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Reversibility of Substrate Adsorption for the Cellulases Cel7A, Cel6A, and Cel7B from *Hypocrea jecorina*

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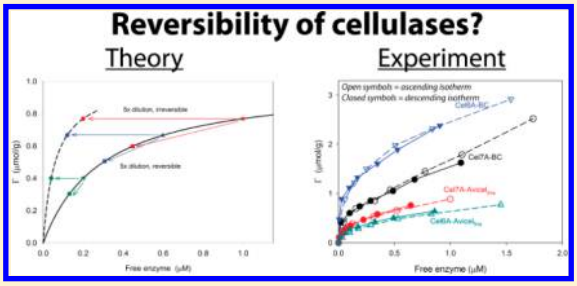
ABSTRACT: Adsorption of cellulases on the cellulose surface is an integral part of the catalytic mechanism, and a detailed description of the adsorption process is therefore required for a fundamental understanding of this industrially important class of enzymes. However, the mode of adsorption has proven intricate, and several key questions remain open. Perhaps most notably it is not clear whether the adsorbed enzyme is in dynamic equilibrium with the free population or irreversibly associated with no or slow dissociation. To address this, we have systematically investigated adsorption reversibility for two cellobiohydrolases (Cel7A and Cel6A) and one endoglucanase (Cel7B) on four types of pure cellulose substrates. Specifically, we monitored dilution-induced release of adsorbed enzyme in samples that had previously been brought to a steady state (constant concentration of free enzyme). In simple dilution experiments (without centrifugation), the results consistently showed full reversibility. In contrast to this, resuspension of enzyme−substrate pellets separated by centrifugation showed extensive irreversibility. We conclude that these enzymes are in a dynamic equilibrium between free and adsorbed states but suggest that changes in the physical properties of cellulose caused by compaction of the pellet hampers subsequent release of adsorbed enzyme. This latter effect may be pertinent to both previous controversies in the literature on adsorption reversibility and the development of enzyme recycling protocols in the biomass industry.



INTRODUCTION

Enzymatic hydrolysis of cellulose is an example of heterogeneous catalysis. It is an atypical example in the sense that it involves a diffusive catalyst (the cellulase) and one diffusive reactant (water), while the other reactant (cellulose) makes up the solid sorbent. Evidently, adsorption of the enzyme must precede catalysis, and many studies have addressed the adsorption of cellulases or their carbohydrate binding modules (CBMs) on different types of celluloses and biomass, both from kinetic and equilibrium points of view (see refs 1 and 2 for reviews). This work has uncovered intricate modes of interaction, and several key questions remain unresolved. Perhaps most noticeably, it is not clear whether adsorption is reversible or not. This question has a number of important ramifications ranging from the feasibility of enzyme recycling in industrial applications to strategies for kinetic modeling the enzymatic process. Regarding the latter, any model must in some way account for the adsorption process as an integral part of the catalytic mechanism, and modeling is made difficult if this step is poorly understood. The controversy regarding reversibility may be illustrated by work on the cellobiohydrolase Cel7A, the most thoroughly studied cellulase. Some reports have concluded that this enzyme (or its carbohydrate binding module, CBM) adsorbs irreversibly[3,4] or almost irreversibly[5] to pure cellulose. Other works have reached the opposite conclusion[6−9] and suggested full reversibility, while still other reports have concluded that the interaction is "partially reversible".[10,11] The molecular origin of the observed irreversibility remains to be fully elucidated, but some reports have suggested that it relies on either strong binding of the CBM or conformational changes in the enzyme.[5,10−12] Different degrees of reversibility, which have also been found for other cellulases than Cel7A,[3,8,12−16] likely relies in part on experimental challenges as well as differences in the structure and properties of the investigated substrates, but in some cases it is also related to vague definitions of "reversibility". From a stringent point of view, a reversible process is constituted of a succession of equilibrium states,[17] i.e., steps for which $\Delta G = 0$. Changes along any other path are irreversible. One consequence of this is that any spontaneous process ($\Delta G < 0$) is irreversible. This is true even if the process can be readily reverted in a subsequent step where conditions (temperature, pressure, or composition) are changed to favor the initial state. In protein adsorption studies, this stringent definition is not normally used and probably not practical. The pivotal point here is whether a dynamic equilibrium is established within the

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experimental time frame and not how it is reached. Consequently, experimental efforts usually focus on how readily adsorbed enzyme can be resolubilized for example following dilution with buffer. In this sense, full reversibility is achieved when the adsorption isotherm measured upon gradual increase of the enzyme concentration (the so-called ascending isotherm) superimposes the isotherm obtained by sequentially diluting a saturated sorbent (the descending isotherm).[18] Rigorously, superposition of the ascending and descending isotherms signifies the absence of hysteresis and not reversibility in the thermodynamic sense, but we will henceforth use the latter term (reversibility) to be in line with the vast majority of earlier literature in the area. This and its relationship to the current experiments are further illustrated in Figure 1.

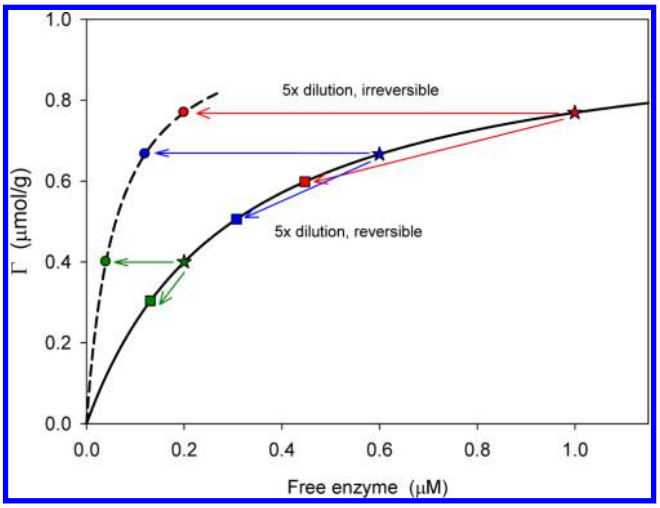

Figure 1. Illustration of how one-step dilution experiments with samples of different initial enzyme concentrations can be interpreted with respect to adsorption reversibility. The solid black line represents a Langmuir isotherm, eq 1, with the parameters Γ_{max} = 1 μmol/g and K_d = 0.3 μM. We consider three samples (star-shaped symbols) with initial free enzyme concentrations of respectively 1 μM (red), 0.6 μM (blue), and 0.2 μM (green). The substrate load in this example is 2 g/L, and all samples are diluted 5-fold. If the adsorption is irreversible, dilution will shift the point horizontally to the left (circles) and define a descending isotherm (dashed line), which is different from the ascending isotherm. For reversible adsorption, the points (squares) will be located on a lower position of the original isotherm, and the ascending and descending isotherms overlap. See text for more details.

In the current work we have investigated the reversibility of cellulose–cellulase interactions through comparisons of ascending and descending binding isotherms. We found that three of the most studied cellulases, Cel7A, Cel 6A, and Cel7B from *Hyrpocrea jerorina* (*Trichoderma reesei*), showed essentially full reversibility on different types of pure cellulose in simple dilution experiments. This suggests the interaction can be characterized as a dynamic equilibrium between a bound and a free enzyme population—at least within the time scales and concentration ranges studied here. We also found that centrifugation of the enzyme–cellulose complex caused significant loss of reversibility, and we speculate that this can be one of the reasons for the divisive literature on cellulase adsorption reversibility.

EXPERIMENTAL SECTION

Materials. All experiments were conducted in 50 mM acetate buffer, pH 5.0. Adsorption and dilution measurements were made in either 2 mL "Protein LoBind" Eppendorf tubes (Eppendorf AG, Hamburg, Germany) or 15 mL "SuperClear" centrifuge tubes (VWR, Leuven, Belgium). These vials were chosen after control experiments (without substrate) had shown that their adsorption of the investigated cellulases was small compared to the experimental scatter (in contrast to a number of other tested vials). The enzymes Cel7A, Cel6A, and Cel7B from *Hypocrea jecorina* were produced heterologously in *Aspergillus oryzae* as described previously.[19−22] Bacterial cellulose (BC) from from *Acetobacter xylinum* was purified from the commercial product "Nata de Coco" (Monika, Fitrite Incorporated, Novaliches Quezon City, Philippines) using the principles of Väljamäe et al.[23] as described elsewhere.[24] Three other types of substrate were made from Avicel PH-101 (Sigma-Aldrich). Avicel consists of microcrystalline cellulose with a crystallinity index I_{Cr} of 0.55−0.70[25,26] and a typical particle size (reported by the manufacturer) of 50 μm. One Avicel-based substrate, henceforth called Avicel$_{coarse}$, was made simply by suspending the powder in buffer. As we were interested in possible effects of particle size, we used a coaxial disperser (IKA ultra-Turrax T8, IKA-Werke GmbH, Staufen, Germany) to prepare Avicel suspensions with smaller particles. Specifically, 10 g/L Avicel$_{coarse}$ in buffer was cooled on ice and dispersed for 5 min at medium intensity (12 000 rpm). This dispersion has a nominal ultimate fineness around 10 μm, and in the following we will call the dispersed substrate, Avicel$_{fine}$. The third substrate based on Avicel PH-101 was regenerated amorphous cellulose (RAC). This was prepared according to a slightly modified[27] version of the method introduced by Zhang and Lynd.[28] All four substrate preparations have previously been characterized[29] with respect to their I_{Cr} and degree of polymerization (DP) using respectively solid-state ^{13}C cross-polarization/magic angle spinning (CP/MAS NMR)[30] and the phenol–sulfuric acid/bicinchoninate method.[31] The crystallinity index was 0.87 for BC, 0.60 for Avicel$_{coarse}$, 0.57 for Avicel$_{fine}$, and <0.05 for RAC. This suggests that RAC is essentially amorphous and that coaxial dispersion of Avicel has limited effect on its crystallinity. The number-average degree of polymerization fell in the range 180−240 glucopyranoside units per cellulose chain for all four substrate preparations studied here.

Enzyme Concentration. The concentration of enzyme in stock solutions was determined from $OD_{280\ nm}$ measurements using the extinction coefficients 86.8 mM^{-1} cm^{-1} for Cel7A, 96.6 mM^{-1} cm^{-1} for Cel6A, and 72.8 mM^{-1} cm^{-1} for Cel7B, which were calculated from the primary structure.[32] Measurements of $OD_{280\ nm}$ were not sufficiently sensitive for adsorption reversibility experiments, and instead we measured intrinsic fluorescence at 280 nm/345 nm (excitation/emission) in a Shimadzu RF-5301PC fluorometer and quantified the output against standard curves made daily in the same buffer.

Standard Dilution Assay. Reversibility of enzyme–substrate interactions was tested in a standard dilution assay, where substrate suspensions in eight vials were added enough enzyme stock to yield total enzyme concentrations (E_0) of respectively 0, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, and 3.00 μM. The load of substrate, S_0 (in g/L), was the same in the eight samples but varied between different trials and for the different types of substrate. Thus, based on preliminary measurements, S_0 was adjusted so that the ascending binding isotherm rolled off toward saturation for the highest studied values of E_0. Typical values for S_0 (after the addition of enzyme stock) were 5 g/L for Avicel$_{coarse}$, 2 g/L for Avicel$_{fine}$, and 0.5 g/L for BC and RAC. The total sample volume was 2000 μL. After the enzyme was added, the samples were mixed on a rotating wheel at 30 rpm for 30 min at room temperature (24 ± 1 °C). The choice of 30 min contact time was based on initial measurements of the adsorption of Cel7A to all four substrates, which showed that the concentration of free enzyme leveled off to a constant value within 3−15 min (depending on the substrate load and enzyme concentration). Based on this, it was concluded that 30 min contact time is long enough to establish a steady state condition (and short enough to ensure limited conversion

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of substrate; see below). Upon removal from the wheel, the suspensions were kept homogeneous through manual agitation, and a 500 μL subset was retrieved. The subset was centrifuged for 3 min at 14000*g*, and the concentration of enzyme in the supernatant was measured and used as the free enzyme concentration before dilution, E_{free}^{ascen} (see eq 4). The remaining 1500 μL was diluted with pure buffer (either 10 or 1.5 mL) and put back on the rotating wheel for 60 min. Finally, the concentration of enzyme in the supernatant of the diluted sample was measured fluorometrically and used as the free enzyme after dilution, $E_{free}^{descend}$ (see eq 5).

In addition to these buffer-dilution experiments we also tested reversibility of Cel7A/Avicel$_{fine}$ interactions in experiments where systems in steady state (i.e., after 30 min contact time) were perturbed by the addition of either more enzyme or substrate. The procedure was the same as above except that instead of diluting with pure buffer after 30 min contact time, we either added 100 μL of 10 μM Cel7A stock or 300 μL of 2 g/L Avicel$_{fine}$.

Dilution Assay with Centrifugation. In the standard dilution assay described above, centrifugation is only used to isolate supernatant (before or after dilution) for measurements of free enzyme concentrations. The enzyme−substrate complex is *not* exposed to centrifugation before the dilution. This is in contrast to many earlier works, which have assessed reversibility by resuspending pelleted cellulose with adsorbed enzyme. To assess possible effects of this, we also tested adsorption reversibility in pellet separated by centrifugation. The procedure followed the standard assay to the point where 2000 μL samples had been mixed for 30 min on the rotating wheel. The samples were then centrifuged for 3 min at 4000*g*, and the supernatant was gently removed and tested for its concentration of enzyme (E_{free}^{ascen}). The pellet was resuspended in 10 mL of buffer, equilibrated for 60 min on the rotating wheel, and tested for free enzyme ($E_{free}^{descend}$) as in the standard assay. We note that ascending isotherms produced either with or without centrifugation follow exactly the same protocol (differences only occur in the subsequent dilution steps). As a result, assessment of effects of centrifugation is based on pairs of identical samples diluted either with or without centrifugation. When such pairs are prepared with different enzyme concentrations the effect can be evaluated over the whole isotherm (cf. Figure 1).

Ion Chromatography. To assess the degree of cellulose conversion, we used ion chromatography with pulsed amperometric detection. Concentrations of soluble sugars were measured in supernatants from samples with the highest enzyme concentration (3 μM) in an ICS-5000 ion chromatograph equipped with a CarboPac PA-10 column and an electrochemical detector (Termo Fisher Scientific Waltham, MA). Samples were eluted with a multistep gradient with 50 mM NaOH (0−4 min), 100 mM sodium acetate + 90 mM NaOH (4−28 min), 450 mM sodium acetate + 200 mM NaOH (28−29 min), and 50 mM NaOH (29−35 min). The results were quantified against standards for glucose, cellobiose, and cellotriose run daily

Theory. To quantitatively illustrate reversibility in dilution experiments, we use the Langmuir isotherm for independent and thermodynamically identical sites. This simple isotherm has often been used to characterize cellulose−cellulase interactions, and we therefore use it as an example here. We stress, however, that the general conclusions regarding shifts or superposition of ascending and descending isotherms, which are discussed in this section, are valid for any adsorption mechanism.

The Langmuir isotherm may be written

$$\Gamma = \Gamma_{max}\frac{E_{free}}{K_d + E_{free}} \tag{1}$$

where Γ_{max} and K_d are the usual Langmuir parameters, i.e., respectively saturation coverage in μmol of enzyme bound per g of cellulose at saturation and the dissociation constant (in μM). The variables Γ and E_{free} are respectively the substrate coverage (μmol of enzyme/g) and the free enzyme concentration (in μM).

In the current context we are interested in dilution experiments, i.e., perturbation of an established adsorption equilibrium by the addition of one dose of buffer. The total concentration of enzyme in the diluted sample, E_0, can be readily calculated from the original and added volumes, and we may write a mass conservation for enzyme as

$$E_0 = E_{free} + E_{bound} \tag{2}$$

In eq 2, E_{bound} is the molar concentration of bound enzyme after dilution and it may be expressed $E_{bound} = \Gamma S_0$, where S_0 is the load of substrate (in g/L). If we insert this expression into eq 2 and combine with eq 1, we get a quadratic equation in E_{free} that relates the free enzyme concentration and the parameters K_d, Γ_{max}, E_0, and S_0, which are all known in a dilution experiment. The (physically meaningful) solution may be written

$$E_{free} = \frac{(K_d + \Gamma_{max}S_0 - E_0) - \sqrt{(K_d + \Gamma_{max}S_0 - E_0)^2 + 4E_0K_d}}{2} \tag{3}$$

Figure 1 exemplifies how eq 3 can be used to illustrate adsorption reversibility for one of the experimental protocols used here (simple dilution without centrifugation). Thus, it is shown how E_{free} and Γ would change upon a 5-fold dilution in the two extreme cases of either fully reversible or fully irreversible adsorption. In the example in Figure 1, the full line is the (ascending) isotherm calculated from eq 1 using Γ_{max} = 1 μmol/g and K_d = 0.3 μM, which are typical values for the adsorption of Cel7A on Avicel.[33] We now consider dilution of three equilibrated samples on this isotherm (indicated by stars) with initial free enzyme concentrations of respectively 1 μM (red), 0.6 μM (blue), and 0.2 μM (green). In this example the load of substrate was 2 g/L. If the interaction is irreversible, dilution of these samples will not change Γ (no release of enzyme). Hence, the only result of the dilution is a 5-fold reduction of E_{free}, and this is indicated by the horizontal arrows and circles in the figure. If this is done for a number of samples with different initial E_{free}, the shifted points will define a new line (dashed line in Figure 1), which is the descending isotherm for a 5-fold dilution experiment in the case of irreversible adsorption. If, on the other hand, the adsorption is fully reversible, E_{free} and Γ after dilution can be calculated from eqs 1 and 3. This is illustrated by sloping arrows and squares in Figure 1, and the most important result is that dilution establishes a new equilibrium condition at a lower position of the original (ascending) isotherm. If the descending isotherm falls between the two extremes in Figure 1, the adsorption is often described as "partially reversible". We will use this one-step dilution principle for several samples with different E_{free} to assess reversibility.

We emphasize that the overall interpretation of superposition and left shift, exemplified here for Langmuir adsorption, is valid regardless of the mode of interaction, and in the current work we will focus on an empirical analysis of reversibility based on these principles. Attempts to resolve the relevance of different adsorption models or the associated mathematical expressions for various cellulase−cellulose systems are beyond the current scope.

RESULTS

The coverage in μmol of enzyme/g of cellulose before dilution,Γ_{ascen}, was calculated as

$$\Gamma_{ascen} = \frac{E_0 - E_{free}^{ascend}}{S_0} \tag{4}$$

where E_{free}^{ascend} is the measured free enzyme concentration before dilution (see methods), and E_0 and S_0 are the total loads of respectively enzyme (μM) and cellulose (g/L). The coverage after dilution, $\Gamma_{descend}$ (descending isotherm), was calculated from the measured free enzyme concentration, $E_{free}^{descend}$, and three volumes. These are the initial sample volume, V_0, the volume of the subset, V_R, retrieved for measurement of E_{free}^{ascend} in the standard dilution assay, and the volume of buffer added in the dilution step, V_{add}. In the analysis of data from the assay

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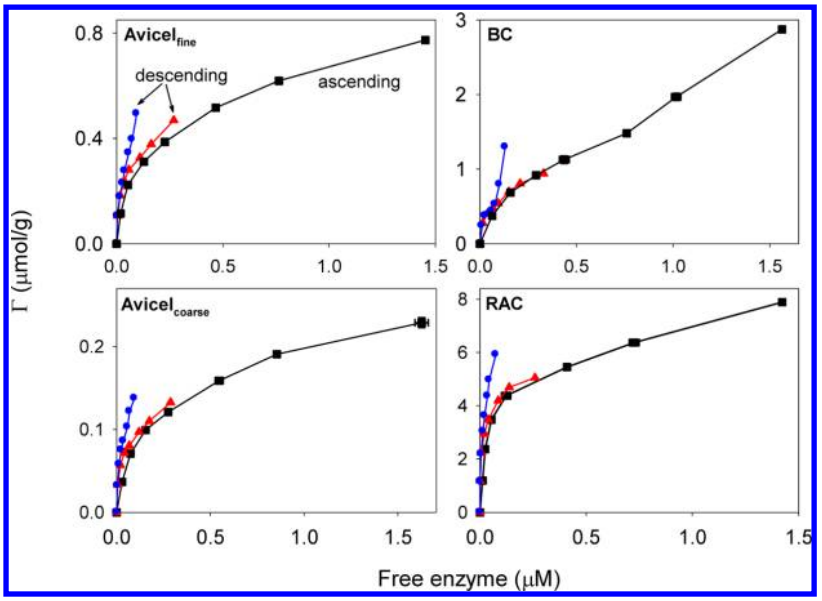

Figure 2. Adsorption reversibility for the cellobiohydrolase Cel7A on four kinds of cellulose: Avicel$_{fine}$, BC, Avicel$_{coarse}$, and RAC. Black squares show data for ascending isotherms, Γ_{ascend}. As the initial conditions where identical in experiments with and without centrifugation, points for the ascending isotherms represent the average of these experiments with (bidirectional) standard deviations. In many cases the standard deviations are comparable to the size of the symbol and difficult to see. Blue circles and red triangles identify descending isotherms, $\Gamma_{descend}$, for assays with and without centrifugation, respectively. The key observation is that $\Gamma_{descend}$ superimposes Γ_{ascend} for the simple assay without centrifugation (red) while $\Gamma_{descend}$ is shifted to the left when the enzyme–substrate complex was pelleted by centrifugation (blue).

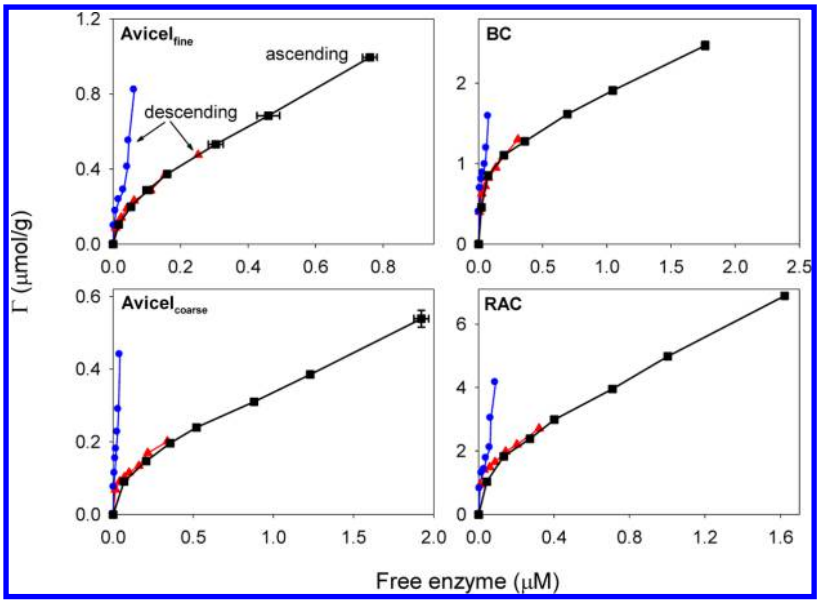

Figure 3. Adsorption reversibility for the cellobiohydrolase Cel6A on four kinds of cellulose: Avicel$_{fine}$, BC, Avicel$_{coarse}$, and RAC. The symbols are the same as in Figure 2.

with centrifugation we assumed that all cellulose was precipitated and that the entire bulk phase is removed with the supernatant (i.e., we neglected the small amount of buffer between the precipitated cellulose particles). Under these conditions $\Gamma_{descend}$ in the assay with centrifugation may be expressed as

$$\Gamma_{descend} = \frac{(E_0 - E_{free}^{ascend})V_0 - E_{free}^{descend}(V_0 + V_{add})}{S_0 V_0} \quad (5)$$

In the standard dilution assay (without centrifugation) some cellulose is removed from the sample together with the subset used for determination of E_{free}^{ascend}, and the expression for $\Gamma_{descend}$ becomes

$$\Gamma_{descend} = \frac{(V_0 - V_R)E_0 - (V_0 - V_R + V_{add})E_{free}^{descend}}{S_0(V_0 - V_R)} \quad (6)$$

We calculated Γ_{ascend} and $\Gamma_{descend}$ according to eqs 4–6 for Cel7A and Cel6A on all four substrates, and for Cel7B on RAC, Avicel$_{fine}$, and Avicel$_{coarse}$ (the Cel7B-BC system was not investigated as Cel7B is known to have low activity against this substrate), and plotted these functions against the free concentration of Cel7A (Figure 2), Cel6A (Figure 3), and Cel7B (Figure 4). In these figures each panel shows results for

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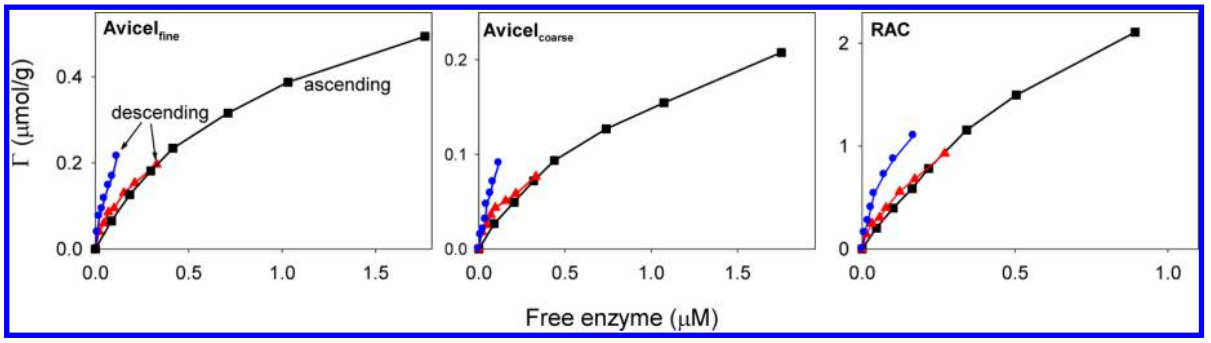

Figure 4. Adsorption reversibility for the endoglucanase Cel7B on three types of cellulose: Avicel$_{fine}$, Avicel$_{coarse}$, and RAC. The symbols are the same as in Figure 2.

one type of substrate. In Figures 2–4 the volumes V_0, V_R, and V_{add} were respectively 2.00 mL, 0.50 mL, and 10 mL, and this corresponds to a 5-fold dilution of the substrate in the experiments with centrifugation and a 6.7-fold dilution of the substrate in the simple dilution experiments. We also tested reversibility at moderate dilution levels for selected enzyme–substrate systems. In this case we only used the simple dilution protocol (no centrifugation), and the volumes V_0, V_R, and V_{add} were respectively 2.00 mL, 0.50 mL, and 1.50 mL (corresponding to a 2-fold dilution of the substrate).

Some noticeable trends were found by comparing the ascending and descending isotherms in Figures 2–4. Thus, all descending isotherms from the simple dilution assay (red triangles) superimposed the ascending isotherms (black squares). This signifies dynamic equilibrium between the adsorbed and free enzyme populations for the 11 investigated systems (cf. Figure 1). In contrast to this, descending isotherms from the assay using resuspension of centrifuged pellet (blue circles) were consistently steeper (i.e., shifted to the left) compared to the corresponding ascending curves (black). As illustrated in Figure 1, this implies (partial) irreversibility of the adsorption process, when the pellet had been separated by centrifugation.

The degree of reversibility in the assay with centrifugation varied for the three investigated enzymes. To asses this we first note that following centrifugation, essentially all free enzyme is removed with the supernatant before the pellet is resuspended. This means that in contrast to the example in Figure 1, the descending isotherm for a fully irreversible interaction will overlay the ordinate. (If there is no dissociation in the diluted sample, the free enzyme concentration will be zero except from a small contribution due to imperfect removal of the supernatant.) This is practically the situation for Cel6A–Avicel$_{coarse}$ in Figure 3, and we conclude that this interaction becomes fully irreversible upon centrifugation. The highest degree of reversibility for centrifuged samples was found for the Cel7B–RAC system (Figure 4). In this case the free enzyme concentration was over 60% of the value predicted from the ascending isotherm. In most other cases this value ranged from 10 to 30%, signifying a predominantly irreversible adsorption.

In addition to the comparisons of ascending and descending curves in Figures 2–4, it is worth noting that the specific binding capacity varies strongly with the physical properties of cellulose. As an example, RAC bound 20–30-fold more Cel7A compared to Avicel$_{coarse}$. This probably reflects differences in the specific accessible surface area of the substrates,[34] but the current data do not allow a detailed analysis.

Results from reversibility tests at moderate (2-fold) dilution are shown in Figure 5. This protocol has the advantage that

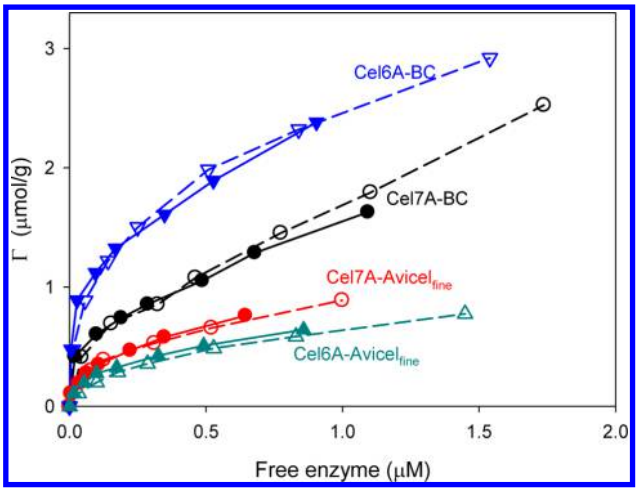

Figure 5. Reversibility of the adsorption for selected enzyme–substrate systems following 2-fold dilution in the standard assay with no centrifugation. Open symbols with dashed lines represent ascending isotherms, and closed symbols with full lines are descending isotherms.

ascending and descending data can be compared over a broader range of enzyme concentrations, and results in Figure 5 confirm the picture from Figures 2–4 as all investigated systems show accordance of ascending and descending data.

The pronounced effect of centrifugation seen in Figures 2–4 underscores that reversibility may depend on the experimental procedure, and in the light of this, we tested different means of perturbing steady-state systems. In Figures 2–4 the strategy was to shift the initial state by the addition of pure buffer, but the perturbation could equally well be brought about by the addition of either enzyme or substrate. We tested the latter two strategies for the Cel7A/Avicel$_{fine}$ system in the simple assay without centrifugation. Adding enzyme inevitably shifts both E_{free} and Γ to higher values (i.e., right and upward in the left panel of Figure 6), while adding more substrate shifts the points in the opposite direction. More importantly, it appeared that the points shifted by either perturbation (open symbols in Figure 6) always fell near the original ascending isotherm (closed symbols), and this implies reversibility also for these alternative means of perturbation.

The concentration of cellobiose in the samples with the highest enzyme load (3 μM) was measured by ion chromatography for all investigated enzyme–substrate systems

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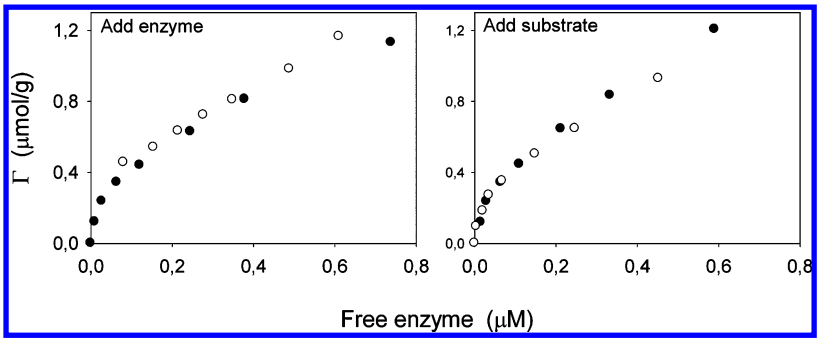

Figure 6. Reversibility of the interaction of Cel7A and Avicel$_{fine}$ tested by adding either more enzyme (left) or substrate (right) to a previously equilibrated enzyme−substrate mixture. This type of perturbation differs from the procedure underlying data in Figures 2−5, where the equilibrated system was diluted by pure buffer. Filled symbols show the original isotherm, and open symbols represent the new condition after the perturbation. It appears that adding enzyme shifts the points upward while adding substrate has the opposite effect. More importantly, these shifted points all remain near the original (ascending) isotherm, and this implies reversibility also for these types of perturbations.

both before dilution and after re-equilibration of the diluted samples (using the standard dilution assay). The degree of substrate conversion calculated from these measurements ranged from 7% for the Cel7B−RAC system to 0.5% for Cel7A−BMCC, with most cases between 1 and 2%. These values are small or comparable to the experimental scatter in the adsorption measurements, and we did not implement a correction in the substrate load but used the initial value, S_0, in the calculations of enzyme coverage (eqs 4−6).

DISCUSSION

Molecular descriptions of enzymatic hydrolysis of cellulose require consideration of a number of distinct reaction steps.[22,35−37] One key process is the association of the enzyme and its insoluble substrate, and in many cases this has been described along the lines of different equilibrium adsorption theories. While the analogy to conventional surface adsorption appears obvious several provisos remain. Most importantly, many studies have found that the interaction is irreversible or partially irreversible (see Introduction) and hence that equilibrium reaction schemes, which underlie simple adsorption models, are not justified. Second, cellulose−cellulase mixtures are reacting systems. This implies that the amount and structure of the sorbent change gradually and hence that equilibrium descriptions will be reasonable only on time scales that are short compared to these changes (and ultimately the full hydrolysis of the solid phase). A third caveat comes from the physical instability of the enzymes. Thus, like any protein conformation, cellulases are prone to irreversible denaturation, and this will complicate the description of the adsorption process. If, as suggested repeatedly,[5,10,12,38] non-native forms bind strongly to the cellulose surface, the concentration of free enzyme (which is the observable in most experiments) will be a complex function of both the affinity of native and denatured forms and the rate of the denaturation process. Indeed, this balance between affinity and stability has often been used to rationalize general aspects of nonspecific adsorption of proteins to solid surfaces.[18,39,40]

In the light of this we have made a systematic study of adsorption reversibility for short contact times where effects of substrate conversion and enzyme denaturation are as small as possible. The results consistently showed accordance of ascending and descending curves in experiments with simple dilution after 30 min. This means that within the experimental conditions used here (i.e., enzyme loads around 1 μM, substrate loads around 1 g/L, and time scales of about 1 h) the adsorption was fully reversible. In other words, there is a dynamic equilibrium between the populations of free and adsorbed enzyme. Indeed, irreversible adsorption is hard to reconcile with the general picture of a rapid initial adsorption followed by a steady state with constant free enzyme concentration which has been reported in many cases.[6,33,36,41−44] Thus, irreversibility implies very low desorption rate, and if the adsorption is relatively fast, as suggested by the rapid initial decline in free enzyme, one would clearly expect continuous buildup on the surface and a concomitant loss of free enzyme rather than a stable free concentration. This latter scenario with a slow, continuous adsorption has been described for cellulase mixtures hydrolyzing newspaper[45] and most recently for Cel7A and Cel7B attacking supported thin films of cellulose.[5] In the latter case, the interaction showed high reversibility for short contact times (minutes), but extensive irreversibility (70−90%) was found already after an hour at room temperature. A similar behavior with gradual buildup of an irreversibly bound population of Cel7A and Cel7B has also been found in a study using bacterial cellulose in suspension.[7] In this case, however, the loss of reversibility (at 30−40 °C) happened on a much slower time scale (days).

The results in Figures 2−4 consistently showed that descending isotherms made by resuspending centrifuged pellets were much steeper than the corresponding ascending isotherms. As illustrated in Figure 1, this is a hallmark of irreversibility. Hence, the shift to the left of the descending isotherms in these figures implies a limited release of adsorbed enzyme in diluted samples over the time scale studied here. As discussed in the Introduction, the precise term for this is hysteresis between the ascending and descending processes, but we have used the commonly accepted term, irreversibility, here. For the cellobiohydrolases Cel7A and Cel6A the results corresponded to a predominately irreversible interaction whereas the endoglucanase Cel7B showed moderate reversibility. We looked for a "dose−response relationship" with respect to the severity of centrifugation but did not find systematic differences between samples centrifuged for 3 min at respectively 1500*g*, 4000*g* (as in Figures 2−4), or 14000*g* (data not shown). Mechanistic analyses of the centrifugation-induced irreversibility await further investigation, but we speculate that compression of the pellet could change the substrate in a way that tends hamper or delay subsequent release of the enzyme. This interpretation may be seen as an analogy to so-called

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hornification that results from lowering the water content in cellulosic suspensions either by drying or wet-pressing. Thus, moderate dehydration of wood pulp (to solid contents above 40−50%) may lead to extensive and irreversible reduction in pore volume and a concomitant reduction in enzymatic digestibility.[46,47] If similar changes occur in pellets made by e.g. centrifugation or filtration,[16] this might impede the subsequent release of enzyme originally adsorbed in wider pores. We note, however, that centrifugation does not *per se* lead to lower surface accessibility because control experiments where the substrate was centrifuged prior to the addition of enzyme showed the same ascending binding isotherm as noncentrifuged substrate. Irrespectively of the molecular origin, the pronounced irreversibility resulting from centrifugation may be pertinent to both cellulase recycling protocols in biomass industries and attempts to reconcile discordant conclusions in earlier literature on cellulase adsorption (see Introduction). Regarding the former, it is clear that the simple cellulose substrates and short time scales studied here only provide information on some of the interactions that govern adsorption of cellulases onto complex biomass. Nevertheless, the results suggest a potential role of mechanical processing and emphasize that centrifugation and possibly other types of compaction could compromise recyclability of cellulases. With respect to deviant conclusions on reversibility in the literature, we note that many earlier works have used resuspension of centrifuged or filtered pellets in their experimental protocols. In the light of the current results it appears that such protocols may have negatively influenced reversibility. However, we emphasize that irreversible adsorption of cellulases has also been reported in simple dilution experiments. In particular, a number of recent studies on the adsorption to supported cellulose films have shown a considerable degree of nonreversibility.[5,10,15] Some of these works have reported a gradually growing population of irreversibly bound enzyme, and we suggest that further studies on the temporal development of reversibility in cellulose suspensions could be fruitful in attempts to understand the process in more detail.

Toward the end of this work, we became aware of a study by Jalak and Väljamäe[48] that suggested irreversibility at very low (nM) enzyme concentrations. Interestingly, enlargements of the dilute range of Figures 2−4 showed a similar behavior for both centrifuged and noncentrifuged samples although these effects were on the verge of the current experimental sensitivity. It appears, however, that future work specifically addressing this low concentration range might be rewarding for a better understanding of adsorption reversibility.

SUMMARY AND CONCLUSIONS

We found superposition of ascending and descending isotherms for the adsorption of three commonly studied cellulases on different types of pure cellulose substrates. The relationship between superposition of isotherms and reversibility was illustrated in Figure 1 for the simple case of Langmuir adsorption (eq 1), but superimposed isotherms signify reversibility in general regardless of the adsorption mechanism. Hence, the results imply that for short contact times (∼1 h) and moderate enzyme and substrate loads (about 1 μM and 1 g/L, respectively), the systems are in a state of dynamic equilibrium. This supports the validity of using simple equilibrium reaction schemes in kinetic models at least for the three cellulases studied here. Other investigations, discussed above, have suggested that this simplified interpretation may not be valid for long contact times and high degrees of conversion. The work also showed that reversibility was mainly lost upon centrifugation. We speculate that this could reflect structural changes such as pore collapse in the cellulose and that it could be one of the reasons for divisive conclusions on reversibility in the literature.

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Notes

The authors declare the following competing financial interest(s): One co-author works at Novozymes, which is a major producer of industrial enzymes.

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