the degree of polymerization (n), an assumption that is likely to be valid for CDAH (Table 1). It is clear from eqn (1) that the observed k_{cat} and K_m values include both productive and non-productive binding terms for the oligomeric substrate whereas the observed k_{cat}/K_m only includes the productive binding terms. Therefore, k_{cat}/K_m values are used to calculate the subsite affinities based on eqn (2), which cannot be used for a system in which k_{int} is dependent on the degree of polymerization of the substrate (the calculation of

Table 3 Subsite structure of *C. lindemuthianum* chitin deacetylase

Subsite affinity was calculated using steady-state kinetic data for the first deacetylation reactions of (GlcNAc)_{2–4} at 30 °C, see text for details. k_{int} is the average value for the intrinsic hydrolytic rate constant calculated using k_{cat} values in Table 1.

Subsite (<i>n</i>) . . .	–2	–1	0	+1
Subsite affinity (– A_n ; kJ · mol ^{–1})	11.0 ± 0.8	1.5 ± 0.4	7.7 ± 0.4	12.5 ± 0.8
$\Delta\Delta G_{(\text{N-acetyl})}$ (kJ · mol ^{–1})	3.3 ± 0.3*	0*	4.0 ± 0.3†	0*
k_{int} (s ^{–1})			7 ± 2	

* Calculated using K_m values at 30 °C reported in [19] where:

$$\Delta\Delta G_{(\text{N-acetyl})} = RT \cdot \ln[K_m(\text{GlcNAcGlcNAcGlcNAcGlcNAc})/K_m(\text{GlcNAc})_4] \text{ for subsite } -2,$$

$$\Delta\Delta G_{(\text{N-acetyl})} = RT \cdot \ln[K_m(\text{GlcNAcGlcNAcGlcNAcGlcNAc})/K_m(\text{GlcNAc})_4] \text{ for subsite } -1,$$

$$\Delta\Delta G_{(\text{N-acetyl})} = RT \cdot \ln[K_m(\text{GlcNAcGlcNAcGlcNAcGlcNAc})/K_m(\text{GlcNAc})_4] \text{ for subsite } +1.$$

† Estimated using the equation $\Delta\Delta G_{(\text{N-acetyl})} = RT \cdot \ln[K_m(\text{GlcNAcGlcNAcGlcNAcGlcNAc})/K_m(\text{GlcNAc})_4]$ where the dominant term in $K_m(\text{GlcNAcGlcNAcGlcNAcGlcNAc})$ is K_{q1} (see text).

subsite affinities is not straightforward for such a system):

$$\begin{aligned} (k_{\text{cat}}/K_m)_n &= k_{\text{int}} \cdot \Sigma K_{n,p} = k_{\text{int}} \cdot \Sigma \exp(-\Delta G_{n,p}^{\text{obs}}/RT) \\ &= k_{\text{int}} \cdot \exp(-\Delta G^{\text{mix}}/RT) \cdot \Sigma \exp(-\Delta G_{n,p}^{\text{bind}}/RT) \\ &= (0.018) \cdot k_{\text{int}} \cdot \Sigma \exp(-\Delta G_{n,p}^{\text{bind}}/RT) \quad (\text{at } 30^\circ\text{C}) \\ &= (0.018) \cdot k_{\text{int}} \cdot \Sigma [\exp(\Sigma -A_i/RT)_{n,p}] \end{aligned} \quad (2)$$

where $\Delta G_{n,p}^{\text{obs}}$ is the observed Gibbs free energy change for one productive binding process ($\Delta G_{n,p}^{\text{obs}} = \Delta G^{\text{mix}} + \Delta G_{n,p}^{\text{bind}}$), ΔG^{mix} is the Gibbs free energy change of mixing for a bimolecular association process in a dilute aqueous solution ($\Delta G^{\text{mix}} = RT \cdot \ln[H_2O]$) and is equal to 10 kJ · mol^{–1} (2.4 kcal · mol^{–1}) [48,49], $\Delta G_{n,p}^{\text{bind}}$ is the intrinsic Gibbs free energy change for the binding process (also referred to as the unitary molecular binding affinity) and $\Delta G_{n,p}^{\text{bind}} = \Sigma A_i$, where ΣA_i indicates the sum of affinities of all subsites occupied by the substrate in the *p*-th binding mode, Σ indicates the sum of all productive complexes for a given substrate, *n* is the degree of polymerization of a given substrate, *R* is the molar gas constant 8.3145 J · mol^{–1} · K^{–1} and *T* is 303.15 K in this study.

The productive binding modes for the substrates (GlcNAc)_{2–4} were determined based on the product structural analysis for the first deacetylation reactions and are shown in Scheme 2. Also shown in the Scheme 2 legend are eqns (3)–(7), which were derived by applying the general eqn (2) to the productive binding modes for (GlcNAc)_{2–4}. The observed value of $(k_{\text{cat}}/K_m)_3$ was divided into three second-order rate constants for the three simultaneous reactions based on the ratios of the three positional isomers of mono-deacetylated (GlcNAc)₃. The values of A_{-2} , A_{-1} and A_{+1} were calculated using appropriate ratios of eqns (3)–(7) (Table 3). The average value of the catalytic subsite affinity A_0 was determined from eqns (3) and (4) using the calculated value of A_{+1} and a k_{int} value of 7 s^{–1} (Table 3). The k_{int} value is the average of k_{cat} values shown in Table 1. The unique distribution of the subsite affinities in CDAH is such that it is subsites –2 and +1 that provide most of the binding free energy (Table 3). This subsite structure dictates the observed pattern of the *n*-dependency of k_{cat}/K_m values: (GlcNAc)₃ occupies three subsites in two of its productive binding modes (Scheme 2); nevertheless, it does not have much more access to the total binding free energy than (GlcNAc)₂, thus the k_{cat}/K_m values are similar for

the two substrates (Table 1). In addition, based on the calculated subsite affinities, theoretical values for the observed dissociation constants with respect to GlcNAc (binding at subsite 0) and (GlcNAc)₂ (binding at subsites –1 and 0) are predicted to be about 3 and 2 M respectively. These values are clearly too high to allow for any significant hydrolytic activity in the presence of CDAH, consistent with our inability to detect any measurable CDAH-catalysed deacetylation of GlcNAc nor the deacetylation of the reducing-end sugar in (GlcNAc)₂.

According to the subsite theory, an apparent subsite affinity, A_i , includes both the unfavourable distortion free energy (positive) produced in the substrate portion bound at that subsite compensated for by the favourable interaction free energy (negative) involved in the binding [33]. Therefore, the apparent A_i value could be small negative or even positive and this does not necessarily imply a lack of interactions between that subsite and the substrate portion occupying it. In the case of CDAH, the small apparent affinities at subsites –1 and 0 may be a result of substrate distortions caused by the binding interactions that may be necessary for catalysis, presumably largely at the N-acetyl group. However, further evidence for possible substrate distortions at these subsites awaits the elucidation of the three-dimensional structure of CDAH. The protein crystallization experiments as well as mutagenesis studies are currently underway. Indeed, substrate distortions at subsites in the vicinity of the catalytic event have been predicted by subsite affinity calculations and verified by X-ray crystallography for enzymes such as hen egg-white lysozyme [50].

Calculated subsite affinities predict the kinetic parameters for the second deacetylation of (GlcNAc)₄

Scheme 3 shows the two productive binding modes of GlcNAcGlcNAcGlcNAcGlcNAc, *p*₁ and *p*₂, to the four-subsite binding cleft of CDAH as suggested by the structures of the di-deacetylated products [19]. These binding modes do not have access to the total available binding free energy because not all four subsites are occupied. This is one factor that causes the subsequent deacetylations to occur much more slowly than the first deacetylation of (GlcNAc)₄ (Figure 2). The estimated association constants, K_{p1} and K_{p2} , were calculated using eqns (9) and (8) (See the legend to Scheme 3) and were 86 and 52 M^{–1} respectively. The prediction is that GlcNAcGlcNAcGlcNAcGlcNAc should also bind to CDAH in a non-productive mode in which all four subsites are occupied with an estimated association constant, K_{q1} , equal to 6402 M^{–1}, as calculated using eqn (10) in Scheme 3. This would result in an observed k_{cat} value that is much lower than k_{int} and an observed K_m value that is similar to the K_m value for (GlcNAc)₄ {from eqn (1), $k_{\text{cat}} = [(K_{p1} + K_{p2})/(K_{p1} + K_{p2} + K_{q1})] \cdot k_{\text{int}}$ and $K_m = 1/(K_{p1} + K_{p2} + K_{q1})$ for this substrate}. The theoretical values of the kinetic parameters k_{cat} , K_m and k_{cat}/K_m for the deacetylation of GlcNAcGlcNAcGlcNAcGlcNAc were therefore calculated for two scenarios: first, only productive binding modes are present (*p*₁ and *p*₂) and second, both productive and non-productive binding occur (*p*₁, *p*₂ and *q*₁). The results are listed in Table 2 with the experimental values determined under steady-state conditions. Comparison of the experimental and the theoretical values clearly indicates the existence of non-productive binding and draws support for the correctness of the calculated subsite affinities, within the limits of experimental error. The non-productive binding of the mono-deacetylated substrate to the enzyme therefore contributes to the temporal separation of the first deacetylation reaction from the subsequent deacetylation reactions, providing an ideal system for the determination of subsite affinities.

Scheme 3 Schematic representations of the productive (p_1 & p_2) and non-productive (q_1) binding modes of GlcNAcGlcNAcGlcNGlcNAc to CDAH

The equations used to calculate the binding constants K_{p1} , K_{p2} and K_{q1} are: eqn 8, $K_{p2} = 0.018 \cdot \exp[-(A_0 + A_{+1})/RT]$; eqn 9, $K_{p1} = 0.018 \cdot \exp[-(A_{-1} + A_0 + A_{+1})/RT]$; eqn 10, $K_{q1} = 0.018 \cdot \exp[-(A_{-2} + A_{-1} + A_0 + A_{+1})/RT]$.

The theoretical value of K_{q1} is an overestimation since it does not account for the absence of the N-acetyl group in subsite 0, i.e. the binding of GlcNAcGlcNAcGlcNGlcNAc in q_1 mode is expected to be somewhat weaker than the binding of (GlcNAc)₄. Therefore, the calculated k_{cat} and K_m are both underestimated (Table 2). However, the theoretical and experimental k_{cat}/K_m values are identical because $k_{cat}/K_m = k_{int} \cdot (K_{p1} + K_{p2})$, which does not include the K_{q1} term. Indeed, this analysis can be used to estimate a $\Delta\Delta G_{(N-acetyl)}$ value for the recognition of the N-acetyl group by the catalytic subsite 0 (Table 3). We also determined $\Delta\Delta G_{(N-acetyl)}$ values for subsites -2, -1 and +1 (Table 3) using the K_m parameters previously reported by Tokuyasu et al. [19] for the other three mono-deacetylated substrates (GlcNGlcNAcGlcNAcGlcNAc, GlcNAcGlcNGlcNAcGlcNAc and GlcNAcGlcNAcGlcNAcGlcN) that have the same productive binding mode as (GlcNAc)₄. These substrates are not directly produced by the action of CDAH on (GlcNAc)₄ and were prepared as described previously [19].

The presumed biological significance of the action pattern of CDAH dictated by its subsite structure

Table 3 lists the three categories of parameters that define the subsite structure of CDAH: subsite affinities, recognition of N-acetyl groups by the subsites and the intrinsic rate constant for the hydrolytic reaction. The pattern of CDAH-catalysed deacetylation of (GlcNAc)₄ shown in Figure 2(B) is a consequence of at least two properties of the enzyme: the distribution of the subsite affinities in the catalytic domain, with subsites -2 and +1 making major contributions, and the recognition of the N-acetyl group by subsites -2 and 0. Another possible determining factor in the action pattern of CDAH may be the direction of access of the substrate into the active site. This unique action pattern of CDAH is quite valuable in regioselective chemo-enzymic synthesis since one specific positional isomer of the mono-deacetylated products of (GlcNAc)₂ or (GlcNAc)₄ can be exclusively produced and isolated.

The plant pathogen *C. lindemuthianum* secretes CDAH during penetration of the plant cells. The enzyme must be involved in modifying the fungal cell wall since the plant cell wall does not contain chitin. It is also known that plants infected by pathogenic fungi can activate a number of different defence responses, one of

which is the production of pathogenesis-related proteins such as endo-chitinases [24–26]. These enzymes can potentially degrade the fungal cell wall unless the chitin constituent is somehow modified and therefore not recognized by chitinase. One form of such modification could presumably be the partial deacetylation of the cell wall chitin by CDAH. But such modification has to be tightly regulated by a finely tuned enzyme. The distribution of the subsite affinities, with subsites -2 and +1 making major contributions, and the organization of the subsites, with the catalytic subsite being internal, ensures the endo-activity of the enzyme, thereby countering the endo-activity of plant chitinase. The recognition of the N-acetyl group only by subsites -2 and 0 ensures that the chitin is only partially deacetylated. If all three non-catalytic subsites recognized the N-acetyl group, the enzyme would not be able to deacetylate the cell wall to a large enough extent in order to effectively inhibit chitinase activity. On the other hand, if none of the non-catalytic subsites could recognize the N-acetyl group, the enzyme would greatly lose its specificity for GlcNAc moieties and would fully deacetylate chitin to chitosan. This must be avoided since plant cells are also known to produce hydrolytic enzymes with chitosanase activity during interactions with invading fungi [51]. In order to provide for effective resistance to plant defence, it seems necessary for the fungus to ensure only partial deacetylation of the cell wall. This is also presumably controlled by another means of regulation that is the inhibition of CDAH by the mono-deacetylated products. This inhibition slows down the full deacetylation of the fungal cell wall during penetration into the plant cell thereby resisting plant defence enzymes with either chitinase or chitosanase activities. Another suggested reason for only partial deacetylation of the fungal cell wall is that full deacetylation would create a polycationic surface that could possibly serve to elicit other plant defence reactions [25]. The actual proof of these speculations would have to come from direct investigations of plant–pathogen interactions.

Why k_{cat} for the first deacetylation reaction and k_{int} do not depend on n in the CDAH system

In the case of a multi-subsite enzyme with polymeric or oligomeric substrates, the absence of n -dependency of the observed k_{cat} values indicates that these substrates either do not form non-productive

complexes with the enzyme or that all such complexes have dissociation constants that are very high relative to those of the productive complexes (from eqn 1, $k_{\text{cat}} = k_{\text{int}}$ if no non-productive binding exists) [52,53]. This latter explanation is indeed the case for the non-productive binding modes of chito-oligosaccharide substrates to the four-subsite cleft of CDAH. There are only three possible non-productive complexes occupying only one or two subsites (subsite +1, subsite -2 or subsites -2 and -1) with expected dissociation constants of 382, 742 and 451 mM respectively as predicted by the subsite affinity distribution of CDAH. These values are too high relative to the range of substrate concentrations used in the kinetic experiments (Figure 1) to allow for any significant formation of non-productive complexes. Values of k_{cat} that are independent of n have been reported in similar studies on other multi-subsite enzymic systems such as exo- α -(1,4)-glucan lyase [54]. The observed k_{cat} values, for the enzyme/substrate systems in which non-productive binding occurs, usually increase with the size of the substrate because smaller substrates statistically have more non-productive binding modes [33,52,53].

The lack of n -dependency of k_{int} implies two things: first, a chemical step must be rate-limiting and not any association/dissociation processes that would probably depend on the size of the substrate/product; and second, there is no subsite co-operativity that would help position the N-acetyl group in the active site thereby promoting catalysis. In the Michaelis complex, the positioning of the N-acetyl group relative to the catalytic residues must be optimal for the smallest substrate (GlcNAc)₂. A more complicated conjecture can also be made about the catalytic mechanism based on the constant value of k_{int} with increasingly larger substrates: a two-step mechanism can be envisioned in which an enzymic intermediate forms (acetyl enzyme) in the first step and then breaks down in the second step with the participation of a water molecule. If the second step is rate-limiting, it is expected that k_{int} would not depend on the size of the substrate (since the amine leaving group has departed). However, the ways in which the energetics of binding of the enzyme to the increasingly larger substrates plays into the catalytic process remain unclear.

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