Measurements of single molecular affinity interactions between carbohydrate-binding modules and crystalline cellulose fibrils†

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Combining atomic force microscopy (AFM) recognition imaging and single molecule dynamic force spectroscopy (SMDFS), we studied the single molecule affinity interactions between the carbohydrate-binding module (CBM) and plant cell wall cellulose using the CBM3a (from Clostridium thermocellum) and CBM2a (from Cellvibrio japonicus) functionalized AFM tips. The binding efficiencies of the CBMs to the cellulose were determined by the binding areas on the crystalline cellulose fibrils surface using the recognition imaging. Several dynamic and kinetic parameters, such as the reconstructed free energy change, energy barrier and bond lifetime constant, were also obtained based on the measured single molecule unbinding forces, which are used to illuminate the affinity of the CBMs binding to the natural and single cellulose surface from a totally different aspect. It was found that CBM3a has a little higher binding efficiency and affinity than CBM2a to both natural and extracted cellulose surfaces and both the CBMs have higher affinities to the natural cell wall cellulose compared to the extracted single cellulose. The in-depth understanding of the binding mechanisms of the CBM–cellulose interactions of this study may pave the way for more efficient plant cell wall degradation and eventually facilitate biofuel production.

Introduction

Plant cell wall degradation by microbial enzymes is an important biological and industrial process to produce environmentally-friendly biofuels.1 Enzymatic hydrolysis of cellulose, the major component of plant cell wall, has potential advantages over traditional chemical processes in terms of higher yields and selectivity, less energy costs as well as milder processing conditions.2 However, due to the complexity of the plant cell wall structures, the hydrolysis mechanism is still not fully understood. It is clear now that plant cell walls are highly complex macromolecular structures consisting of various polysaccharides held together by interacting not only with each other through extensive networks but also with other non-polysaccharide polymers overlaying outside.3,4 This complex structure, therefore, severely hinders the loosening and degradation of the plant cell wall by enzymes. To facilitate the accessibility of the enzymes to the compacted plant cell wall carbohydrates, cell wall hydrolases have a complex molecular architecture consisting of catalytic modules and non-catalytic, substrate-targeting carbohydrate-binding modules (CBMs).5 Hence, the efficiency and affinity of CBMs binding to plant cell walls directly affect the degradation process. CBMs are usually grouped into three types based on the topology of the binding sites. Typically, the binding site of type A CBMs (such as CBM3a derived from Clostridium thermocellum6 and CBM2a derived from Cellulomonas fimi7) comprises a planar hydrophobic surface which recognizes crystalline polysaccharides such as cellulose and chitin.8

CBM binding to cellulose, the very first step of the enzymatic hydrolysis of cellulose, is one of the most significant carbohydrate–protein interactions.9–12 Protein–carbohydrate interactions have long been proven to play crucial roles in biological activities such as protein folding and diverse cell functions.13–15 Carbohydrates are involved not only in many essential recognition processes in biology and physiology, but also in degradations of plant and biofuel conversion in nature. Therefore, measuring the single molecular CBM–cellulose interaction may provide a better understanding of mechanisms of protein–carbohydrate interactions at the single-molecule level quantitatively.
The mechanisms of CBM–cellulose interactions have been studied extensively. With the current progress in this topic, researchers increasingly focus on the molecular basis of the CBM–cellulose interactions.\textsuperscript{16} Especially some molecular modeling and simulation work has been reported to mimic the dynamic process of the CBM–cellulose complex using Trichoderma reesei cellobiohydrolase I and parallel cellulose chains.\textsuperscript{17–19} However, the results from simulation work are inconclusive and need more experimental supports. For instance, the binding and unbinding processes of CBM molecules to the large crystalline surface of cellulose microfibrils still need tedious work to build the model and it takes a lot of computing resources and time to simulate the equilibrium process.\textsuperscript{17} Different modeling approaches and force fields generated different structures and behavior of the crystalline cellulose microfibril.\textsuperscript{20} Especially, the binding processes of different families of CBMs to the intact microcrystalline cellulose surface are still missing. On the other hand, traditional bulk experimental measurements quantitatively showed a wide range of the affinity of different CBMs on the crystalline or amorphous cellulose substrates, with association constant $K_a$ ranging from $10^5$ M$^{-1}$ to $10^7$ M$^{-1}$.\textsuperscript{21,22} weaker than the $K_a$ of antigen–antibody interactions which can be as high as $10^{12}$ M$^{-1}$. The reported data of these traditional methods are based on the measurements of the ensemble that contains a large amount of molecules mixed together and the obtained data are averaged by a statistical mechanism, which could lead to incomplete, even contradictory conclusions. For instance, according to the review work done by Peter Tomme et al.,\textsuperscript{23} CBM2a exhibited high affinities for bacterial microcrystalline cellulose (BMCC) ($K_a = 3.2 \times 10^8$ M$^{-1}$), but the $K_a$ of CBM3 to BMCC varied from a lower value of $1.7 \times 10^6$ M$^{-1}$ to a much higher value of $2.9 \times 10^9$ M$^{-1}$. Melean and co-workers also reported in 2002 that the $K_a$ of CBM2a to BMCC was $3.2 \times 10^6$ M$^{-1}$, but the $K_a$ of CBM3 to BMCC was only $1.0 \times 10^6$ M$^{-1}$.\textsuperscript{21} In order to reconcile the above discrepancy, it is necessary to study the CBM–cellulose binding at the single molecule level, which should reveal the real reaction dynamic and kinetic mechanisms. 

There are several widely used single molecule techniques for direct measurement of intermolecular forces, such as optical tweezers,\textsuperscript{24} bio-membrane force probes (BFP),\textsuperscript{25} and atomic force microscopy (AFM) single-molecule dynamic force spectroscopy (SMDFS).\textsuperscript{26,27} Such experiments allow for estimating unbinding forces and dissociation rate constants, even for mapping energy landscapes.\textsuperscript{27} Among these techniques, AFM can also be used to image the sample surface with ultrahigh resolution, down to single molecules and atomic levels. Indeed, AFM has been used to directly visualize the surface structure of plant cell walls with nanometer resolution.\textsuperscript{28} Using a sharp, bare AFM tip, the morphology and surface roughness of the plant cell wall were precisely determined. However, the information such as the chemical components of the cell wall surface and their distributions cannot be recognized. Recently, AFM recognition imaging was combined with the dynamic force measurements using functionalized AFM tips to map the substrate components. This technique can be used to measure intra-molecular unfolding forces of individual molecules and inter-molecular forces between various specific reaction molecules.\textsuperscript{26,29–31} Due to the complexity of the plant cell wall surface structures, this AFM technique provides a more comprehensive approach than conventional bulk solution ones. The CBM functionalized AFM tip first locates the cellulose using recognition imaging and then measures the interactions between the CBM and cellulose using SMDFS. Therefore it offers more accurate data and eventually a better understanding of the mechanisms of CBM–cellulose interactions.

In this study, we mapped the natural poplar cell wall surface and extracted single crystalline cellulose by recognition imaging, and consequently measured unbinding forces\textsuperscript{32} between the CBM and cellulose using the CBM3a (derived from Clostridium thermocellum Scaffoldin CipA) and CBM2a (derived from Cellulibrio japonicus Xyn10A) functionalized AFM tips, respectively. To reveal the binding mechanisms, we also estimated the energy barriers, dissociation rate constants, extension lengths, bond lifetimes and constructed the free energies for the interactions of these two CBMs.

**Experimental**

**Preparation of recombinant CBM3a and CBM2a (ESI-I)**

The recombinant CBM3a and CBM2a used in this study were provided by Complex Carbohydrate Research Center, University of Georgia. The preparation procedure of recombinant CBM3a was described in detail in our previous work.\textsuperscript{32} The recombinant CBM2a was derived from Cellulibrio japonicus Xyn10A (CjXyn10A) with a molecular weight of 12.328 and an estimated pI of 8.08.

**Poplar cell wall sample preparations for recognition imaging and unbinding force measurement**

The natural poplar cell wall preparation has been reported previously.\textsuperscript{32} The pretreatment of the extracted single crystalline cellulose followed a widely used procedure.\textsuperscript{33} Briefly, 1 mg of micromotted poplar slice was treated by a mixture of NaOH and Na$_2$S solution (1% w/v and 15.5 mL, respectively) at 80 °C for 1.5 h (bath ratio: 1:30). The sample was then bleached by sodium chloride (1.7% w/v, 5 mL) at 80 °C for 2.5 h in the presence of an acetate buffer (0.135 g NaOH and 0.375 mL glacial acetic acid in 5 mL distilled water). The bleached cellulose fibers were centrifuged 8 times with purified water (SORVALL BioFuge Pico Microcentrifuge) and then dried in air at room temperature. Further, the fibers were hydrolysed in sulfuric acid (64% w/w, 1 mL) at 60 °C for 30 min under strong agitation. The reaction was stopped by addition of 2 mL cold purified water. The diluted suspension is centrifuged at 10 000 rpm for 10 min to obtain the precipitates. The last two steps were repeated (around 10 times) until a turbid suspension was obtained. The suspension was collected and dialyzed using a micro dialyzer (QuixSep) for 5 h. Finally, the suspension was sonicated for 10 min and stored at 4 °C with addition of 0.05% sodium azide.

To prepare the AFM sample for recognition imaging and force measurement, poly-$\epsilon$-lysine hydrobromide (MW 53 900, 0.1 wt%, 10 $\mu$L) was dropped onto a freshly cleaved mica surface. After 5 min, the mica surface was gently washed using
200 μL purified water 4 times using a 200 μL pipette and dried in air. The pretreated poplar suspension (0.1 wt%, 6 μL) was dropped onto the modified mica surface. After 3 min, the mica surface was washed using 200 μL purified water 3 times to remove the extra cellulose. After drying in air, the mica surface was further incubated with sodium glutamate (NaGlu) solution (10 wt%, 10 μL) for 10 min and then washed gently using 200 μL purified water 4 times. Finally, the air-dried mica was fixed into an AFM flow cell and then filled with 0.3 mL Tris-Cl buffer (10 mM Tris-Cl and 150 mM NaCl, pH = 7.5) for recognition imaging and force measurement.

AFM tip functionalization

The AFM tip functionalization with CBM3a has been introduced in our previous paper. Here the AFM tip with CBM2a was functionalized in a similar way. Briefly, the gold-coated CS-10 silicon AFM tips (Nanoscience Instruments, nominal spring constant of about 0.1 N m⁻¹) were first immersed in the HS-PEG2000-NTA crosslinker (Nanocs Inc.) (0.2 mg mL⁻¹, 300 μL) for 3 h, and moved to NiCl₂ (10 mM, 20 μL) for 30 min, at room temperature. The tips were then washed repeatedly and immersed in 400 μL Tris-Cl buffer (10 mM Tris-Cl and 150 mM NaCl, pH 7.5) with addition of CBM2a (27 μg mL⁻¹, 6 μL). The solution was then kept at 4°C for 8 h. The modified tips were finally washed thoroughly in Tris-Cl buffer for future imaging.

Experimental setup for recognition imaging and force measurement

The simultaneous topography and recognition images were obtained using Top magnetic AC (TopMAC) mode using a PicoTREC controller under a PicoPlus Molecular Imaging system (Agilent Technologies, Santa Clara, CA). The method is based on detecting a small shift in the peak value of the cantilever deflection signal that occurs when a tip-tethered molecule (CBM) binds to a target (cellulose) on the surface, bridging the gap between the surface and an oscillating tip. During the recognition imaging of the cellulose, the force–distance curves (F–D curves) were also recorded. Over 300 representative F–D curves were used under at each loading rate and processed using a home-designed Labview program to construct the histograms. Gaussian fitting was used to identify the values of the most probable unbinding forces. The whole system was shielded from environmental interference using a PicoPlus Isolation Chamber.

Results and discussions

CBM3a and CBM2a both belong to “type A” carbohydrate-binding modules and are proven to bind specifically to crystalline cellulose. Using the CBM-functionalized AFM tips, we first imaged the CBM–cellulose binding events on the crystalline cellulose surface. Fig. 1 shows the topographic and recognition images of both the crystalline cellulose of natural poplar cell walls and extracted single cellulose using CBM3a (a–d) and CBM2a (e–h) functionalized AFM tips.

The topographic images in Fig. 1 clearly show that the crystalline cellulose fibrils on the natural cell wall are closely packed (a, e) while the extracted cellulose fibrils are well isolated (c, g). The surface areas recognized by both CBM3a and CBM2a are shown as dark signals which indicate the specific binding (b, d, f, h). On the natural cell wall cellulose...
surface, the binding sites lay closely to each other due to the compact crystalline cellulose structure (b, f). However, on the extracted single cellulose microfibril sample, the binding sites were found to orderly align along the cellulose microfibril surface (d, h). Since impurities exist on the natural cell wall surface, such as hemicellulose, pectin and other non-carbohydrate residues, certain small areas on the cellulose surface may not be recognized by the CBM-functionalized AFM tip. For example, the red frames in Fig. 1(a), (b), (e) and (f) highlight the small areas on the crystalline cellulose surface with probable impurities. Insets are the enlarged areas in the red frames and the representative cellulose microfibrils are labelled with white, dashed lines. The recognition signals along the same cellulose microfibrils (white, dashed lines) are interrupted as shown in Fig. 1(b) and (f) (insets) due to the existence of impurities.

To further determine the binding efficiencies of CBM3a and CBM2a, the binding sites on the surface of extracted single cellulose microfibrils were recognized by the above two binding modules and their topographic and recognition images were compared. Fig. 2 exhibits a small area of extracted single cellulose microfibrils recognized by the CBM3a-functionalized AFM tip in topographic (a) and recognition (b) images. The extracted cellulose microfibrils in the topographic image (a) show clear recognition signals in the corresponding recognition image (b).

To identify the individual binding sites, we analysed the cross-section profiles of two representative extracted cellulose microfibrils marked a–a’, b–b’ in (a) and c–c’, d–d’ in (b). Fig. 2(c) and (d) compare the cross-section profiles of the two labelled extracted single cellulose microfibrils marked in Fig. 2(a) and (b). Each recognition event (unbinding event) is highlighted by a vertical red, dashed line, which indicates the corresponding topographic and recognition signals. The black curve shown in Fig. 2(e) is one representative F–D curve measured on the extracted single cellulose microfibril at point A in Fig. 2(a), which is corresponding to A’ in Fig. 2(b). The black curve in Fig. 2(e) was measured on the mica substrate at point B, corresponding to point B’ in Fig. 2(b). The overlay of these two curves indicates that the interaction measured between the CBM3a molecule on the AFM tip and the cellulose surface was specific, since no interaction was detected between the CBM3a and mica surface.

As shown in Fig. 2(c) and (d), the smallest intervals in the cross-section profiles are measured to be 5–8 nm, which is consistent with the result we reported in the past. The binding site of a single CBM3a molecule has proven to directly come into contact with 6–7 glucose units (3–4 nm in width) on the surface of cellulose microfibrils. Therefore, a separation distance of 2–5 nm between two binding sites can be deduced.

To compare the binding efficiencies of CBM3a and CBM2a, we measured the binding area percentage (BAP) on both natural cellulose microfibril and extracted single cellulose microfibril surfaces. The BAP is defined as the ratio of the bound area on single cellulose microfibrils (nm²) to the total surface area of single cellulose microfibrils (nm²). The cellulose bound area to CBM3a or CBM2a was considered as the dark area generated by the recognition signal from recognition images, and the

![Fig. 2](https://example.com/fig2.png)

**Fig. 2** Topographic (a) and recognition (b) images of extracted single cellulose microfibrils with cross-section analysis (c) and (d) on smaller surface areas. The images of (a) and (b) are obtained using the CBM3a-functionalized AFM tip. The curves in (e) represent F–D curves measured (under the loading rate of 40 nN s⁻¹) at point A in (a), which is corresponding to point A’ in (b), and point B in (a) corresponds to point B’ in (b). The F–D curve A (A’) (in red) shows the specific interaction between the CBM3a-functionalized tip and the extracted single cellulose microfibril surfaces; while the curve B (B’) (in black) shows no interaction between the tip and the mica surface.
apparent surface area of cellulose included the entire bright area of cellulose from topographic images. These area values were calculated using Picoscan software. The BAP was calculated on 20 cellulose microfibrils from both natural and extracted samples (see ESI-II). The results show that BAPs of CBM3a and CBM2a to the natural cell wall cellulose surface are 56.7 ± 10.0% and 47.0 ± 12.8%, respectively. The BAPs of CBM3a and CBM2a to the extracted cellulose surface are found to be a little larger, with 73.7 ± 11.1% and 63.6 ± 13.5%, respectively. The data show that CBM3a has about 10% more bound area than that of CBM2a on both natural cell wall cellulose and extracted single cellulose microfibrils. This difference on the BAP at the single-molecule level is mainly due to the detailed molecular structures of these two binding modules and their cellulose binding conformations.

Based on previously published work, CBM3a has 10 conserved residues participating in the cellulose binding activity, with five of them (W118, R112, D56, H57, and Y67) in the planar strip and another five of them as anchors (N16, Q110, S12, N10, and S113). Among these residues, H57 and Y67 can form strong hydrogen bonds with the glucose units of cellulose, and in turn stabilize the binding conformation. Other residues can also contribute to possible hydrogen bonding, electrostatic interactions, and van der Waals interactions to help orientate the CBM3a active residues to the right locations on the cellulose surface. The CBM2a structure in the literature shows 8 conserved residues involving in binding, with four of them (W17, N87, W54, and W72) in the planar strip, and another four (N15, N24, Q83, and Q52) as anchors. For the CBM2a used in our experiment, we found that the residues N15, W17, W54, and W72 were conserved in the similar positions in the secondary structure, but other active residues changed. For the planar strip, N87 helps W54 form the correct orientation on the cellulose surface; while for the anchor residues, only N15 keeps in the secondary structure. Therefore, the CBM2a we used may not show affinity as strong as that reported in the literature. Compared to CBM3a, the contact area between CBM2a and cellulose in the literature is smaller than that between CBM3a and cellulose; therefore, the overall binding affinity of CBM3a in our experiments is supposed to be higher than that of CBM2a. The topographic and recognition images of extracted single cellulose microfibrils using the CBM2a-functionalized AFM tip with cross-section analysis are also provided in the supporting information (see ESI-III†), which shows less binding events than the CBM3a-cellulose interaction. Moreover, CBM3a and CBM2a show 17% and 16.6% more bound area on natural cell wall cellulose than that on extracted cellulose, respectively, which may be resulted from the more impurities blocking the CBMs from binding to the cellulose crystalline surface.

To reveal the binding mechanisms, we measured the single molecule binding affinity determined by the F–D curves. Since in the traditional bulk experiments, the affinity (described as association constant $K_a$) of different CBMs on various carbohydrate substrates is usually measured from an average value of a large amount of CBM molecules. All these results are based on statistical calculations involving numerous protein molecules each time, which will introduce various sources of uncertainties when compared with the study of the affinity and binding mechanisms of a single CBM molecule on the cellulose surface. The single molecule interaction measurements can provide data to determine the in-depth dynamic and kinetic mechanisms of the CBM–cellulose interactions, which has not been reported yet.

Based on the study of molecular interactions of biological molecules with dynamic force spectroscopy, it has been widely accepted that weak non-covalent bonds have limited lifetimes and can break apart when the applied external forces last long enough times. According to Bell’s model, the reaction unbinding rate or off rate increases exponentially with the external force. Therefore, to calculate the off rates of the CBM–cellulose interactions on the cellulose surface, we measured the unbinding forces at 11 incremental loading rates from 0.5 nN s$^{-1}$ to 500 nN s$^{-1}$. Fig. 3 shows the 11 force histograms, each of which was constructed from over 300 F–D curves (an example is shown in Fig. 2(e)) for both CBM3a–extracted single cellulose microfibril (a) and CBM2a–extracted single cellulose microfibril (b) interactions. The histograms are arranged in the 3-D figure with the most probable unbinding force calculated using Gaussian fitting (force values labelled on top). We observed that, at the loading rates higher than 300 nN s$^{-1}$, the unbinding force reached its saturation, which indicates the minimum force to overcome the energy barrier ($E_0$) of CBM–cellulose non-covalent interactions instantaneously. Some representative F–D curves at selected loading rates of CBM3a and CBM2a–extracted single cellulose microfibril interactions are listed in ESI-IV.†

We also determined the lifetime of the bond under external force ($t_F$). The $t_F$ value is calculated from the equation derived based on Bell’s model,

$$t_F = t_{off} \frac{F - x_0}{a_T} = \frac{1}{k_{off}} \frac{F - x_0}{a_T},$$

where $F^*$ is the unbinding force (pN), $x_0$ is the barrier length (nm), $t_{off}$ is the natural lifetime of the bond without external forces.
force (s), $k_{\text{off}}$ is the dissociation rate or off rate (s$^{-1}$), $T$ is the effective temperature (300 K) and $k_B$ is Boltzmann’s constant. Due to the thermal activation, the non-covalent bonds have modest lifetimes which are gradually shortened under external force. It has already been predicted that the bond lifetime depends on the rapidity of applied forces. The most probable unbinding force will determine the most probable binding lifetime at the specific force loading rate ($\tau$). Therefore, we can establish a relationship between the measured unbinding force and the affinity between CBM and cellulose. The unbinding forces under a series of loading rates determine the $k_{\text{off}}$ and the larger the $k_{\text{off}}$ value, the lower the affinity. Taking CBM3a–extracted single cellulose microfibril interactions as an example, the plot of unbinding force and $\tau$ versus the logarithmic coordinates of loading rates is shown in Fig. 4(a). The data points for unbinding forces are marked in black squares and the data points for $\tau$ are marked in red triangles. The $\tau$ decreases dramatically from 7.92 s under 0.5 nN s$^{-1}$ to 0.01 s under 300 nN s$^{-1}$.

The force spectroscopy reflects only the non-equilibrium state of the CBM–cellulose interactions since the $F$–$D$ curve is measured at a certain loading rate. The most probable unbinding force, when plotted as a function of $\ln(\tau)$, can be used to reconstruct the energy landscape along the unbinding pathway. According to Jarzynski’s equality, the free energy change of the CBM3a–extracted single cellulose microfibril interactions under the equilibrium state can be reconstructed. The free energy profile was rebuilt based on 20 $F$–$D$ curves and the reconstructed free energy change for CBM3a–extracted single cellulose microfibril interactions was calculated to be 27.06 kcal mol$^{-1}$ or 45.33 $k_B T$ (Fig. 4(b)). The reconstructed free energy change of CBM2a–extracted single cellulose microfibril interaction is 25.21 kcal mol$^{-1}$ or 42.51 $k_B T$, for CBM3a–natural cellulose interaction is 29.86 kcal mol$^{-1}$ or 50.02 $k_B T$ and for CBM2a–natural cellulose interaction is 28.61 kcal mol$^{-1}$ or 48.02 $k_B T$ (see Table 1 and ESI-V1). The results show a very similar affinity of the two CBM molecules to the cellulose surface. Nevertheless, some differences can be identified for the two CBMs and for the natural and extracted cellulose microfibrils: the CBM3a–natural cell wall cellulose microfibril complex has the highest free energy change and the CBM2a–extracted single cellulose microfibril complex has the lowest one. Specifically, the CBM3a–cellulose complexes have higher free energy change than that of CBM2a–cellulose complexes, which can be contributed to a larger contact area of CBM3a to the cellulose surface because of more CBM3a binding residues and their wider occupancy of the binding interface. The proposed reason for both CBM–natural cellulose complexes to have higher free energies than those of CBM–extracted single cellulose microfibril complexes is that the crystalline structure of the extracted single cellulose microfibrils may be partially damaged during the extraction procedure. This may lead to the consequence that only partial binding residues of the CBMs can bind to the damaged crystalline cellulose surface so that the binding of CBMs to the extracted cellulose is not so tight as to the intact, natural cellulose.

Due to the small differences in free energy change, we took a close look at the $\tau$ values of different CBM–cellulose complexes to compare their kinetic properties. At the lowest loading rate of 0.5 nN s$^{-1}$ and the highest loading rate of 300 nN s$^{-1}$ before the saturation, the CBM3a–extracted single cellulose microfibril interaction shows the longest bond lifetime of 7.92 s and 0.01 s, respectively, while the CBM3a–natural cell wall cellulose shows the shortest bond lifetime of 6.74 s and 0.01 s, respectively (see ESI-V1). Moreover, the CBM2a–extracted single cellulose microfibril complex shows a slightly shorter bond lifetime of 7.19 s and 0.01 s than that of the CBM2a–natural cell wall complex, which is 7.88 s and 0.01 s, respectively. The calculated and experimental $\tau$ values of the four sets of experiments are plotted with the 9 loading rates before the saturation and the results are listed in ESI-V1. These results exhibit a good agreement between the calculated and experimental $\tau$ values. The differences in bond lifetime among the four complexes are not very pronounced. Specifically, CBM3a exhibits a shorter bond lifetime on natural cellulose and a longer bond lifetime on extracted single cellulose microfibrils than that of CBM2a. The possible reason is that the major binding residues on the CBM3a planar strip (mentioned above) are more sensitive to impurities on the natural cell wall than those of CBM2a. In contrast, the planar strip of CBM3a can maintain relatively strong interactions with the extracted single cellulose microfibrils although some parts of the crystalline structure on the cellulose microfibril surfaces have changed. For CBM2a, the impurities on the natural cell wall have less influence on the planar strip and anchor residues, but they require more regular cellulose crystalline surfaces than CBM3a for stable interactions.

### Table 1

<table>
<thead>
<tr>
<th>CBM</th>
<th>Natural</th>
<th>Extracted</th>
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<tbody>
<tr>
<td>x0 (nm)</td>
<td>0.64</td>
<td>0.63</td>
</tr>
<tr>
<td>$E_b$ (kcal mol$^{-1}$)</td>
<td>15.12</td>
<td>14.23</td>
</tr>
<tr>
<td>$k_{\text{off}}$ (s$^{-1}$)</td>
<td>0.0081</td>
<td>0.0099</td>
</tr>
<tr>
<td>$\Delta F$ (kcal mol$^{-1}$)</td>
<td>29.86</td>
<td>28.61</td>
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**Fig. 4** (a) The unbinding force and $\tau$ vs. loading rate plots for the interactions between CBM3a and extracted single cellulose microfibrils. The data points for the unbinding force are marked with black squares and the data points for $\tau$ are marked in red triangles. The unbinding forces after saturation are marked with blue, dashed lines. (b) The weighted average $F$–$D$ curve and the work integral used to obtain the reconstructed free energy change (27.06 kcal mol$^{-1}$ or 45.33 $k_B T$) for CBM3a–extracted single cellulose microfibril interactions. The averaged $F$–$D$ curve is marked in blue and the free energy profile is marked in black.
The dissociation rates and energy barrier \( E_b \) can also provide a theoretical support for our dynamic and kinetic study on the CBM–cellulose interaction mechanism. To calculate the \( E_b \) for all of the CBM3a–natural/extracted single cellulose microfibril and CBM2a–natural/extracted single cellulose microfibril interactions, 100 \( F-D \) curves were collected at the loading rate of 100 nN s\(^{-1}\) for each CBM–cellulose system. According to Bell’s model, a constant force \( F \) applied to a distance \( x_b \) will lower the energy barrier.\(^{38}\) The \( x_b \) defined here in our study is the energy barrier width larger than which the bond is broken. The \( E_b \) here was estimated using \( E_b = \frac{1}{2} F x_b \), where \( F x_b \) is the unbinding force and \( L \) is the stretching distance of the non-covalent bond during the unbinding event.\(^{42}\) The kinetic off rate (\( k_{\text{off}} \)) can provide insight into the occurrence of the bond, bond strength and relaxation time. The \( k_{\text{off}} \) of CBM–cellulose interaction listed in Table 1 is calculated by the linear fitting (red line in Fig 4(a)) of unbinding forces before the saturation. The fitted unbinding forces agreed with Bell’s one-barrier bond dissociation model.\(^{36}\) The complete results of calculations are listed in Table 1. The CBM3a–natural cell wall cellulose complex shows the lowest off rate (with \( k_{\text{off}} \) of 0.0081 s\(^{-1}\)) and highest energy barrier (with \( E_b \) of 15.12 kcal mol\(^{-1}\)) and the CBM2a–extracted single cellulose microfibrils show the highest off rate (with \( k_{\text{off}} \) of 0.0099 s\(^{-1}\)) and lowest energy barrier (with \( E_b \) of 13.73 kcal mol\(^{-1}\)). The off rate of CBM–cellulose interactions measured in this study is much larger than that of streptavidin and biotin (1.67 \( \times \) 10\(^{-3}\) s\(^{-1}\)), which is one of the strongest protein–ligand interactions.\(^{43}\) Therefore this result shows a much weaker interaction between CBM and cellulose. As discussed previously about the free energy change, the larger CBM3a–cellulose contact area enhances the binding process although the differences are still not very significant.

Based on the above analysis, we conclude that the CBM3a molecule has slightly higher binding efficiency and affinity than those of CBM2a molecules to both natural and extracted cellulose surface at the single-molecule level. Furthermore, both CBM3a and CBM2a have higher affinities to the natural cell wall cellulose than those to the extracted single cellulose microfibrils. The bond lifetime analysis at different loading rates showed the detailed differences of the kinetic properties of these two CBMs at the single-molecule level. The same analysis method can be applied to other CBM–cellulose systems and reveal possible significances of different CBMs, as well as the details of different cellulose hydrolysis processes.

Conclusions

In this work, we studied the carbohydrate-binding module and plant cell wall cellulose interactions using a family 3 CBM and a family 2 CBM on both natural poplar plant cell walls and extracted single cellulose microfibrils. The CBM molecules are functionalized on a pre-coated AFM tip and the unbinding forces are measured using atomic force microscopy based single-molecule dynamic force spectroscopy (SMDFS). Different from the widely used traditional bulk experiments, we determined several dynamic and kinetic parameters, such as reconstructed free energy change, energy barrier, and bond lifetime, based on the measured single molecule unbinding forces, which were used to estimate the affinities of the CBMs to the natural and extracted cellulose microfibril surface from a single-molecule perspective. Generally, a CBM3a single molecule has a slightly higher binding efficiency and affinity than the CBM2a molecule to both natural and extracted cellulose surfaces. Both CBMs have higher affinities to the natural cell wall cellulose microfibrils compared to the extracted single cellulose microfibrils. As one of the most important carbohydrate–protein interactions, the CBM–cellulose interaction studied using SMDFS will offer a radical approach to provide more detailed information on not only the plant cell wall degradation but also on other single molecule interaction systems. Understanding the binding of CBM to the carbohydrate substrate is essential not only for its fundamental importance toward cell wall degradation but also as a motivation for designing new potential enzymes.

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