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Investigation of the Function of Mutated Cellulose-Binding Domains of *Trichoderma reesei* Cellobiohydrolase I

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ABSTRACT **The function of the cellulose-binding domain (CBD) of the cellobiohydrolase I of *Trichoderma reesei* was studied by site-directed mutagenesis of two amino acid residues identified by analyzing the 3D structure of this domain. The mutant enzymes were produced in yeast and tested for binding and activity on crystalline cellulose. Mutagenesis of the tyrosine residue (Y492) located at the tip of the wedge-shaped domain to alanine or aspartate reduced the binding and activity on crystalline cellulose to the level of the core protein lacking the CBD. However, there was no effect on the activity toward small oligosaccharide (4-methylumbelliferyl β-D-lactoside). The mutation tyrosine to histidine (Y492H) lowered but did not destroy the cellulose binding, suggesting that the interaction of the pyranose ring of the substrate with an aromatic side chain is important. However, the catalytic activity of this mutant on crystalline cellulose was identical to the other two mutants. The mutation P477R on the edge of the other face of the domain reduces both binding and activity of CBHI. These results support the hypothesis that both surfaces of the CBD are involved in the interaction of the binding domain with crystalline cellulose.**

Key words: cellobiohydrolase, cellulose degradation, substrate binding, cellulose-binding domain

INTRODUCTION

Cellulose is an insoluble polymer composed of long, linear chains of β-1,4-linked glucose units. Hydrogen bonding and van der Waals forces create an effective interchain binding network associating the single chains to elementary fibrils which then further aggregate to larger bundles forming ordered crystals. The extreme crystallinity of cellulose is the major factor limiting its enzymatic hydrolysis and also makes biotechnical exploitation of this abundant biopolymer difficult. Nature has solved this problem by evolving a number of multienzyme systems in which each of the components has a different role in the total hydrolysis process.[1–3] A detailed understanding of the mode of action of the individual enzyme components is, however, still lacking.

The best characterized cellulolytic enzyme system today is without doubt that of the filamentous fungus *Trichoderma reesei*. It hydrolyzes crystalline cellulose by using two secreted exoglucanases (cellobiohydrolases, CBH) and at least two endoglucanases (EG).[4–9] Biochemical studies have shown that exoglucanases are required for the complete breakdown of crystalline cellulose and that tight binding of the enzymes is essential for its efficient hydrolysis. In addition, the synergism of exoglucanases and endoglucanases is most pronounced when tightly binding endoglucanases are combined with exoglucanases, which further emphasizes the importance of tight adsorption in the cellulase function.[1,2,10,11]

Comparison of the primary structures and various biophysical and biochemical studies of *T. reesei* cellulases revealed a common structural organization consisting of a large catalytic domain joined by a flexible, O-glycosylated linker peptide to a smaller cellulose-binding domain (CBD).[12–22] This two domain structure—as also found in many fungal and bacterial cellulases—has been shown to be essential for their full activity on insoluble, crystalline sub-

Abbreviations: MUL, 4-methylumbelliferyl β-D-lactoside; DNS, dinitrosalisylic acid; CBH, cellobiohydrolase; EG, endoglucanase; CBD, cellulose-binding domain; AFOS, alkaline phosphatase; Enzymes: endoglucanase, 1,4-β-D-glucanohydrolase (EC 3.2.1.4); exoglucanase, 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91).

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strates.[14] It has further been demonstrated that the CBD binds to cellulose both by itself and as a part of the intact enzyme.[21,22] The core enzyme, without this domain, has much reduced activity on crystalline cellulose.

There are two hypotheses to explain this observation.[2,20,23] One is that the CBD simply binds to cellulose and therefore raises the effective substrate concentration with respect of the catalytic domain. The more interesting hypothesis is that the CBD plays more active role in breaking the crystals and liberating cellulose chains from the crystal.

The 3D structure of the synthetic C-terminal binding domain of CBHI has been determined earlier.[24] The domain folds into a wedge-like structure with one face remarkedly flat and hydrophilic. Interestingly, the other face of the wedge is clearly more hydrophobic. Since the crystalline cellulose has flat, layered structure with hydrogen bonding in the plane of the layer and van der Waals forces holding the layers together, both surfaces of the tail domain could interact with the substrate.[25] In this paper we report the effects of specific amino acid changes in the CBD of CBHI on the binding and activity on crystalline cellulose.

MATERIALS AND METHODS

Strains and Vectors

The *cbh1* cDNA was derived from the plasmid pTTc01.[26] Bluescribe M13+ vector (Vector Cloning Systems, San Diego, CA) was used for site-directed mutagenesis in *E. coli* TG2 (recA$^-$ derivative of JM101, a kind gift of Dr. P. Thomas). After mutagenesis the cellulase genes were expressed in *Saccharomyces cerevisiae* yeast strain AH 22[27] (*leu2–3 leu2–112 his4–519 can1 gal2 cir*$^+$) using expression vector pMA91[28] as described by Penttilä et al.[29]

DNA Techniques

Site-directed mutagenesis was performed using an in vitro mutagenesis kit (Amersham, UK). The mutated fragments were sequenced by dideoxy sequencing according to Zagursky et al.[30] Standard methods were used in other DNA manipulations.[31] Yeast transformation was carried out using published protocols.[32]

Expression and Purification of the CBHI Mutants

Yeast cells were grown for 3 days in shake flasks using rich (nonselective) YEP-D-medium.[33] The cells were centrifuged down (5,000*g* for 10 min) and the supernatant was treated with 10 g/liter Bentonite (Berkbond no. 2, Steetle, Minerals Division, Milton Keynes, UK) at pH 4.0 at room temperature for 1 hr. The Bentonite was removed by centrifugation (5,000*g* for 10 min) and the solution was concentrated with Pellicon laboratory cell system using PT 10 membranes (Millipore, USA). After desalting with Biogel P-6 (Bio-Rad, USA), the protein was bound to DEAE-Sepharose fast flow (Pharmacia, Sweden) equilibrated in 5 mM His-HCl buffer pH 6.2. CBHI protein was eluted with linear salt gradient (0–0.5 M NaCl). The sample was further concentrated (PM10 membranes, Amicon, USA) and the buffer was changed in Biogel P-10 to 50 mM NaAc pH 5.2 containing 0.1 M glucose. Finally the sample was purified with thiocellobioside-based affinity chromatography.[34] The homogeneity of the product was checked on SDS-polyacrylamide gels.[35]

Measurement of the Enzyme Activity

4-Methylumbelliferyl β-D-glycoside of lactose (0.15 mM) was used to determine the activity of CBHI on soluble substrate as described previously.[36,37] Activity on crystalline insoluble cellulose was measured by determination of liberated reducing sugars (as D-glucose equivalents) by the dinitrosalisylic acid method.[38] *Acetobacter xylinum* cellulose suspension (1 mg/ml) was incubated in end-over-end mixer with the enzyme for 3 hr at 50°C (50 mM NaAc pH 5.0) and the reaction was stopped by filtration through 0.45-μm Durapore^R membranes (Millipore, USA).

Protein concentration was calculated according to the molar absorption coefficient for CBHI and CBHI core protein (73 000 M^{-1} at 280 nm).[14] The absorbance was measured from UV spectrum corrected for light scattering. Alternatively the protein concentration was measured at 205 nm.[39] The protein content during the purification was routinely monitored with Bio-Rad protein assay kit (Bio-Rad, USA).

Adsorption Studies

The binding studies were done by using 0.5 mg/ml crystalline cellulose suspension and initial protein concentrations from 0.1 to 4.5 μM. The free concentration in solution in equilibrium after 1 hr incubation was determined after filtration with Durapore membrane (Millipore, USA) by spectrofluorimetry (Emission 280 nm/bandwidth 3 nm, excitation 350 nm/bandwidth 20 nm) using a standard curve measured with purified protein. The amount of the bound protein was calculated from the initial concentration. Experiments were done in duplicates and at least two independent sets of experiments were performed.

Endoglycosidase-H Treatment

Digestions with endoglycosidase-H were carried out under conditions recommended by the manufacturer (Boehringer).[40]

Detection of the CBHI Proteins With Monoclonal Antibodies

Dilution series of the protein (1–100 ng in 50 mM NaAc pH 5.0) was pipetted onto nitrocellulose filter

Fig. 1. The overall structure of CBHI cellulose-binding domain. The hydrophilic surface is partly created by three conserved tyrosines (indicated by arrows). The mutated residues are labeled.

with BIODOT SF blotting apparatus (Bio-Rad, USA). The protein was detected with monoclonal antibodies cI-89,[41] H-4,[42] and C271[41] followed by commercial AFOS-labeled anti-mouse IgG (Sigma) followed by staining with Protoblot^R system (Sigma, USA).

RESULTS

Design of the Mutants

The amino acid sequences of the four cellulose-binding domains of *T. reesei* cellulases share 70% amino acid identity. Comparison of these sequences with the three-dimensional structure of the CBHI CBD[24] reveals three conserved tyrosines (Y474, Y492, Y493, corresponding to residues Y13, Y31, and Y32 according to Kraulis et al.[24]) lined up to form part of the flat hydrophilic surface (Fig. 1). Y492 and Y493 are strictly conserved in all *T. reesei* cellulases but Y474 is substituted by tryptophan in CBHII and EGII. It has been shown earlier by chemical modification that tyrosine residues are important for the binding of CBHI to cellulose.[8] Interestingly, the spacing between the tyrosines (and the tryptophans) is equal to that between every second glucose unit in the cellulose crystal. Y492 is located in a loop that forms the structurally distinct, sharp tip of the domain. The hydroxyl of the phenolic side chain of tyrosine could form a hydrogen bond with the sugar oxygens and the sugar pyranose ring could interact with the aromatic ring with van der Waals forces when stacked face to face. The other face of the CBD is more hydrophobic in character. Its van der Waals surface is also relatively flat but has a slight indentation in the middle.

Schematic representation of the two hypotheses suggested for the function of the CBHI CBD is shown in Figure 2. The first question that can be asked is whether both surfaces of the CBD are involved in the hydrolysis of cellulose or whether the flat hydrophilic surface alone is sufficient for full activity. If, however, the CBD does play a role in displacing cellulose chains from the crystal, it is likely that both surfaces would be important and a residue at the tip of the wedge, such as Y492, could have an important function.

Therefore, the aromatic Y492 was mutagenized to three different residues: a histidine (Y492H, aromatic), an aspartate (Y492D, negative, nonaromatic), and an alanine (Y492A, noncharged, nonaromatic). In order to investigate the importance of the hydrophobic surface on cellulose binding, proline 477 (corresponding to residue P16 in ref. 24) located at one edge of the surface was chosen for mutagenesis. P477 is conserved in all *Trichoderma* cellulases except in EGI, where it is replaced by a cysteine. Careful examination of the side chain interactions of an arginine mutation on the hydrophobic surface indicated that mutation at P477 would most unlikely cause unfavorable contacts and alter the folding of the mutated CBD. The P477R mutation introduces an extended side chain with a positive charge on the hydrophobic face of the domain thereby breaking the surface.

In addition to these point mutations, a truncated CBHI enzyme, lacking the linker-CBD region, was created by deleting the cDNA coding for amino acids 433–497. The truncated mutant is similar to the core domain obtained by papain digestion after residue 430 of the native *Trichoderma* enzyme.[14]

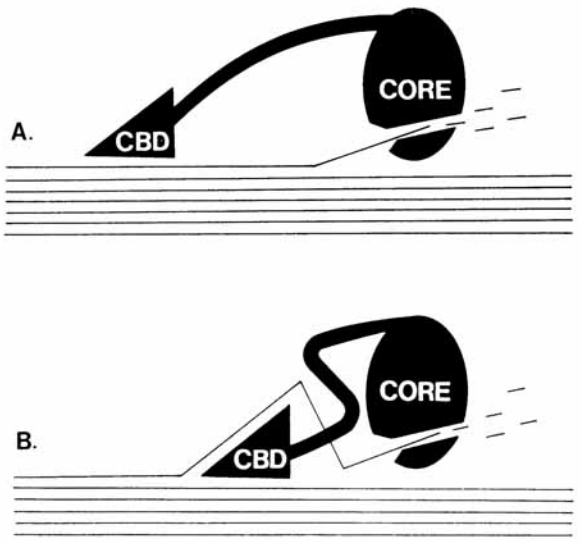

Fig. 2. Hypotheses of the function of the cellulose-binding domain. (**A**) Crystal binding site model. (**B**) Crystal disrupting model.

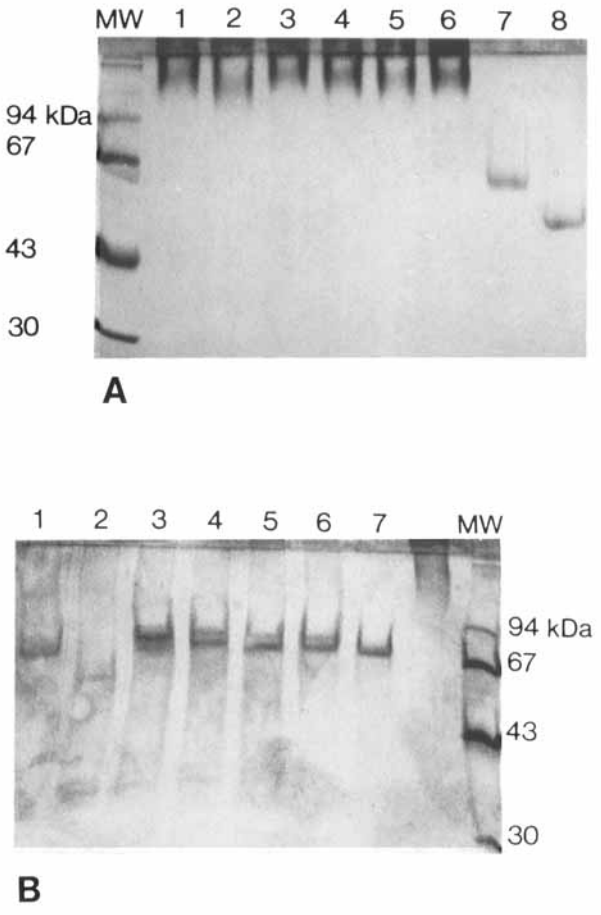

Fig. 3. SDS-electrophoresis of CBHI wild-type and mutant enzymes produced in yeast. One microgram samples were loaded on the gel. Silver staining was used to detect the protein. Lanes: 1. CBHI produced in yeast; 2. CBHI core produced in yeast; 3. Mutant P477R; 4. Mutant Y492A; 5. Mutant Y492D; 6. Mutant Y492H; 7. CBHI produced in *Trichoderma;* 8. CBHI core produced in *Trichoderma.* MW, molecular weight marker. (**A**) Nondigested samples. (**B**) Samples digested with endoglycosidase H.

Structural Properties of the Mutant Proteins

The yeast *Saccharomyces cerevisiae* has been used earlier as a host for the production of active fungal cellulases.[29,43,44] In this study the production levels of the wild type and all of the mutated enzymes secreted to the culture medium were roughly identical (≈ 5 mg/liter after 3 days cultivation).

All forms of the yeast produced CBHI appear much more extensively glycosylated than the corresponding wild-type enzyme from *Trichoderma* as evidenced by a smear in SDS gels (Fig. 3A). The overglycosylation in the yeast produced CBHI can be largely removed by treatment with endoglycosidase H (Fig. 3B) whereafter a distinct band appears in the gel. Comparison of the apparent molecular weights of the deglycosylated and undigested core proteins and native CBHI produced in yeast and *Trichoderma* reveals that most of the overglycosylation occurs at the N-glycosylation sites of the core protein. Therefore, it is likely that little or no overglycosylation occurs at O-glycosylation sites of the connecting linker region, where it could interfere with the function of the CBD.

Monoclonal antibodies specific to different epitopes of CBHI structure were used in Western blot analysis to probe the structures of the mutated proteins (Table I). Two of the antibodies, H-4[42] and CI-89,[41] are specific for two different epitopes in the tail domain and c271[41] recognizes specifically the core domain. As seen in Table I, cI-89 reacted with the P477R mutant but not with any of the Y492 mutants. This is consistent with epitope mapping data of cI-89 and the 3D structure of the domain, which show that this antibody recognizes the C-terminal loop containing the Y492. The detection limit in the blots for the P477R mutant is somewhat higher than that of the control enzymes. This might be due to minor structural change or to the possibility that the hydrophobic face is part of the recognition site of the antibody. The H-4 antibody recognized all of the Y492 mutants with detection limits identical to the wild-type enzymes, but did not recognize the mutant P477R. This suggests that the H-4 epitope must be on the hydrophobic face of the CBD. Since the structure of the CBD is stabilized by two disulfide bridges[24] and all of the mutant proteins reacted with at least one of the CBD-specific monoclonals, major structural changes in the mutant domains seem very unlikely.

Enzymatic Activities of the Mutant Proteins

Comparison of the specific activities of the wild-type and mutated CBHI on small, soluble oligosaccharide derivative (4-methylumbelliferyl lactoside)

TABLE I. The Detection Limit in ng Protein for Recognition of CBHI Mutants by Different Monoclonal Antibodies*

Enzyme	Antibody		
	cI-89	H-4	c271
Trichoderma			
Wild-type	5	20	10
Core	N†	N	10
Yeast			
Wild-type	5	50	10
Core	N	N	10
Y492A	N	50	10
Y492H	N	50	10
Y492D	N	50	10
P477R	50	N	10

*cI-89 and H-4 are specific for the cellulose-binding domain; c271 detects the core domain.
†N, no reaction.

TABLE II. Activity of CBHI Mutant Enzymes and the Amount of Bound Protein in the Activity Experiments as Deduced From the Binding Saturation Curves*

Enzyme	MUL 10^{-1} (kat/mol)	Crystalline cellulose (10^{-2} kat/mol)	Protein bound (nmol/g)
Trichoderma			
Wild-type	1.3 ± 0.1	7.0 ± 0.1	1200
Core	1.7 ± 0.1	1.4 ± 0.1	580
Yeast			
Wild-type	1.1 ± 0.2	3.0 ± 0.1	820
Core	1.3 ± 0.1	1.7 ± 0.2	320
Y492A	1.7 ± 0.1	1.8 ± 0.1	540
Y492H	1.3 ± 0.2	1.7 ± 0.1	710
Y492D	1.6 ± 0.2	1.8 ± 0.1	530
P477R	1.4 ± 0.1	1.5 ± 0.1	420

*The activity is measured on 4-methylumbelliferyl-β-D-glucoside of lactose (MUL, 0.15 mM) and on crystalline cellulose (1 mg/ml). 1 kat corresponds to 1 mol of free methylumbelliferon or 1 mol glucose produced in 1 sec, respectively.

did not reveal any significant differences (Table II). If anything, the core protein and tail mutants of CBHI exhibit somewhat increased activity relative to the corresponding wild-type enzymes.

The activities on crystalline cellulose are clearly more complicated to interpret. It is clear that the expression in yeast results in 2- to 3-fold decrease in the activity and binding of wild-type CBHI relative to the native enzyme from *Trichoderma* (Table II). The expression system does not influence the activity of the CBHI core protein. However, if the core and intact CBHI are both produced in yeast, a clear difference can still be seen in their activities and binding on crystalline cellulose. All of the CBD mutants show enzyme activities similar to that of the core protein indicating that these mutations reduce the activity of CBHI on crystalline cellulose.

The Adsorption of the Mutant Proteins Onto Crystalline Cellulose

Binding of the proteins on crystalline cellulose was characterized by measuring the dependence of the amount of bound protein on the free protein concentration. Low temperature was used in the binding experiments to exclude hydrolysis during the incubations, since it has been shown that the adsorption of CBHI on cellulose is not affected by temperature.[45] The binding isotherms (Fig. 4) indicate that CBHI protein saturates the cellulose surface at high enzyme concentration and that the total amount of CBHI bound in saturation is increased by the cellulose-binding domain. The binding curves show that all mutations introduced resulted in reduced binding compared to the wild-type enzyme (Fig. 4). The Y492H mutation affected the binding less than the rest of the mutations, which had binding curves closer to those of the core protein.

The Scatchard plot constructed from the binding data of the wild-type protein is concave (Fig. 5). The plots of the core protein and all of the mutants studied are also concave, which indicates that the interaction cannot be explained by single binding site model. In addition, careful comparison of the Scatchard plots and also the saturation curves reveals that the wild-type CBD increases the binding of CBHI on crystalline cellulose most effectively at very low protein concentration by increasing the amount of "high affinity" binding sites.

DISCUSSION

Degradation of the highly ordered crystalline cellulose represents a significant challenge to enzymes. It has been shown earlier that in *Trichoderma,* CBHI is the key enzyme in the disintegration of the cellulose microfibrils[46,47] and initial disruption of crystalline regions is needed for the hydrolysis.[2] The role of the CBD of CBHI on the adsorption and activity has been well demonstrated.[14,22,45] Moreover, the presence or absence of the CBD *T. reesei* CBHI has no effect on its binding or activity on phosphoric acid swollen amorphous cellulose indicating specificity for crystalline cellulose.[14] In this paper we show that the interaction of the CBD with crystalline cellulose can be destroyed by single amino acid changes.

Interaction of Native and Core CBHI With Crystalline Cellulose

The binding interactions observed with wild-type CBHI produced in yeast or in *Trichoderma* are heterogeneous as evidenced by concave Scatchard plots of the binding data (Fig. 5). The data cannot be fitted to single or double binding site models. Although the bacterial cellulose used in this study is 100% crystalline (as determined by X-ray diffraction), it is not structurally homogeneous.[48] Therefore it is pos-

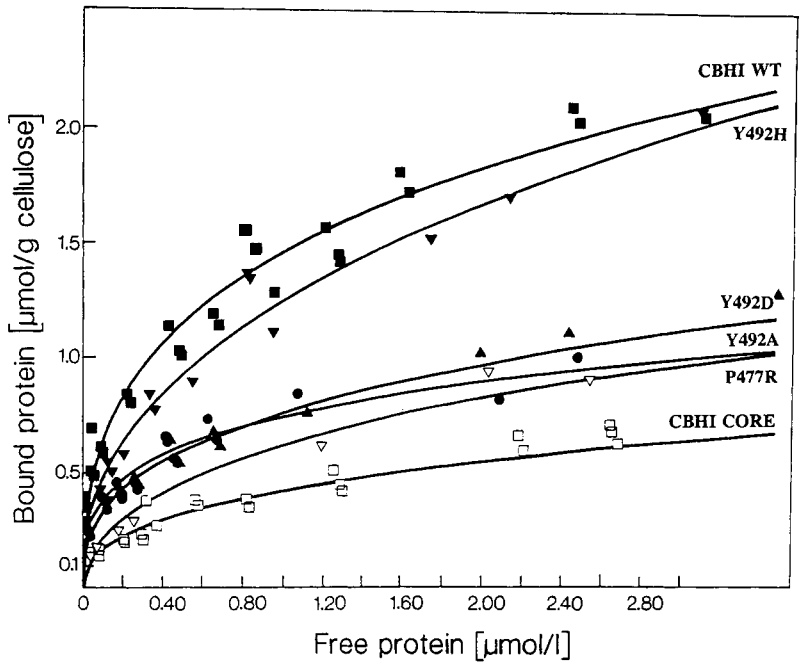

Fig. 4. Comparison of the binding isotherms of the mutants, CBHI core protein, and CBHI wild type protein produced in *Saccharomyces cerevisiae;* 0.5 mg/ml bacterial cellulose was used as the substrate. (■) Wild-type CBHI, (□) CBHI core protein, (●) mutant Y492A, (▼) mutant Y492H, (▲) mutant Y492D, (▽) mutant P477R. Some data points not shown for clarity.

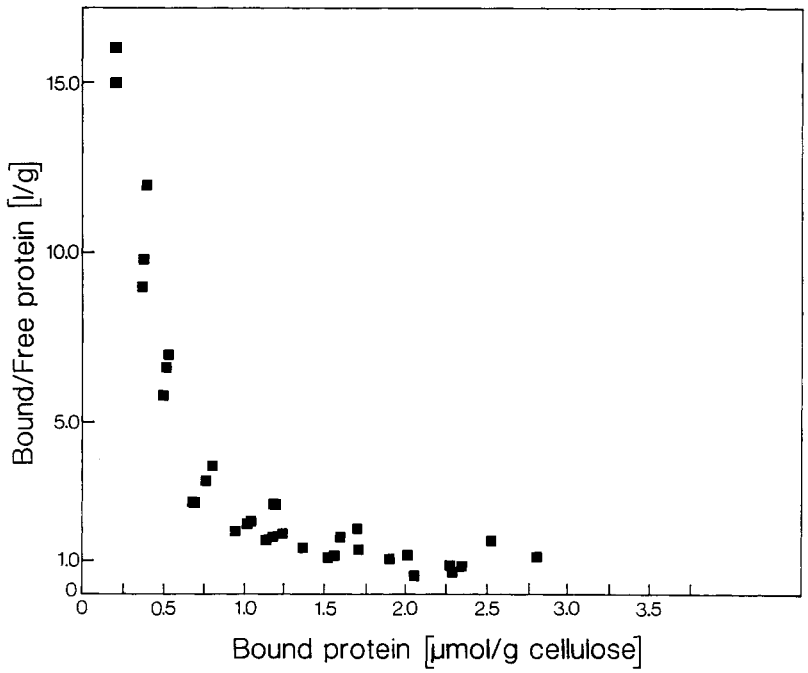

Fig. 5. Scatchard plot of the binding data of wild-type CBHI.

sible that the enzyme can bind to different locations along the crystal with different affinities. Moreover, it cannot be excluded that the heterogeneous glycosylation in the yeast produced CBHI interferes with the binding process. An alternative—so far speculative—way to explain the binding data is negative

cooperativity. Increase in the enzyme concentration at one position would reduce the affinity and more molecules could move to another position along the substrate.

Since the number of possible binding sites is unknown, the saturation curves were compared semiquantitatively and no apparent affinity constants were calculated. Interpretation of the binding data of the wild-type CBHI suggests that in low enzyme concentrations the enzyme binds crystalline cellulose with high affinity and in high enzyme concentrations lower affinity binding also occurs. The results are consistent with the results obtained from similar binding studies with CBHI and CBHI core using semicrystalline cellulose (Avicel).[22,45]

Structural Integrity of the CBD Mutants

In order to confirm the structural integrity of the mutated CBDs their structures will be determined. However, the analysis of the mutant structures by molecular modeling shows that the mutations do not generate any unfavorable steric interactions. Moreover, the CBD structure is stabilized by two intramolecular disulfide bridges. Also the monoclonal antibody data supports the view that the structure of the mutated CBDs is intact. The *Saccharomyces cerevisiae* host overglycosylates CBHI, which decreases the activity and binding of CBHI on native cellulose. Nevertheless, we feel confident that the heterologous proteins can be used in the preliminary characterization of the mutants since the control enzymes (wild-type CBHI and CBHI core protein) have been expressed in the same host.

Effects of the CBD Mutations on the Binding and Activity of CBHI

The reduction of both the binding and activity of CBHI on crystalline cellulose following the mutations at the Y492 strongly suggests it has an important role in the function of the CBD. The mutants Y492A and Y492D behave quite similarly to the isolated core domain of CBHI indicating that the aromatic ring on the tip of the domain is needed for full activity and binding. It is interesting that while the mutant Y492H binds better than the other mutants studied, the enzymatic activities on bacterial cellulose of all the four mutants are nearly identical (Table II). It is therefore possible that efficient binding depends largely on the hydrophobic interactions between the glucose pyranose rings and the aromatic rings of both tyrosine and histidine side chains but the hydroxyl present in the Tyr-31 is required for the full enzymatic activity.

The reduced binding and activity of the P477R mutant suggests that the intact hydrophobic surface of the CBD may indeed be required for full activity of CBHI on crystalline cellulose. The penetration of the CBD between the glucose chains could perhaps stabilize the newly formed fibril surfaces and prevent the reassociation of the glucose chains, thereby facilitating the solvation of the solid substrate. Alternatively, the effects of the mutation could conceivably come out through electrostatic effects or minor structural changes translated to the hydrophilic surface.

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