Tryptophan 272: an essential determinant of crystalline cellulose degradation by *Trichoderma reesei* cellobiohydrolase Cel6A

Anu Koivula[a], Tiina Kinnari[a], Vesa Harjunpää[b], Laura Ruohonen[a], Anita Teleman[b], Torbjörn Drakenberg[b], Juha Rouvinen[1,c], T. Alwyn Jones[c], Tuula T. Teeri[a,*]

[a] *VTT Biotechnology and Food Research, P.O. Box 1500, FIN-02044 VTT, Espoo, Finland*
[b] *VTT Chemical Technology, P.O. Box 1401, FIN-02044 VTT, Espoo, Finland*
[c] *Department of Molecular Biology, BMC, P.O. Box 590, S-75124 Uppsala, Sweden*

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Abstract ***Trichoderma reesei*** **cellobiohydrolase Cel6A (formerly CBHII) has a tunnel shaped active site with four internal subsites for the glucose units. We have predicted an additional ring stacking interaction for a sixth glucose moiety with a tryptophan residue (W272) found on the domain surface. Mutagenesis of this residue selectively impairs the enzyme function on crystalline cellulose but not on soluble or amorphous substrates. Our data shows that W272 forms an additional subsite at the entrance of the active site tunnel and suggests it has a specialised role in crystalline cellulose degradation, possibly in guiding a glucan chain into the tunnel.**

Key words: Cellulase; Crystalline cellulose; Mutagenesis; Oligosaccharide; Sugar binding site

1. Introduction

Cellulose is composed of unbranched polymers of D-glucose residues linked by β-1,4-glycosidic bonds. The adjacent glucan chains pack together by hydrogen bonding, resulting in a crystalline structure. Cellulose is biodegradable by a variety of microbes, always relying on the activities of many different cellulolytic enzymes (reviewed in [1]). *Trichoderma reesei* produces two cellobiohydrolases (Cel7A, formerly CBHI, and Cel6A, formerly CBHII) (EC 3.2.1.91; new nomenclature presented in [2]). Both degrade crystalline cellulose very efficiently, releasing cellobiose from the chain ends [3–5]. Besides the two cellobiohydrolases, *T. reesei* produces several endoglucanases (EC 3.2.1.4) which cleave cellulose chains at random (reviewed in [6]).

T. reesei Cel7A and Cel6A, as well as many other cellulases have separate catalytic and cellulose-binding domains (CBDs) [1,7,8]. Removal of the CBD impairs the activity of Cel7A and Cel6A on crystalline cellulose whereas their catalytic domains remain fully active on soluble substrates [8]. High resolution crystal revealed that stable loops on the surface of both enzymes form extended active site tunnels for substrate binding and catalysis [9–11]. According to the current hypothesis, a single glucan chain is introduced at the tunnel entrance and threads through the entire tunnel with eventual release of product, cellobiose, from the end of the tunnel [4,5,9–11]. Both Cel7A and Cel6A appear to be processive enzymes, catalysing several bond cleavages without dissociation from the polysaccharide chain [9–15]. Endoglucanases homologous to Cel7A and Cel6A lack the long loops over the active site, and therefore have more open active sites allowing access to internal glycosidic bonds along the cellulose chains [9,10,16,17]. To understand how a cellobiohydrolase with a tunnel shaped active site can act on a solid substrate, one has to envision a mechanism for solubilising single glucan chains from the cellulose surface for introduction into the active site tunnel for catalysis to occur.

The active site tunnel of Cel6A contains four structurally defined glucosyl binding sites, designated −2 to +2 (earlier nomenclature A–D) (Fig. 1) [9,18]. The non-reducing end of a cellulose chain binds to subsite −2, which has to be occupied for the hydrolysis to occur ([19,20] and our unpublished results). The general acid catalysis takes place between the subsites −1 and +1 with an inversion of the configuration at the anomeric carbon [9,21,22]. Stacking of sugar rings with aromatic amino acids is frequently observed in binding sites of proteins that interact with sugars [23]. In the active site of Cel6A, binding of the glucose rings is mediated by hydrogen bonds and, in the subsites −2, +1 and +2 by ring stacking interactions with the tryptophan side chains W135, W367 and W269, respectively (Fig. 1A) [9]. Crystal-crystal contacts blocking the tunnel entrance of Cel6A have so far prevented its co-crystallisation with oligosaccharides longer that 4 glucose units. However, enzymatic characterisation of Cel6A suggests protein-sugar interactions extending further than the 4 subsites so far identified [15]. We were, therefore, intrigued by W272, an exposed tryptophan on a surface loop of Cel6A, close to the 'entrance' of the enclosed catalytic tunnel (Fig. 1B). Molecular modelling suggested that this residue could stack with a sixth glucose ring in a cellulose chain, provided the cellulose chain were to twist between the subsite +2 and the proposed new subsite +4 (Fig. 1A,B). Here, we have investigated the role of this putative subsite +4 in the substrate binding and catalysis by Cel6A.

2. Materials and methods

2.1. Production and purification of the Cel6A wild-type and mutant proteins

Mutations W272A (TGG→GCG) and W272D (TGG→GAC)

*Corresponding author. Present address: Department of Biochemistry and Biotechnology, Kungliga Tekniska Högskolan, S-10044 Stockholm, Sweden. Fax: (46) (8) 790 6689.
E-mail: Tuula@biochem.kth.se

[1]Present address: Department of Chemistry, University of Joensuu, P.O.Box 111, FIN-80101 Joensuu, Finland.

Abbreviations: Ac, acetate; BMCC, bacterial microcrystalline cellulose; CBD, cellulose-binding domain; CBH, cellobiohydrolase; DP, degree of polymerisation; Glc_1-Glc_6, glucose-cellohexaose; wt, wild-type

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were introduced into the *cel6A* cDNA by a polymerase chain reaction (PCR) [24]. The nucleotide sequence of the whole fragment subjected to PCR was confirmed. The mutated *cel6A* cDNA was cloned under the *cel7A* (formerly *cbh1*) promoter of the fungal expression vector as described [18].

The Cel6A mutant constructions were transformed into *Trichoderma* strain ALKO2877, devoid of the *cel6A* and *egl7A* genes, and the mutant proteins W272A and W272D were produced in the *Trichoderma* essentially as described earlier [18]. Desalted culture supernatant was purified with ion-exchange, affinity chromatography [8] and finally with immunoaffinity chromatography [25]. Cel6A wild type (wt) was produced and purified as described earlier [18]. The catalytic domains of Cel6A wild type, W272D and W272A enzymes were produced by papain (Sigma) digestion and purified with a MonoQ column (Pharmacia, Sweden) as described earlier [18]. The purity of all protein preparations was checked and verified by SDS-PAGE and Western blot [25]. In addition, the contaminating cellulase activities were measured on various substrates essentially as described [25].

Cel6A wild-type concentration in a purified preparation was determined from UV absorbance at 280 nm using a molar extinction coefficient, $\varepsilon = 80\,500\ M^{-1}\ cm^{-1}$ for the intact enzyme and $\varepsilon = 75\,000\ M^{-1}\ cm^{-1}$ for the catalytic domain [26]. For the W272D and W272A mutants, a molar absorptivity value for tryptophan (5550 $M^{-1}\ cm^{-1}$) [27] was subtracted resulting in $\varepsilon = 74\,950\ M^{-1}\ cm^{-1}$ for the intact proteins and $\varepsilon = 69\,450\ M^{-1}\ cm^{-1}$ for the respective catalytic domains.

2.2. Preparation and hydrolysis of amorphous and crystalline cellulose

Bacterial microcrystalline cellulose (BMCC) was prepared from Nata de Coco (Reyssons Food Processing, The Philippines) as described by Gilkes et al. [28] resulting in a preparation with DP of 240. The solvent regenerated, amorphous cellulose was prepared from Avicel (Fluka AG, Switzerland) as described by Isogai and Atalla [29].

The enzymatic activities on BMCC and amorphous cellulose were determined by shaking the intact enzymes (final concentration of 1.4 μM) and substrate (0.7 mg/ml) at 27°C in 40 mM sodium acetate, pH 5.0. Samples were taken at designated time points and the reaction was stopped by adding half the reaction volume of a stop-reagent containing 9 vol. of 94% ethanol and 1 vol. of 1 M glycine, pH 11. The soluble sugars were analysed by HPLC after filtering the samples through Millex GV 0.22-mm units (Millipore) and using soluble oligosaccharides as standards as described ([18,19], see also below).

2.3. Binding studies on crystalline cellulose

The adsorption studies were carried out in 30 mM NaAc (pH 5.0) as described [30]. The concentration of the BMCC suspension was 2 mg/ml in the experiments with the catalytic domains and 1 mg/ml with the intact enzymes. The free protein concentration in solution in the equilibrium was measured after 90-min incubations at +4°C by filtering the samples through 0.22-mm Millex GV membranes (Millipore) and measuring the protein concentration with spectrofluorimetry (Shimadzu RF-5000, Japan). A separate standard curve was prepared for each protein. The amount of the bound protein was calculated from the initial protein concentration.

2.4. Kinetics on small soluble cello-oligosaccharides

Hydrolysis experiments on cello-oligosaccharides (cellotriose-cellohexaose) were performed in 10 mM NaAc buffer, pH 5.0 at 27°C. Samples were taken at intervals, stopped and analysed by HPLC (Waters Millipore, Milford, MA, USA) equipped with a refractive index detector as described [15,18]. The column used for the separation of the oligosaccharides was Aminex HPX-42A (Bio-Rad) and deashing cartridges (Micro-Guard, Bio-Rad) were used as pre-columns. Kinetic constants for W272 mutant were calculated by a non-linear regression data analysis program or by fitting the whole progress curve [15,18,19].

3. Results and discussion

In order to explore potential protein-sugar interactions in the hypothetical subsites +3 and +4 at the tunnel entrance, a cellohexaose molecule was model-built into the binding site. The modelling was based on previously determined complex structures of Cel6A with different ligands and inhibitors binding to the subsites −2 to +2 ([9]; Zou, J.-Y., Kleywegt, G., Driguez, H., Koivula, A., Teeri, T.T. and Jones, T.A., in preparation). According to the complex structures, the cellulose chain is twisted about 20° between subsites +1 and +2. The modelling suggested another twist of 70° over the subsites +2 and +4, in which case a few protein-sugar interactions could be predicted in the subsites +3 and +4. These included hydrogen bonding of the side-chain of N275 to the glucose rings at the +3 and +4 subsites and the side-chain of T228 to the sugar ring at subsite +3 as well as water-mediated hydrogen bonds from the side chains of Q363 and N229 to the glucose unit at subsite +3. Besides the hydrogen-bonding interactions, the solvent exposed side-chain of W272 could stack against the a-face of the glucose ring at subsite +4. In order to investigate the functional role of the proposed subsite +4, W272 was mutated either to an alanine (W272A) or to an aspartic acid (W272D). The mutated enzymes were produced in *Trichoderma* and purified.

3.1. Hydrolysis of small soluble oligosaccharides

The catalytic constants of the mutated enzymes were first determined on soluble cello-oligosaccharides containing 3–6 glucose units (Table 1). The data shows that mutations at W272 enhance the rate of hydrolysis of some but not all oligosaccharides while the corresponding specificity constants (k_{cat}/K_m) remain unaltered. Our earlier data suggested that at least Glc_3 and Glc_5 experience non-productive binding modes in the active site of Cel6A [15,19]. The apparent increase in catalytic rates hereby obtained for the two mutants imply that

Table 1
Kinetic parameters for the hydrolysis by the intact Cel6A wild type and the W272 mutants on cello-oligosaccharides in 10 mM NaAc buffer, pH 5.0 at 27°C

Substrate	Cel6A wild type[a]			W272A[b]			W272D[b]		
	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
Glc_3	3.7 ± 0.6	17 ± 5	0.22 ± 0.10	24 ± 6	67 ± 5	0.35 ± 0.11	24 ± 6	67 ± 5	0.35 ± 0.11
Glc_4	246 ± 30	2.6 ± 0.5	95 ± 30	300 ± 60	ND[c]	ND	240 ± 60	ND	ND
Glc_5	66 ± 12	1.3 ± 0.4	51 ± 25	480 ± 120	(∼20)[d]	(∼24)[d]	480 ± 120	16 ± 5	30 ± 17
Glc_6	840 ± 120	14 ± 6	60 ± 34	≥900	ND	ND	≥900	ND	ND

[a]Kinetic constants for Cel6A wild type have been published earlier [15].
[b]Kinetic constants for W272 mutant were calculated by a non-linear regression data analysis program or by fitting the whole progress-curve [15,18,19].
[c]ND = not determined.
[d]K_m value is an estimate based on measuring the initial rates at four different substrate concentrations.

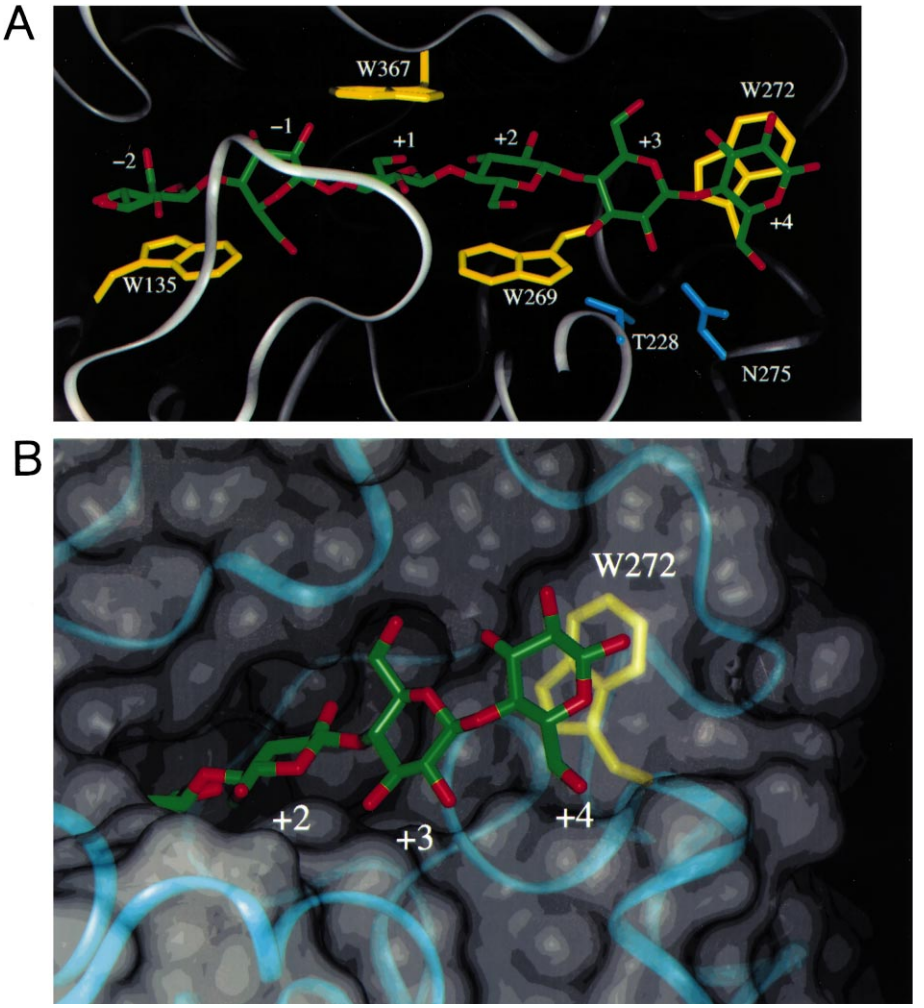

Fig. 1. A: Binding of a cellohexaose molecule into the active site tunnel of *T. reesei* Cel6A as revealed by complex structures with different ligands and inhibitors in the subsites −2 to +2 ([9]; Zou, J.-Y., Kleywegt, G., Driguez, H., Koivula, A., Teeri, T.T. and Jones, T.A., in preparation) and subsequent model building to extend the chain to the putative subsite +4. The subsite number for each sugar ring and the identity of relevant protein side chains are indicated by the white labels. The tryptophan side chains shown (W135, W367 and W269) or predicted (W272) to interact with the sugar rings are drawn in yellow, and the residues T228 and N275 proposed to form hydrogen bonds at sites +3 and +4 are drawn in turquoise. B: A solvent-accessible-surface-view of the entrance of the active site tunnel of Cel6A. A bound cellohexaose molecule is included to illustrate its partial enclosure within the tunnel and the side chain of W272 is shown to demonstrate its location on a loop readily exposed to the solvent.

the side chain of W272 may be responsible for at least some of the non-productive binding modes and consequently it contributes to the formation of +4 subsite. The k_{cat}/K_m value for Cel6A wild type increases until Glc_4 but no longer on Glc_5 or Glc_6 (Table 1). Sugar binding in the subsites +4 and +3 is thus not required for transition state stabilisation, and removal of the side-chain of W272 has no impact on the catalytic efficiency of Cel6A per se.

3.2. Degradation of amorphous and crystalline cellulose

Due to the insolubility and complexity of polymeric crystalline and amorphous cellulose, the kinetics of cellulases on these substrates does not correspond to typical Michaelis-Menten behaviour. However, both *T. reesei* cellobiohydrolases produce primarily soluble sugars from the cellulose chain ends and only slowly reduce the degree of polymerisation (DP) which is indicative of insoluble product formation [4,5,31,32]. The hydrolysis of polymeric substrates can thus be followed by measuring the soluble products formed and a profile of the products can be obtained by HPLC. Here we used solvent regenerated (amorphous) cellulose and bacterial microcrystalline cellulose (BMCC). The major difference between the two substrates is the level of order: amorphous cellulose is mostly disordered cellulose while BMCC presents essentially a crystalline surface for the enzyme to act on. Cellobiose was the major soluble product formed in 48 h from both substrates by the wild-type Cel6A and the two mutants (Figs. 2A and 3A). Owing to the conformation of a cellulose chain and the limitations imposed by the tunnel shaped active site of Cel6A, small amounts of cellotriose and – upon its hydrolysis – glucose, were also observed after the hydrolysis of amorphous cellulose (Fig. 2B,C) [9,15]. In the beginning of the hydrolysis by wild-type Cel6A, the formation of cellotriose was faster than its hydrolysis, and the maximum cellotriose concentration is reached after 30 min (Fig. 2C). For the mutants the maximum cellotriose concentration was lower (Fig. 2C), which is in agreement with its faster rate of hydrolysis by the mutants (Table 1). On BMCC, the amounts of glucose

detected were even lower (Fig. 3B), and the amounts of cellotriose approached the detection limit (1–3 mM) (for wild-type Cel6A) or could not be detected (for W272 mutants) (data not shown). No other intermediate products (Glc_4–Glc_6) were observed on either BMCC or amorphous cellulose.

The apparent degradation rates were calculated from the initial Glc_2 formation for the wild-type Cel6A (based on data presented in Figs. 2A and 3A). The values obtained on amorphous cellulose (10/min), and especially on crystalline cellulose (2–3/min), were at least an order of magnitude lower than the corresponding rates on a fully soluble substrate, Glc_4 (246/min). This and parallel work on bacterial cellulases [33] suggest that the bottle neck in the degradation of amorphous and particularly of crystalline cellulose is not the catalytic efficiency per se but the ability of the enzyme to access its substrate, i.e. a single glucan chain on a well organised crystal.

Mutagenesis of W272 did not lead to any major changes in the initial degradation rates on amorphous cellulose (10/min for Glc_2 formation for both mutants) and only a minor drop

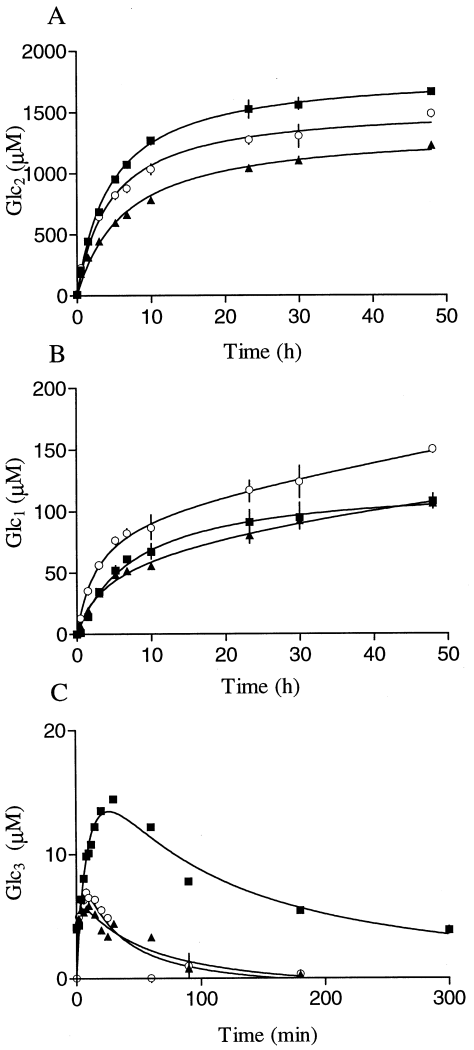

Fig. 2. The formation of cellobiose, Glc_2 (A), glucose, Glc_1 (B) and cellotriose, Glc_3 (C) in the hydrolysis of amorphous cellulose by intact Cel6A wild type (■), W272A (▲) and W272D (○) mutants at 27°C. The soluble products were measured at 14–19 different time points. The error bars indicate the reproducibility of the data in two independent series of experiments.

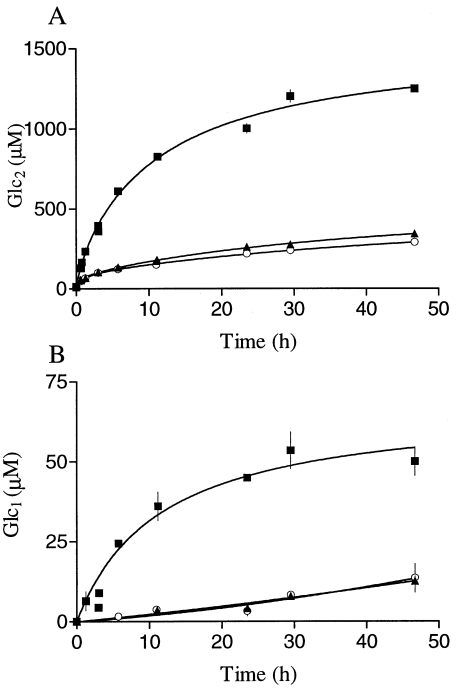

Fig. 3. The formation of Glc_2 (A) and Glc_1 (B) in the hydrolysis of bacterial microcrystalline cellulose (BMCC) by intact Cel6A wild type (■), W272A (▲) and W272D (○) mutants at 27°C. The experiments were performed in the same way as the hydrolysis of amorphous cellulose (see text for details). The soluble products were measured at 10–12 different time points. The error bars are included to indicate the reproducibility of the data of three independent measurements.

was seen in the overall catalytic activity after 48 h (Fig. 2A). In contrast, both mutations led to a dramatic decrease in activity during hydrolysis of BMCC. As shown in Fig. 3, the amount of cellobiose produced by both mutants was only about 20–25% of that produced by the wild-type enzyme throughout the whole hydrolysis experiment. Extrapolation of the data in Fig. 3A reveals that it takes the wild-type Cel6A 30–40 min to produce a final concentration of 170 mM cellobiose, whereas both the W272 mutants need 11–12 h to reach the same product concentration.

Although it is now clearly established that W272 interacts with oligosaccharides, this interaction is not a major contribution for the binding capacity of Cel6A on crystalline cellulose. This is evident from Fig. 4, showing that mutations introduced at the W272 had no influence on the binding isotherm of the intact enzyme nor its isolated catalytic domain. Loss of activity of the two mutants on crystalline cellulose is thus not due to reduced binding, nor were any significant differences observed in the stabilities of the three forms of the enzyme (data not shown).

3.3. *Role of tryptophan W272 in crystalline cellulose degradation*

The catalytic domains of cellulases and other glycosyl hydrolases have been classified into families, each with conserved protein fold and stereochemistry of hydrolysis [1,34]. The catalytic domain of Cel6A belongs to the glycosyl hydrolase family 6, which currently contains 17 proteins of bacterial and fungal origin [1,33]. Sequence and structural comparisons reveal that the residue corresponding to W272 in Cel6A is

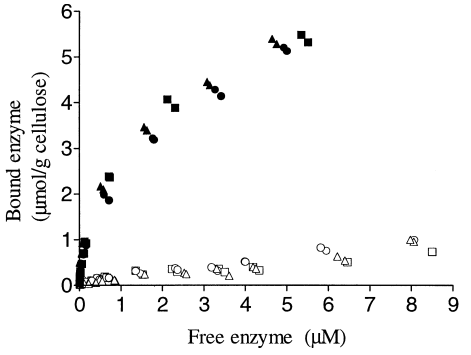

Fig. 4. The equilibrium binding isotherms of Cel6A wild type and W272A and W272D mutants on BMCC. The values shown are the average of experiments carried out in duplicate. Symbols: intact Cel6A wild type (■), W272A (▲) and W272D (●); catalytic domain of Cel6A wild type (□), W272A (△) and W272D (○).

conserved in all of the cellobiohydrolases but that there is a deletion – including the tryptophan – in all of the endoglucanases so far sequenced in that cellulase family. Thus, this residue is apparently of no importance for endoglucanases binding to and hydrolysing bonds in the middle of the long polymeric chains of cellulose. In the case of the cellobiohydrolase, Cel6A, assumed to act from the end of a cellulose chain, mutagenesis of W272 caused a dramatic decrease in activity on crystalline cellulose without any apparent effect on binding or stability. It is generally recognised that the catalysis per se is not rate limiting in the hydrolysis of crystalline cellulose (see above and [33]), but the rate limiting step has not been identified. Apart from the discovery of cellulose-binding domains, the identification of W272 here is the first example of a site exclusively required for the degradation of crystalline cellulose. Since this residue is clearly not essential for activity on non-crystalline substrates, we propose that W272 contributes to the rate-limiting step of Cel6A on crystalline cellulose. The location of the tryptophan on a solvent accessible loop very near the open entrance of the active site tunnel (see Fig. 1B), allures us to speculate that it is the residue needed for an initial contact with a single glucan chain end at the cellulose surface. The potential hydrogen bond forming residues (N275, T228, Q363 and N229) at subsites +3 and +4 might be important for the subsequent movement of the chain end further into the active site of Cel6A. Furthermore, the overall twist proposed to take place in the glucan chain bound to subsites +1 to +4 is partially dictated by W272 at site +4 and by W269 at site +2 (see Fig. 1). Very similar, although larger twists have recently been observed upon substrate binding to the other *T. reesei* cellobiohydrolase, Cel7A [11], implying that the stabilisation of such substrate conformations by the enzyme may be a major contribution to the crystal breaking capability of cellobiohydrolases in general.

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