Substrate specificity of endoglucanase A from *Cellulomonas fimi*: fundamental differences between endoglucanases and exoglucanases from family 6

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Values of k_{cat} and K_m for the hydrolysis of cellotetraose, cellotriose, β -cellobiosyl fluoride and various β -aryl cellobiosides by endoglucanase A (CenA) from *Cellulomonas fimi* indicate that specific binding interactions between the reducing-end glucose residues of cellotetraose and cellotriose and the enzyme at the transition state provide enormous stabilization, endowing glucose with the 'effective leaving group ability' of 2,4-dinitrophenol. As has been seen with several other inverting glycosidases, CenA hydrolyses the 'wrong' anomer of its glycosyl fluoride substrate, α -cellobiosyl fluoride, according to non-Michaelian kinetics. This indicates that CenA carries out this hydrolysis by a mechanism involving binding of two substrate molecules in the active site [Hehre, Brewer and Genghof (1979) J. Biol. Chem. 254, 5942–5950] in contrast with that reported for

cellobiohydrolase II, another family-6 enzyme [Konstantinidis, Marsden and Sinnott (1993) Biochem. J. 291, 833–838]. The pH profiles for wild-type CenA indicate that k_{cat} for CenA depends on the presence of both a protonated group and a deprotonated group for full activity, consistent with the presence of an acid and a base catalyst at the active site. By contrast, the profile for the Asp252Ala mutant of CenA shows a dependence only on a base-catalytic group, thereby confirming the role of Asp-252 as an acid catalyst. These results show that hydrolysis by CenA occurs by a typical inverting mechanism involving both acid and base catalysis, as first proposed by Koshland. It also suggests that endoglucanases from family 6 may function by fundamentally different mechanisms from exoglucanases in this family.

INTRODUCTION

Micro-organisms that degrade cellulose generally secrete many different cellulases which act synergistically to hydrolyse the substrate. For instance, the Gram-positive soil bacterium *Cellulomonas fimi* produces an array of cellulases when grown on cellulose [1]. The genes encoding four endoglucanases (EC 3.2.1.4) (CenA, CenB, CenC and CenD) [2–6], two exoglucanases (EC 3.2.1.91) [cellobiohydrolases A and B (CbhA and CbhB)] [7,8] and an exoglucanase/xylanase (EC 3.2.1.8) (Cex) [9] from *C. fimi* have been cloned in *Escherichia coli* and sequenced. These proteins all have the ability to bind to cellulose.

The endoglucanase CenA has been well characterized and is a member of family 6 of cellulases and xylanases [10]. An interesting feature of family 6 is that it contains both endoglucanases and exoglucanases which show definite differences in amino acid sequence, the exoglucanases having extra amino acid insertions [8,11–13]. The X-ray-crystallographic structures for two family-6 enzymes, CbhII, an exoglucanase from *Trichoderma reesei*, and E2, an endoglucanase from *Thermomonospora fusca*, have been solved [12,13]. Both enzymes have a central α/β barrel structure similar to that of triose phosphate isomerase, with virtually identical topologies. Although very similar overall, there are significant differences between the two enzymes consistent with the fact that one is an endoglucanase whereas the other is an

exoglucanase. The active site of CbhII is enclosed by two extended surface loops which form a tunnel about 2 nm (20 Å) in length [12]. The C-terminal surface loop is absent from E2 whereas the N-terminal loop is present but is pulled back so that it no longer covers the active site [13]. The tunnel-shaped active site in CbhII is thought to restrict access of long cellulose polymers, thus imparting exoglucanase activity only. In E2, this restriction is not a problem so that the active site is fully accessible to the cellulose polymers, and the enzyme therefore acts as an endoglucanase.

All family-6 enzymes so far tested hydrolyse the β -1,4-glucosidic bond with inversion of anomeric configuration. The inverting mechanism (Scheme 1) involves protonation of the glycosidic oxygen of the scissile bond by an acidic amino acid residue (general acid catalyst) with concerted attack of a water molecule at the anomeric carbon. The nucleophilicity of this water molecule is greatly increased through deprotonation by a basic amino acid residue (general base catalyst). The partial positive charge formed at the anomeric carbon in the transition state is stabilized through resonance with the ring oxygen. This gives the transition state significant oxocarbenium ion character which is stabilized by electrostatic interactions with the nearby carboxylate side chains and by specific binding interactions with the sugar in its half-chair conformation (Scheme 1). In general, only glutamate and aspartate residues act directly as general acid or base catalysts in glycosidases [14–16]. The general acid and

Abbreviations used: Cen, endoglucanase from *Cellulomonas fimi*; Cbh, cellobiohydrolase; Cex, exoglucanase from *Cellulomonas fimi*; E2, endoglucanase from *Thermomonospora fusca*; Cel, cellulase from *Clostridium thermocellum*; 2,4-DNPC, 2,4-dinitrophenyl β -cellobioside; 3,4-DNPC, 3,4-dinitrophenyl β -cellobioside; 2,5-DNPC, 2,5-dinitrophenyl β -cellobioside; α - or β -CF, α - or β -cellobiosyl fluoride; S_0 , initial substrate concentration.

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Scheme 1 Typical inverting mechanism for hydrolysis of β -glucosides by β -glucosidases

R indicates a glycosyl residue.

base catalysts have been identified in CenA as Asp-252 and Asp-392 respectively through mutation of conserved amino acids and kinetic analysis [17]. Thus whereas the activity (k_{cat}) of the Asp252Ala mutant was reduced 220 000-fold for CM-cellulose, it was actually slightly increased for 2,4-dinitrophenyl β -cellobioside (2,4-DNPC). Such behaviour is consistent with Asp-252 being the acid catalyst since its presence is essential for substrates with poor leaving groups, but not for those with leaving groups needing no assistance. Indeed the k_{cat}/K_m value for hydrolysis of CM-cellulose is some 10-fold higher with the mutant than the wild-type, indicating improved binding of this anionic substrate to an enzyme active site missing a carboxyl(ate) group. Identification of Asp-392 as the base catalyst was based on the fact that activities with both substrates 2,4-DNPC and CM-cellulose were reduced enormously, consistent with a critical role for that residue. Interestingly, slightly greater residual activity was seen with CM-cellulose than with 2,4-DNPC, possibly indicating that the substrate carboxyl groups in CM-cellulose provide some 'substrate-assisted catalysis'. Although these catalytic residues are essential for hydrolysis, much of the rate enhancement that is due to enzyme catalysis probably comes from hydrogen-bonding and hydrophobic and electrostatic interactions between the substrate and amino acid residues farther removed from the scissile bond. These residues are responsible for binding the substrate, particularly at the transition state, thereby significantly lowering the activation barrier [15].

Although all glycosidases hydrolyse by either a 'retaining' or 'inverting' mechanism as described, some inverting glycosidases have the ability to hydrolyse certain artificial substrates with the 'wrong' anomeric configuration. For these substrates, hydrolysis occurs with overall retention of anomeric configuration. This has been shown to occur for the hydrolysis of many glycosyl fluorides with the wrong anomeric configuration, including β -maltosyl fluoride by β -amylase (EC 3.2.1.2) from sweet potato [18] and α -xylosyl fluoride by a β -xylosidase (EC 3.2.1.37) from *Bacillus pumilus* [19]. In all cases, the glycosyl fluoride with the 'correct' anomeric configuration was hydrolysed with 'normal' inversion of configuration. A mechanism to explain these observations, in which two molecules of substrate bind at the active site, has been proposed by Hehre et al. [18] (Scheme 2). The nucleophilic hydroxy group of one substrate molecule, bound in the 'aglycone' site, directly attacks at the anomeric centre of the other substrate bound normally, in a virtual reverse reaction. This then forms the 'normal' substrate which is rapidly hydrolysed. Evidence for this mechanism comes from the dependence of the reaction rate on the square of the substrate concentration and, in some cases, on the observed accumulation of the intermediate [20]. CbhII, the inverting family-6 exoglucanase from *T. reesei*, also

Scheme 2 Proposed mechanism for the hydrolysis of α -CF by the Hehre mechanism

hydrolyses α -cellobiosyl fluoride (α -CF) [21], but appears to do so according to regular Michaelis-Menten kinetics. Sinnott and co-workers [21] thus propose that hydrolysis by CbhII occurs by a mechanism different from that proposed by Hehre, one involving an S_Ni reaction.

This paper presents data to show that CenA, a member of the same family as CbhII, behaves as a typical inverting glycosyl hydrolase. It also points out major differences between the exoglucanases and endoglucanases of family 6 and provides further insight into the role of Asp-252, the putative acid catalyst.

MATERIALS AND METHODS

Chemicals, substrates and enzymes

All chemicals used were of analytical or HPLC grade and were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.) or BDH (Poole, Dorset, U.K.) unless otherwise indicated. Cellotriose and cellotetraose were obtained from Seikagaku Corporation (Tokyo, Japan). Substrates 2,4-DNPC, 2,5-dinitrophenyl β -cellobioside (2,5-DNPC) and 3,4-dinitrophenyl β -cellobioside (3,4-DNPC) were gifts from Dedreia Tull from the Department of Chemistry, University of British Columbia. All enzymes were produced and purified as described previously [17].

Synthesis of α - and β -CFs

Reactions were monitored by TLC using Merck Kieselgel 60 F₂₅₄ aluminium-backed sheets. Compounds were detected by charring with 10% H₂SO₄ in methanol or with 10% ammonium molybdate in 2 M H₂SO₄. Flash chromatography was performed on short columns (10–15 cm) of Merck Kieselgel 60 (230–400 mesh) under a positive pressure with specified eluents. ¹H- and ¹⁹F-NMR spectra were recorded on a Bruker AC 200 spectrometer. ¹H- and ¹⁹F-NMR spectra recorded in ²H₂O are quoted relative to external 4,4-dimethyl-4-silapentane-1-sulphonate and trifluoroacetic acid (δ 0.00) respectively. Hepta-*O*-acetyl- α -cellobiosyl fluoride was prepared by a modification of the procedure of Jünemann et al. [22] by treatment of cellobiose octa-acetate with 3:7 pyridinium poly(hydrogen fluoride) (Aldrich Chemical Co.), initially at –20 °C and then for 4 h at room temperature. Work-up followed by crystallization from ethanol gave the pure product with m.p., ¹H- and ¹⁹F-NMR spectra identical with those reported. The NMR spectra indicated that it was free of β -fluoride. The hepta-acetate was deacetylated by treatment with a saturated solution of ammonia in methanol at 0 °C for 6 h. Concentration followed by flash chromatography [ethyl acetate/methanol/water (16:4:1, by vol.)] gave α -CF as a solid which was crystallized from methanol/ether, m.p. 270 °C (dec.; darkens at 160 °C). (Found: C, 41.56; H, 6.27. C₁₂H₂₁FO₁₀ requires C, 41.86; H, 6.15%). The product was homogeneous by TLC and NMR. ¹⁹F NMR (188 MHz, ²H₂O): δ 74.1 (dd, *J*_{1,F} 53.6, *J*_{2,F} 26.0 Hz). ¹H NMR (200 MHz, ²H₂O): δ 5.66 (dd, *J*_{1,F} 53.6, *J*_{1,2} 2.7 Hz, H1), 4.49 (d, *J*_{1,2} 7.8 Hz, H1'). Hepta-*O*-acetyl- β -cellobiosyl fluoride, prepared by the method of Micheel et al. [23], was deacetylated and purified as described above to give β -CF as an amorphous solid. ¹⁹F NMR (188 MHz, ²H₂O): δ 67.2 (dd, *J*_{1,F} 53.0, *J*_{2,F} 12.8 Hz). ¹H NMR (200 MHz, ²H₂O): δ 5.23 (dd, *J*_{1,F} 53.0, *J*_{1,2} 7.1 Hz, H1), 4.47 (d, *J*_{1,2} 7.8 Hz, H1'). The β -fluoride was quite labile and the ¹H-NMR spectrum indicated the presence of approx. 8% cellobiose which increased over time on storage. Before use the amount of hydrolysis was determined by ¹H NMR and by fluoride ion analysis. Samples were not used for kinetic analysis if they contained more than 20% hydrolysis product.

Determination of kinetic parameters for the hydrolysis of cellotriose and cellotetraose

Initial rates of hydrolysis of cellotriose and cellotetraose were determined by incubating appropriate substrate concentrations

in potassium phosphate buffer (5 mM, pH 7.0) at 37 °C until thermally equilibrated. Reactions were initiated by the addition of 20 μ l of enzyme (total volume 1 ml); after 30 min, reactions were stopped by addition of 100 μ l of H₂SO₄ (1 M). The cellobiose produced was quantified with an HPLC system (model DX500; Dionex, Sunnyvale, CA, U.S.A.) using an anion-exchange column (Carbopac PA1) and a pulsed amperometric detector (ED40). A gradient of NaOH, sodium acetate and water was used as described previously [24]. Data were analysed using the Dionex Peaknet software package, and kinetic parameters were determined using the program GraFit 3.0 (Sigma).

Determination of kinetic parameters for the hydrolysis of nitrophenyl cellobiosides

All steady-state kinetic studies for aryl cellobioside hydrolysis were performed by recording changes in absorbance using either a Hitachi U 2000 spectrophotometer (Tokyo, Japan), with a temperature-controlled cell holder, or a Pye–Unicam PU-8800 spectrophotometer, equipped with a temperature-controlled circulating water bath. Hydrolysis was monitored at appropriate wavelengths using absorption coefficients previously described [25]. Initial rates of hydrolysis were determined by incubating solutions of the appropriate substrate concentrations in 50 mM potassium phosphate buffer (pH 7.0)/0.1% BSA at 37 °C within the spectrophotometer until thermally equilibrated. Reactions were initiated by the addition of enzyme, and release of phenol product was monitored at the appropriate wavelength. Values of *K*_m and *k*_{cat} were determined by measuring initial rates of hydrolysis at seven to ten different substrate concentrations, which generally ranged from 0.2 to 5 times the *K*_m value. Values for *K*_m and *k*_{cat} were determined by non-linear regression analysis using the program GraFit 3.0.

Determination of kinetic parameters for hydrolysis of α - and β -CFs

Initial rates of hydrolysis of both α - and β -CF were determined by incubating appropriate substrate concentrations in potassium phosphate buffer (50 mM, pH 7.0) at 30 °C until thermally equilibrated and reactions were initiated by the addition of 20 μ L of enzyme (total volume 500 μ l). Release of fluoride was monitored using a fluoride electrode (Fluoride Ion Combo-electrode; VWR, Toronto, Ontario, Canada) coupled to a pH-meter (VWR). The electrode was calibrated using standard solutions of NaF (10, 1 and 0.1 mM). Kinetic parameters were determined using the program GraFit 3.0.

pH-dependence studies

Kinetic parameters for hydrolysis of 2,4-DNPC were determined for wild-type CenA and the Asp252Ala mutant over the range pH 4.5–9.5 at increments of 0.5 pH unit. Sodium succinate buffer (50 mM, containing 0.1% BSA and 150 mM NaCl) was used from pH 4.5 to 6.0, potassium phosphate buffer (50 mM, containing 0.1% BSA and 150 mM NaCl) was used from pH 6.0 to 8.0 and glycylglycine buffer (50 mM, containing 0.1% BSA and 150 mM NaCl) was used from pH 8.0 to 9.5. A stock solution (111.1 mM buffer, 0.22% BSA and 333.3 mM NaCl) of each buffer was prepared and 50 ml of each was added to 40 ml of water. NaOH or HCl was added to bring the buffer to the correct pH value and water was added to adjust the total volume to 100 ml. The actual pH of each buffer was determined using a pH-meter (Accumet 925 pH Ion Meter; Fisher Scientific, Ottawa, Ontario, Canada). Molar absorption coefficients were determined

for 2,4-DNPC from pH 4.5 to 6.5 by making dilutions of a stock solution of 2,4-DNPC in each buffer. Initial rates of hydrolysis were determined by adding 900 μ l of the appropriate buffer, 90 μ l of substrate and 10 μ l of enzyme to a 1 ml cuvette. Rates were measured for six to ten different substrate concentrations, which generally ranged from 0.2 to 5 times the K_m value. Values of k_{cat} , K_m and pK_a were determined using GraFit 3.0. Duplicate determinations were carried out at pH values where the buffers employed overlapped. Identical values were determined for the two buffer systems.

RESULTS AND DISCUSSION

Values of k_{cat} and K_m for the hydrolysis of cellotetraose, cellotriose, β -CF and various β -aryl cellobiosides by CenA are shown in Table 1.

Cellotetraose is an excellent substrate for CenA with a k_{cat} value of 220 s^{-1} . This value is quite typical for glycoside hydrolases, which commonly have k_{cat} values of the order of 10^2 – 10^3 s^{-1} [21]. Hydrolysis of cellotriose by CenA is significantly slower, with a k_{cat} value that is 755-fold lower and a K_m value that is five times higher than for cellotetraose. Based on crystal structures for E2 from *Th. fusca* and CbhII from *T. reesei* [12,13], as well as on binding studies with small soluble substrate analogues [12,13,26,27], the active site of family-6 enzyme consists of four subsites (A to D), with hydrolysis occurring between subsites B and C. CenA hydrolyses cellotetraose to cellobiose only, whereas cellotriose is hydrolysed to glucose and cellobiose [28]. Presumably, cellotetraose is able to completely fill all four binding subsites whereas cellotriose binds in subsites A–C. Binding in all four subsites is obviously very important for full realization of catalytic potential in CenA because when subsite D remains empty, both K_m and k_{cat} values are significantly affected. By comparison, the k_{cat} value for hydrolysis of cellotetraose by CbhII (3.3 s^{-1}) is only 100-fold greater than that for cellotriose [21], indicating a somewhat lower requirement for binding in subsite D in that case.

Whereas the rates of hydrolysis of 2,4-DNPC and β -CF are comparable with that for cellotriose, the rates for the other aryl cellobiosides with leaving groups of higher pK_a than 2,4-dinitrophenol are considerably lower. Further, these rates are much lower than that for hydrolysis of cellotetraose. Given that the inherent leaving-group abilities of these phenols and of fluoride are much greater than those of the glucose and cellobiose leaving groups from cellotriose or cellotetraose, it is evident that specific interactions between these 'aglycone' sugars and the enzyme accelerate reaction rates enormously by stabilizing the transition state. It is unlikely, however, that specific aglycone–enzyme interactions significantly assist the hydrolysis

Figure 1 Rate of hydrolysis of α -CF by CenA as a function of S_0 (a) and the double-reciprocal plot ($1/\text{rate}$ versus $1/S_0$) (b)

The inset graph in (a) shows data at low substrate concentration.

of the artificial substrates. Therefore the enzyme, by use of specific binding interactions, improves the inherent leaving-group ability of the glucose moiety (alcohol $pK_a \approx 16$) enormously such that it becomes about as good a leaving group as 2,4-dinitrophenol ($pK_a = 4$). Although this increase in effective leaving-group ability is quite large, that for the cellobiose moiety (from the value for cellotetraose) is approx. 1000-fold greater. A measure of the effective leaving-group ability in this case can be obtained by using the Brønsted plot (not shown) of $\log(k_{cat}/K_m)$ for each aryl cellobioside substrate versus its aglycone pK_a . Although the quality of this plot is not high ($\rho = 0.89$), its large slope ($\beta_{lg} = 0.9$) indicates substantial charge development on the leaving oxygen at the transition state, consistent with almost complete bond cleavage and little proton donation. Assuming that alkyl alcohols fall on the same plot as phenols, then extrapolation of this plot to a value of $k_{cat}/K_m = 1800 \text{ mM}^{-1} \cdot \text{s}^{-1}$, as measured for cellotetraose, indicates that the effective leaving group ability of cellobiose is that of a phenol of $pK_a \approx 0$. This represents an enormous effective activation of the substrate by the enzyme occasioned by transition-state binding interactions.

Hydrolysis of α -CF did not occur by regular Michaelian kinetics. Plots of rate versus S_0 and $1/\text{rate}$ versus $1/S_0$ for α -CF (where S_0 is initial substrate concentration) are upwardly concave, suggesting that hydrolysis is substrate activated (Figure 1). Additional plots of rate versus S_0^2 and $1/\text{rate}$ versus $1/S_0^2$

Table 1 Hydrolysis of various substrates by CenA

Substrate	Aglycone pK_a	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
β -CF	3.2	0.92	0.60	1.5
2,4-DNPC	4.0	0.37	0.17	2.2
2,5-DNPC	5.1	0.012	0.48	0.025
3,4-DNPC	5.4	0.00072	0.13	0.0057
p-NPC*	7.2	0.00078	0.41	0.0018
Cellotriose	≈ 16	0.27	0.58	0.47
Cellotetraose	≈ 16	220	0.11	1800

* p-nitrophenyl β -cellobioside.

Figure 2 Rate of hydrolysis of α -CF by CenA as a function of S_o^2 (a) and the double-reciprocal plot ($1/\text{rate}$ versus $1/S_o^2$) (b)

The inset graph (a) shows data at low substrate concentration.

(Figure 2) confirm that CenA hydrolyses α -CF by the standard Hehre mechanism (Scheme 2). This is in contrast with results for CbhII of family 6, which apparently hydrolyses α -CF with typical Michaelis–Menten kinetics [21]. This may reflect fundamental differences between oxoglucanases and endoglucanases within this family. Sinnott and co-workers [21] have proposed a fundamentally different mechanism for hydrolysis by CbhII which lacks the requirement for a general base catalyst. Crystal structure data for CbhII may support this since no candidate general base catalyst is apparent [12,21]. However, in the case of CenA, there is certainly no need to postulate a mechanism different from that postulated by Koshland [29]. Further, crystal-structure data for the closely related endoglucanase E2 suggest two aspartate residues in a position to act as general acid and base catalysts, and mutations of the corresponding amino acids in CenA coupled with kinetic analysis have confirmed this [17].

To confirm further the presence of both an acid and a base catalyst in CenA, the pH-dependences of both $k_{\text{cat.}}/K_m$ were determined for both wild-type CenA and the acid catalyst mutant, Asp252Ala [17], using 2,4-DNPC as substrate. Unfortunately, comparable analysis cannot be performed with the general base mutant, Asp392Ala, because it is completely inactive [17]. Plots of $k_{\text{cat.}}$ versus pH for CenA and Asp252Ala are shown in Figure 3 and plots of $k_{\text{cat.}}/K_m$ versus pH in Figure 4. Within the confines of the normal assumptions [30,31], the former ($k_{\text{cat.}}$ versus pH)

Figure 3 Plot of $k_{\text{cat.}}$ versus pH for hydrolysis of 2,4-DNPC by CenA (○) and Asp252Ala (□)

The pH profile curves were fitted to the data points directly using GraFit 3.0. Resulting pK_a estimates are indicated.

provides information on ionizations in the ES complex and the latter ($k_{\text{cat.}}/K_m$ versus pH) provides information on ionizations in the free enzyme. Enzyme activity remained constant over time at each pH value, indicating that all enzymes were stable under these conditions. Increasing ionic strength by the addition of 150 mM NaCl slightly decreased the activity of both proteins at pH 7.0. pK_a values were determined for each apparent ionization and are shown on the Figures.

The pH profiles for hydrolysis of 2,4-DNPC by wild-type CenA are classic bell-shaped curves, indicating that CenA requires a protonated group and a deprotonated group for full activity. The group which must be protonated is probably the general acid catalyst (pK_a 5.9, free enzyme; pK_a 6.7, substrate-bound enzyme), whereas the deprotonated group is likely to be the general base (pK_a 5.7, free; pK_a 6.3, substrate bound). Even with substrates such as 2,4-DNPC, hydrolysis of which does not require general acid catalysis, deprotonation of the acid catalyst reduces activity. This is probably due to electrostatic destabilization of the departing 2,4-dinitrophenolate. The pK_a values of these two groups are very similar for both free enzyme and substrate-bound enzyme, presumably reflecting the relative symmetry of the transition state. The pK_a values of the acid and base catalyst are higher than those of free glutamate or aspartate residues, probably because of the presence of nearby negatively charged or hydrophobic groups in the enzyme, both of which would destabilize the deprotonated forms of the acids and thus raise their pK_a values, making the enzyme optimally active at the required pH value. The binding of substrate appears to raise the apparent pK_a values of these groups even further, most likely by

Figure 4 Plot of k_{cat}/K_m versus pH for hydrolysis of 2,4-DNPC by CenA (○) and Asp252Ala (□)

The pH profile curves were fitted to the data points directly using GraFit 3.0. Resulting pK_a estimates are indicated.

exclusion of water from the active site, thus making it a more hydrophobic environment.

In contrast with the bell-shaped curves of the wild-type enzyme, the log k_{cat} versus pH profile for Asp252Ala (Figure 3) more closely approximates that of a single ionization of pK_a equal to 6.4. This would suggest that the basic ionization, that for the group which must be in its protonated form, has been removed. In the case of substrates requiring acid catalysis, this would be due to removal of such assistance. In the case of DNPC, this is due to removal of repulsive electrostatic interactions with the departing dinitrophenolate. Gratifyingly, this is exactly what would be expected for the acid catalyst mutant, thereby nicely confirming the assignment.

In conclusion, this study has shown that CenA is a very typical inverting enzyme which requires both acid and base catalysis for hydrolysis of substrates. This is in contrast with CbhII, an

exoglucanase from the same family which may function by a different mechanism.

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