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Free Energy Diagram for the Heterogeneous Enzymatic Hydrolysis of Glycosidic Bonds in Cellulose*

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Background: Heterogeneous enzyme catalysis is common but has rarely been rationalized through free energy diagrams. Results: The thermodynamic properties of stable and activated cellulase complexes are reported. Conclusion: The rate of enzyme-substrate complexation is entropy-controlled, whereas dissociation is controlled by enthalpy. Significance: Supposedly, this is the first elucidation of the transition states for the complexation and dissociation steps of a cellulase.

Kinetic and thermodynamic data have been analyzed according to transition state theory and a simplified reaction scheme for the enzymatic hydrolysis of insoluble cellulose. For the cellobiohydrolase Cel7A from *Hypocrea jecorina* (*Trichoderma reesei*), we were able to measure or collect relevant values for all stable and activated complexes defined by the reaction scheme and hence propose a free energy diagram for the full heterogeneous process. For other Cel7A enzymes, including variants with and without carbohydrate binding module (CBM), we obtained activation parameters for the association and dissociation of the enzyme-substrate complex. The results showed that the kinetics of enzyme-substrate association (*i.e.*, formation of the Michaelis complex) was almost entirely entropy-controlled and that the activation entropy corresponded approximately to the loss of translational and rotational degrees of freedom of the dissolved enzyme. This implied that the transition state occurred early in the pathway where the enzyme has lost these degrees of freedom but not yet established extensive contacts in the binding tunnel. For dissociation, a similar analysis suggested that the transition state was late in the path where most enzyme-substrate contacts were broken. Activation enthalpies revealed that the rate of dissociation was far more temperature-sensitive than the rates of both association and the inner catalytic cycle. Comparisons of one- and two-domain variants showed that the CBM had no influence on the transition state for association but increased the free energy barrier for dissociation. Hence, the CBM appeared to promote the stability of the complex by delaying dissociation rather than accelerating association.

Free energy diagrams are undoubtedly one of the most useful and fundamental ways to illustrate and explain enzyme catalysis, and essentially any biochemistry textbook uses profiles of free energy and reaction coordinates to introduce enzyme function. However, in the case of enzyme activity at interfaces (*i.e.*, heterogeneous enzyme catalysis), free energy diagrams have rarely been used. This may appear surprising in light of the common use of such diagrams within related fields, including catalysis in inorganic interfaces, and further analyses of free energy barriers appear promising in mechanistic studies of interfacial enzyme catalysis, which is common both in vivo (1) and in technology (2, 3). In the current work, we present free energy profiles for the enzymatic hydrolysis of cellulose with special emphasis on activation parameters for complexation and dissociation of enzyme and the insoluble substrate. Specifically, we investigated cellobiohydrolases from the glycoside hydrolase family 7. These enzymes utilize the retaining catalytic mechanism, which is similar to that seen in many other glycoside hydrolase families and understood on an atomistic level (4, 5). Cellobiohydrolases have recently attracted particular research interest, which is at least in part driven by their role in different present day challenges, including the distribution of carbon between soil and atmosphere (6, 7) and the utilization lignocellulosic feedstock in sustainable industries (8). Here, we examine the wild-type and some variants of two cellobiohydrolases (both called Cel7A) from *Hypocrea jecorina* and *Rasamsonia emersonii*.

One key element of the aforementioned retaining mechanism is a transient glycosyl enzyme intermediate that is flanked by oxocarbenium ion-like transition states (4), and the properties of these species for the specific case of Cel7A hydrolyzing cellulose have recently been discussed on the basis of different types of molecular modeling (9–14). This theoretical work has provided important information on the structure and processive movement of the cellulose strand in the 5-nm-long binding tunnel, which houses the active site, and suggested (12, 14) that formation of the intermediate (so-called glycosylation) is the rate-limiting step in the catalytic reaction sequence. One study computed a catalytic rate constant around 11 s⁻¹ for glycosylation (14), and this is in line with experimental attempts to single out $k_{cat}$ for Cel7A acting on insoluble cellulose (15–18). In most cases, however, the maximal specific rate at quasi-steady state for a monocomponent cellobiohydrolase hydrolyzing insoluble cellulose at room temperature is 1 or 2 orders of magnitude lower than this (16, 19, 20). This was confirmed in the companion article (71), which showed maximal specific

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Free Energy Diagram for Cellobiohydrolase Cel7A

rates, \( p V_{\text{max}}/E_0 \) (subscript p in front of the parameter indicates its relationship to the processive Scheme 1 in the companion article), of about 0.1 s\(^{-1}\) at room temperature for the investigated Cel7A enzymes. The divergence between \( k_{\text{cat}} \) (defined as the turnover of the Michaelis complex in the inner catalytic cycle) and the maximal steady state rate probably relies on the heterogeneous process for Cel7A, which in addition to the catalytic steps that parallel those seen for glycoside hydrolases acting on soluble substrates includes adsorption to and dissociation from the insoluble substrate. Thus, before reaching the Michaelis complex, the enzyme must adsorb to the interface, diffuse laterally to find an attack site, and translocate a sizable piece of the cellulose strand (~10 glucosepyranose units) from the crystalline substrate into the binding tunnel. Different arguments have been made that this multifaceted association process or its reversal (dissociation) at the end of a processive run could be much slower than the inner catalytic cycle and hence that the on- or off-rate, rather than the actual catalysis, could be rate-limiting for cellobiohydrolases (16, 20–25). This latter interpretation would reconcile the disparity of rate-limiting for cellobiohydrolases (16, 20–25). This latter could be much slower than the inner catalytic cycle and hence the maximal stead state rate probably relies on the overall reaction rate at quasi-steady state. Unfortunately, molecular details of the association and dissociation steps are not nearly as well understood as the catalytic cycle. This is at least in part because adsorbed species cannot be crystallized and hence structural changes along the adsorption (and desorption) reaction path remain mostly unknown.

To elucidate some aspects of the association and dissociation steps, we combined kinetic and thermodynamic data from the companion article (71), additional measurements reported here, and literature data and analyzed these data along the lines of the transition state theory. Based on this, we have proposed a free energy diagram for the course of the heterogeneous catalysis and estimated enthalpic and entropic contributions to the free energy changes. As indicated above, structural and kinetic information on adsorption and desorption is still too elusive to design and analyze a full heterogeneous reaction scheme composed by elementary reactions (akin to those describing the catalytic cycle (14)). Instead, we used a coarser approach, which merges some putative steps and describes processive catalysis by three composite rate constants and a processivity number (26, 27). Incomplete models such as this have previously proven useful in attempts to assess properties of stable and activated complexes along the reaction path of complicated enzyme reactions (28–30), although exact correlations between free energy and structure obviously cannot be established when elementary steps have been lumped together in composite reactions.

The analysis showed that complexation (formation of the Michaelis complex) and dissociation of Cel7A were limited by comparable free energy barriers but that the balance of entropic and enthalpic contributions was very different for these two steps. This provided some fundamental information on the nature of the transition states, the role of the carbohydrate binding domain (CBM),\(^2\) and the sensitivity to temperature of these two steps.

\(^2\) The abbreviations used are: CBM, carbohydrate binding domain; CORE, catalytic domain; PS5, pre-steady state; \( H_{\text{CBM}} \), cellobiohydrolase Cel7A from \( H.\

Experimental Procedures

Enzymes—We used the same cellobiohydrolases as in the companion article (71), and procedures regarding cloning, expression, purification, and concentration determination are described there. The enzymes were Cel7A wild types from, respectively, \( R.\) emersonii (\( Re \)) and \( H.\) jecorina (\( Hj \)) (often identified by the name of its anamorph, \( Trichoderma reesei \)). The latter is a two-domain enzyme with a catalytic domain (CORE) module and a CBM, whereas the former has a one-domain architecture with no CBM. We also investigated a one-domain variant of the \( Hj\) enzyme without linker and CBM and a chimera composed of the \( Re\) enzyme and the linker and CBM from \( Hj\) Cel7A. In the following, we will use the same abbreviations as in the companion article (71) for these four enzymes, i.e. \( Hj_{\text{CORE}}, Re_{\text{CORE}}, Hj_{\text{CORE}}\), and \( Re_{\text{CBM}}\), respectively.

Product Profile—To estimate processivity, we measured the concentrations of products generated by the four enzymes at temperatures from 10 to 50 °C (i.e. the same temperature range used for the kinetic measurements in the companion article (71)). The reactions were started by the addition of 80 μl of enzyme stock solution to 920 μl of preheated or -cooled Avicel solution in 2-ml Eppendorf tubes. The final enzyme concentration was 0.40 μM as in the kinetic experiments in the companion article (71). All samples were incubated for 1 h with orbital shaking at 1100 rpm and subsequently quenched by 100 μl of 1.0 M NaOH. Following centrifugation for 3 min at 3500 rpm at 5 °C (Hereaus Multifuge 3 S-R), the concentrations of glucose, cellobiose, and cellotriose in the supernatants were quantified in a Dionex ICS-5000 ion chromatograph (Thermo Fisher Scientific, Waltham, MA). Details of the chromatographic procedure have been described previously (31). A blank (without enzyme) was included and subtracted for all measurements.

Pre-steady State Kinetics—To elucidate the temperature dependence of the catalytic rate constant, \( k_{\text{cat}} \), we investigated the pre-steady state kinetics of \( Hj_{\text{CBM}} \) on Avicel (the same substrate as in the companion article (71)). We measured the production of cellobiose in real time with an amperometric biosensor functionalized with the oxidoreductase cellobiose dehydrogenase from \( \text{Phanerochaete chrysosporium} \) (32) and analyzed the results as described previously (15, 27). We used the experimental temperatures 5, 25, and 50 °C, and the enzyme concentration was 150 nM. Duplicate measurements were performed with 5–10 different Avicel loads between 0.5 and 5 g/ liter at the three temperatures.

Results

The aim of this work was to elucidate changes in free energy, enthalpy, and entropy of stable and activated complexes along a previously suggested reaction path for processive cellulases (26, 27). For the current use, we made the additional assumption that consecutive processive steps will be thermodynamically similar, and we hence only include one catalytic step in the diagram. This means that the reaction sequence we want to describe may be written as shown in Scheme 1.
The catalytic step incorporates the hydrolytic reaction, which is the rate-determining step of the reaction. The rate constants for these steps can be derived from the kinetic parameters and the temperature dependence of the rate constants can be calculated using the Arrhenius equation.

\[
\ln k = E_A / R \times (1 / T) + \ln A
\]

where \(E_A\) is the activation energy, \(R\) is the gas constant, and \(A\) is the pre-exponential factor. The rate constants for the association and dissociation steps can be calculated using similar equations.

The main purpose of measuring processivities in the current context is to determine the processivity of enzymes with a CBM around room temperature. The processivity is defined as the number of cellobiose units hydrolyzed per substrate binding event. The processivity number, \(n_p\), is related to the rate constants \(k_{cat}\) and \(k_{off}\) by the following equation:

\[
n_p = k_{cat} / k_{off}
\]

where \(k_{cat}\) is the forward rate constant and \(k_{off}\) is the reverse rate constant. The processivity can be plotted as a function of temperature, as shown in Fig. 2.

The processivity shows little dependence on temperature for the exocellulase Cel48A from Thermobifida fusca, which has a CBM. However, enzymes with a CBM have moderately higher processivity than their one-domain analogs.

### Free Energy Diagram for Cellobiohydrolase Cel7A

The free energy diagram for Cellobiohydrolase Cel7A is shown in Fig. 1. The diagram illustrates the energy profile of the enzymatic reaction, showing the free energy changes at each step. The free energy changes are calculated using the following equations:

\[
\Delta G = -RT \ln \left( \frac{k_B T}{k_B}\right)
\]

where \(k_B\) is the Boltzmann constant, \(T\) is the temperature, and \(R\) is the gas constant.

The free energy diagram shows that the reaction proceeds through a series of conformational changes, including adsorption to the surface, binding of the substrate, and hydrolysis. The reaction is exergonic, with a negative free energy change.

### FIGURE 2. Temperature dependence of the rate constants \(k_{cat}\) (left ordinate) and \(k_{off}\) (right ordinate) calculated from Equations 1 and 2 using data from the companion article (71). Triangles identify enzymes with a CBM, and circles are for one-domain variants.

In summary, the study provides a detailed analysis of the enzymatic reaction of Cellobiohydrolase Cel7A, including the energy profile, rate constants, and processivity. The results are consistent with earlier studies and provide insights into the mechanism of cellulose degradation by this enzyme.
of a transition state in a diffusion-controlled association process is outside the original scope of the theory. However, it has been argued that transition state theory may be applied to protein-protein association in solution (41, 42), and in the current work, we used a similar approach for the adsorption on the substrate. Equation 3 is the simplified form of the Eyring equation, which assumes that the transmission coefficient, $\kappa$, is 1, and this means that the extrathermodynamic contribution to the activation free energy, $\Delta G_{\text{extra}}$, is neglected. It appears from Equations 1 and 2 that both $k_{\text{on}}$ and $k_{\text{off}}$ are inversely proportional to $n$, and these rate constants (Fig. 2) are therefore sensitive to possible systematic errors in the processivity measurements, which were mentioned above. However, the logarithmic conversion to $\Delta G^\circ$ in Equation 3 tends to dampen the effect of this, and even if the processivity number was wrong by as much as a factor of 2, it would only change $\Delta G^\circ$ by about 1.7 kJ/mol. As comparisons of current $n$ values and earlier measurements (see above) suggested quite good accordance, imprecise $n$ values do not appear to be a major cause of error on the activation parameters. Transition state free energies calculated under the assumption that $\Delta G_{\text{extra}} = 0$ (sometimes called phenomenological activation free energies (43)) for association ($\Delta G^\circ_{\text{assoc}}$) and dissociation ($\Delta G^\circ_{\text{dissoc}}$) are plotted as a function of temperature for each of the four investigated enzymes in Fig. 3.

The results in Fig. 3 show that the CBM had no effect on $\Delta G^\circ_{\text{assoc}}$. Specifically, the average difference in $\Delta G^\circ_{\text{assoc}}$ between one- and two-domain enzymes at the same temperature was $0.3 \pm 0.7$ kJ/mol. Similarly, we did not detect significant differences in $\Delta G^\circ_{\text{dissoc}}$ when comparing enzymes from H. jecorina and R. emersonii (all association data in Fig. 3 overlap). It follows that neither the CBM nor natural adaptation to higher temperatures appeared to affect the free energy barrier of association in this study. For dissociation, however, we found a small but significant increase in activation free energy for enzymes with CBM ($\Delta G^\circ_{\text{dissoc}}$ was $2.6 \pm 0.8$ kJ/mol higher on average for two-domain enzymes in Fig. 3). The most conspicuous result in Fig. 3 was a much stronger temperature dependence of $\Delta G^\circ_{\text{assoc}}$ compared with $\Delta G^\circ_{\text{dissoc}}$. To further elucidate this, we plotted $\ln(h_{\Delta G^\circ/k_B T})$ against $1/T$ in Fig. 4. This is a so-called Eyring plot, which has a slope of $-\Delta H^\circ/R$ where $\Delta H^\circ$ is the activation enthalpy as defined in transition state theory. It appears that the (linear) Eyring plots in Fig. 4 were much steeper for dissociation compared with association. The shallow slope for association meant that that $\Delta H^\circ_{\text{assoc}}$ was very low ($15–20$ kJ/mol) with no distinctive differences between the four investigated enzymes. This similarity between the different enzyme variants suggested that the CBM did not affect the enthalpy of the transition state for the association step. For the dissociation process, the transition state enthalpies were much higher with $\Delta H^\circ_{\text{dissoc}}$ values between 60 and 70 kJ/mol. In this case, a moderate effect of the CBM could be singled out because $\Delta H^\circ_{\text{dissoc}}$ for enzymes with a CBM was $5–10$ kJ/mol higher than for the corresponding one-domain enzymes. Based on the above estimates of, respectively, free energies and enthalpies of activation, the entropic contributions $T\Delta S^\circ_{\text{assoc}} = \Delta H^\circ_{\text{assoc}} - \Delta G^\circ_{\text{assoc}}$ and $T\Delta S^\circ_{\text{dissoc}} = \Delta H^\circ_{\text{dissoc}} - \Delta G^\circ_{\text{dissoc}}$ were calculated. These results consistently showed that this entropic contribution was large and negative ($-70$ to $-80$ kJ/mol) for association and quite small ($-20$ to $-25$ kJ/mol) for dissociation.

Catalysis—Previous work has suggested that the catalytic rate constant for Cel7A acting on insoluble substrate is $5–10$ s$^{-1}$ around room temperature (14–18). Surprisingly, the temperature dependence of this parameter has not been reported previously for processive cellulases. Many studies have investigated the temperature dependence of the hydrolytic rate, but as argued above, the actual catalysis is unlikely to be rate-limiting under most conditions, and it follows that changes in the observed overall rate do not necessarily provide any insight into
we found $k_{\text{cat}}$ values of $1.6 \pm 0.2$ and $10.2 \pm 2.1 \text{ s}^{-1}$, respectively. Independent regressions were conducted for duplicate measurements at 5–10 different substrate loads, and the stated $k_{\text{cat}}$ values are averages and standard deviations for all regressions at a given temperature. We have previously analyzed correlation coefficients for this type of regression for room temperature data (15, 16) and found a moderate mutual interdependence of $k_{\text{cat}}$ and $k_{\text{on}}$. This correlation became worse at higher temperature, and this was a main cause of the larger standard deviation at 50 °C. Insertion of $k_{\text{cat}}$ values in Equation 3 showed that the free energy of activation for the catalytic step, $\Delta G_{\text{cat}}^{\text{act}}$, increased linearly from $67 \pm 0.6 \text{ kJ/mol}$ at 5 °C to $73 \pm 0.8 \text{ kJ/mol}$ at 50 °C. An Eyring plot of the catalytic rate constants (not shown) was near linear ($R^2 = 0.97$) and gave an activation enthalpy, $\Delta H_{\text{cat}}^{\text{act}}$ of $29 \pm 5 \text{ kJ/mol}$, and the entropic contribution at room temperature, $T \Delta S_{\text{cat}}^{\text{act}} = \Delta H_{\text{cat}}^{\text{act}} - \Delta G_{\text{cat}}^{\text{act}}$, was therefore about $-40 \text{ kJ/mol}$.

Thermodynamic Data—Thermodynamic information on adsorption and hydrolysis is also required for the diagram (see Fig. 6). The net affinity of Cel7A for insoluble cellulose has been investigated in several earlier studies. Most recently, Jalak and Väljamäe (45) suggested a multimode binding of Cel7A to crystalline cellulose and reported dissociation constants, $K_p$, of 3 and 400 nM, respectively, for two binding modes, which showed complete or partial substrate occupancy of the active tunnel. Other studies using a two-site Langmuir model have found dissociation constants in the 10–200 nM range for the high affinity site (18, 46–48), and the affinity of long cellobioigosaccharides for a catalytically deficient variant of Cel7A was also in this range (49). Hence, the best estimate for a dissociation constant of the Michaelis complex for Cel7A appears to be in the low to mid nM range. Using $\Delta G_{\text{assoc}}^{\text{act}} = -RT \ln(1/K_p)$, this translates into a standard free energy changes of association, $\Delta G_{\text{assoc}}^{\text{act}}$, of $-40$ to $-45 \text{ kJ/mol}$. The enthalpy change for the association process, $\Delta H_{\text{assoc}}^{\text{act}}$, can be estimated by a Van’t Hoff analysis (i.e. plots of $\ln(K(T))$ versus $1/T$) using either $K_{\text{on}}$ or $K_{\text{off}}$, which are reported in the companion article (71). This approach suggested $\Delta H_{\text{assoc}}^{\text{act}}$ values of $-40$ to $-50 \text{ kJ/mol}$, and a similar value was found by direct (calorimetric) measurements of $\Delta H$ for the binding of celloctaose to H. jecorina Cel7A around room temperature (49). As the values of $\Delta G_{\text{assoc}}^{\text{act}}$ and $\Delta H_{\text{assoc}}^{\text{act}}$ are comparable in size, the entropic contribution to association, $T \Delta S_{\text{assoc}}^{\text{act}} = \Delta H_{\text{assoc}}^{\text{act}} - \Delta G_{\text{assoc}}^{\text{act}}$, at room temperature must be close to zero. The last process we need to consider is the hydrolysis of the glycosidic bond. The enthalpy change for the hydrolysis of glycosidic bonds in Avicel is weakly exothermic with a $\Delta H_{\text{hydrolyd}}$ of about $-4 \text{ kJ/mol}$ (50). We are not aware of any experimental data on the standard free energy change, $\Delta G_{\text{hydrolyd}}^{\text{act}}$, for the conversion of Avicel (or any other type of cellulose) into cellobiose. Goldberg and co-workers (51) found that $\Delta G_{\text{hydrolyd}}^{\text{act}}$ for the $\beta$-1,4-glycosidic bond in cellobiose was about $16 \text{ kJ/mol}$ at room temperature and further reported that the free energy change per hydrolyzed glucosidic bond was in general quite constant in longer oligosaccharides. Based on this, we use $\Delta G_{\text{hydrolyd}}^{\text{act}} = -16 \text{ kJ/mol}$ in the diagram below. The lack of direct measurements of this parameter obviously puts some provisos on the interpretation of this step, but we note
that the value of $\Delta G_{\mathrm{hydro}}^0$ would have to be very different before it would affect any of the interpretations.

**Discussion**

For the wild type $H_j{\text{CBM}}$, we now have room temperature estimates of 15 parameters characterizing stable and activated complexes defined by Scheme 1, and this is illustrated in the diagram in Fig. 6. For the other enzymes, $R_p{\text{CORE}}, R_p{\text{CBM}}$ and $H_j{\text{CORE}}$, we have data for association and dissociation (but not catalysis), and we will return to a comparative discussion of this below. First, we discuss some aspects of the diagram for $H_j{\text{CBM}}$.

**Association**—The Michaelis complex ($EC_m$) has almost the same entropy but a much lower enthalpy than the free enzyme and substrate ($E + C_m$), and enthalpy is hence the main driving force for association around room temperature (Fig. 6). Kinetically, association is governed by a sizable activation free energy ($\Delta G_{\mathrm{act}}^a \approx 92 \text{ kJ/mol}$), which is dominated by the entropic contribution ($-T\Delta S_{\mathrm{act}}^a \approx 77 \text{ kJ/mol}$; note that to facilitate visual comparisons of enthalpic and entropic contributions in Fig. 6, the latter are given as their negative values, $-T\Delta S$). Interestingly, estimates based on the principles proposed in the seminal study by of Page and Jencks (52) suggest that this $-T\Delta S_{\mathrm{act}}^a$ value is comparable with the entropic penalty associated with losses of rotational and translational degrees of freedom of the enzyme. The exact entropic cost of freezing out these degrees of freedom has been debated subsequently (53–56), but most values fall in this range. This coincidence suggests that the transition state is located early in the path of complexation where the associated enzyme has lost these degrees of freedom but not yet established extensive contacts with the cellulose strand in the binding tunnel. These contacts are associated with comprehensive hydrogen bonding (14, 57, 58) and dehydration of hydrophobic surfaces (49), which will tend to lower both the enthalpic (red trace) and entropic (green trace) contributions in Fig. 6. We suggest that these contacts are established later in the path of association as the strand moves forward and gradually fills the binding tunnel and thus brings the enthalpy to a large negative value and $-T\Delta S$ back to approximately zero (Fig. 6). The interpretation of a transition state early in the path of association is also in accord with the low activation enthalpy, $\Delta H_{\mathrm{act}}^a$ as little rearrangement of hydrogen bonding (and other interactions) has occurred at this stage. A probable structural interpretation could be that the transition state of association is the initial threading where the end of the cellulose strand must be dislodged from the crystal and moved into the mouth of the binding tunnel. Previous work also supports the notion that early complexes in the association path could be rather unstable. Thus, the work required to abstract a glucopyranose unit from crystalline cellulose is quite high (5–14 kJ/mol depending on position and crystal structure), whereas the affinity in the first part of the catalytic tunnel only contributes $-3$ to $-7$ kJ/mol per glucopyranose unit to the standard free energy of binding (49, 59). These numbers underscore that contact interactions in the binding tunnel are unlikely to compensate for the sizable loss in translational entropy until late in the association.
path where the strongly interacting subsites near the exit of the tunnel (49, 57, 60) become filled. One interesting aspect of an early transition state governed by entropy is that $k_{\text{on}}$ is expected to be quite insensitive to interactions in the tunnel (as most of these interactions are established during the downhill part of the free energy trace subsequent to the transition state).

As a result, protein engineering that modifies such interactions is predicted to exert only moderate changes of $k_{\text{on}}$. This prediction was confirmed in a recent study of a Cel7A mutant where a tryptophan in the middle of the tunnel had been replaced by alanine. This enzyme variant showed a markedly reduced affinity for the substrate, but the reduction was almost entirely due to a faster rate of dissociation, whereas $k_{\text{on}}$ only decreased by about 27% (61).

**Catalysis**—The free energy barrier for catalysis was about 69 kJ/mol at room temperature, and this is in good accordance with theoretical studies, which have found $\Delta G_{\text{cat}}$ values of 65 and 73 kJ/mol (12, 14). The entropic contribution was dominating ($-\Delta S_{\text{cat}} = 40$ kJ/mol), although $\Delta H_{\text{cat}}$ was only moderately smaller (29 kJ/mol). Unlike for association and dissociation, the free energy landscape for the inner catalytic cycle of Cel7A has been extensively studied on an atomic level (9–14). Comparisons of these results and the current observation of a dominant entropic contribution may hint that fixation of the Glu-212 side chain and strain in the substrate chain around subsite −1 could be important destabilizing effects in the transition state. However, proper complementation of the comprehensive theoretical work on catalysis awaits more sophisticated experimental approaches such as measurements of kinetic isotope effects or linear free energy relationships (62–65).

**Dissociation**—The free energy barrier of dissociation, $\Delta G_{\text{dissoc}} \approx 87$ kJ/mol, was comparable with the barrier of association, but in sharp contrast to association, it was dominated by the enthalpic contribution, $\Delta H_{\text{dissoc}} \approx 58$ kJ/mol. This together with the observation that the enthalpy of the transition state for dissociation (last maximum in the red trace in Fig. 6) was on the same level as the dissociated components suggests that the transition state is located late in the dissociation path with few remaining enzyme-substrate contacts. One direct consequence of the comparably high $\Delta H_{\text{dissoc}}$ is that dissociation is accelerated much more by increasing temperatures than the two other steps. To illustrate this, we calculated activation energies, $E_a = \Delta H^a + RT$, and inserted the results in the Arrhenius equation. This showed that changing from room temperature (where many activity studies are conducted) to the more industrially relevant 50 °C increases the rate constants for association ($k_{\text{on}}$), catalysis ($k_{\text{cat}}$), and dissociation ($k_{\text{off}}$) by factors of 1.7, 2.6, and 6.7, respectively. This strong preferential activation of dissociation may lead to unexpected kinetic changes when the temperature is raised, and some aspects of this were discussed in the companion article (71). Preferential temperature activation of dissociation has also been observed for a β-glucosidase acting on soluble substrate, and in this case, it led to a change in the rate-determining step around 20 °C (66). Below this temperature, dissociation of the enzyme-substrate complex was rate-limiting, but at higher temperatures, formation of the complex became the bottleneck. This parallels the rapid growth in the dissociation rate found here, although we did not see evidence of a change in the rate-limiting step. The late location of the transition state for dissociation implies that most enzyme-substrate contacts must be broken to reach it, and it follows that $k_{\text{off}}$ is predicted to depend strongly on these interactions. This is in contrast to the entropy-controlled $k_{\text{on}}$ (discussed above), which was suggested to be less dependent on the extent of substrate contact. This predicted behavior of $k_{\text{on}}$ and $k_{\text{off}}$ was confirmed both in comparisons of enzymes with and without CBM in the current work and in an earlier study of a mutant with weakened substrate interactions in the binding tunnel (61). In both cases, deletion of substrate interactions led to a distinctive rise in $k_{\text{off}}$ and little or no change in $k_{\text{on}}$. This suggests that $k_{\text{off}}$ may be tuned in a controlled way through engineering of targets such as the binding tunnel, loops, CBM, or linker glycans, which have recently also been shown to play a role in substrate interactions (67). This contribution from glycans to substrate interactions also points toward relevance of the organism used in heterologous expression. Thus, variation in the glycosylation pattern of Cel7 enzymes expressed in different organisms has previously been shown to exert a marked effect on enzyme efficiency (68, 69), and this could potentially rely on interactions that reduce $k_{\text{off}}$.

**Comparative Analysis of Different Enzymes**—The data in Figs. 2 and 3 provide activation parameters for association and dissociation for all four enzymes, $H_{\text{CBM}}$, $R_{\text{CORE}}$, $H_{\text{OFF}}$, and $R_{\text{CBM}}$, and comparative analysis of this appears to be of interest particularly regarding possible roles of the CBM. For association, the results showed that the binding module did not change any of the measured activation parameters. This strongly suggests that the transition state of association was unaffected by CBM-cellobiose interactions. For dissociation, however, we found that the CBM increased the free energy barrier by 3–4 kJ/mol (Fig. 2) and that this difference was due to an increased enthalpic contribution (Fig. 3). This reflects that $k_{\text{off}}$, but not $k_{\text{on}}$, was affected by the CBM, and as the stability constant of the enzyme-substrate complex is the ratio of the on- and off-rate constants, these observations showed that the well-known increment in substrate affinity generated by the CBM (48, 70) is the result of slower dissociation, whereas the rate of association remained mostly unaffected by the CBM. The difference in the standard free energy of association ($\Delta G_{\text{assoc}}$) for one- and two-domain enzymes derived from the current data was about 4 kJ/mol, and this affinity contribution of the CBM corresponds to earlier reports (48, 61).

**Conclusions**—We have used kinetic and thermodynamic data to construct a free energy diagram for the hydrolysis of a β-1,4-glycosidic bond in insoluble cellulose and assessed enthalpic and entropic contributions to the activation free energies. The diagram was based on a reaction scheme that separates the complex heterogeneous process into three basic steps: association, catalysis, and dissociation. This type of simplification is currently necessitated by limitations in both structural and quantitative experimental data for association and dissociation. Rate-limiting steps were only assessed on an empirical level by simple comparisons of rates under typical conditions. This suggested that around room temperature the bottleneck was dissociation, but as this step was accelerated much more by tem-
temperature than the others, the importance of dissociation for the overall rate lessened at higher temperatures (see the companion article (71)). Despite the limitations associated with an incomplete model picture, it was possible to elucidate different mechanistic aspects. We found that the kinetics of forming the Michaelis complex was primarily governed by large, unfavorable entropy but low enthalpy activation barrier has previously been found for a processive chitinase acting on insoluble substrate (25). This distribution of the enthalpic and entropic contributions to ΔG° meant that the on-rate only increased slowly with temperature, and on a structural level, it suggested that the transition state occurred early in the path of the association where the enzyme has lost rotational and translational degrees of freedom but not yet established extensive interactions in the binding tunnel. For dissociation, the enthalpic contribution to the activation free energy was dominant, and this suggested that the transition state for this step was also close to the free i.e. late in the dissociation path at a stage where most enzyme-substrate interactions had been broken. This high activation enthalpy also meant that the rate of dissociation increased much more with temperature than the rate of the two preceding steps. The CBM had no detectable effect on the activation parameters for association, but it increased the free energy barrier of dissociation. This meant that the higher affinity brought about by the CBM relies on a delay of dissociation rather than an increased rate of association.

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