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

Henrik Zakariassen, Vincent G.H. Eijsink, Morten Sørlie *

Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science, N-1432 Ås, Norway

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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.

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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

* Corresponding author. Tel.: +47 64965902; fax: +47 64965901.
E-mail address: morten.sorlie@umb.no (M. Sørlie).

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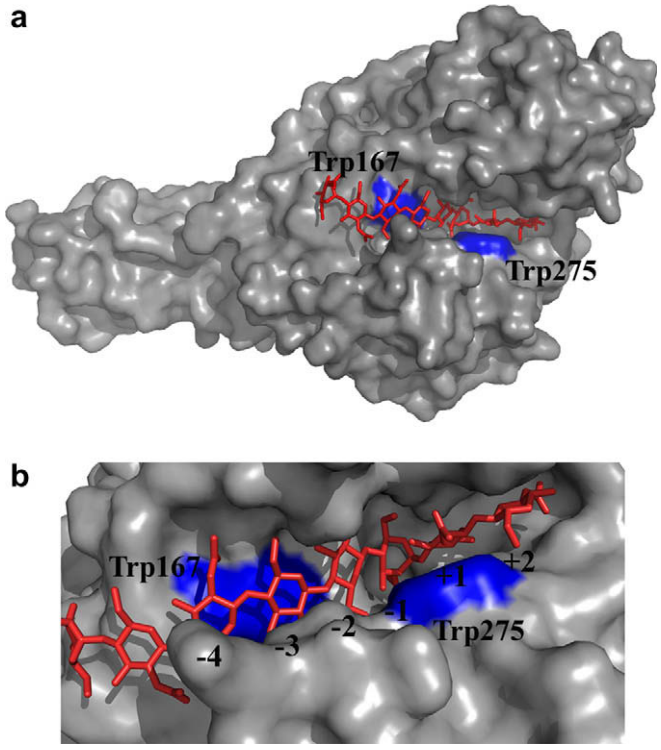

Fig. 1. Protein surface representation of ChiA-E315Q with bound $(GlcNAc)_8$ (a) and a close-up view of the active site and the $(GlcNAc)_8$ ligand (b) (Protein Data Bank code 1EHN) (Papanikolau et al., 2001). $(GlcNAc)_8$ is shown as red sticks and the surfaces of the Trp^{167} and the Trp^{275} residues 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.

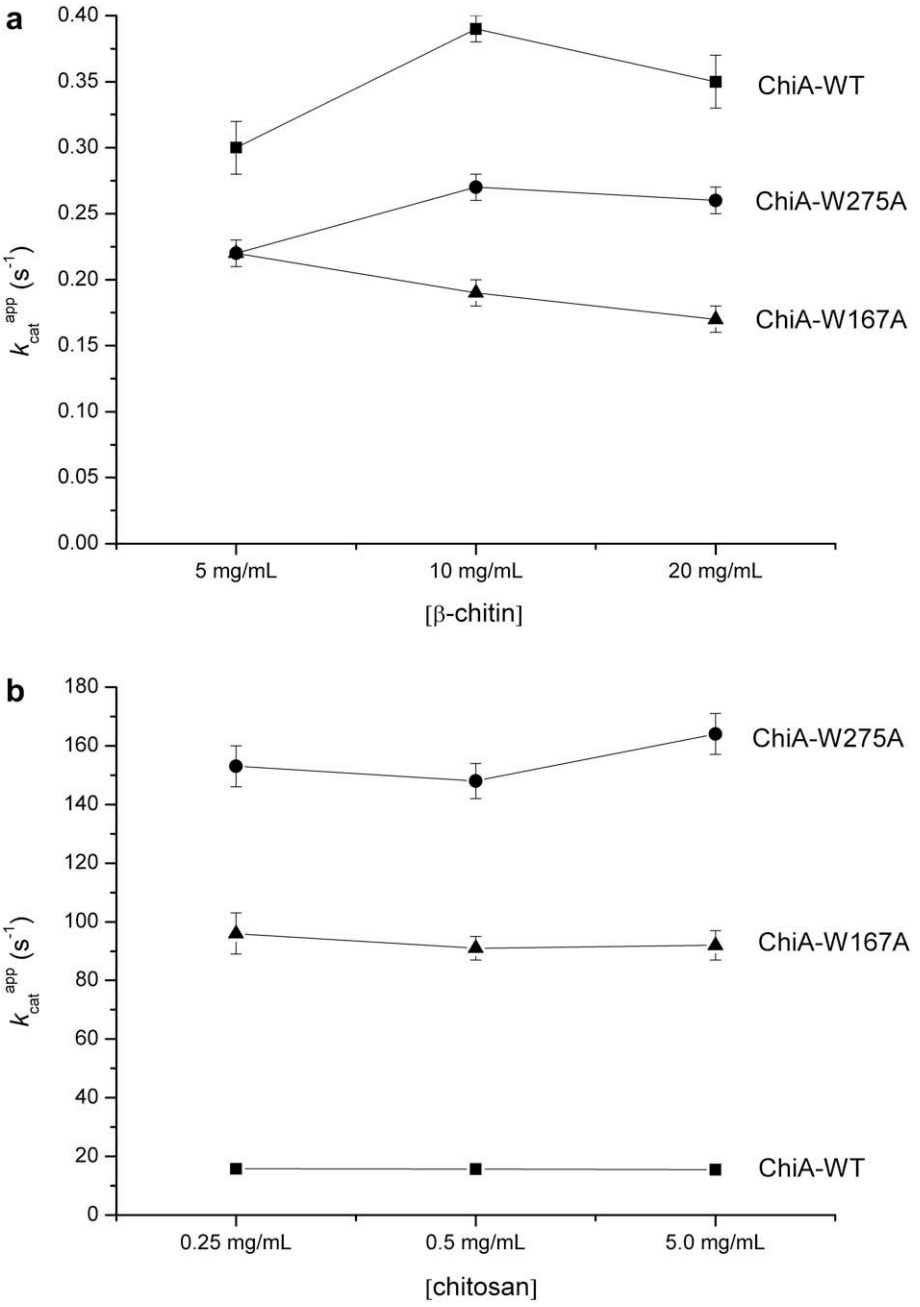

Fig. 2. The figures show initial rates of the hydrolysis of β-chitin (a) and chitosan (b) by ChiA-WT, ChiA-W275A, and ChiA-W167A at different substrate concentrations, at 26.5 °C. The error bars represent the standard deviations of at least two independent experiments (see Section 2 for details).

$1/T$) was performed using the Origin v7.0 software (OriginLab Corporation). $\Delta H^{\ddagger}$ was determined from the slope of this line ($-\Delta H^{\ddagger}/R$). $\Delta S^{\ddagger}$ was determined from the relationship described in Eq. (3):

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (3)$$

3. Results and discussion

Generally, productive binding to glycoside hydrolases yields two products, one in the glycon site (P_{glycon}) and one in the aglycon site ($P_{aglygon}$). For another hydrolytic event to take place, these products need to be displaced. During processive hydrolysis, the oligomeric (often dimeric) product ($P_{aglycon}$ or P_{glycon}, depending on the directionality of the enzyme; $P_{aglycon}$ in ChiA) dissociates either unassisted or by displacement by the polymeric product (P_{glycon} in ChiA) that slides by two sugar units to become the new substrate. A non-processive hydrolysis would require complete dissociation of both P_{glycon} and $P_{aglycon}$ either unassisted or assisted by a new, incoming substrate (Fig. 4).

The activation parameters for the chitinase-catalyzed hydrolysis of β-chitin and chitosan, shown in Table 1, were derived from Eyring analysis of the temperature dependence of the apparent catalytic rate constants (k_{cat}^{app}) (Figs. 3 and 5). For hydrolysis of β-chitin, all three ChiA variants showed low activation enthalpy changes ($\Delta H^{\ddagger}$) and relatively large negative changes in activation entropy ($\Delta S^{\ddagger}$), characteristic for a bimolecular (associative) rate-limiting step. Furthermore, ChiA-WT had a 1.5-fold higher k_{cat}^{app} compared to the ChiA-W275A and ChiA-W167A mutants ($0.54 \pm 0.04\ s^{-1}$ vs. $0.37 \pm 0.01\ s^{-1}$ and $0.34 \pm 0.02\ s^{-1}$, respec-

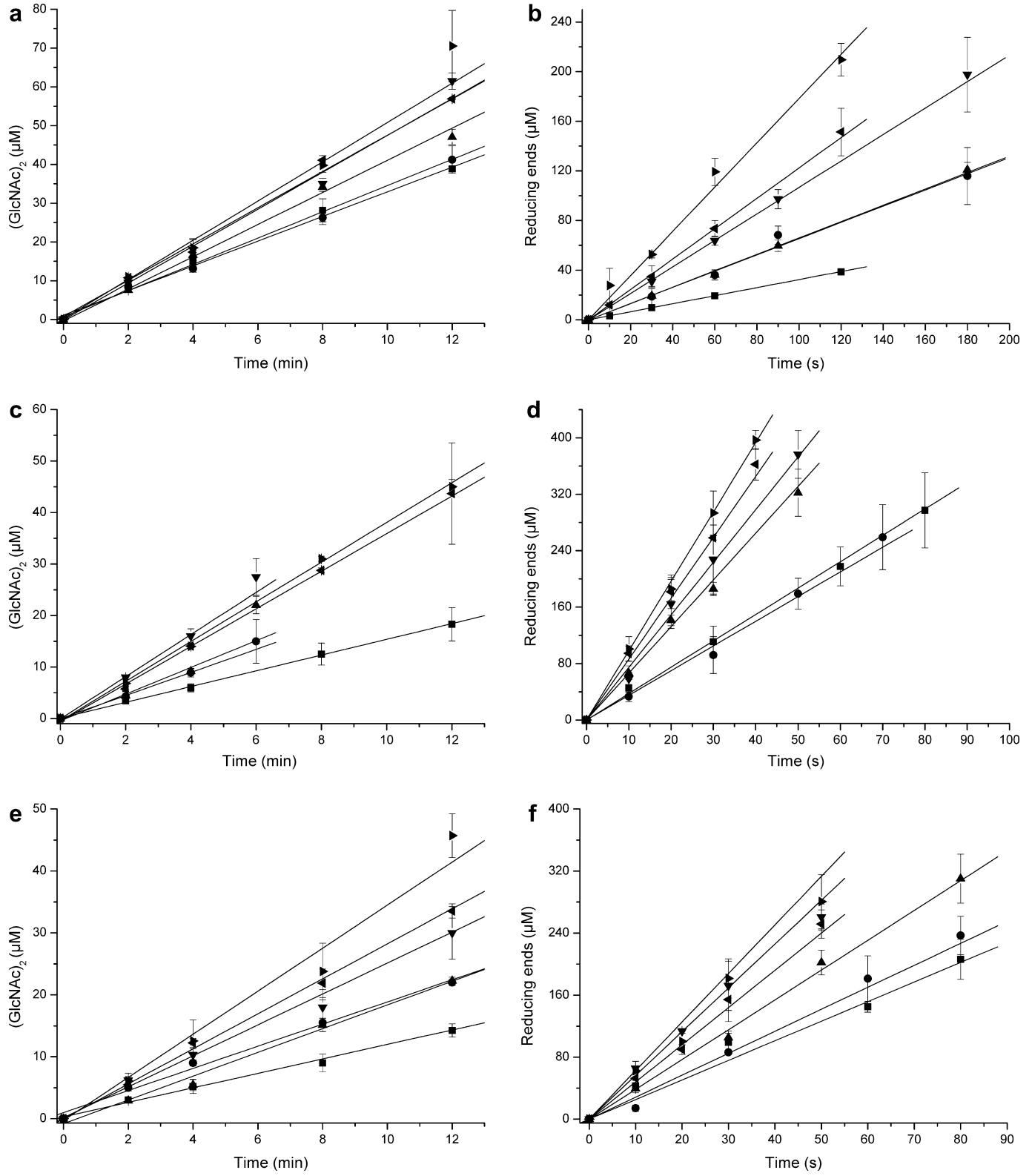

Fig. 3. Time course of the degradation of β-chitin by ChiA-WT (a), ChiA-W275A (c), and ChiA-W167A (e) and of chitosan by ChiA-WT (b), ChiA-W275A (d), and ChiA-W167A (f), at different temperatures (18 °C, squares; 23 °C, circles; 26.5 °C, triangles, bottom down; 30 °C, triangles, bottom up; 33.5 °C, triangles, bottom right and 37 °C, triangles, bottom left). The error bars represent the standard deviations of at least two independent experiments (see Section 2 for details).

tively), due to a more favorable $\Delta H^{\ddagger}$ (4.7 ± 0.5 vs. 7.4 ± 0.6 and 8.7 ± 0.6 kcal/mol, respectively), in accordance with having more aromatic residues available for association to the insoluble polymer. It should be noted that these k_{cat}^{app} values are derived from the initial linear stage of the reaction where the easily accessible amorphous fraction of the substrate is degraded. The non-linear parts of the product formation curves show that the negative effect of the two mutations on the efficiency of chitin turnover is in fact

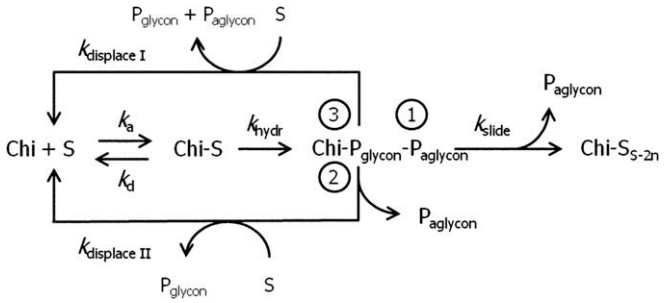

Fig. 4. Scheme over the proposed mechanism for chitosan hydrolysis by ChiA. Chi, chitinase; S, substrate; P_{glycon}, product on the glycon side; $P_{aglycon}$, product on the aglycon side; S_{S-2n}, new substrate resulting from productive binding of S, hydrolysis of S, followed by sliding of the polymeric P_{glycon} by two or a multiple (n) of two sugar moieties to form a new productive complex; k_a, k_d, k_{hydr}, k_{slide} and $k_{displace}$ are rate constants for association, dissociation, and hydrolysis of S, sliding of P_{glycon}, and displacement of products by incoming new S, respectively. The numbers in circles indicate three possible paths referred to in the text. Path 1 is the processive mechanism; paths 2 and 3 are non-processive and differ with respect to the involvement of incoming S in release of the dimeric product, $P_{aglycon}$.

Table 1
Activation parameters for substrate hydrolysis in 50 mM sodium acetate buffer, pH 6.1.

Enzyme	k_{cat}^{app} [a]	$\Delta G^{\ddagger}$ [b,c]	$\Delta H^{\ddagger}$ [b]	$-T\Delta S^{\ddagger}$ [b,c]
β-Chitin				
ChiA-WT	0.54 ± 0.04	18.6 ± 0.1	4.7 ± 0.5	13.9 ± 0.4
ChiA-W275A	0.37 ± 0.01	18.8 ± 0.1	7.4 ± 0.6	11.4 ± 0.6
ChiA-W167A	0.34 ± 0.02	18.9 ± 0.1	8.7 ± 0.6	10.2 ± 0.6
Chitosan				
ChiA-WT	40 ± 2	15.9 ± 0.1	15.2 ± 0.6	0.7 ± 0.5
ChiA-W275A	233 ± 20	14.8 ± 0.1	9.6 ± 0.7	5.2 ± 0.5
ChiA-W167A	130 ± 17	15.2 ± 0.1	7.9 ± 0.7	7.3 ± 0.7

[a] s^{-1}.
[b] kcal/mol.
[c] T = 37 °C.

more dramatic. In contrast to the wild-type enzyme the two mutants are not capable of completely degrading the β-chitin substrate (Zakariassen et al., 2009). Taken together, these findings show that substrate binding is the rate-limiting step in insoluble β-chitin hydrolysis and that Trp^{167} and Trp^{275}, not unexpectedly, play a role in this process.

Changing the substrate from insoluble β-chitin to water-soluble chitosan led to remarkable changes in the activation parameters of ChiA-WT-catalyzed hydrolysis. Firstly, the activation entropy change is reduced dramatically ($-T\Delta S^{\ddagger}$ from 13.9 ± 0.4 to 0.7 ± 0.5 kcal/mol; Table 1), suggesting that enzyme-substrate association is no longer the rate-limiting step. Secondly, the activation enthalpy change is significantly increased ($\Delta H^{\ddagger}$ from 4.7 ± 0.5 to 15.2 ± 0.6 kcal/mol). The values of the activation parameters are compatible with sliding of P_{glycon} and displacement of $P_{aglycon}$ (Path 1 in Fig. 4) being the rate-limiting step. The entropic term is small because the substrate is already in the active site and because water molecules involved in the enzyme-substrate interaction remain "trapped" in the interaction. For sliding, all weak interactions between the P_{glycon} and the enzyme need to be temporarily disrupted (Varrot et al., 2003), explaining the large activation enthalpy change.

In the degradation of chitin, processivity comes in after the rate-limiting step and is thus not amenable to kinetic analysis. This is different in the case of chitosan. Residue Trp^{167} is characteristic for ChiA. It stacks with the sugar moiety in the −3 subsite in the glycon part of the active site (Fig. 1) and its mutation to alanine results in an enzyme variant that is no longer processive (Zakariassen et al., 2009). As seen in Table 1, $\Delta H^{\ddagger}$ for ChiA-W167A is smaller than for ChiA-WT (7.9 ± 0.7 vs. 15.2 ± 0.6 kcal/mol) while the entropic term has become much more unfavorable ($-T\Delta S^{\ddagger}$ = 7.3 ± 0.7 vs. 0.7 ± 0.5 kcal/mol). These changes in activation parameters are in accordance with the loss in processivity. Firstly, in the non-processsive ChiA-W167A mutant, the unfavorable enthalpic effect of displacement of P_{glycon} and $P_{aglycon}$ is partly compensated by the formation of new intermolecular interactions between the incoming substrate and the enzyme (Paths 2 and 3 in Fig. 4), leading to a reduced $\Delta H^{\ddagger}$. Moreover, it has been shown that the enthalpy change of the hydrophobic stacking interaction between a tryptophan and a sugar is approximately 4 kcal/mol (Zolotnitsky et al., 2004), meaning that deletion of one sugar-interacting tryptophan, would also contribute to lowering the $\Delta H^{\ddagger}$. Secondly, in this non-processive enzyme variant most catalytic cycles would involve association with a new substrate. This is entropically more demanding than sliding of P_{glycon} and dissociation of $P_{aglycon}$ in the processive mechanism, explaining the larger entropy term for the W167A mutant.

Trp^{275} stacks with a sugar moiety in the +1 subsite in the aglycon part of the active site (Fig. 1). The ChiA-W275A mutant is still processive albeit not as much as the wild type (Zakariassen et al., 2009). Since the stacking interaction between a tryptophan and the sugar in the +1 subsite is removed, it is expected that dissociation of $P_{aglycon}$ will be easier in the ChiA-W275A mutant, meaning that path 2 (Fig. 4) is this mutant's most probable alternative for the processive path 1. In accordance with its "mixed" (partly processive) character, the activation parameters for the ChiA-W275A mutant ($\Delta H^{\ddagger}$ = 9.6 ± 0.7 kcal/mol; $-T\Delta S^{\ddagger}$ = 5.2 ± 0.5 kcal/mol) represent intermediate values between the extremes of processive ChiA-WT ($\Delta H^{\ddagger}$ = 15.2 ± 0.6 kcal/mol; $-T\Delta S^{\ddagger}$ = 0.7 ± 0.5 kcal/mol) and non-processive ChiA-W167A ($\Delta H^{\ddagger}$ = 7.9 ± 0.7 kcal/mol; $-T\Delta S^{\ddagger}$ = 7.3 ± 0.7 kcal/mol).

Processivity requires a "sticky" enzyme because this increases the probability for processive sliding of P_{glycon} rather than complete dissociation. Our data show that aromatic residues are crucial for achieving this "stickiness", as previously suggested by crystallographic studies (Varrot et al., 2003). Weakening of the Trp^{167}- or Trp^{275}-sugar interactions not only reduces processivity but also leads to an increase in chitosan-hydrolyzing activity (Table 1) (Zakariassen et al., 2009). The present study shows that this is a consequence of a more favorable $\Delta H^{\ddagger}$ that more than compensates the unfavorable entropic effects of the changed mechanism of action. These effects on the activation parameters are consistent with release of $P_{aglycon}$ and sliding of P_{glycon} being the rate-limiting step in chitinase-catalyzed hydrolysis of chitosan (as opposed to enzyme-substrate association which is rate-limiting when insoluble chitin is the substrate). In the case of chitosan, the substrate and the enzyme are in the same phase and freely diffusible. Association rates are likely to be high and single polymer chains would, therefore, be continuously available for enzyme-binding. Under these conditions, reduced product affinities are catalytically beneficial as is indeed observed for the less processive ChiA-W167A and ChiA-W275A mutants.

4. Conclusion

In this study we show how activation parameters and rate-limiting steps for polysaccharide hydrolysis by ChiA depend on the type of substrate. The values of the determined activation parameters indicate a bimolecular (associative) rate determining step for the degradation of insoluble crystalline β-chitin while the rate-limiting step is associated with product-displacement and release when the substrate is changed to the water-soluble polymeric chitin-derivative chitosan. Furthermore, exploiting the unique experimental opportunities provided by chitosan, we show that processivity coincides with dominance of the enthalpic term in the activation barrier, and that the entropic term becomes

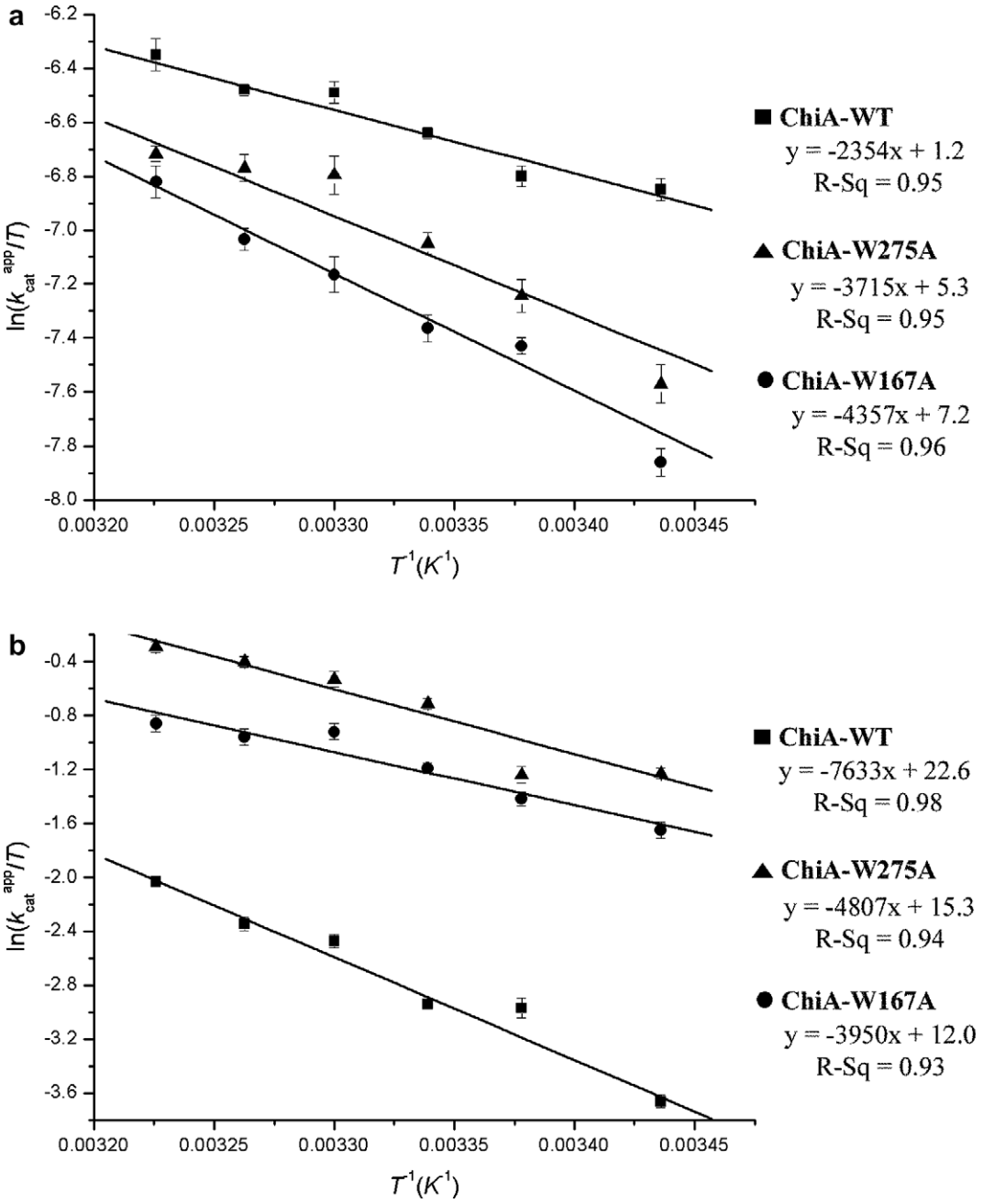

Fig. 5. Eyring plots for the hydrolysis of β-chitin (a), and chitosan (b) by ChiA. The error bars represent the standard deviations of at least two independent experiments (see Section 2 for details).

more prominent in site-directed mutants displaying reduced processivity.

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