The surface diffusion rate of bacterial cellulases from *Cellulomonas fimi* on cellulose was quantified using fluorescence recovery after photobleaching analysis. Studies were performed on an endo-β-1–4-glucanase (Cex), an endo-β-1–4-glucanase (CenA), and their respective isolated cellulose-binding domains (CBDs). Although these cellulose-binding domains bind irreversibly to microcrystalline cellulose, greater than 70% of bound molecules are mobile on the cellulose surface. Surface diffusion rates are dependent on surface coverage and range from a low of 2 × 10^{-11} to a maximum of 1.2 × 10^{-10} cm^2/s. The fraction of mobile molecules increases only slightly with increasing fractional surface coverage density. Results demonstrate that the packing of *C. fimi* cellulases and their isolated binding domains onto the cellulose surface is a dynamic process. This suggests that the exclusion of potential CBD binding sites on the cellulose due to steric effects of neighboring bound CBDs may not fully explain the apparent negative cooperativity exhibited in CBD adsorption isotherms. Comparison with the kinetics of cellulase hydrolysis of crystalline substrate suggests that surface diffusion rates do not limit cellulase activity.

Cellulases, amylases, chitanases, and other enzymes involved in the hydrolysis of insoluble polysaccharides are typically modular enzymes with a distinct substrate-binding domain joined to a catalytic domain. The substrate-binding domains appear to assist in the hydrolysis of insoluble substrates, because lower activities are generally observed following their removal by proteolysis or genetic manipulation. Cellulose-binding domains (CBDs) are found in most fungal and bacterial cellulases. They can be classified into 10 families on the basis of amino acid similarities. Family II is the largest. The CBDs of seven cellulases and xylanases including CenA, an endo-β-1–4-glucanase, and Cex, a mixed endo-β-1–4-glucanase/xylanase, from the bacterium *Cellulomonas fimi* belong to this family.

Previous experiments show that CBD_{Cex} and CBD_{CenA} do not dissociate from cellulose after binding (3–5). This suggests that the enzymes must freely diffuse across the substrate surface to gain access to susceptible bonds, since enzyme activity is enhanced by the presence of a CBD. To test this hypothesis, we have determined the mobility of Cex, CenA, and their isolated CBDs on a cellulose surface using fluorescence recovery after photobleaching (FRAP). Enzymes or CBDs, labeled with a fluorescent tag, were adsorbed to sheets of crystalline cellulose microfibrils prepared from the cell walls of *Valonia ventricosa*, a small unicellular marine alga. A well-defined region of the surface-bound fluorescent molecules was then irreversibly photobleached using a high intensity laser pulse. Recovery of fluorescence in the bleached region was subsequently monitored by confocal laser scanning microscopy to determine the diffusive mobility of the bound molecules. The results of this study provide unequivocal evidence for surface diffusion of *C. fimi* cellulases on crystalline cellulose and give new insights into the process of cellulase adsorption and the mechanism of cellulose hydrolysis.

FRAP has been used previously to measure the diffusion of α-d-glucan maltohydrolase on insoluble starch (6) and of collagenase adsorbed to a peptide substrate covalently coupled to an insoluble support (7). In each of these systems, surface mobility of the adsorbed enzyme was thought to arise because the proteins made multiple binding contacts with the sorbent surface. Individual bonds at contact points were presumed to be weak enough to permit diffusion across the surface, but the ensemble of bonds maintained the protein at the surface. Indeed, it was suggested that the combined effect of multiple weak interaction sites on a single molecule and the dynamics of protein structure and unfolding oscillations could result in a molecular motion similar to that observed for a centipede (8).

**EXPERIMENTAL PROCEDURES**

**Protein Production and Purification**—The genes encoding the exoglycanase Cex or the isolated CBD_{Cex} were subcloned into the pTZE07 vector and expressed in *Escherichia coli* JM101 (9). The gene fragments encoding the catalytically inactive mutant of the endoglucanase CenA (D252A) and the isolated CBD_{CenA} were subcloned into the vector pUC18 and expressed in *E. coli* JM101 (10). Fermentations were carried out in a 20-liter Chemap fermenter at 37 °C. Cellulases and their isolated CBDs were purified by affinity chromatography on Avicel PH101 (FMC; County Cork, Ireland), a microcrystalline form of cellulose (11). Contaminating oligosaccharides from the Avicel affinity column were removed by size exclusion chromatography on a Superose-12 column (Pharmacia; Uppsala, Sweden) (5).

**Protein Labeling with Fluorescein**—CBD_{Cex} has only two amino groups that can react with fluorescein isothiocyanate (FITC): the N terminus and a single, surface-exposed lysine residue. Neither is on or near the putative binding face of the CBD (12). CBD_{Cex} also has two potential reaction sites that are sufficiently removed from the binding face so that FITC labeling does not influence adsorption characteristics. Proteins were labeled by standard procedures (13). Briefly, 0.15 mg of FITC was added per mg of protein at 1 mg of protein/ml. The pH was adjusted to approximately 9 to initiate the reaction, and the solution was gently mixed in the dark at 4 °C for 5 h. The labeled protein was then be hereby marked " in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: CBD, cellulose-binding domain; FRAP, fluorescence recovery after photobleaching; CLSM, confocal laser scanning microscope; FITC, fluorescein isothiocyanate; PMT, photomultiplier tube; BMCC, bacterial microcrystalline cellulose.
Surface Diffusion of Cellulases on Cellulose

FRAP is typically performed using a laser spot focused through a microscope on the surface to be investigated. The laser is equipped with a shutter that permits the rapid attenuation of beam intensity so that recovery can be monitored following bleaching. Confocal laser scanning microscopes (CLSMs) have recently been used for FRAP analysis (e.g., Refs. 16 and 17). Confocal laser scanning microscopy has the significant advantage of permitting recovery monitoring at a defined image plane of the specimen. For relatively slow transport processes, a CLSM without a rapid laser attenuation shutter can be used for FRAP analysis. Slower recovery processes also permit the acquisition of entire image planes, which may include several bleached regions on the sample. In principle, this permits the determination of diffusive anisotropy across the surface under investigation.

For FRAP analysis, a 0.06% transmission filter was placed in the laser path in front of the instrument’s standard filter set to attenuate the laser for recovery monitoring. The neutral density filter wheel on the Bio-Rad instrument was set to 3 (3% transmission) during all imaging scans. An image collected prior to bleaching was used to normalize fluorescence intensities to prebleach levels. The CLSM was then electronically zoomed (zoom = 8) so that only a small region of the surface was illuminated during laser scanning. One scan was performed at this high zoom to produce a large, bleached reference region. The CLSM zoom was then returned to its normal setting (zoom = 2), and the neutral density filter wheel on the Bio-Rad instrument set to 0 (100% transmission). During the active laser bleaching and shutter opening (~100 ms each) sequences, six bleached spots were produced for FRAP analysis (Fig. 3). The neutral density filter was then returned to the 3 position, and recovery monitoring was initiated. Fluorescence intensity was monitored until greater than 95% of fluorescence recovery had occurred.

RESULTS

Fig. 1A shows images of a mounted cellulose sheet stained with the fluorescein-labeled binding domain of the exoglucanase Cex (CBD_cex–FITC). The parallel array microfibril structure and uniformity of the surface is evident. V. ventricosa cellulose has a very high degree of crystallinity (>95%; Ref. 16) and a high binding capacity for CBDSs. Uniform, flat regions on the cellulose surface were selected for photobleaching experiments. Fig. 1B shows a cross-section perpendicular to the surface at the axis indicated in Fig. 1A. The sheets of V. ventricosa cell wall prepared for our studies are approximately 1 μm thick. The axial resolution of the confocal microscope under our imaging conditions is on the order of 1 μm. All fluorescence signals collected during recovery monitoring thus arise within or very near the cellulose sheet.

To ensure that binding of labeled and unlabeled CBDSs was equalized, binding isotherms were measured for mixtures containing 10, 20, and 50% labeled CBD_cex. The affinity constants and saturation capacities of the CBD on cellulose for the mixtures were in quantitative agreement, indicating that FITC labeling of the CBD did not affect its binding properties. Quantitative fluorescence microscopy and isotherm analysis showed that CBD-FITC fluorescence intensity/mol of bound protein
was independent of surface concentration, indicating that FITC self-quenching at these surface concentrations is not significant.

Fig. 2 shows the binding isotherm for FITC-labeled CBD\textsubscript{Cex} on disrupted cellulose fibers. The use of fluorescently labeled protein permitted data to be collected at much lower concentrations than have been reported previously. The adsorption isotherm data was analyzed by nonlinear regression using a model that includes two classes of binding sites (5). Table I reports regressed binding constants and capacities. The saturation capacity of \textit{V. ventricosa} cellulose was determined independently using a mixture of labeled and unlabeled protein. Thus, three parameters were regressed from the adsorption isotherm: high and low affinity constants and fraction of high affinity sites. The binding parameters for CBD\textsubscript{Cex} agree well with earlier values determined using unlabeled CBD and bacterial microcrystalline cellulose derived from \textit{Acetobacter xylinum} (5).

Previous studies indicate that the binding of CBD\textsubscript{Cex} to bacterial microcrystalline cellulose (BMCC) is effectively irreversible (5). The binding characteristics of CBD\textsubscript{Cex} to BMCC and \textit{V. ventricosa} cellulose are very similar (Table I), consistent with the highly crystalline nature of these two substrates. However, it was not certain that the binding of CBD\textsubscript{Cex} to \textit{V. ventricosa} cellulose is also irreversible. Because irreversible association is a critical condition in our interpretation of the FRAP measurements, this question was examined in detail. First, FITC-labeled CBD was adsorbed to cellulose sheets according to the protocol for FRAP analysis. Following washing, the protein-loaded cellulose was equilibrated with 50 mM phosphate buffer. No fluorescence could be detected in the equilibrated buffer solution after 8 h of incubation, indicating that no CBD had been released from the surface. To demonstrate that CBD was not released from the cellulose surface during FRAP, the bottom side of the microscope slide well was sealed with a coverslip bearing a second unlabeled sheet of \textit{V. ventricosa} cellulose. This surface acted as the capture surface for any CBD that might desorb from the target cellulose surface during FRAP. After a series of FRAP measurements, the top sheet with bound protein and the bottom sheet, which initially had no protein adsorbed to it, were imaged using the confocal microscope. The confocal aperture was adjusted for a depth of field of approximately 1 \(\mu\)m so that fluorescence from opposing walls was excluded. The fluorescence intensity of the unlabeled cellulose sheets did not increase during FRAP experiments, confirming that the adsorption of these CBDs to crystalline cellulose is effectively irreversible under these conditions.

**Photobleaching Analysis of CBD-FITC on Crystalline Cellulose**—Fig. 3 shows typical images recorded for FRAP analysis of FITC-labeled CBD\textsubscript{Cex} on \textit{V. ventricosa} cellulose. Fluorescence intensity measurements of the surface just prior to bleaching (Fig. 3A) were used to normalize subsequent measurements to prebleach intensities. Gaussian profile spots were bleached with a series of high intensity laser pulses in the pattern shown (Fig. 3B), and the fluorescence intensity was recorded over time by successive imaging of the bleached spots and surrounding area. Approximately 7 min after bleaching, substantial fluorescence recovery is evident in the bleached spots and at the

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**Table I**

| Sorbent          | Association affinity constant | Capacity |
|------------------|-------------------------------|----------|
|                 | \(\mu\)M \textsuperscript{-1} | \(\mu\)mol/g  |
| \textit{V. ventricosa} cellulose | \(K_1 \times 10^{6}\) | 5.0  |
|                  | \(K_2 \times 10^{6}\) | 1.2  |
| BMCC (5)         | \(K_3 \times 10^{6}\) | 3.43 |
|                  | \(K_4 \times (\pm 0.6) \times 10^{6}\) | 0.9 (\pm 0.05) |

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**Fig. 1**. 
\textit{V. ventricosa} cell wall sheet labeled with CBD\textsubscript{Cex}-FITC.
A, images of the mounted cellulose sheet stained with the fluorescein-labeled binding domain from the exoglycanase Cex (CBD\textsubscript{Cex}-FITC). The \(\sim 0.5\) \(\mu\)m fibers of packed microfibrils are evident. These fibers are stacked into lamella oriented at right angles. Several orthogonal layers make up each sheet. B, a cross-section of the surface at the axis indicated in the figure. The sheet is approximately 1.0 \(\mu\)m thick. The image was collected with a \(\times 60\) (N.A. 1.4) lens, and the scale bar represents 5 \(\mu\)m. The images are the average of three successive scans at a zoom of 2. The confocal aperture on the Bio-Rad 600 was set to 3.

**Fig. 2**. \textit{Valonia}-CBD-FITC isotherms. Shown is a typical isotherm prepared with FITC-labeled CBD\textsubscript{Cex}. Binding parameters derived from a two-site model for binding are in good agreement with our earlier studies using unlabeled CBD and bacterial microcrystalline cellulose. About 85% of the binding has a high apparent affinity of about 50 \(\mu\)M \textsuperscript{-1}. The remaining 15% of the sites have a lower affinity of about 1.0 \(\mu\)M \textsuperscript{-1}. \textit{Valonia} cellulose binds about 6.2 \(\mu\)mol of CBD\textsubscript{Cex}/g of cellulose. The inset shows a semilog plot of the isotherm data and the fitted model. Points are means of triplicate binding reactions.
Surface Diffusion of Cellulases on Cellulose

The diffusion coefficient is related to the estimated characteristic diffusion time $\tau_d$ by the equation,

$$D = \frac{\omega^2}{4\tau_d}$$

where $\omega$, the half-width of the Gaussian profile (at $e^{-2}$ times the spot profiles depth), was obtained by regression of the initial bleach spot profile. The mobile fraction $R$ was determined from the long time recovery intensity by the equation,

$$R = \frac{[F_i(t) - F_i(0)]}{[F_i(\infty) - F_i(0)]}$$

where $F_i(\infty)$ is the effective infinite time recovery, $F_i(0)$ the fluorescence just after bleaching, $F_i(t)$ the fluorescence intensity prior to bleaching, and $F_i(\infty)$ the fluorescence intensity just after bleaching.

Fig. 4B shows normalized FRAP results for CBD in a 60% maximal surface coverage. Under these conditions, the two-

$\omega$,
dimensional diffusion coefficient for CBD_{Cex} on crystalline cellulose is $6.0 \pm 0.9 \times 10^{-11}$ cm$^2$/s. This diffusion coefficient is more than 4 orders of magnitude slower than the free solution diffusion rate of $10^{-6}$ cm$^2$/s estimated from the Einstein equation for a globular protein with a mean diameter of 3 nm. If diffusion is strictly stochastic (i.e., no preferred direction or orientation), this diffusion rate corresponds to a cellulose unit-cell transit time of approximately 0.18 ms.

Based on the maximum recovery of fluorescence, the mobile fraction of CBD_{Cex} on the crystalline cellulose surface is $65 \pm 5\%$. Therefore, on the time scale of these experiments, the majority of the CBD adsorbed to the lattice surface is mobile. FRAP analysis repeated on cellulose sheets stored in buffer at 4 °C for 48 h yielded similar diffusion parameter estimates. There were no obvious changes in the morphology of the cellulose surface or microfibril packing with incubation time, indicating that the CBD had not significantly altered the cellulose structure.

Control experiments were performed to examine the effect of void spaces and the resulting potential for hindered diffusion due to molecular sieving within the cellulose fibril network of the *V. ventricosa* cellulose sheets. FITC-labeled myoglobin was prepared as described above. Myoglobin was selected because it is similar to CBD_{Cex} in size (17.5 kDa) and has no measurable affinity for crystalline cellulose. Mounted cellulose sheets were incubated for 4 h with FITC-labeled myoglobin at a molar concentration 5 times the saturation level for CBD_{Cex} and then washed in 50 mM phosphate buffer. No increase in cellulose surface fluorescence was observed, indicating that no FITC-myoglobin was bound to, or trapped within, the microcrystalline cellulose fibril network. Identical results were obtained when the cellulose sheet was preincubated with unlabeled CBD_{Cex}, indicating that the CBDs did not modify the cellulose microfibril structure. FRAP experiments performed with unwashed cellulose sheets containing free FITC-myoglobin gave fluorescence recovery rates at least 3 orders of magnitude faster than those observed for CBDCs bound to the cellulose surface. This indicates that the fibril network of the *V. ventricosa* cellulose sheet imposes little hindrance to solution diffusion of the labeled protein molecule, presumably because the void space is made up of pores much larger than individual protein molecules.

A series of FRAP experiments were performed with three different objective lenses (×60, 40, and 20) to create a range of initial bleach spot diameters to determine whether the observed fluorescence recovery for bound CBD_{Cex} in FRAP experiments was due to surface diffusion or exchange between bound and unbound CBDs. For a diffusion-limited process, the characteristic fluorescence recovery time varies with the square of the half-width of the initial bleach spot diameter (see Equation 2). For a recovery process dominated by exchange from solution, two possible rate-limiting cases must be considered. If the desorption step is rate-limiting, $\tau_d$ will be independent of $\omega$. If the desorption step is not rate-limiting, $\tau_d$ will be scaled linearly with $\omega^2$, and the slope will yield a diffusion coefficient on the order of $10^{-6}$ to $10^{-7}$ cm$^2$/s. As expected for a diffusion-limited process, $\tau_d$ scales linearly with $\omega^2$ (Fig. 5). From Equation 2, the slope of the line in Fig. 5 yields a diffusion coefficient of $3.0 \times 10^{-11}$ cm$^2$/s. This value is in good agreement with the diffusion coefficient for CBD_{Cex} on crystalline cellulose determined using nonlinear regression fitting of Axelrod’s series solution to the measured recovery curve.

At shorter recovery times. As a consequence, the estimated mobile fraction of molecules would increase as $\omega$ decreases. However, in our experiments the mobile fraction of adsorbed CBDs was independent of the initial bleach spot size (data not shown). Therefore, a single characteristic time constant adequately characterizes the observed fluorescence recovery. The second-order dependence of recovery time on bleach spot size and the independence of the mobile fraction on characteristic recovery time strongly support our contention that fluorescence recovery results from surface diffusion of CBDs adsorbed onto microcrystalline cellulose.

**Surface Diffusion Rate as a Function of Bound CBD Surface Coverage Density**—Fig. 6 shows diffusion coefficients and mobile fractions regressed from FRAP measurements of CBD_{Cex} on *V. ventricosa* cellulose at various fractions of the maximal surface coverage ($\Gamma_{\text{max}}$). Measured binding isotherms for CBDs on the prepared sheets were used to estimate fractional surface coverage densities. The maximal protein loading was 0.4 nmol of CBD_{Cex}/cm$^2$ of cellulose. The average sheet thickness was approximately 1.0 μm as determined by imaging several cross-sections using a confocal microscope. Each sheet therefore represents a total cellulose volume of approximately $1.2 \times 10^{-5}$ cm$^3$ of cellulose, or approximately 147 μg of cellulose/cm$^2$ of cellulose sheet based on a crystalline cellulose density of 1.5 g/cm$^3$. These results give a binding capacity of 5.5 μmol of CBD/g of Valonia cellulose. This capacity agrees well with values from isotherms prepared using disrupted cellulose sheets, indicating that most of the surface of the undisrupted sheet is available for binding.

The diffusion rate of CBD_{Cex} increases with surface coverage up to a $\Gamma_{\text{max}}$ of $-0.9$, after which the estimated diffusion rate decreases as the surface becomes saturated (Fig. 6A). At low surface coverages, the diffusion rate is about $3.0 \times 10^{-11}$ cm$^2$/s, increasing to a maximum of about $1.2 \times 10^{-10}$ cm$^2$/s at $\Gamma_{\text{max}}$.
were found to increase with G. Table II presents recovery results for two different C.

density, the mobile fraction reaches a maximum of about 85%. The mobile fraction is approximately 60%. At high surface coverage, the fraction of CBD Cex as a function of G max. Points are means of 12 individual spot FRAP analysis; error bars show ±1 S.E. Diffusion rates were found to increase with Γ/Γ max. B shows the estimated mobile fraction of CBD Cex as a function of Γ/Γ max. Points are means of 12 individual spot FRAP analysis; error bars show ±1 S.E. At low surface coverages, the mobile fraction is approximately 60%. As the surface coverage density increases, the mobile fraction increases to a maximum of about 80%.

A catalytically inactive mutant of CenA was therefore used to prevent surface degradation (10). This mutant binds substrate with wild-type affinity but is unable to cleave substrate because the acid catalyst Asp-252 is mutated to alanine. Diffusion coefficients and mobile fractions are presented for equivalent molar concentrations of protein. In both cases, the whole enzyme has a significantly higher diffusion rate than the isolated binding domain, with Cex having a higher diffusion rate than CenA. The mobile fraction of CenA is about 85% compared with 65% for Cex. The mobile fractions appear to be a function of the CBD domain and do not depend upon whether the domain is isolated or part of the enzyme.

**DISCUSSION**

Both CBD Cex and CBD CenA are family II cellulose-binding domains (2). CBD Cex binds irreversibly to crystalline cellulose; dilution of the free CBD at otherwise constant conditions does not result in desorption over time (5). How then does the bound enzyme find available substrate when it is distributed across the cellulose surface? Our FRAP results indicate that the irreversibly adsorbed enzyme finds reactive β-1,4-glucopyranoside linkages by diffusing in two dimensions across the cellulose surface. Surface diffusivities of Cex and the inactive mutant of CenA are similar; both diffuse about 30% faster than their respective isolated binding domain.

The three-dimensional solution structure of CBD Cex has recently been solved by NMR (12). The molecule has a compact β-barrel motif with no helix content. These results and others (20) implicate three Trp residues (Trp-54, -72, and -17) exposed on a planar face of the molecule in binding interactions with cellulose. This putative binding face presents a cluster of hydrophobic residues flanked by hydrogen bond donors and acceptors to the cellulose surface. This motif is preserved across the CBD type II family. Using titration microcalorimetry, we recently demonstrated that dehydration effects dominate the driving force for binding of CBD Cex to crystalline cellulose (5). We proposed that a CBD-cellulose complex is formed when a number of the hydrophobic residues along the CBD binding face make sufficient contact to dehydrate both the binding face and the underlying sorbent. Dehydration of the interface facilitates the formation of hydrogen-bonding pairs between the protein and the cellulose surface. Each hydrogen bond interaction has a modest affinity and dissociation rate, but the sum of interactions results in irreversible association.

The observed surface mobility of adsorbed CBD brings into question the validity of our previous model, based on a two-dimensional extension of the steric exclusion theory of McGhee and von Hippel (21), for CBD Cex adsorption to crystalline cellulose (11). Steric exclusion theory assumes that adsorbed protein molecules are static, which is not supported by our FRAP results. A large fraction of bound CBD molecules are mobile...
and can therefore redistribute on the surface so that binding site exclusion does not occur and close packing of adsorbed CBDs is possible. Therefore, we conclude that a two-site adsorption model is more appropriate to explain CBD-cellulose adsorption data.

Several groups have noted a concentration dependence of diffusion coefficients for proteins in bilipid membranes (e.g., Refs. 22 and 23) or bound nonspecifically at surfaces (24). In each of these studies, the rate of surface diffusion decreased with increasing concentration of protein. For single sorbate diffusion on a homogeneous surface containing a single class of adsorption sites, these results are supported by theory, which predicts near 0 order dependence at low surface coverage with a strong decrease in surface diffusivity as the sorbate surface concentration approaches the jamming limit (i.e. surface saturation) (25). In accordance with this simple theory, measured surface diffusivities for CBD_{Cav} on crystalline cellulose (Fig. 5) are insensitive to surface concentration at G_\text{max} < 0.4. A marked drop in the diffusion coefficient is also observed with increasing surface coverage near surface saturation. However, in contrast to theory, we observe an increase in the diffusion coefficient with increasing surface coverage over the range 0.4 < G_\text{max} < 0.9. Clearly, this system is not well described by the simple model of a single self-diffusing species on a homogeneous crystalline lattice.

The failure to capture this effect with existing simple diffusion models based on a single diffusing species on a homogeneous binding surface suggests that the crystalline cellulose surface presents a heterogeneous array of binding sites. Adsorption isotherms for CBD_{Cav} on crystalline cellulose are well fit by a model that recognizes two distinct classes of binding sites on the cellulose surface (5). Model predictions are in quantitative agreement with isothermal titration calorimetry data for the binding event and in qualitative agreement with fluorescence microscopy images of CBD_{Cav}-FITC bound to crystalline cellulose at low and high surface coverages. At monolayer coverage, about 20% of the adsorbed protein is bound to lower affinity sites.

FRAP analysis measures the mean self-diffusion rate. The increase in CBD diffusion rate observed with increasing G_\text{max} could therefore be the result of averaging between a self-diffusion rate of CBDs on high affinity sites, diffusing at –3 x 10^{-11} cm^{2} s^{-1}, and an increasing fraction of CBDs bound at lower affinity sites and thus diffusing at a much higher rates. This interpretation is consistent with the two-site model for adsorption. The observed results are reproduced in simulations with 20% of binding interactions of a lower affinity type such that the second diffusion coefficient is 2 orders of magnitude greater than that for proteins bound to high affinity sites. This higher rate is in the same range as that reported for bovine serum albumin adsorbed nonspecifically to a poly(methylmethacrylate) film (24).

Geometric considerations of the lamella structure of the V. ventricosa cell wall indicate that some fraction of bound CBDs may move axially with respect to the scanning laser probe. Electron microscopy of CBDs absorbed at low surface coverage suggests that CBDs have a preference for crystal edges or for one of the crystalline faces of the cellulose microfibril (26). Thus, if the CBD has a preference for the 220 crystal plane, preferentially oriented parallel to the laser scanning axis, the increase in diffusion coefficient with G_\text{max} may be a result of the unequal partitioning of CBDs between the two crystal faces at lower G_\text{max}.

Molecules that diffuse on surfaces parallel to the laser scanning axis will appear to diffuse more slowly because translation along the laser axis is not observable in our experiments (although fluorescence intensity is). Our experiments do not allow one to determine whether there is diffusion direction anisotropy resulting, for instance, from the migration of CBDs along the cellulose fiber axis.

Relatively little can be said about the nature of the immobile fraction of CBD molecules. The mobile fraction of bound CBD molecules was ~70% and increased only slightly with increasing G_\text{max}. The immobile species may be due to the existence of sites that promote a very slow diffusion rate or to the trapping of adsorbed CBDs on chain ends or discontinuities in the cellulose crystal.

The role of the CBD in the activity of cellulases is not completely clear. Removal of the CBD by proteolysis or genetic manipulation reduces cellulase activity on insoluble substrates but not on soluble analogues. We have shown previously that the family II CBDs of Cex and CenA disrupt the structure of Ramie cotton fibers and release small particles from Avicel and cotton (26). Since the CBDs have no hydrolytic activity, we attribute these effects to the disruption of noncovalent binding between fibers or particles. In the present study, we saw no evidence that the CBDs or enzymes disrupted the regular structure of the V. ventricosa cellulose sheets. It seems unlikely that the CBDs can penetrate this matrix. Thus, the disruptive effects of CBDs on fibers and Avicel particles presumably relates more to macroscopic structures with weaker interactions.

CBDs can target the catalytic domain of a cellulase to its substrate, thereby increasing the local enzyme concentration. Surface diffusion of the CBD-bound enzyme would then allow the enzyme to search the cellulose surface for accessible glucosidic linkages. Little is known about substrate accessibility on the surface of crystalline cellulose. Presumably, the marked differences in the activity of endoglucanases on crystalline substrates relate in a large part to their ability to disengage microfibrils from the crystalline array and thus access new sites; however, the processivity of the enzyme may also be important. For example, the C. fimi endoglucanases CenA, CenB, and CenD display almost equal activity on Avicel, but their activities on highly crystalline BMCC vary by 2 orders of magnitude (28). Each of these enzymes has a family II CBD, so the differences in activity cannot be attributed to the CBD.

Since the spacing of accessible cleavage sites on the cellulose surface is not known, we cannot determine unequivocally whether or not the rate of surface diffusion limits cellulase activity. However, based on our measurements, this seems unlikely. At the diffusion rates reported here, the CBD will traverse several hundred lattice units on the cellulose crystal in 1 min. CenA has only moderate activity on crystalline cellulose with 0.23 mol of reducing sugar being released per mol of enzyme per min for BMCC degradation (28). Other C. fimi cellulases are more active on crystalline cellulose substrate, with turnover rates up to ~10.0 mol of reducing sugar/mol of enzyme/min. These low rates suggest that surface diffusion of CBDs does not limit substrate catalysis.

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