Binding and Movement of Individual Cel7A Cellobiohydrolases on Crystalline Cellulose Surfaces Revealed by Single-molecule Fluorescence Imaging*§

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Background: Molecular level mechanisms underlying cellulose hydrolysis by cellulases remain poorly understood.

Results: The majority of cellobiohydrolase molecules on cellulose surfaces were stationary.

Conclusion: There is a need for improved understanding of cellulose properties resulting in large fractions of stalled cellulases.

Significance: Dynamic single-molecule imaging of cellulases provides insights on productive/nonproductive binding and surface diffusion on cellulose.

The efficient catalytic conversion of biomass to bioenergy would meet a large portion of energy requirements in the near future. A crucial step in this process is the enzyme-catalyzed hydrolysis of cellulose to glucose that is then converted into fuel such as ethanol by fermentation. Here we use single-molecule fluorescence imaging to directly monitor the movement of individual Cel7A cellobiohydrolases from Trichoderma reesei (TrCel7A) on the surface of insoluble cellulose fibrils to elucidate molecular level details of cellulase activity. The motion of multiple, individual TrCel7A cellobiohydrolases was simultaneously recorded with ~15-nm spatial resolution. Time-resolved localization microscopy provides insights on the activity of TrCel7A on cellulose and informs on nonproductive binding and diffusion. We measured single-molecule residency time distributions of TrCel7A bound to cellulose both in the presence of and absence of cellobiose the major product and a potent inhibitor of Cel7A activity. Combining these results with a kinetic model of TrCel7A binding provides microscopic insight into interactions between TrCel7A and the cellulose substrate.

Lignocellulosic biomass is the most abundant biological material on earth. It has been projected that the available land resources of the United States are sufficient for producing the biomass needed for cellulosic biofuels to meet 30% of the nation’s transportation fuel requirements by the middle of this century (1). However, at present, the high cost of producing such cellulosic biofuels prevents their widespread use. Processes for the biochemical conversion of lignocellulosic biomass to biofuels are typically comprised of three steps (2): (i) pretreatment: cellulose is separated from the plant cell wall matrix and rendered into a form more susceptible to enzyme degradation; (ii) enzyme degradation: a mixture of cellulases and other enzymes catalyzes the hydrolysis of cellulose fibrils, a chemically stable assembly of cellulose molecules having a degree of polymerization of 100–20,000 to its monomer component, glucose, a simple sugar (3); and (iii) fermentation: microbial conversion of glucose to biofuels such as ethanol and other useful hydrocarbon products. Currently, the high cost of the second step is a roadblock to the economical, large scale conversion of lignocellulosic biomass to fuel. The enzyme-catalyzed hydrolysis of cellulose is complex; it entails the synergistic action of enzymes known collectively as cellulases consisting of endoglucanases and exoglucanases on the insoluble substrate. According to the consensus model of synergistic cellulose catalysis, endoglucanases randomly break accessible cellulose chains; exoglucanases engage free ends of cellulose chains and processively move along the chains releasing primarily cellobiose, a glucose dimer, and to a lesser extent monomeric glucose and longer glucose polymers (up to 6-mers) into solution. Other enzymes, β-glucosidases, catalyze the cleavage of these soluble products to monomeric glucose.

Cellulases secreted by the filamentous fungus Trichoderma reesei have received the most scientific and commercial attention to date. This organism is known to produce two cellobiohydrolases, Cel7A (CBH I) and Cel6A (CBH II). Here we use the newer CAZy classification system to denote these enzymes (4); their older names are given in parentheses. These cellulases have similar structures consisting of a large (~450 amino acids) catalytic domain connected by a ~30-amino acid peptide linker to a small (~35 amino acids) cellulose-binding module. T. reesei Cel7A (TrCel7A), a processive cellobiohydrolase (~50–60% of total protein secreted by T. reesei), is known to degrade cellulose from the reducing ends of the chains, whereas...
the somewhat less processive Cel6A (≈15–20% of total protein secreted by T. reesei) shows activity toward nonreducing chain ends. The different catalytic functions of these cellulases have been attributed to differences in the structures of their catalytic domains.

In contrast to homogeneous solution phase catalysis, it is well known that the overall efficiency of this heterogeneous catalysis process depends on factors in addition to the catalytic rates of the cellulases, including: cellulase adsorption, desorption, and diffusion rates on the insoluble cellulose substrate and the processivity of exoglucanase-catalyzed hydrolysis of individual cellulose molecules. To date, in large part because of limitations of the bulk analysis methods used for its study, this heterogeneous reaction is poorly understood. Here we have used time-resolved, single-molecule fluorescence imaging to monitor the binding and movement of individual TrCel7A molecules on highly crystalline cellulose isolated from Cladophora sp. algae. We compare the binding behaviors of TrCel7A cellulohydrolase from Trichoderma under various conditions that are either conducive to or inhibitory to cellulase catalysis. Additionally we have measured the distribution of residence times of individual TrCel7A molecules bound to the cellulose substrate both in the presence of and in the absence of cellobiose.

**EXPERIMENTAL SECTION**

**Enzyme Purification and Labeling**—For the present study, TrCel7A was purified from a commercially available source, Celluclast (Novozymes), following a variation of previously described methods (5, 6). An affinity purification step using a p-aminophenylcelllobioside matrix (7) was included to ensure complete removal of endoglucanases in the purified TrCel7A preparation. The purified cellulase was labeled with a cyanine fluorophore (Cy5; GE Healthcare) according to procedures specified by the manufacturer, with some modification. The labels are functionalized with N-hydroxysuccinimidyld ester that reacts with the primary amines of lysine residues on the enzyme. To control the dye to protein ratio of the fluorescently labeled cellulase, the labeling reaction was done under less than favorable conditions, i.e., lower pH (pH 7.5–8.0) and reduced dye to protein ratio (1:2). SDS-PAGE confirmed the purity of both the unlabeled and Cy5-labeled TrCel7A (Fig. 1A). Single-molecule photobleaching step measurements of the labeled protein showed that the labeling scheme successfully limited the number of dyes attached to the individual enzyme to one or two (Fig. 1, B and C). Similarly labeled cellulases have previously been shown to retain their original activity on cellulose (8, 9). This was confirmed for our labeled enzymes with activity assays.

**Enzyme Activity Assay**—The activities of both labeled and unlabeled TrCel7A on insoluble Cladophora sp. cellulose were compared using the 2,2'-bicinchoninic acid assay to measure the reducing sugars produced by cellulase catalyzed hydrolysis of the substrates. 40 μmol of TrCel7A and Cy5-TrCel7A cellulase per gram of cellulose were incubated with 1.5 and 0.75 mg/ml of Cladophora sp. cellulose at room temperature for 18 and 114 h. The solutions were spun down to remove the insoluble fibers, and the supernatant was used to perform the colorimetric 2,2'-bicinchoninic acid assay (10). The results (Fig. 2A) were base line-corrected for background absorptions caused by the presence of the enzyme and substrate. Cellulase activities were also measured using the fluorogenic substrate 4-methylumbelliferyl-β-D-celllobioside (Sigma-Aldrich) as described previously (11). Briefly, 0.1 mM of substrate was incubated at room temperature with either TrCel7A or Cy5-TrCel7A at concentrations of 125 and 500 nm in 50 mM sodium acetate (pH 5). Cleavage of the substrate produces a fluorescence signal to indicate the cellulase activity. 1 M sodium carbonate (pH 10) was used to quench the hydrolysis reaction. The fluorogenic assay was used to measure the activities of all the enzymes used in this study, both unlabeled and labeled. This assay was also used to measure verify inhibition of cellulase activities by cellobiose as well as to verify the retention of cellulase activity in the presence of glucose, a major component of the oxygen-scavenging system used to reduce photobleaching of the Cy5 fluorophore in the single-molecule imaging experiments (Fig. 2C).

**Cellulose Sample and Labeling**—Purified cellulose from the green algae Cladophora sp. was prepared as previously described (12). Because the cellulose was isolated from the organism using a sulfuric acid treatment, we soaked it in a mild solution of hydrochloric acid (0.1 M HCl) with 5 min of incubation in a sonicator bath to remove sulfur groups left by the treatment. Dispersed suspensions of the cellulose fibrils were obtained using a series of ultrasonication steps totaling 30 min in 50 mM sodium acetate buffer (pH 5). For fluorescence imaging of the cellulose fibrils, the cellulose was labeled with dichloro-
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FIGURE 2. Activities of TrCel7A and Cy5-TrCel7A against soluble and insoluble substrates. A, the activities of the labeled and unlabeled TrCel7A against the insoluble Cladophora sp. cellulose were compared at two time points (18 and 114 h) using the colorimetric bicinchoninic acid assay to detect released sugars as described under "Experimental Procedures." Open circle, TrCel7A + 0.75 mg/ml cellulose; solid circle, Cy5-TrCel7A + 0.75 mg/ml cellulose; solid triangle, TrCel7A + 1.5 mg/ml cellulose; open triangle, Cy5-TrCel7A + 1.5 mg/ml cellulose. B, the activities of TrCel7A and Cy5-TrCel7A against the soluble substrate 4-methylumbelliferyl-β-D-cellobioside measured at four time points (15, 30, 45, and 60 min). Open circle and solid line, 500 nM TrCel7A; open triangle, 125 nM TrCel7A; open inverted triangle, 125 nM Cy5-TrCel7A. The concentration of 4-methylumbelliferyl-β-D-cellobioside was fixed at 0.1 mM. C, the activity of Cy5-TrCel7A against the soluble substrate 4-methylumbelliferyl-β-D-cellobioside was measured in the presence of various components. Open circle and solid line, pH 5 (standard conditions); open square and dashed line, pH 5 + 110 mM glucose; open triangle and dashed line, pH 5 + 20 mM cellobiose. Other components of our imaging buffer (i.e., Trolox, glucose oxidase, and catalase in the oxygen scavenger system) had negligible effect on the enzyme activity (data not shown).

FIGURE 3. A, atomic force microscopy height (left panel) and amplitude (right panel) images of cellulose from Cladophora sp. fibrils on a glass coverslip (10 × 10 μm² image area). The range of the height image is 0–300 nm. The smaller fibrils are ~1–3 μm long, ~100–400 nm wide, and ~10–40 nm high. B, differential interference contrast image. C, corresponding fluorescence image of DTAF-labeled cellulose. The scale bars in B and C are 50 μm.

triarylaminofluorescein (DTAF; Sigma-Aldrich)² according to the protocol described previously (13, 14). Differential interference contrast and fluorescence images of the DTAF-labeled cellulose verified the specificity of the DTAF labeling for the cellulose (Fig. 3, B and C). Cellulose (Sigma-Aldrich) was purchased and used without further purification.

Single-molecule Imaging and Analysis—A suspension of cellulose fibrils was introduced into the imaging chamber, which was fabricated from a quartz slide coupled with a coverslip (inner volume, ~10 μl), and incubated overnight. After washing to remove unbound fibrils, the imaging surface was blocked with BSA by treatment with 1 mg/ml of BSA solution for 15 min. The BSA blocking was required to reduce nonspecific interactions between the cellulose and the glass surface. Without BSA blocking, significant nonspecific binding of enzyme to the glass surface was observed. It has been reported that BSA only weakly interacts with various celluloses including delignified celluloses similar to one used in this study (15, 16). Therefore, we expect BSA to have a negligible effect on the interactions between cellulases and cellulose. Enzyme samples were preincubated under the various conditions used (pH 5, pH 5 + 20 mM cellobiose, or pH 10) for 30-300 s prior to their introduction into the imaging chamber. We refer to reactions conducted at pH 5 as the “standard condition,” indicating conditions conducive to enzyme hydrolysis. Picomolar concentrations of labeled enzyme were introduced into the imaging chamber for the fluorescence imaging experiments. Single-molecule imaging was performed using prism type total internal reflection fluorescence microscopy (supplemental Fig. S1). Laser excitation at 633 and 488 nm was used to excite the Cy5-labeled cellulases and DTAF-labeled cellulose fibrils, respectively. A ×60 1.2 NA water immersion objective (UPlanS Apo; Olympus) was used to image the emission from the sample surface (~54 × 27 μm area) onto an electron multiplying charge coupled device camera (Photon Max; Princeton Instruments). Laboratory-constructed dual view optics and appropriate emission filters (Semrock) were used to form a pair of images centered on the emissions of the fluorescein, and Cy5 fluorophores used to label the cellulose and cellulase, respectively. The overall magnification resulted in a pixel size of 106 nm. Image sequences were collected at integration times of 0.1 s (10 frames/s) and 1 s (1 frames/s) over intervals of up.

² The abbreviations used are: DTAF, dichlorotriazinyl aminofluorescein; CD, catalytic domain; CBM, cellulose binding module; NP, nonproductive; P, productive.
to 1200 s. The excitation lasers were blocked except during image acquisition to avoid photobleaching the samples. Image data were collected from previously unilluminated regions of the sample surface (supplemental Fig. S3). Buffer solutions consisting of 50 mM sodium acetate or 50 mM glycerol were used to fix the pH at 5 and 10.4, respectively. Oxygen scavenging components consisting of glucose, glucose oxidase, catalase, and Trolox™ (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were added to the imaging buffers to improve the photostability of the Cy5 fluorophore used to label TrCel7A. The activity assay using 4-methylumbelliferyl-β-D-cellobioside showed that these additional components had no effect on cellulase activity. All of the imaging experiments were performed at room temperature.

The image data were initially visualized and analyzed using ImageJ software (National Institutes of Health) and a program written in IDL (ITT Visual Information Solutions) and then further analyzed using the Igor Pro (Wave Metrics) and Matlab (Mathworks) programs. Trajectories of individual fluoroscently labeled TrCel7A molecules were generated by frame by frame analysis of the image data. In each frame, spatially isolated fluorescence spots with intensities above a preset threshold were identified. The centroid positions of these spots were determined from fits to two-dimensional Gaussians over pixels within a ∼700-nm radius of the peak intensity pixel. The spatial registration between the two image channels was done by two-dimensional polynomial mapping to subpixel accuracy (17). Mapping coefficients were generated from calibration images of 0.1-μm diameter fluorescent beads (TetraSpeck™; Invitrogen) with emission spectra covering both channels. Using the centroid positions of spots identified during an initial analysis, time trajectories of the spot fluorescence intensities were calculated by summing the signal intensity in 5 × 5-pixel regions around the spot centroid positions. These time histories were used to compile the binding time distributions shown in Fig. 7.

RESULTS

Single-molecule fluorescence imaging was used to monitor the binding and movement of individual, fluoroscently labeled TrCel7A celllobiohydrolases on crystalline cellulose substrates. Fluorescence microscopy permits precise measurement of the positions of fluorescent molecules by localization, in the image plane, of the centroids of the point spread functions of optically resolved molecules (18–20). In the limit of zero background noise, the localization precision scales as ω/√N, where ω is the standard deviation of the point spread function, and N is the number of detected fluorescence photons comprising the point spread function. In our imaging setup, ω = ~180 nm, and we detect several thousand photons per fluorescent molecule per second. In the absence of background, detection of 1000 photons from a single fluorescent molecule would permit its localization to well under 10 nm. However, in practice, localization precision is substantially degraded by the presence of noise (background emission from the sample and CCD camera readout noise) in the image (19, 20). Under our imaging conditions, background noise limits the localization precision to ~15 nm. This “super-resolution” optical method provides resolution approaching that of scanning electron microscopy, while enabling time-resolved imaging under physiological conditions.

Specific Binding of Cellulase Enzyme on Cellulose Substrates—To render cellulases fluorescent, TrCel7A was labeled with one or more cyanine fluorophores via surface-exposed lysine residues on its catalytic domain. Our cellulose substrate was highly crystalline cellulose fibrils isolated from Cladophora sp. algae. Atomic force microscopy imaging (Fig. 3A) showed that the smaller cellulose fibrils were ~1–3 μm in length, ~100–400 nm wide, and ~10–40 nm high. The larger fibril structures were visible using conventional light microscopy; however, the smaller cellulose fibrils were not. Therefore, it was necessary to verify that observed cellulases were actually bound to the cellulose substrate (and not the glass substrate) and moved along the fibrils. We tested the binding specificity of the cellulases to the cellulose in our imaging setup. Because the total internal reflection fluorescence microscopy imaging setup provides laser excitation ~100 nm beyond the glass-water interface, it can be used to monitor the binding of fluorescently labeled enzymes to the ~10–40 nm high cellulose substrates immobilized to the glass substrate (supplemental Fig. S1). For colocalization experiments, cellulose was fluorescently labeled with DTAf as described under “Experimental Procedures.” Fig. 4 and supplemental Video S1 show that Cy5-TrCel7A binds specifically to cellulose and that super-resolution localization imaging of the positions of individual molecules bound to the cellulose can be reconstructed (Fig. 4C). The colocalization of the DTAf-labeled cellulose emission and Cy5-TrCel7A spot positions (Fig. 4, A and B) verifies the specific binding of enzyme to the cellulose substrate (also see supplemental Fig. S2).

Fluorescently labeled cellulases were observed to bind to and desorb from the immobilized cellulose substrate during data collection. This behavior is shown in supplemental Video S1. Fig. 4B shows the composite image formed by the summation of 1200 1-s frames to show the extent of enzyme binding on the cellulose substrate. The super-resolution image of the same area (Fig. 4C) reconstructed from the accumulated centroid positions of single Cy5-labeled TrCel7A celllobiohydrolases bound to the cellulose fibrils shows the distribution of cellulase binding to the cellulose. We observed that the cellulases bind over the entire lengths of the fibrils with no preference for either the ends or middle of the fibrils. However, some areas showed extensive binding, whereas others showed little or no binding. For example, comparing the locations of the cellulose

FIGURE 4. Super-resolution imaging of Cy5-TrCel7A bound to cellulose. A, composite, false color image of single Cy5-TrCel7A molecules (red spots) bound to DTAf-labeled cellulose fibers from Cladophora sp. algae (green). Each resolved spot shows the position of a single Cy5-TrCel7A molecule. B, composite, false-color image of Cy5-TrCel7A (red) binding to DTAf-labeled cellulose fibrils (green) constructed by summing 1200 consecutive 1-s frames. C, super-resolution image of the same area reconstructed from centroid positions of individual Cy5-TrCel7A molecules bound on the fibrils. The scale bar is 2 μm.

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FIGURE 5. Trajectory of an individual TrCel7A molecule moving along a cellulose fibril. A, x-y coordinates of the molecule’s trajectory at indicated times. B, temporal plot of displacement distance from the initial location of the molecule. C, corresponding velocity distribution over the time. The trajectory is obtained from the image data shown in supplemental Video S3.

fibrils and the distribution of cellulase binding in Fig. 4, there are areas of fibrils where there were very few cellulase binding events over the course of 1200 s.

We observed that TrCel7A bound rapidly to the cellulose and remained bound to the cellulase fibrils even after extensive washing with buffer solution. Our results are consistent with electron microscopy imaging studies of Cel7A binding to microcrystalline cellulose from Valonia macrophysa (21, 22). In addition, there were occasional observations of movement of TrCel7A on the cellulose, as demonstrated in supplemental Fig. S4 and the supplemental videos.

Dynamics of TrCel7A on Cellulose Substrates—We used single-molecule trajectories obtained from fluorescence imaging data to characterize the movement (e.g., diffusive versus linear motion) of individual TrCel7A molecules on the cellulose substrate. Because we collected image sequences at frame rates as high as 10 per second, we were able to monitor the binding (see supplemental Video S2) and movement (see supplemental Video S3) of individual cellulases on the cellulose substrate. We observed that, under standard conditions (conducive to hydrolysis at pH 5), the majority (>90%) of the TrCel7A molecules bound to the cellulose were stationary within our ~15-nm localization precision. In addition, we observed that a similar fraction (~5%) of enzymes showed a “stop and go” type of motion, undergoing a rapid, sliding movement between the slow/stationary periods. Fig. 5 shows an example of this type of motion for an individual TrCel7A molecule moving on a cellulose fibril (see supplemental Video S3). The time history of the motion of this celllobiohydrolase molecule can be interpreted as follows. In seconds 0–4, the enzyme in the solution approached the cellulose surface and bound weakly as evidenced by its relatively large velocity (25–30 nm/s) and concomitantly high position variance during this time. In seconds 5 and 6, the enzyme moved rapidly (~350 nm/s) along the cellulose fibril. Given the high speed of movement, we interpret this as a rapid diffusional motion rather than processive movement associated with hydrolysis of the cellulose substrate. In seconds 7–59, the enzyme stopped its rapid movement and bound tightly to the cellulose substrate as evidenced by its small position variance. During this stationary phase, the enzyme’s position variance was due to the ~15-nm localization precision of our measurement. A possible interpretation of this behavior is that the enzyme bound to an active hydrolysis site and began slow, processive hydrolysis of the cellulose substrate. Given the range of turnover rates (~1–4 per second) expected for TrCel7A, this enzyme likely did not processively hydrolyze the substrate over the entire time, ~50 s, it was tightly bound to the substrate because it would have moved ~50–200 nm over this time. Its position change during this time was within our localization precision (~15 nm) so its processivity in this instance was ~15 turnovers or less. During seconds 60–72, the enzyme molecule again began to move rapidly along the cellulose fibril, stopping briefly at several sites before finally desorbing from the surface. The movement of the enzyme is summarized in Fig. 5. Fig. 5A is a plot of the enzyme’s trajectory on the cellulose substrate. Fig. 5B shows the displacement of the enzyme from its initial binding position versus time. The “stop and go” movements are evidenced by the abrupt changes in displacement. Fig. 5C is a plot of the enzyme’s velocity versus time. The enzyme moved at a speed of ~350 nm/s during its first large displacement and at an average speed of ~84 nm/s during its subsequent stop and go movements. We again emphasize that only a small fraction (~5%) of bound spots show rapid movements between stationary periods, whereas majority of the spots remained stationary within our 15-nm localization precision. More of these events are shown in supplemental Fig. S5 and supplemental Videos S4 and S5.

Occasionally we observed an interesting phenomenon where a second enzyme would bind in close proximity to a previously bound enzyme (see supplemental Videos S6 and S7). This was evidenced by the appearance of elongated, oval spot shapes caused by the binding of two or more enzymes in close proximity that evolved into a single, brighter round spot as one enzyme moved even closer to the other (see supplemental Video S8). This binding mode is often accompanied by a rapid (~100 nm/s) codirectional, sliding movement of the spots that may indicate the concerted movement of two or more enzymes to the next stopping point.

TrCel7A Binding Behavior in the Presence of Inhibitor—To test the effect of a catalyst inhibitor on cellulase binding, TrCel7A was preincubated with cellobiose in the buffer before flowing into the imaging chamber containing the cellulose. Cellobiose, the major product of TrCel7A activity, is known to be an effective inhibitor of Cel7A activity (23). Our bulk assay using the fluorogenic substrate (4-methylumbelliferyl-β-D-cellobioside) also confirmed that the activities of unlabeled and labeled TrCel7A decreased by more than 10-fold in the presence of 20 mM cellobiose (Fig. 2C). In the imaging experiments at pH 5 in the presence of 20 mM cellobiose, we observed a slight reduction in the number of TrCel7A molecules bound to the cellulose substrate compared with that observed under standard conditions (pH 5). Moreover, in the presence of cellobiose, we observed an increase in the number of TrCel7A molecules that moved substantial distances (>100 nm) along the cellulose fibrils. In one example, Fig. 6 shows TrCel7A enzyme moving ~700 nm along the fibril in the presence of cellobiose. This movement is shown in supplemental Video S9, composed of three separate image sequences that show similar motions by three enzyme molecules at different times (at ~5, ~350, ~440, and ~760 s) of observation. This movement was similar to the stop and go motion we occasionally observed (Fig. 5) for TrCel7A under standard conditions without cellobiose, the main differences being that in the presence of cellobiose, the distance moved during rapid diffusional steps increased (~160...
nm) and the time spent between steps decreased (supplemental Fig. S5).

**Binding Time Distributions of TrCel7A Bound to Cellulose**—Fig. 7 shows measured distributions of residence times of single TrCel7A cellobiohydrolases bound to the cellulose substrate. For each individual TrCel7A, the residence time was assessed as the time during which the enzyme remained at a given location before desorbing or diffusing on the cellulose. Under standard conditions (pH 5), 1025 TrCel7A molecules were observed (Fig. 7A), whereas 1035 TrCel7A molecules were measured in the presence of cellobiose (Fig. 7B). At pH 5, the TrCel7A binding time distribution (Fig. 7A) shows a biexponential decay. The time constants and relative amplitudes of the two decay components are \( \tau_a = 30 \text{ s} \) (84%) and \( \tau_b = 173 \text{ s} \) (16%), giving an amplitude-weighted average lifetime of 53 s. At pH 5, the presence of 20 mM cellobiose (Fig. 7C) exhibited increased mobility similar to that observed for TrCel7A under standard conditions (pH 5). We also found that the residence time of the bound enzyme decreased compared with that observed for TrCel7A at pH 5 in the presence of 20 mM cellobiose. The surface coverage of bound enzyme decreased as compared with that observed for TrCel7A at pH 5 in the presence of cellobiose (see supplemental Video S10).

**DISCUSSION**

Super-resolution imaging of fluorescently labeled TrCel7A bound to cellulose fibrils from Cladophora sp. (Fig. 4) showed spatial heterogeneity in the degree of binding of the enzyme to the substrate. The origin of this binding heterogeneity is unknown. Given that TrCel7A is known to hydrolyze cellulose from the reducing end of the cellulose polymer, one possible explanation is that the observed binding heterogeneity reflects the distribution of productive binding sites (free reducing ends) on the cellulose fibrils. Another possibility is that where little to no TrCel7A was observed, the hydrophobic faces of the fibrils where the family 1 CBMs of TrCel7A have been shown to localize (25) are not exposed. However, we cannot discount the possibility that areas that show no binding are physically inaccessible to the enzyme. Further experimentation, for example comparison of TrCel7A binding behaviors on cellulose substrates having different crystalline morphologies (e.g., Cellulose II and Cellulose III) and controlling the orientation of the fibrils, is needed to answer this question.

Under conditions conducive for enzyme-catalyzed hydrolysis (pH 5), we observed that the majority (>90%) of the TrCel7A molecules were stationary to within ~15 nm, the lateral resolution in our images. Presumably these cellobiohydrolases were either nonproductively bound to the substrate or had stalled after completing a limited number of catalytic turnovers. Given the ~15-nm lateral resolution in our images and the expected ~1-nm displacement of the enzyme along the substrate per turnover, only the movement of an enzyme executing more than ~15 turnovers can be tracked in our images. As a result, we cannot differentiate between low processivity (<15 turn-

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**FIGURE 6. Diffusional motion of TrCel7A in the presence of 20 mM cellobiose.** A TrCel7A molecule moving along a cellulose fibril decorated with several immobile TrCel7A molecules is shown. A, in the images, a TrCel7A molecule (indicated with a white arrow) binds to a cellulose fibril between 0 and 1 s and moves ~700 nm along the fiber until it desorbs from the fiber between 11 and 12 s. The scale bar in the upper left image is 2 \( \mu \text{m} \). B–D, corresponding plot of the centroid positions of the moving TrCel7A molecule (B), velocity histogram (C), and displacement distance from the initial binding site (D). See supplemental Video S9.

**FIGURE 7. Binding time histograms of TrCel7A.** The TrCel7A data show biexponential decays under standard (pH 5) conditions (A): \( \tau_a = 30 \text{ s} \) and \( \tau_b = 173 \text{ s} \), which corresponds to average binding time of \( \langle \tau_{TrCel7A} \rangle = 53 \text{ s} \), and in the presence of cellobiose (B): \( \tau_a = 9.5 \text{ s} \) and \( \tau_b = 145 \text{ s} \), which corresponds to average binding time of \( \langle \tau_{TrCel7A} \rangle = 14 \text{ s} \).
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over) catalysis events and stationary, nonproductive binding events in our images. Igarashi et al. (26, 27) observed the movement of individual TrCel7A cellobiohydrolases on the surface of Cellulose I with high speed atomic force microscopy and showed that the enzyme moves up to ~50 nm with an average speed of ~3.5 nm/s. A significant difference between our work and that of Igarashi et al. is that our experiments were performed at >1000-fold lower (subnanomolar) enzyme concentration than the micromolar enzyme concentration used by Igarashi et al. This may account for our different results; at the low enzyme concentrations we used, the highly processive events observed by Igarashi et al. may be exceedingly rare.

Other recent studies measured TrCel7A processivities that were much lower (~1–100 (28) and ~10 (29, 30)) than the degree of polymerization in native cellulosomes (~1000–100000) or the intrinsic processivity (~500–4000) estimated for this enzyme. This low processivity was attributed to limitations (obstacles) imposed by the substrate morphology on enzyme catalysis (28). The lower processivity also explains the predominance of stationary enzymes in our images under standard conditions. Kinetic measurements by Westh and co-workers (29) showed that the majority of TrCel7A molecules (>70% of the total enzyme pool) are stalled on the insoluble substrate after only ~30 s following the addition of the enzyme. Our results are also consistent with recent work by Sugimoto et al. (31), who analyzed and compared the adsorption of a fusion protein analog of TrCel7A and that of intact TrCel7A on crystalline cellulose from Cladophora sp. Their results suggest that only a small fraction of the cellobiohydrolases bound to the cellulose may be truly active. Fluorescence imaging by Moran-Mirabal et al. (9) also observed no sustained surface diffusion of fluorocein labeled bacterial cellulases (Cel5A, Cel6B, and Cel9A from Thermobifida fusca) on bacterial microcrystalline cellulose.

A small population of the isolated TrCel7A molecules (~5%) exhibited rapid, sliding movements between slow/stationary periods on the cellulose (Fig. 5 and supplemental Fig. S5). We attribute this stop and go sliding movement to a population of weakly bound TrCel7A not engaged in processive hydrolysis. Although we cannot discount the possibility that the observed “sliding” movement is due to rapid dissociation and rebinding of the TrCel7A molecule along the substrate, the molecule cannot be moving very far above the substrate because it would become invisible because of the small (~100 nm) penetration depth the excitation intensity into the aqueous phase imposed by the total internal reflection excitation geometry used in our imaging setup. Such incidences of stop and go sliding motion increased under conditions inhibiting TrCel7A activity (in the presence of cellobiose or at pH 10). Moreover, the mobility of bound TrCel7A increased (see Fig. 6, supplemental Fig. S5, and supplemental Videos S9 and S10), and average binding lifetime was reduced (Fig. 7B). We note here that the stop and go motion observed in our experiments is distinct from that observed by Igarashi et al. (27), who previously attributed the stop and go motion observed on a smaller length scale (~50 nm) in their experiments to the formation and resolution of enzyme “traffic jams” caused by obstacles encountered on the cellulose substrate during enzyme-catalyzed hydrolysis. For hydrolysis to occur, the reducing end of the cellulose chain must be threaded into the catalytic tunnel of the CD and bound to the catalytic site. We hypothesize that this conformation corresponds to the “tight binding” (stationary) mode we observed for the majority of the TrCel7A under standard conditions (pH 5) and is represented by the population of TrCel7A with the longer binding time (173 s) observed in these experiments (Fig. 7A). In contrast, we hypothesize that the population with the shorter residence time of 30 s (Fig. 7A) corresponds to TrCel7A bound only by the cellulose binding module (CBM). When binding through the CD is inhibited either by the cellobiose inhibitor or by alkaline conditions, the enzyme remains bound to the substrate through its CBM. This binding mode is weaker, as evidenced by our observations of increased diffusive motion (Fig. 6 and supplemental Video S9) and reduced binding lifetimes from 30 to 9.5 s (Fig. 7B). Moreover, an increase in the fraction of the overall bound TrCel7A population with the shorter residence time (84–97%) was observed, indicating that more of the TrCel7A enzymes were weakly bound in the presence of cellobiose. Our observation that TrCel7A binds to cellulose either in the presence of the cellobiose inhibitor or under alkaline conditions is consistent with previous studies that showed that the binding affinity of the TrCel7A CBM to the cellulose substrate is relatively insensitive to the presence of cellobiose (32, 33) or to pH change in the range of 3–10 (24).

**Kinetic Model of TrCel7A Binding**—The biphasic distribution of the binding lifetimes of TrCel7A on cellulose (Fig. 7) supports our hypothesis of two binding modes (tight binding and weaker binding) of TrCel7A on cellulose. We modeled these two binding modes as two kinds of complexes formed by TrCel7A on cellulose: the CBM binding to hydrophobic regions on the cellulose surface to produce nonproductive (NP) complexes and the CD binding to the reducing ends of the cellulose chains leading to the formation of multivalent productive (P) complexes with chain ends (Fig. 8). The lifetime of the NP complex bound to regions of cellulose fibrils other than the reducing ends is related to the rate of desorption of the CBM from the cellulose surface (k_{off}).
The desorption rate obtained using this model depicted in Fig. 8, under steady state conditions, the lifetimes shown in Fig. 7 (two binding lifetimes). Considering the model active site architecture associated with processive enzymes.

Conclusion—Time-resolved, super-resolution single-molecule fluorescence imaging revealed the complex nature of cel lulase activity on crystalline cellulose substrates. Our imaging results showed that under conditions conducive to hydrolysis (pH 5), the majority (>90%) of the TrCel7A cellulohydrolase molecules were stationary to within the ~15-nm resolution of our measurement, whereas ~5% of the population translated with rapid, linear, sliding motion. We interpret these populations as a majority of enzymes that were nonproductively bound or stalled after completing less than ~10 catalytic turnovers and a small fraction that were weakly bound, thus diffusing rapidly along the cellulose surface. Moreover, we found that competitive inhibition by cellobiose promoted more rapid desorption of nonproductively bound TrCel7A (bound only by its CBM), while simultaneously increasing the likelihood of “productive engagement” by TrCel7A (binding by both CD and CBM). The results accentuate the need for improved understanding of substrate properties that result in large fractions of nonproductively bound or physically stalled cellulases. Our results demonstrate that single-molecule imaging will be a powerful tool to directly assess the effectiveness of substrate processing strategies to improve the efficiency of cellulases on cellulose.
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