Single-molecule Imaging Analysis of Elementary Reaction Steps of *Trichoderma reesei* Celllobiohydrolase I (Cel7A) Hydrolyzing Crystalline Cellulose I$_\alpha$ and III$_i$*

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**Background:** The catalytic mechanism of *Trichoderma reesei* celllobiohydrolase I (TrCel7A) is still unclear.

**Results:** TrCel7A exhibited similar reaction kinetics during crystalline cellulose I$_\alpha$ and III$_i$ hydrolysis.

**Conclusion:** Not differences in kinetic parameters but surface properties of the crystalline cellulose influence the susceptibilities of cellulose I$_\alpha$ and III$_i$ to hydrolysis by TrCel7A.

**Significance:** Single-molecule measurements further our understanding of TrCel7A mechanism.

*Trichoderma reesei* celllobiohydrolase I (TrCel7A) is a molecular motor that directly hydrolyzes crystalline celluloses into water-soluble cellobiose. It has recently drawn attention as a tool that could be used to convert cellulosic materials into biofuel. However, detailed mechanisms of action, including elementary reaction steps such as binding, processive hydrolysis, and dissociation, have not been thoroughly explored because of the inherent challenges associated with monitoring reactions occurring at the solid/liquid interface. The crystalline cellulose I$_\alpha$ and III$_i$ were previously reported as substrates with different crystalline forms and different susceptibilities to hydrolysis by TrCel7A. In this study, we observed that different susceptibilities of cellulose I$_\alpha$ and III$_i$ are highly dependent on enzyme concentration, and at nanomolar enzyme concentration, TrCel7A shows similar rates of hydrolysis against cellulose I$_\alpha$ and III$_i$. Using single-molecule fluorescence microscopy and high speed atomic force microscopy, we also determined kinetic constants of the elementary reaction steps for TrCel7A against cellulose I$_\alpha$ and III$_i$. These measurements were performed at picomolar enzyme concentration in which density of TrCel7A on crystalline cellulose was very low. Under this condition, TrCel7A displayed similar binding and dissociation rate constants for cellulose I$_\alpha$ and III$_i$, and similar fractions of productive binding on cellulose I$_\alpha$ and III$_i$. Furthermore, once productively bound, TrCel7A processively hydrolyzes and moves along cellulose I$_\alpha$ and III$_i$, with similar translational rates. With structural models of cellulose I$_\alpha$ and III$_i$, we propose that different susceptibilities at high TrCel7A concentration arise from surface properties of substrate, including ratio of hydrophobic surface and number of available lanes.

Enzyme activity on insoluble substrates is a common but poorly understood phenomenon in biological systems. The action of cellulytic enzymes on cellulose is an important example of heterogeneous biocatalysis that has been the focus for ample research aimed at reducing the cost of lignocellulose-derived sugars for the production of biofuels (1–4). Fungal cel lulases that hydrolyze crystalline cellulose share a common two-domain structure consisting of a catalytic domain and a cellulose binding domain, which promotes degradation by mediating adsorption of the cellulases on the cellulose surface (5, 6). Recent studies using high speed atomic force microscopy (HS-AFM)$^2$ have revealed that celllobiohydrolase I (Cel7A) from *Trichoderma reesei* (TrCel7A) is a linear molecular motor, and productive binding to the reducing end of the crystalline cellulose results in translational movement to the nonreducing end (7, 8). However, the detailed mechanisms of action of TrCel7A, including kinetic constants of elementary reaction steps such as productive and nonproductive bindings, processive hydrolysis, and dissociation are not yet completely understood (Fig. 1).

In addition to the elementary reaction steps of TrCel7A, it has been reported that the crystalline form of cellulose itself strongly affects hydrolysis by TrCel7A (9). Cellulose I$_\alpha$ and III$_i$ are major commercial substrates that have received a lot of scientific and industrial attention lately. Treatment of cellulose I$_\alpha$ (naturally occurring crystalline cellulose) with supercritical ammonia results in conversion to cellulose III$_i$ (10). Crystalline cellulose I$_\alpha$ and III$_i$ are allomorphs that differ in their hydrogen bonding network and stacking interaction patterns responsible

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2 The abbreviations used are: HS-AFM, high speed atomic force microscope (microscopy); Cel7A, celllobiohydrolase I; Cy$_3$-TrCel7A, Cy$_3$-labeled *Trichoderma reesei* celllobiohydrolase I; pNPG, p-nitrophenyl-β-D-glucoside; pNPL, p-nitrophenyl lactoside; TrCel7A, *Trichoderma reesei* celllobiohydrolase I; TIRFM, total internal reflection fluorescence microscope (microscopy).
for holding their cellulose chains together (10–13). Recent works have reported that conversion of cellulose Iα into Iβ enhances its enzymatic hydrolysis rates by up to 5-fold with micromolar enzyme concentrations (8, 9, 14). Several studies have recently proposed that “decrystallization” of individual chains from the surface of crystalline cellulose is the key elementary step determining the susceptibility (15–17). Weaker intrasheet hydrogen bonding is considered to be one of the possible reasons for increased glucan chain flexibility and a lower thermodynamic barrier to individual chain extraction from the surface of cellulose Iβ (9, 14–17). However, the actual mechanism underlying the increased susceptibility of cellulose Iβ remains unclear. With the HS-AFM, we have previously revealed that TrCel7A causes molecular congestion, or “traffic jams,” on the surface of crystalline cellulose (8). Therefore, the traffic jams will also affect the susceptibility to hydrolysis by TrCel7A.

In this study, we determined that susceptibility of cellulose Iα and Iβ to hydrolysis by TrCel7A is highly dependent on enzyme concentration. Although cellulose Iβ was more susceptible than cellulose Iα at micromolar enzyme concentration, no difference was observed at a nanomolar enzyme concentration. Furthermore, by means of single-molecule fluorescence imaging with total internal reflection fluorescence microscopy (TIRFM) and HS-AFM, we have directly visualized single TrCel7A molecules on cellulose Iα and Iβ. The kinetic constants for elementary reaction steps of TrCel7A were determined and compared at picomolar enzyme concentration in which traffic jams do not occur on crystalline cellulose surfaces (Fig. 1). In all kinetic parameters measured, such as the binding rate constant, dissociation rate constant, and translational rate coupled with processive hydrolysis, large differences between cellulose Iα and Iβ were not observed. Based on our results and the previously reported structural models of cellulose Iα and Iβ (9–13), we propose that different susceptibilities of cellulose Iα and Iβ to hydrolysis by TrCel7A at high enzyme concentration arise from the surface properties of crystalline cellulose including ratio of hydrophobic surface and number of available lanes.

**Experimental Procedures**

**Construction of TrCel7A Mutant-encoding Plasmid and Transformation into Trichoderma reesei**—The recombinant *T. reesei* strain producing the mutant TrCel7A (S128C) was prepared as described previously (18, 19).

**Production of Mutant TrCel7A**—The recombinant *T. reesei* was grown on a potato dextrose agar plate for 2 weeks, and the spores were collected in 0.9% NaCl solution. The spores were inoculated in 2 liters of Kremer and Wood medium (20) containing 2% cellulose (Sigmacell Cellulose Type 50; Sigma-Aldrich) as the sole carbon source in a jar fermenter with a 5-liter working volume (Takasugi Seikakusho). The temperature was 37 °C, and the pH was maintained at 5.0 with phosphoric acid and potassium hydroxide. The reaction mixture was stirred at 200 rpm, with air supply at 3.0 liters/min. After 8 days of cultivation, the culture supernatant was separated from cellulose and mycelia by using a glass filter membrane. Extracellular proteins were concentrated by Kivick Laboratory 10-kDa cut cassettes (GE Healthcare).

**Purification of Mutant and Wild-type TrCel7A**—Ammonium sulfate was added to the extracellular protein solution of the recombinant *T. reesei* to a final concentration of 1 M, and fractionation was carried out on a phenyl-Toyopearl 650s column (column volume: 70 ml; Tosoh) equilibrated with 20 mM sodium acetate buffer (pH 5.0) containing 1 M ammonium sulfate. Proteins were eluted with a reverse linear gradient of 1–0 M ammonium sulfate in a total volume of 140 ml. The protein concentration of each fraction was estimated with a protein assay kit (Bio-Rad) according to the manufacturer’s instructions. The purity of each fraction was confirmed by SDS-PAGE, and fractions containing proteins of ~60 kDa were collected. The buffer of enzyme solution was changed to 20 mM potassium phosphate (pH 7.0) and injected onto the DEAE-Toyopearl 650s column (column volume 150 ml; Tosoh) equilibrated with the same buffer. Proteins were eluted with a 1650-ml linear gradient of 0–130 mM KCl. Protein concentration and purity were analyzed by same methods, and fractions containing proteins of approximately 60 kDa were collected. Finally, ammonium sulfate was added to the protein solution to a final concentration of 1 M, and fractionation was carried out on a phenyl-Toyopearl 650s column (column volume 70 ml) equilibrated with 20 mM sodium acetate buffer (pH 5.0) containing 1 M ammonium sulfate. Proteins were eluted with a reverse linear gradient of 1–0 M ammonium sulfate in a total volume of 700 ml. Protein concentration and purity were analyzed by same methods, and the hydrolysis activity of *p*-nitrophenyl lactoside (pNPL) and *p*-nitrophenyl-β-D-glucoside (pNPG) were measured. Aliquots of 20 μl were incubated with 20 μl of 10 mM pNPL or pNPG, 20 μl of 1 M sodium acetate buffer (pH 5.0), and 140 μl of water. The addition of 20 μl of 2 M Na2CO3 stopped the reaction, and the absorbance of the reaction mixture was measured at 405 nm (ε405 = 16,608 M−1cm−1). pNPL or pNPG hydrolytic activity (units) was calculated using the standard curve of *p*-nitrophenol. The fractions containing ~60-kDa enzyme with pNPL-hydrolyzing activity but without pNPG-hydrolyzing activity were collected and equilibrated with 20 mM sodium acetate buffer (pH 5.0). Wild-type TrCel7A was purified from Celluclast (Novozymes) by the same methods described above. The concentration of TrCel7A was measured by absorbance at 280 nm. The molecular extinction coefficient, 88,250 M−1cm−1, was determined from quantitative amino acid analysis (21).
**Elementary Reaction Steps of Cellobiohydrolase I (Cel7A)**

**Cellulose Preparation**—Highly crystalline cellulose was prepared from green algae *Cladophora* spp. as described previously (22). The cellulose suspension was washed with water and stored at 4°C. Crystallinity of cellulose substrates was estimated to be 99% by x-ray powder diffraction (23).

**Preparation of Cy3-TrCel7A**—*TrCel7A*(S128C) mutant (380 μM, 100 μl) in 50 mM MOPS (pH 7.0) containing 50 mM KCl was mixed with Cy3-maleimide monoreactive dye (118 mM, GE Healthcare) in dimethyl sulfoxide and reacted for 3 days at room temperature. Unreacted Cy3-maleimide was removed by ultrafiltration (Vivaspin 500 5K PES membrane; VIVAPRODUCTS). The labeling ratio of Cy3 to *TrCel7A*(S128C) was 90% (Fig. 2). For Cy3, the molecular extinction coefficient of ε = 150,000 M⁻¹cm⁻¹ at 550 nm was used for concentration determination.

**Hydrolysis Activity Measurement**—The hydrolysis activities were measured by estimating the amount of reaction products via HPLC. The hydrolytic activities of wild-type *TrCel7A* and Cy3-*TrCel7A* against crystalline cellulose Iα and IIII were determined by mixing each enzyme (final concentration of 0.1 μM or 2.0 μM) and 0.1% substrate for 2 min at 25°C in 50 mM sodium acetate (pH 5.0). The amount of released cello-oligosaccharides was determined by high performance anion exchange chromatography as described previously (24). Glucose (Fluka), cellobiose, cellotetraose, and cellopentaose (Serva), and cellotriose and cellohexaose (Seikagaku) were used as standards. In all cases, cellulbiose was the major soluble product generated.

**Single-molecule Fluorescence Imaging Analysis**—For single-molecule fluorescence imaging, the sample chamber was constructed using a glass coverslip (thickness, 0.12–0.17 mm; Matsunami Glass) cleaned with 10M KOH. The cellulose suspension was spin-coated (3000 rpm, 10 s) on the coverslip and observed. Single-molecule fluorescence imaging was performed using objective-type TIRFM with isotropic illumination (25, 26), constructed on an inverted microscope (IX71; Olympus). Cy3 was excited with 532-nm laser (DPGL-2100F; Photop Suwtech). By introducing the ring-shaped laser beam from the peripheral region of the back aperture of the objective lens (PlanApoN60×TIRFM, numerical aperture = 1.45; Olympus), a nonpolarized evanescent field was formed on the glass surface. The laser power before the objective lens was set at 0.14 μW/μm². The illuminated area was 44 μm in diameter (1.5 × 10⁴ μm² in area). Image sequences were collected with an exposure time of 0.2 s (5 frames/s) for 120–160 s after the addition of Cy3-*TrCel7A* solution on the glass coverslip. The images were analyzed using ImageJ software (National Institutes of Health).

**RESULTS**

**Different Susceptibilities of Cellulose Iα and IIII to Hydrolysis by *TrCel7A* Are Highly Dependent on Enzyme Concentration**—First, we measured hydrolysis activities of *TrCel7A* against cellulose Iα and IIII at two different enzyme concentrations, 0.1 μM and 2 μM, fixed initial substrate concentration (0.1% [w/v]), and fixed reaction time (2 min). At 2 μM *TrCel7A*, hydrolysis activities of 6.1 ± 0.2 min⁻¹ and 20.6 ± 0.5 min⁻¹ (mean ± S.D., n = 3) against cellulose Iα and IIII, respectively, were obtained (Table 1). As previously reported, cellulose IIII was more susceptible than cellulose Iα (8, 9, 14). However, at 0.1 μM, *TrCel7A* exhibited nearly as high hydrolysis activities against cellulose Iα and IIII, 18.7 ± 2.1 min⁻¹ and 15.0 ± 1.9 min⁻¹ (mean ± S.D., n = 3), respectively. The increase in susceptibility of cellulose Iα at low *TrCel7A* concentration strongly suggests that the density
of TrCel7A molecules on the surface of cellulose Iα affect their activity.

Hydrolysis Activity of Cy3-TrCel7A against Cellulose Iα and IIIα—For single-molecule fluorescence imaging analysis, a mutant of TrCel7A(S128C) was generated and conjugated with a fluorescent dye, Cy3. The labeling ratio was 90% (Fig. 2). Cy3-labeled TrCel7A (Cy3-TrCel7A) showed hydrolysis activity comparable with those of the wild-type against cellulose Iα and IIIα and similar dependence on the enzyme concentration (Table 1). These results ensure that the mutation and Cy3 conjugation did not affect the enzyme activity.

Binding Specificity of Cy3-TrCel7A for Cellulose Iα and IIIα—Typical examples of the bright-field image of cellulose Iα and IIIα microfibrils and single-molecule fluorescence image of Cy3-TrCel7A are shown in Fig. 3. Some thick bundles of cellulose Iα and IIIα microfibrils were visible under a conventional bright-field microscope (Fig. 3A). Cellulose IIIα tended to display higher contrasts than did cellulose Iα, suggesting the different number of microfibrils in a bundle. Binding of Cy3-TrCel7A to cellulose Iα and IIIα was highly specific (Fig. 3, B and C, 30 pm Cy3-TrCel7A in solution). Fig. 3B shows single-fluorescence image frames obtained with an exposure time of 0.2 s. The density of Cy3-TrCel7A on the cellulose surface was very low, and individual molecules were clearly visible. Therefore, under this low enzyme concentration condition, traffic jams of Cy3-TrCel7A do not occur. Fig. 3C illustrates the accumulated fluorescence images constructed by summing 600 consecutive images recorded at 5.0 frames/s. Virtually no binding events to the glass surface were observed. During observation, binding

**TABLE 1**

|   | Hydrolysis activity (min⁻¹) |
|---|-----------------------------|
|   | Wild-type (2 μM) | Wild-type (0.1 μM) | S128C-Cy3 (2 μM) | S128C-Cy3 (0.1 μM) |
| Cellulose Iα | 6.1 ± 0.2 | 18.7 ± 2.1 | 4.8 ± 0.1 | 17.7 ± 1.0 |
| Cellulose IIIα | 20.6 ± 0.5 | 15.0 ± 1.9 | 14.7 ± 0.3 | 16.8 ± 0.6 |

* Mean ± S.D.

**FIGURE 3.** Binding specificity of Cy3-TrCel7A to cellulose Iα and IIIα. A, bright-field images of cellulose Iα (left) and IIIα (right) microfibrils. B, single-molecule fluorescence images of Cy3-TrCel7A (30 pm in solution) bound to the surface of cellulose Iα (left) and IIIα (right), obtained with exposure time of 0.2 s. C, fluorescence images of many Cy3-TrCel7A molecules bound to cellulose Iα (left) and IIIα (right), constructed by summing 600 consecutive images recorded at 5.0 frames/s. In A–C, same image fields for cellulose Iα and IIIα are shown. The image sizes were 6.4 μm × 12.7 μm and 3.9 μm × 9.8 μm for cellulose Iα (left) and IIIα (right), respectively. D, examples of time course of fluorescence intensity. Time courses of the region of interest (10 × 10 pixels, 1 pixel = 86 nm) on cellulose Iα and IIIα, are shown. The single-step increases and decreases in the fluorescence intensity time course correspond to the binding and dissociation events of single Cy3-TrCel7A molecules, respectively.
**Elementary Reaction Steps of Cellobiohydrolase I (Cel7A)**

**TABLE 2**
Peak and mean values of $k_{on}$ for Cy3-TrCel7A against cellulose I$_a$ and III$_i$
Measurement buffer, 50 mM sodium acetate (pH 5.0); TrCel7A, 14 pm; initial cellulose concentration, 0.02% (w/v); temperature, 25 °C ± 1 °C.

| Peaks         | Primary$^*$ | Secondary$^*$ | Tertiary$^*$ | Mean$^*$ |
|---------------|-------------|---------------|--------------|----------|
| Cellulose I$_a$ | $7.3 \times 10^3 \pm 2.9 \times 10^2$ | $1.3 \times 10^4 \pm 1.5 \times 10^4$ | $2.0 \times 10^4 \pm 2.8 \times 10^4$ | $1.6 \times 10^4 \pm 7.5 \times 10^4$ |
| Cellulose III$_i$ | $9.5 \times 10^3 \pm 1.2 \times 10^4$ | $1.7 \times 10^4 \pm 7.6 \times 10^4$ | $2.6 \times 10^4 \pm 7.3 \times 10^4$ | $2.3 \times 10^4 \pm 1.1 \times 10^4$ |

$^*$ The peak value ± S.E. of the fitting with the Gaussian.
$^a$ The mean value ± S.D. of all data.

**TABLE 3**
Summary of kinetic constants for Cy3-TrCel7A against cellulose I$_a$ and III$_i$
Measurement buffer, 50 mM sodium acetate (pH 5.0); temperature, 25 °C ± 1 °C.

| Kinetic constants | Cellulose I$_a$ | Cellulose III$_i$ |
|------------------|----------------|------------------|
| $k_{on}$ (M$^{-1}$μm$^{-2}$s$^{-1}$) | $3.0 \times 10^{10} \pm 7.6 \times 10^{9}$ | $6.4 \times 10^{10} \pm 1.5 \times 10^{10}$ |
| $k_{off}$ (s$^{-1}$) | $0.12 \pm 0.01^c$ | $688^b \pm 0.14^c$ |
| (Fraction) | $48%$ | $57%$ |
| $k_{on}NP$ (s$^{-1}$) | $0.86 \pm 0.03^c$ | $688^b \pm 0.74^c$ |
| (Fraction) | $52%$ | $43%$ |
| $k_{oN}$ (μm/s) | $5.0 \pm 2.0^b$ | $72^b \pm 5.1 \pm 1.9$ |

$^* The peak value ± S.D. of the fitting with the Gaussian.
$^b$ Number of cellulose microfibrils.
$^c$ The mean ± S.E. of the fitting with the double exponential decay functions.
$^d$ Number of TrCel7A molecules.
$^e$ The peak value ± S.D. of the fitting with the Gaussian.

and dissociation events of Cy3-TrCel7A on the cellulose surface were easily observed (Fig. 3D). The examples of the time course of fluorescence intensity are shown in Fig. 3D. Single-step increase and decrease correspond to the binding and dissociation of single Cy3-TrCel7A, respectively. From these images and time courses, we estimated the binding rate constant ($k_{on}$) and the dissociation rate constant ($k_{off}$) of Cy3-TrCel7A (see Figs. 5A and 6 and Tables 2 and 3).

**The Binding Rate Constant of Cy3-TrCel7A for Cellulose I$_a$ and III$_i$**—We estimated the binding rate constant $k_{on}$. We first defined $k_{on}$ as the number of Cy3-TrCel7A bound to cellulose microfibrils in a unit Cy3-TrCel7A concentration, unit cellulose length, and unit time (M$^{-1}$μm$^{-2}$s$^{-1}$). The concentration of Cy3-TrCel7A in solution was first set at 14 pm, and image sequences were obtained for analysis. Then, concentration was increased to 10 nm to confirm the shape of cellulose I$_a$ and III$_i$ microfibrils. Even at 10 nm, binding of Cy3-TrCel7A was highly specific (Fig. 4A). At 10 nm Cy3-TrCel7A, some microfibrils displayed high saturated fluorescence intensity above the dynamic range of the detector (EMCCD camera) (Fig. 4B). We considered these highly fluorescent microfibrils as large bundles and excluded them from the analysis. Fig. 5A shows the distribution of $k_{on}$ for Cy3-TrCel7A on cellulose I$_a$ and III$_i$. The distributions of $k_{on}$ showed multiple peaks and could be fitted by multiple Gaussians with peak positions separated almost equally (Table 2).

To investigate the origin of multiple peaks, we simultaneously observed cellulose I$_a$ (Fig. 5B, left) and III$_i$ (Fig. 5B, right) microfibrils with TIRFM (Fig. 5B, top left) and HS-AFM (Fig. 5B, top right and bottom) (27). The fluorescence images were obtained by adding a high concentration (10 nm) of Cy3-TrCel7A in solution. Simultaneous imaging revealed that even the dim, apparent single microfibrils in fluorescence images are bundles of several microfibrils in HS-AFM images. Therefore, we concluded that multiple peaks in $k_{on}$ distribution represent the number of cellulose microfibrils in a bundle, and the minimum peak corresponds to the $k_{on}$ for a single microfibril (Fig. 5A, indicated by arrows). The $k_{on}$ values for a single microfibril of cellulose I$_a$ and III$_i$ were determined to be $7.3 \times 10^8$ M$^{-1}$μm$^{-2}$s$^{-1}$ and $9.5 \times 10^8$ M$^{-1}$μm$^{-2}$s$^{-1}$, respectively.

From the HS-AFM images, we also measured distributions of the diameter of a single microfibril of cellulose I$_a$ and III$_i$ (Fig. 4A). Cellulose I$_a$ and III$_i$ microfibrils indicated by multiple Gaussians with peak positions separated almost equally (Fig. 4B). The fluorescence images of cellulose I$_a$ (left) and III$_i$ (right) microfibrils in the presence of 10 nm Cy3-TrCel7A. Image sizes are 14.6 μm × 14.6 μm. B, line profiles of fluorescence intensity indicated in A. Cellulose I$_a$ and III$_i$ microfibrils indicated by line 3 showed high, saturated fluorescence intensity, and were excluded from the analysis.
The distributions were well fitted with a single Gaussian, and the peak positions of 24 nm and 21 nm for cellulose I and III, respectively, were obtained. The average ± S.D. of the diameter for cellulose I and III were 24.0 ± 7.3 nm (n = 48) and 25.6 ± 15.7 nm (n = 72), respectively. The diameter of a single microfibril of cellulose I was similar to that of cellulose III, although in our measurement, the diameter was defined as the half-maximum full-width of the HS-AFM image and the

FIGURE 5. Measurement of the binding rate constant for Cy3-TrCel7A. A, distributions of the binding rate constant (k_{on}) of Cy3-TrCel7A for cellulose I (left) and III (right). The distributions of k_{on} were fitted by three Gaussians (from 0 to 2.4 × 10^9 M^{-1}μm^{-1}s^{-1} for cellulose I, and from 0 to 3.3 × 10^9 M^{-1}μm^{-1}s^{-1} for cellulose III). Arrows indicate the minimum peaks that correspond to k_{on} for single microfibrils. The positions of peaks are summarized in Table 2. The bin width is 1.5 × 10^9 M^{-1}μm^{-1}s^{-1}. The number of cellulose microfibrils analyzed was 64 and 169 for cellulose I and III, respectively. The concentration of Cy3-TrCel7A was 14 pM. B, simultaneous observation of cellulose I (left) and III (right) microfibrils with TIRFM (top left) and HS-AFM (top right). The fluorescence images were obtained by adding 10 nM Cy3-TrCel7A in solution to bind to cellulose microfibrils. The sizes of fluorescence (top left) and HS-AFM (top right) images were 6.0 μm × 6.0 μm. The magnified HS-AFM images indicated by squares in the top are also shown (bottom). The size of magnified HS-AFM image was 200 nm × 200 nm. C, distributions of the diameter of single microfibril of cellulose I (left) and III (right). The distributions were fitted by single Gaussians. The peak values were 24 nm and 21 nm for cellulose I and III, respectively. The average ± S.D. of the diameter were 24.0 ± 7.3 nm (n = 48) and 25.6 ± 15.7 nm (n = 72) for cellulose I and III, respectively. The bin width is 4 nm. All measurements were carried out in 50 mM sodium acetate buffer (pH 5.0) at 25 °C.
actual size was slightly smaller. The $k_{on}$ values normalized to the unit area of cellulose using the peak values of the diameter were $3.0 \times 10^{10} \text{M}^{-1}\mu\text{m}^{-2}\text{s}^{-1}$ and $4.5 \times 10^{10} \text{M}^{-1}\mu\text{m}^{-2}\text{s}^{-1}$ for cellulose I$_a$ and III$_r$, respectively (Table 3).

**The Dissociation Rate Constant of Cy3-TrCel7A for Cellulose I$_a$ and III$_r$**—Next, we estimated the dissociation rate constant $k_{off}$. Fig. 6 shows the distributions of duration times for Cy3-TrCel7A bound to cellulose I$_a$ and III$_r$. For each individual TrCel7A, the duration time was defined as the time the enzyme remained on the cellulose surface before dissociation. For both cellulose I$_a$ and III$_r$, the distribution could be fitted by double exponential decay functions better (the squares of the correlation coefficient ($R^2$)) were 0.996 and 0.994 for cellulose I$_a$ and III$_r$, respectively) than by single exponential decay functions (the values of $R^2$ were 0.975 and 0.973 for cellulose I$_a$ and III$_r$, respectively). Therefore, there were two distinct binding components, which are represented by long and short duration times. We considered that the component with a long duration time corresponds to the productive binding, in other words, binding to the reducing end and processive movements on cellulose, and that with short duration time corresponds to the nonproductive binding events, respectively (see also “Discussion”). From the fitting, the dissociation rate constants for productive binding ($k_{off}^{P}$) were estimated to be $0.12 \pm 0.01 \text{ s}^{-1}$ and $0.14 \pm 0.01 \text{ s}^{-1}$ (mean $\pm$ S.E. of fitting) for cellulose I$_a$ and III$_r$, respectively (Table 3). Similarly, the dissociation rate constants for nonproductive binding ($k_{off}^{NP}$) were $0.86 \pm 0.03 \text{ s}^{-1}$ and $0.74 \pm 0.05 \text{ s}^{-1}$ for cellulose I$_a$ and III$_r$, respectively. The rate constant for photobleaching of Cy3 was 0.021 s$^{-1}$ in our experimental condition (data not shown) and much lower than $k_{off}$ values, indicating the effect of photobleaching is negligible. The relative fractions of productive binding to total binding events were 48 and 57% for cellulose I$_a$ and III$_r$, respectively. Both the values of $k_{off}$ and the ratio of productive binding for cellulose I$_a$ and III$_r$ were similar.

**DISCUSSION**

In the previous biochemical assay, cellulose I$_a$ and III$_r$ exhibit large differences regarding their susceptibility to hydrolysis by TrCel7A (9). However, in the present study, we found that susceptibility of cellulose I$_a$ and III$_r$ to TrCel7A is highly dependent on the enzyme concentration in the solution, and hydrolysis activities of TrCel7A against cellulose I$_a$ and III$_r$ are similar at low nanomolar concentrations (Table 1). With single-molecule imaging analysis, we also determined kinetic constants for elementary reaction steps of cellulose I$_a$ and III$_r$ hydrolysis by TrCel7A (Fig. 1). The results are summarized in Table 3. As suggested by the biochemical assay performed at nanomolar
enzyme concentration, no significant differences were observed in all kinetic constants for TrCel7A against cellulose I<sub>a</sub> and III<sub>I</sub>. The binding modes of Cel7A to cellulose have been implicated as an important factor limiting efficient biocatalysis (2, 28, 29). The \( k_{on} \) against single microfibril of cellulose III<sub>I</sub> was only 1.5 times larger than that against cellulose I<sub>a</sub> (Fig. 5A and Table 3). Note that the mean values of all \( k_{on} \) for cellulose I<sub>a</sub> and III<sub>I</sub> were \( 1.6 \times 10^9 \pm 7.5 \times 10^8 \text{m}^{-1} \text{g}^{-1} \text{min}^{-1} \) (mean ± S.D., \( n = 64 \)) and \( 2.3 \times 10^9 \pm 1.1 \times 10^9 \text{m}^{-1} \text{g}^{-1} \text{min}^{-1} \) (mean ± S.D., \( n = 169 \)), respectively (Table 2), and the differences were <2-fold. Therefore, even if our attribution of the multiple peaks in \( k_{on} \) distribution to the number of microfibrils in a bundle is not correct, the conclusion will not change. The values of \( k_{on} \) for productive binding (or initial-cut product rate) have been reported in previous biochemical studies of Cel7A and crystalline cellulose from different sources (30, 31), although the definition of \( k_{on} \) in previous studies \((\text{g/liter})^{-1} \text{min}^{-1} \) g/liter corresponds to the substrate concentration) is different from ours \( (\text{m}^{-1} \text{g}^{-1} \text{min}^{-1}) \) or \( \text{m}^{-1} \text{g}^{-1} \text{sec}^{-1} \). The previously reported \( k_{on} \) values are almost similar and \( \sim 10^{-2} \) (g/liter) \( \text{min}^{-1} \) (30, 31). The substrate concentrations used in the previous studies were \( \sim 1 \) g/liter, which corresponds to the binding rate of \( \sim 1 \times 10^{-2} \text{ sec}^{-1} \) per reducing end. The previously reported \( k_{on} \) values were obtained at \( 10^{-2} \)–\( 10^{-3} \) M enzyme concentrations, and the corresponding \( k_{on} \) values in our study will be \( 10^{-2} \)–\( 10^{-3} \) \( \mu \text{m}^{-2} \text{sec}^{-1} \) or \( 10^{-4} \)–\( 10^{-5} \) \text{nm}^{-2} \text{sec}^{-1} \). Considering the fraction of productive binding (0.05), area of \( \beta \)-glucose unit \( (\sim 0.2 \text{ nm}^2) \), and degree of polymerization of the cellulose \( (\sim 10^4) \) used in our study (32), this would correspond to the binding rate of \( 10^{-2} \)–\( 10^{-3} \) \text{ sec}^{-1} \) per reducing end. Thus, the \( k_{on} \) values obtained in our study are similar or slightly larger compared with those previously reported.

TrCel7A exhibited very similar \( k_{off} \) values on cellulose I<sub>a</sub> and III<sub>I</sub> in the present study (Fig. 6 and Table 3). These values were \( 10^{-3} \)–\( 10^{-4} \) times larger than those obtained by previous biochemical assays (28, 30). This can be attributed to the use of Cel7A and/or crystalline cellulose from different sources because our recent HS-AFM observation of TrCel7A on cellulose III<sub>I</sub> obtained almost similar \( k_{off} \) \( (0.20 \text{ sec}^{-1}) \) (33). According to the distribution of duration times on the cellulose surface, binding events of TrCel7A on both cellulose I<sub>a</sub> and III<sub>I</sub> included roughly half of productive binding that accompanies processive hydrolysis. Thus, there were no large differences in the binding modes of TrCel7A to cellulose I<sub>a</sub> and III<sub>I</sub>. As described above, degree of polymerization of the cellulose used in our study is \( \sim 10^3 \) (32). Therefore, the density of the reducing end on the surface of crystalline cellulose will be \( 10^{-3} \) per \( \beta \)-glucose unit, and the fraction of TrCel7A that finds the reducing end will be \( 10^{-3} \) when TrCel7A binds randomly to the cellulose surface. This implies that TrCel7A binds to the reducing end with remarkable specificity.

The \( k_{tr} \) values for TrCel7A were almost the same when comparing cellulose I<sub>a</sub> and III<sub>I</sub> (Fig. 7). Considering the length of a cellobiose unit (\( \sim 1 \text{ nm} \)), the \( k_{tr} \) represents not only the translational rate but also turnover rate of TrCel7A against cellulose I<sub>a</sub> and III<sub>I</sub>, and can be estimated as \( 5.0 \text{ sec}^{-1} \) and \( 5.1 \text{ sec}^{-1} \), respectively. Furthermore, the values of \( k_{off} \) correspond to the time constants of 8.3 s and 7.1 s for cellulose I<sub>a</sub> and III<sub>I</sub>, respectively. Therefore, the expected values of processivity for Cy3-TrCel7A on cellulose I<sub>a</sub> and III<sub>I</sub> are estimated to be 42 (5.0 s\(^{-1}\) \times 8.3 s) and 36 (5.1 s\(^{-1}\) \times 7.1 s), respectively. These values are comparable with those of TrCel7A on bacterial cellulose previously determined biochemically (34), and on cellulose III<sub>I</sub> obtained by our recent HS-AFM observation (33). The good accordance of processivity supports our interpretation that the long component of \( k_{off} \) corresponds to the productive binding.

Our results indicate that the effect of conversion of cellulose I<sub>a</sub> into III<sub>I</sub> on the susceptibility at high enzyme concentration does not come from the different \( k_{on} \), \( k_{tr} \), and \( k_{off} \). One possible factor determining the susceptibility is the decrystallization step as previously proposed (15–17). However, dependence of cellulose I<sub>a</sub> susceptibility on TrCel7A concentration is not apparently consistent with the notion that decrystallization is a rate-limiting step (Table 1). If the decrystallization is slow on cellulose I<sub>a</sub>, a transient pause before translational movement will occur after initial binding to the reducing end. Although we could not resolve decrystallization step from translational movement in our single-molecule fluorescence imaging, the decrystallization step may be directly visualized with high resolution HS-AFM observation. It will be an interesting issue to be investigated in the future study.

In addition to the decrystallization as an elementary reaction step (9, 14–17), the force generated by TrCel7A in the decrystallization step will be another interesting issue to be resolved. To understand chemo-mechanical coupling and force generation mechanisms of TrCel7A as a molecular motor, visualization of the steps and pauses for moving TrCel7A is indispensable (35). This will require single-molecule measurements with subnanometer and microsecond spatio-temporal resolution (36). Although HS-AFM is a powerful technique that can directly visualize movements and conformational changes of molecular motors with high spatial resolution (8, 37), temporal resolution is limited to several tens of milliseconds (38). Single-molecule analysis of TrCel7A with high spatio-temporal resolution with optical microscopy can be achieved with larger probes such as colloidal gold, which enables high image contrast (39, 40).

The diameters of cellulose I<sub>a</sub> and III<sub>I</sub> microfibrils used in our study were almost the same (Fig. 5C). However, it has been reported that cellulose I<sub>a</sub> and III<sub>I</sub> have different crystalline forms (8, 11–13). Cellulose I<sub>a</sub> has narrow hydrophobic 110 surfaces between wide hydrophilic 100 and 010 surfaces, whereas cellulose III<sub>I</sub> has wide moderately hydrophobic 100 surfaces in addition to narrow hydrophilic 110 surface and wide hydrophilic 010 surface (Fig. 8A) (8, 11–14). Considering that TrCel7A primarily binds to hydrophobic surfaces, a large moderately hydrophobic surface in cellulose III<sub>I</sub> will increase number of accessible reducing ends in single microfibril. Therefore, actual \( k_{on} \) for the single reducing end may be different between cellulose I<sub>a</sub> and III<sub>I</sub> as proposed in previous study (16). It is also highly probable that cellulose III<sub>I</sub> has an increased number of available lanes for TrCel7A movement relative to cellulose I<sub>a</sub>. This would relieve the traffic jams of TrCel7A on cellulose III<sub>I</sub>. However, quantitative comparison of the degree of traffic jams
between cellulose Iα and IIIβ at defined enzyme concentration was difficult due to strong and significant nonspecific bindings of TrCel7A to the graphite used as a surface to which cellulose Iα and IIIβ were immobilized in HS-AFM observation.

Finally, we propose our current model of different susceptibilities of cellulose Iα and IIIβ to hydrolysis by TrCel7A. At low TrCel7A concentration (picomolar to nanomolar) (Fig. 8B, top), accessible reducing ends for TrCel7A binding and lanes for TrCel7A translational movement are sufficient on both cellulose Iα and IIIβ, and both cellulose Iα and IIIβ are susceptible. At high concentration (micromolar), however, TrCel7A will easily cause shortage in available reducing ends and/or traffic jams on cellulose Iα, but not on cellulose IIIβ, which results in low susceptibility of cellulose Iα (Fig. 8B). Therefore, optimization of enzyme concentration and hydrophobicity of the crystalline cellulose surface will be essential for efficient industrial production ofcellulosic biofuels with TrCel7A.

FIGURE 8. Model of different susceptibilities of cellulose Iα and IIIβ to hydrolysis by TrCel7A. A, schematics show cross-sections of cellulose Iα (left) and IIIβ (right). B, compared with cellulose Iα (left), cellulose IIIβ (right) has a largely increased number of accessible reducing ends for TrCel7A binding and lanes for TrCel7A translational movement. At low TrCel7A concentration (picomolar to nanomolar) (top), accessible reducing ends and lanes are sufficient on both cellulose Iα and IIIβ. At high TrCel7A concentration (micromolar) (bottom), shortage of reducing ends and/or traffic jams occurs on cellulose Iα, but not on cellulose IIIβ.

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**Elementary Reaction Steps of Cellobiohydrolase I (Cel7A)**

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