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Signatures of activation parameters reveal substrate-dependent rate determining steps in polysaccharide turnover by a family 18 chitinase

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ABSTRACT

Glycoside hydrolases play an important role in the degradation of biomass such as cellulose and chitin. Many of these enzymes act by a processive mechanism, which is generally considered favorable because it improves substrate-accessiblity. Recently we showed that this only applies to insoluble substrates. Towards more soluble and accessible substrates, processivity may in fact reduce the catalytic activity. Here, we describe kinetic studies showing how the type of substrate, insoluble or soluble, affects the activation parameters and rate determining steps for catalysis by the processive two-domain chitinase A (ChiA) from *Serratia marcescens*. The activation parameters show a large entropic activation barrier, indicative of a bimolecular (associative) rate determining step, for the degradation of insoluble crystalline β-chitin. For the water-soluble polymeric chitin-derivative chitosan, the rate determining step is associated with product-displacement and release. Furthermore, the degree of processivity is reflected in the activation parameters for chitosan hydrolysis; increase in processivity results in increase in activation enthalpy change and decrease in activation entropy change.

1. Introduction

Glycoside hydrolases (glycosidases) are important enzymes in nature that cleave glycosidic bonds. Based on sequence similarity, glycoside hydrolases have been classified in the CAZy database (Cantarel et al., 2009) which now holds 115 different families. Family 18 contains chitinases, which break down chitin, an insoluble linear 1,4-β-linked polymer of *N*-acetyl-β-D-glucosamine (GlcNAc). Chitin is the second most abundant biopolymer in nature, and is a major structural component of the exoskeleton of insects and crustaceans and the cell walls of some fungi. Like cellulases, chitinases face the daunting task of productively binding to an insoluble crystalline polymer. To achieve efficient degradation of the recalcitrant substrate many cellulases and chitinases have evolved to be processive (Horn et al., 2006a; Teeri, 1997).

Processivity is not easy to analyze experimentally, but for family 18 chitinases convenient experimental systems exist that recently have been exploited to create novel insight into the mechanism of processivity (Eijsink, Vaaje-Kolstad, Vårum, & Horn, 2008; Horn et al., 2006a; Zakariassen et al., 2009).

For example, it was shown that mutation of aromatic residues close to the catalytic centre of the processive two-domain ChiA from *Serratia marcescens* (Sikorski, Sørbotten, Horn, Eijsink, & Vårum, 2006) (Trp^{167} → Ala and Trp^{275} → Ala, see Fig. 1) reduces processivity as assessed by studying hydrolysis of the water-soluble polymeric chitin-derivative chitosan (Zakariassen et al., 2009). Interestingly, while these less processive variants showed reduced efficiency of crystalline β-chitin degradation, they showed clearly increased activity towards chitosan. These results indicate that the rate-limiting steps for the degradation of insoluble and soluble polymeric substrates are different, as previously suggested for both chitinous (Horn et al., 2006a) and cellulosic substrates (Harjunpää et al., 1996; Zhang & Wilson, 1997). To substantiate these indications by experiment, we have determined the activation parameters for the degradation of insoluble crystalline β-chitin and water-soluble chitosan by *S. marcescens* ChiA and two mutants with reduced processivity, ChiA-W167A and ChiA-W275A.

2. Experimental

2.1. Materials

Squid pen β-chitin (180 μm) was purchased from France Chitin (Marseille, France). Characterization of β-chitin from several squid species has shown that the number of average degree of polymerization (DP_n) is very high (in the order of thousands), and that the crystallinity index (C.I.) is approximately 80% (Chandumpai, Singhpibulporn, Faroongsarng, & Sornprasit, 2004; Jaworska, Sakurai, Gaudon, & Guibal, 2003; Susana Cortizo, Berghoff, & Alessandrini, 2008). The degree of acetylation was 92% ($F_A = 0.92$) (Karlsen, Heggset, & Sørlie, 2010). Chitosan, with a degree of *N*-acetylation

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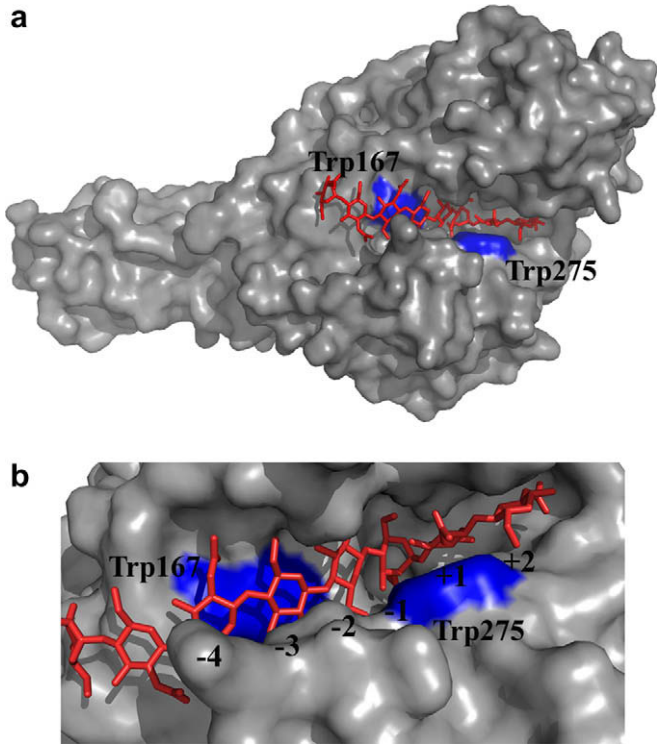

Fig. 1. Protein surface representation of ChiA-E315Q with bound $(GlcNAc)_8$ (a) and a close-up view of the active site and the $(GlcNAc)_8$ ligand (b) (Protein Data Bank code 1EHN) (Papanikolau et al., 2001). $(GlcNAc)_8$ is shown as red sticks and the surfaces of the Trp^{167} and the Trp^{275} residues are shown in blue. The numbers indicate the subsites to which the sugar-moieties are bound. ChiA has four (−4 to −1) glycon and two (+1 and +2) aglycon subsites. The chitin binding domain (ChBD) extends the active site toward the non-reducing end of the substrate; dimeric products are thought to be released from the +1 and +2 aglycon subsites (Horn et al., 2006b; Hult, Katouno, Uchiyama, Watanabe, & Sugiyama, 2005; Uchiyama et al., 2001; Zakariassen et al., 2009).

of 63% (F_A = 0.63), an intrinsic viscosity, [η] of 730 mL/g, and with a DP_n of 800, was prepared by homogeneous *N*-deacetylation of milled (1.0 mm sieve) shrimp shell chitin (Sannan, Kurita, Ogura, & Iwakura, 1978) and was converted to the chitosan hydrochloride salt (Draget, Vårum, Moen, Gynnild, & Smidsrød, 1992). This procedure results in a chitosan with a random distribution of *N*-acetylated and de-*N*-acetylated units (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991).

2.2. Enzymes

The wild type chitinase gene *chia* from *S. marcescens* strain BJL200 (Brurberg, Eijsink, & Nes, 1994) and mutant variants of *chia* were expressed in *Escherichia coli* TOP10 (Invitrogen, CA, USA) by growing cells for 16–18 h at 37 °C in Luria–Bertani medium containing 50 μg/mL ampicillin. Site-directed mutations in the *chia* gene were prepared using the QuickChange site-directed mutagenesis kit supplied by Stratagene (La Jolla, CA, USA), as described previously (Zakariassen et al., 2009). Periplasmatic extracts were produced as previously described (Brurberg, Nes, & Eijsink, 1996) and were purified using chitin affinity column chromatography, as described by Zakariassen et al. (2009). Enzyme purity was verified by SDS–PAGE and protein concentrations were determined using the Qubit Protein Assay (Invitrogen, Carlsbad, CA, USA).

2.3. Degradation of β-chitin

Hydrolysis of β-chitin (10 mg/mL) was carried out in 50 mM sodium acetate, pH 6.1, containing 0.1 mg/mL bovine serum albumin and enzyme at a concentration of 170 nM. The reaction mixtures were incubated in water baths at 18, 23, 26.5, 30, 33.5 and 37 °C. Reaction samples of 50 μL were withdrawn at regular time intervals, and the enzyme was inactivated by adding 50 μL of acetonitrile. All reactions were performed at least in duplicate and all samples were stored at −20 °C until further analysis. Because the substrate is in large excess (10 mg/mL chitin corresponds to a dimer concentration in the order of 25 mM), substrate saturating conditions were assumed. Use of reaction mixtures (at T = 26.5 °C) with an even higher chitin concentration (20 mg/mL) gave similar initial rates (Fig. 2A), confirming that substrate concentrations indeed were saturating. The samples were analyzed by isocratic high performance chromatography using an Amide-80 column (Tosoh Bioscience, Montgomeryville, PA), coupled to a UltiMate 3000 high performance liquid chromatography system from Dionex (Sunnyvale, CA). The liquid phase consisted of 70% acetonitrile, with a flow rate of 0.7 mL/min. Twenty microliters samples were injected using an UlitMate 3000 autosampler (Dionex). Eluted oligosaccharides were monitored by recording absorption at 210 nm. Chromatograms were collected and analyzed using the Chromeleon 6.8 software (Dionex). Since in all cases $(GlcNAc)_2$ represented more than 95% of the total amount of degradation products [GlcNAc and $(GlcNAc)_2$], only $(GlcNAc)_2$ peaks were subjected for data analysis and used for quantification of the extent of chitin degradation. Samples with known concentrations of $(GlcNAc)_2$ were used to prepare a standard curve. The initial rates were calculated using linear regression of $(GlcNAc)_2$ concentrations vs. time (at least four data points) in the initial phase of the reaction (Fig. 3A, C and E).

2.4. Degradation of chitosan

Hydrolysis of chitosan (0.5 mg/mL) was carried out in 50 mM sodium acetate, pH 6.1, containing 0.1 mg/mL bovine serum albumin and enzyme at a concentration of 43 nM. The reaction mixtures were incubated in water baths at 18, 23, 26.5, 30, 33.5 and 37 °C. Reaction samples of 200 μL were withdrawn at regular time intervals, and the enzyme was inactivated by adding 200 μL 0.5 M NaOH. The concentrations of the newly formed reducing ends were determined by using the 3-methyl-2-benzothiazolinone (MBTH) method, as described previously (Horn & Eijsink, 2004). Samples with known concentrations of $(GlcNAc)_2$ were used to prepare a standard curve. Reactions (performed at T = 26.5 °C) with 10 times higher concentrations of chitosan (5 mg/mL) gave the same initial rates (Fig. 2B), showing that substrate concentrations were saturating. The initial rates were calculated using linear regression of reducing end concentrations vs. time (at least four data points) in the initial phase of the reaction (Fig. 3B, D and F). All reactions were performed at least in duplicate.

2.5. Eyring analysis

To obtain the activation parameters for the chitinase-catalyzed hydrolysis of β-chitin and chitosan, two forms of the Eyring equation were used (Eqs. (1) and (2)):

$$\Delta G^{\ddagger} = -RT\ln(k_{cat}h/k_bT) \quad (1)$$

$$\ln(k_{cat}/T) = \ln(k_B/h) + \Delta S^{\ddagger}/R - \Delta H^{\ddagger}/RT \quad (2)$$

where k_{cat} is the measured rate of the reaction, $\Delta G^{\ddagger}$ is the activation free energy, $\Delta S^{\ddagger}$ is the activation entropy, $\Delta H^{\ddagger}$ is the activation enthalpy, h is the Planck's constant, k_B is the Boltzmann's constant, R is the gas constant, and T is the absolute temperature. $\Delta G^{\ddagger}$ was determined from using Eq. (1). Furthermore, the determined k_{cat}-values were fitted to the linear form of the Eyring Eq. (2) where the linear regression of the points of the Eyring plot ($\ln k_{cat}/T$ vs.