4-Methyl-7-thioumbelliferyl-β-D-cellobioside: A Fluorescent, Nonhydrolyzable Substrate Analogue for Cellulases†

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ABSTRACT: The kinetics of cellulose binding and hydrolysis by cellulases is not well understood except at steady-state conditions. For use in studies of cellulase pre-steady-state and steady-state kinetics, we have prepared 4-methyl-7-thioumbelliferyl-β-D-cellobioside (MUS-CB), a ground-state nonhydrolyzable analogue of the fluorescent cellulase substrate 4-methylumbelliferyl-β-D-cellobioside (MU-CB). MUS-CB is not hydrolyzed by the catalytic domain of cellulase E1 from *Acidothermus cellulolyticus* under conditions where this enzyme rapidly degrades MU-CB. Thermodynamic parameters describing the steady-state binding of MUS-CB to *Thermobifida fusca* cellulase Cel6A are similar to those for MU-CB, indicating that MUS-CB can be used in place of MU-CB to study binding events in the Cel6A active-site cleft. In the pre-steady-state, MUS-CB binds to Cel6A by a simple, one-step bimolecular association reaction. It is anticipated that similar thio-containing 4-methylumbelliferyl compounds will have applications in studies of other enzyme systems.

Cellulases are enzymes that degrade cellulose (a linear polymer of D-glucose) into small glucose oligomers by hydrolysis of its β-(1→4) glycosidic bonds (*1*, *2*). Enzymatic hydrolysis of cellulose is of considerable interest because of its potential application in converting plant biomass (a renewable resource) into glucose. This glucose could subsequently be used as feedstock for the production of ethanol or other chemicals and could have a major impact on the reduction of the atmospheric production of CO_2 (*3*, *4*). The term "cellulase" includes enzymes that have been classed into 13 structural families, as defined on the basis of protein sequence comparisons (*5*). These enzymes can have widely different activities on cellulosic substrates. Small cellulose-derived glycosides containing fluorescent or chromophoric leaving groups (such as 4-methylumbelliferone (MUF)[1] or *p*-nitrophenol) are common cellulase substrates, and the varying activities of cellulases on these substrates show some correlation with a particular enzyme's gene family (*6*).

Much is known about the catalytic mechanisms of cellulases (and glycoside hydrolases in general), and these enzymes have been extensively studied by steady-state kinetics and mutagenesis (*7*, *8*). However, there are few papers in the literature describing cellulase pre-steady-state kinetics. The most extensive study of this topic examined the pre-steady-state binding of glycosides by *Trichoderma reesei* Cel6A (*9*) but did not investigate their hydrolysis. Other papers in the literature describe pre-steady-state substrate hydrolysis by glycoside hydrolases such as β-glucosidases (*10*) and xylanases (*11*, *12*) but not by cellulases. By themselves, the steady-state kinetic parameters k_{cat}, K_m, and k_{cat}/K_m that are known for various cellulases are insufficient to describe the fast (non-rate-limiting) steps of catalysis. Because enzyme mutations are as likely to affect fast reaction steps as they are to affect slow ones, there is a need to characterize all steps in a cellulase reaction pathway (using a combination of steady-state and pre-steady-state kinetics) so that the full impact of amino acid mutations can be assessed.

Glycosides containing the 4-methylumbelliferyl reporter group (MU-glycosides) are well suited for these studies, as their interaction with cellulases results in two distinct fluorescence signals. Compounds such as 4-methylumbelliferyl-β-D-cellobioside (MU-CB) possess a weak emission maximum at 370 nm that is quenched upon binding to cellulases. This quench has been used to measure steady-state dissociation constants (K_d) (*13*, *14*) and the pre-steady-state rate constants for MU-CB binding to cellulases (*9*) under conditions where there is no substrate hydrolysis. With cellulases, the use of extrinsic fluorophores such as MU-CB is more sensitive than intrinsic tryptophan fluorescence. Tryptophans are common carbohydrate-binding residues in cellulases and other proteins (*15*), with some cellulase catalytic domains containing 10 or more. This abundance of tryptophan can complicate the fluorescence signal compared to that of MU-glycosides, which generally bind to cellulases in a 1:1 ratio.

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[1] Abbreviations: MUF, 4-methylumbelliferone; MU, 4-methylumbelliferyl; MU-CB, 4-methylumbelliferyl-β-D-cellobioside; MUS, 7-mercapto-4-methylumbelliferone; MUS-CB, 4-methyl-7-thioumbelliferyl-β-D-cellobioside; E1cd, the catalytic domain of *Acidothermus cellulolyticus* endocellulase E1; Cel6Acd, the catalytic domain of *Thermobifida fusca* endocellulase Cel6A; k_{cat}, turnover number; K_m, Michaelis constant; K_d, dissociation constant; K_{eq}, equilibrium constant; k_{obs}, apparent pre-steady-state rate constant; M, molar; NMR, nuclear magnetic resonance spectroscopy; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; λ_{max}, wavelength of maximal intensity; F, relative fluorescence; $F_{initial}$, relative fluorescence at $T = 0$.

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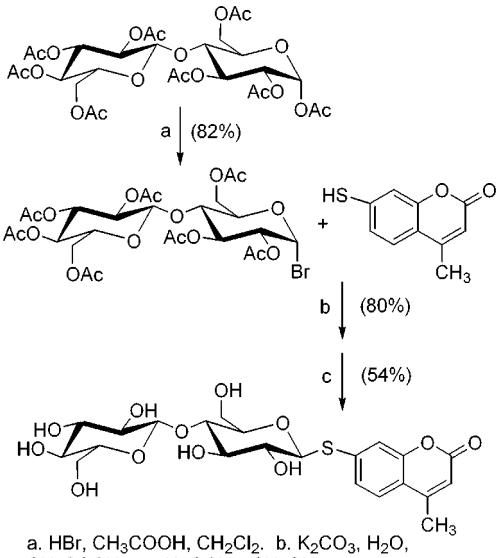
Scheme 1: Synthesis of 4-Methyl-7-thioumbelliferyl-β-D-cellobioside (MUS-CB)



When MU-CB is cleaved by cellulases, the product 4-methylumbelliferone (MUF) is much more fluorescent than MU-CB and has a new fluorescence emission at 450 nm. This MUF fluorescence has been used to measure the steady-state activity of many glycoside hydrolases (*14*, *16*) and is potentially useful for studies of cellulase pre-steady-state "burst" kinetics.

One difficulty with using MU-glycosides in this manner is that the compounds are being used to perform two tasks that are mutually exclusive. The measurement of catalytic rates requires that MU-glycosides be hydrolyzed, while the measurement of noncatalytic binding interactions requires that MU-glycosides remain intact. Thus, a complete cellulase kinetic analysis requires MU-glycoside ground-state analogues that bind in the same manner as do their parent compounds but that are unable to be cleaved. It has been shown (*17*) that glycosides in which the glycosidic oxygen atoms between sugars have been replaced with sulfur are not cleaved by glycoside hydrolases. These thio-oligosaccharides bind to their respective enzymes with high affinity and have been used in a variety of X-ray crystallographic studies probing the structure of cellulase enzyme/substrate complexes (*18*). In this contribution, we describe the synthesis of 4-methyl-7-thioumbelliferyl-β-D-cellobioside (MUS-CB) and show that it is a useful ground-state substrate analogue for MU-CB.

MATERIALS AND METHODS

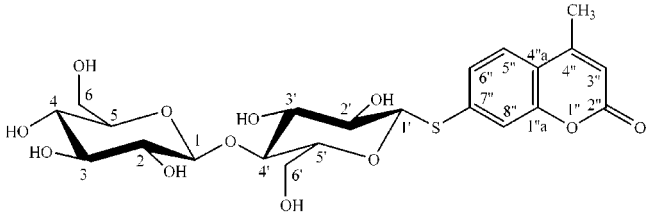
Synthesis. General. The protocol for the synthesis of 4-methyl-7-thioumbelliferyl-β-D-cellobioside (MUS-CB) is based on procedures for related compounds (*19*, *20*) and is shown in Scheme 1. HBr and α-D-cellobiose octaacetate were from Aldrich, and 7-mercapto-4-methylumbelliferone was from Fluka. Reactions were monitored by HPLC using a Partsil 10 ODS-3 column (Whatman) with a flow rate of 1.0 mL/min and detection at 254 nm. The column mobile phase was a mix of methanol/acetonitrile/0.012 M KH_2PO_4(aq) (43:40:17, v/v/v). Elemental analysis was performed by Quantitative Technologies, Inc. (Whitehouse, NJ). NMR spectra were determined for a solution of MUS-CB (0.35 M) in dimethyl sulfoxide-d_6 (neat) at 25 °C. One-dimensional ^{1}H- and ^{13}C NMR spectra were collected on a 400 MHz spectrometer by Spectral Data Services (Champaign, IL), while two-dimensional COSY and ^{13}C HSQC spectra were measured on a Bruker DRX 500 MHz spectrometer at the Center for Advanced Research in Biotechnology (Rockville, MD).

*2,3,6,2′,3′,4′,6′-Hepta-O-acetyl-α-cellobiosyl Bromide (**1**)*. HBr (30 mL of a 30 wt % solution in acetic acid) was added to a cold solution containing 2.00 g (2.95 mol) of α-D-cellobiose octaacetate that was dissolved in 25 mL of CH_2Cl_2. The reaction mixture was stirred for 5 h at 0 °C. The mixture was then diluted with 50 mL of CH_2Cl_2 and washed (2× each) with equal volumes of ice-cold aqueous 10% $KHSO_4$, aqueous saturated $NaHCO_3$, and deionized water. The reaction mixture was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The final product **1** (1.648 g, 82% yield) was either used immediately without further purification or stored under argon at −20 °C.

*4-Methyl-7-thioumbelliferyl-β-D-cellobioside (**2**)*. 7-Mercapto-4-methylumbelliferone (0.20 g, 1.04 mmol) and K_2CO_3 (0.15 g, 1.08 mmol) were suspended in 7 mL of deionized water, and **1** (0.73 g, 1.04 mmol) was dissolved in 25 mL of acetone. The two solutions were mixed, and the reaction was stirred overnight in the dark at room temperature. The solvent was subsequently removed under reduced pressure, and the resultant residue was dissolved in CH_2Cl_2. The solution was washed as before and dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The acetylated product (0.675 g, 80% yield) was dissolved in a minimal amount of ethyl acetate and further purified by flash column chromatography on silica gel (70−230 mesh, Sigma). The column (2.1 × 40 cm) was eluted with a mixture of ethyl acetate/hexane (1:1, v/v). Column fractions containing the pure compound (as determined by HPLC) were pooled, and the solvent was removed under reduced pressure. The resulting white solid (0.188 g) was deacetylated by treatment with 6 mM sodium methoxide in methanol (15 mL) for 4 h at room temperature. The solution was treated with Dowex 50W-X8 (H^+) resin, concentrated to <5 mL, and allowed to crystallize. The deacetylation product **2** (0.64 g, 54% yield) was over 99.5% pure as determined by HPLC; mp 232.1−232.7 °C. Anal. Calcd for $C_{22}H_{28}O_{12}S$: C, 51.16; H, 5.46; S, 6.21. Found: C, 51.03; H, 5.21; S, 6.33.

Cellulase Activity Assays. The catalytic domain of endocellulase E1 from *Acidothermus cellulolyticus* (E1cd) was from the National Renewable Energy Laboratory, Golden, CO (*21*). The catalytic domain of *Thermobifida fusca* endocellulase Cel6A (Cel6Acd, formerly called E2cd) was prepared as described (*14*). MU-CB (prepared as described (*16*)) was a gift from M. Claeyssens at the State University of Ghent, Belgium. UV/vis spectra were collected on a Hewlett-Packard 8452 diode array spectrophotometer at 25 °C. Concentrations of MUS-CB and MU-CB stock solutions were calculated from their absorbances at 316 nm in 50 mM sodium acetate, pH 5.5 buffer using $\epsilon = 13\,600$ M^{-1} cm^{-1} (*16*). Cellulase concentrations were determined by measuring their absorbances at 280 nm using extinction coefficients calculated from the protein sequence as described (*22*, *23*). The relative ability of cellulases to hydrolyze MUS-CB and

Table 1: NMR Assignments for MUS-CB



^{13}C NMR Chemical Shifts (δ)

Glc	C-1	C-2	C-3	C-4	C-5	C-6
	103.1	73.35	76.48	70.05	72.20	61.05
	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
	85.22	76.81	76.26	79.77	78.88	60.19

MeUmb	C-2″	C-3″	C-4″	C-5″	C-6″	C-7″	C-8″	C-1a″	C-4a″	Me
	159.7	113.5	153.1	125.5	124.3	140.6	115.4	153.1	117.5	18.1

^{1}H NMR Chemical Shifts (δ)

Glc	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	OH-2	OH-3	OH-4	OH-6
	4.31	3.04	3.18	3.08	3.22	3.44	3.72	5.28	5.07	5.04	4.65
	H-1′	H-2′	H-3′	H-4′	H-5′	H-6a′	H-6b′	OH-2′	OH-3′		OH-6′
	4.95	3.23	3.47	3.43	3.55	3.65	3.78	5.66	4.87		4.73

MeUmb	H-3″	H-5″	H-6″	H-8″	Me
	6.34	7.68	7.40	7.44	2.42

MU-CB was determined by incubating each glycoside with *A. cellulolyticus* E1cd. Assays were performed in 25 mM sodium acetate, pH 5.5 using one of the following two methods. In the first method, MUS-CB and MU-CB (1.5 mM each) were incubated with 38 nM E1cd for 24 h at 25 °C, and the extent of hydrolysis was visualized by thin-layer chromatography as described (*24*). In the second method, MUS-CB and MU-CB (100 μM each) were incubated with 3.8 nM E1cd at 50 °C. Aliquots (30 μL) of the reaction mixtures were taken at $T = 0$, 30, and 60 min and 24 h, combined with 70 μL of acetonitrile, and immediately frozen at −80 °C. For these samples, the extent of hydrolysis was determined by HPLC using an Alltech Partisil 10 PAC column with an acetonitrile/water (70:30, v/v) mobile phase using flow rate = 0.5 mL/min and with detection at 316 nm.

Binding of MUS-CB by T. fusca Cel6Acd. All fluorescence experiments were performed on an SLM 8000C spectrofluorimeter modernized by OLIS, Inc. (Bogart, GA) in 50 mM sodium acetate, pH 5.5 buffer. Data were plotted and fit using KaleidaGraph v. 3.51 (Synergy Software). The steady-state binding of MUS-CB to cellulases was investigated by titrating a solution of MUS-CB with *T. fusca* Cel6Acd at six temperatures in the range 7.0−30.2 °C as described (*14*). Excitation was at 330 nm, and the resultant quenching of MUS-CB fluorescence at 400 nm was used to calculate the dissociation constant (K_d) for the MUS-CB−Cel6Acd interaction at each temperature. The thermodynamics of the steady-state binding interaction was determined from a plot of ln K_{eq} vs $1/T$ according to the van't Hoff equation, $\ln K_{eq} = -\Delta H°/RT + \Delta S°/R$ (*25*).

The pre-steady-state binding of MUS-CB by Cel6Acd was investigated at 25 °C under pseudo-first-order conditions using the OLIS-modified SLM 8000C equipped with an OLIS-USA stopped-flow apparatus. The dead-time of the instrument was approximately 3.1 ms as determined according to procedures described in (*26*). In the stopped-flow experiments, chamber A contained various concentrations of Cel6Acd (15.0−44.1 μM), while chamber B contained 1.8 μM MUS-CB. All samples also contained 50 mM sodium acetate, pH 5.5 buffer. Mixing of samples was achieved by a nitrogen-driven piston at 100 psi, and fluorescence was monitored at 400 nm after excitation at 316 nm. The measurement at each substrate concentration was repeated 3−4 times, and the fluorescence traces were fit to a monoexponential decay using eq 1

$$F = F_{\text{initial}} - A e^{-k_{\text{obs}} t} \quad (1)$$

where A is the amplitude of the observed fluorescence change. Calculated values of k_{obs} were plotted as a function of Cel6Acd concentration and fit to eq 2 using KaleidaGraph.

$$k_{\text{obs}} = k_{-1} + k_{+1}[\text{Cel6Acd}] \quad (2)$$

RESULTS

MUS-CB was synthesized from α-D-cellobiose octaacetate and 7-mercapto-4-methylumbelliferone (MUS) as shown (Scheme 1). The complete ^{1}H- and ^{13}C NMR assignments for MUS-CB are given in Table 1. The anomeric region of the ^{1}H NMR spectrum of MUS-CB displays a signal at δ 4.95 (d, 1 H, J 9.4 Hz) for the proton on C1′ adjacent to the glycosidic sulfur and a signal at δ 4.31 (d, 1 H, J 8.0 Hz) for the proton on C1 adjacent to the glycosidic oxygen, confirming the β-configuration of both glycosidic bonds.

The absorbance spectrum of MUS contains a broad peak at $\lambda_{max} = 360$ nm, which is changed to a peak at $\lambda_{max} = 326$ nm in MUS-CB (not shown). It was determined that the extinction coefficient for MUS-CB at 316 nm is essentially the same (±10%) as that for MU-CB, $\epsilon = 13\,600$ M^{-1} cm^{-1}

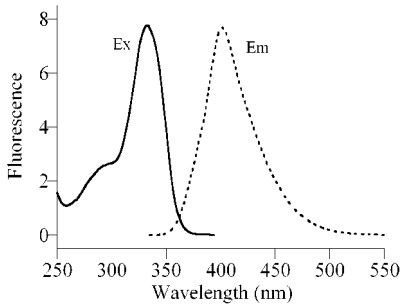


FIGURE 1: Fluorescence excitation (solid line) and emission (dashed line) spectra of MUS-CB.

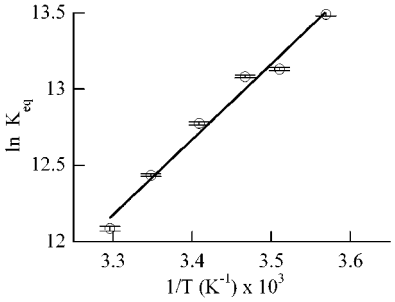


FIGURE 2: Van't Hoff plot for the binding of MUS-CB by *T. fusca* Cel6Acd.

(*16*). This result came from curve fits for the steady-state binding titrations, as the fits converged properly only if the concentration of MUS-CB present was the same as that calculated using the above value of ϵ (when using a precisely known concentration of Cel6Acd and assuming a 1:1 binding interaction). The fluorescence excitation spectrum of MUS-CB displays a maximum at 330 nm, while the fluorescence emission spectrum has a maximum at 400 nm (Figure 1). Interestingly, the fluorescence of MUS is several orders of magnitude less intense than that of MUS-CB, and the two compounds possess the same fluorescence excitation and emission maxima. This situation is unlike that with MUF and MU-CB where MUF has a more intense fluorescence emission than does MU-CB and where the emission maximum of MU-CB is blue-shifted by 80 nm relative to that of MUF.

The relative ability of cellulases to hydrolyze MUS-CB and MU-CB was examined by incubating each glycoside with *A. cellulolyticus* E1cd. E1cd has high activity on soluble substrates such as MU-CB and has been commonly assayed on the similar chromophoric substrate *p*-nitrophenyl-β-D-cellobioside (*21*). Upon incubation of 1.5 mM MUS-CB and MU-CB with E1cd for 24 h, TLC analysis showed that MU-CB had been completely hydrolyzed to MUF and cellobiose while MUS-CB showed no reaction. Similarly, HPLC analysis showed that 100 μM MUS-CB (retention time = 6.7 min) was not cleaved by 3.8 nM E1cd after 24 h at 50 °C while under the same conditions E1cd completely converted 100 μM MU-CB (retention time = 6.8 min) to MUF (retention time = 5.4 min) in less than 30 min.

The ability of MUS-CB to bind to cellulases was investigated by fluorescence titrations with *T. fusca* Cel6Acd. The steady-state binding interactions of this enzyme with a series of MU-glycosides including MU-CB have been determined (*14*), providing a system for the direct evaluation of the effects of the oxygen-to-sulfur change in MUS-CB. Although like E1cd this enzyme is able to hydrolyze MU-CB, the activity of Cel6A on MU-CB is so poor ($k_{cat} = 2.3 \times 10^{-4}$ sec^{-1} at 37 °C (*14*)) that the more active E1cd was used to characterize cellulolytic activity on MUS-CB. The fluorescence emission of MUS-CB at 400 nm is quenched upon binding to Cel6Acd, and this quench was used to determine the dissociation constant (K_d) for the MUS-CB−Cel6Acd interaction at six temperatures in the range 7.0−30.2 °C. The thermodynamics of the steady-state binding interaction was determined from a van't Hoff plot of the binding data (Figure 2), and the results (compared with the published data for MU-CB) are summarized in Table 2. At 25 °C, MUS-CB binds to Cel6Acd with an affinity ~3-fold weaker than that of MU-CB. The enthalpy of MUS-CB binding by Cel6Acd is slightly less favorable (by ~6 kJ/mol) than that for MU-CB, although both are still strongly favorable. Similarly, the entropy of MUS-CB binding is ~12 J/mol K less unfavorable than that for MU-CB, although this difference falls within experimental error.

Table 2: Dissociation Constants and Thermodynamic Parameters for the Interaction of MUS-CB and MU-CB with *T. fusca* Cel6Acd

	MUS-CB	MU-CB[b]
K_d, M[a]	3.98×10^{-6}	1.42×10^{-6}
$\Delta G°$, kJ/mol[a]	−30.9	−33.4
$\Delta H°$, kJ/mol	-41.5 ± 2.8	-47.3 ± 2.9
$\Delta S°$, J/mol K	-34.5 ± 9.7	-46.8 ± 10.0

[a] $\Delta G°$ and K_d values calculated at 25.0 °C. [b] Data for MU-CB from (*14*).

The kinetics of MUS-CB binding by Cel6Acd was determined by stopped-flow experiments under pseudo-first-order conditions using excess Cel6Acd. As shown in Figure 3A, the binding of MUS-CB by Cel6A is rapid, and in all cases, the observed data fit to a monoexponential progress curve. In control experiments, the mean relative fluorescence of MUS-CB alone (0.90 μM after mixing) was 7.53, of Cel6Acd alone (22.05 μM after mixing), 0.06, and of buffer alone, 0.01. Under the conditions used, complete saturation of MUS-CB with Cel6Acd could not be attained. However, the magnitude of the residual fluorescence at the end of the exponential decay in Figure 3A is equal to the fraction of unbound MUS-CB present at equilibrium (as calculated using the K_d value from the steady-state data). The apparent rate constants for binding (k_{obs}) increased linearly with enzyme concentration (Figure 3B). As a result, a one-step, bimolecular association reaction can be proposed:

$$\text{Cel6A} + \text{MUS-CB} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [\text{Cel6A*MUS-CB}] \qquad (3)$$

The calculated association and dissociation rate parameters for this interaction are $k_{+1} = 1.34 \pm 0.19 \times 10^7$ M^{-1} sec^{-1} and $k_{-1} = 80 \pm 30$ s^{-1}. Although the standard deviations for some of the data in Figure 3B are large, the K_d value calculated from the stopped-flow data (using $K_d = k_{-1}/k_{+1}$) is 6.0×10^{-6} M, similar to the value of 3.98×10^{-6} M derived from the steady-state data.

DISCUSSION

The results presented here show that MUS-CB is a useful nonhydrolyzable substrate analogue for the study of cellulases. The preparation of MUS-CB is straightforward (Scheme

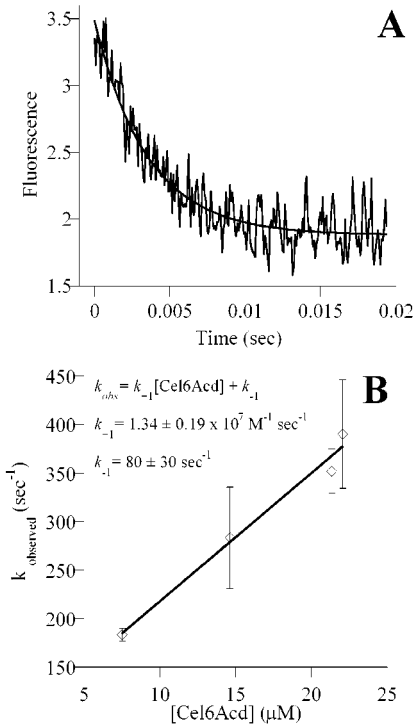


FIGURE 3: Stopped-flow fluorescence data for the binding of MUS-CB by *T. fusca* Cel6Acd. In panel A, the concentrations after mixing were 0.90 μM MUS-CB and 14.6 μM Cel6Acd, and the data were fit to a monoexponential decay (smooth line through the data), giving an observed rate constant k_{obs}= 282 s^{-1}. In panel B, the pseudo-first-order binding data were plotted as a function of Cel6Acd concentration, with standard deviations given by vertical bars. A linear fit of the data (solid line) yielded the results as shown.

1) and yields the final product in quantities sufficient to be used in a variety of biochemical analyses. Cellulase activity assays show that *A. cellulolyticus* E1cd (which displays significant activity on the oxygen-containing parent compound MU-CB) has no detectable activity on MUS-CB, which is consistent with results for other cellulases with thio-containing oligosaccharides (*17*) and indicates that the replacement of oxygen (in MU-CB) with sulfur (in MUS-CB) forms a glycosidic bond that is unable to be cleaved by these enzymes. The oxygen-to-sulfur change also makes several changes to the fluorescence emission spectra of MUS and MUS-CB relative to those for MUF and MU-CB. For this contribution, the most important change is that the fluorescence emission maxima of MUS and MUS-CB are the same (both at 400 nm), while those of MUF and MU-CB are different (at 370 and 450 nm, respectively). The reason for this spectral difference is unclear, and its effect is potentially complicating, as it precludes the direct fluorimetric method to distinguish between enzyme-bound and cleaved MUS-CB. However, because one can use other methods (HPLC, UV/vis spectra) to confirm that MUS-CB cannot be degraded by a particular enzyme (as shown here with E1cd), the potential uses of MUS-CB in fluorescence binding experiments are not affected.

The interaction of MUS-CB with *T. fusca* Cel6Acd in the steady state shows that MUS-CB binds to the enzyme in a manner very similar to that of MU-CB. The MUS-CB data fit well to an algorithm (*27*) describing a 1:1 binding interaction. This algorithm had been used previously to fit data for the binding of a variety of MU-glycosides by Cel6Acd (*14*). When the strength of the MUS-CB−Cel6Acd binding interaction was examined as a function of temperature, the resulting van't Hoff plot (Figure 2) was linear and permitted calculation of the thermodynamic parameters of binding (Table 2). These data show that the MUS-CB dissociation constant (at 25 °C, calculated from $\Delta H°$ and $\Delta S°$) is only slightly weaker (~3-fold) than that of MU-CB. This small difference due to replacing the glycosidic oxygen with sulfur is consistent with that observed for *T. reesei* Cel6A, as this enzyme binds the ligand MU-GlcSGlc with an affinity (K_d = 2.9 μM) similar to that for MU-CB (K_d = 5.0 μM) (*9*). While the slightly weaker affinity of *T. fusca* Cel6Acd for MUS-CB is larger than the error between duplicate measurements (previously estimated at 10−15%), for this enzyme it is much smaller than that seen with other changes of MU-glycoside structure. For example, changing from MU-CB to MU-lactose (moving the nonreducing O-4 hydroxyl from equatorial to axial) causes a 300-fold decrease in binding affinity (*14*).

It has been shown that Cel6A contains four glycosyl-binding subsites in its active-site cleft, labeled (from the nonreducing end) −2, −1, +1, and +2, with cleavage occurring between −1 and +1 (*28*). MU-CB binds to the active site of Cel6A with its cellobiose in subsites −2 and −1 and MUF in subsite +1 (*14*). This previously published data also indicates that subsite −2 binds a glucosyl unit with high affinity and that the sterically constrained subsite −1 distorts its bound glucosyl unit to assist catalysis. On the basis of the results presented in this report, we believe that MUS-CB binds to Cel6A in the same position as does MU-CB. By extrapolation from the through-space lengths of β-1,4-glycosidic bonds in thio-oligosaccharides cocrystallized with *Clostridium cellulolyticum* Cel48F (*18*), the replacement of oxygen with sulfur would shift the MU group in MUS-CB ~0.5 Å farther away from cellobiose than is found with MU-CB. It seems unlikely that this slight increase in the glycosidic bond length would be enough to shift the position of ligand-binding to Cel6Acd because alternative binding modes for MU-CB would either lose the favorable binding interaction in subsite −2 or force the large MU group to fit into the sterically constrained −1 subsite. Because Cel6Acd is highly sensitive to structural changes in the glycosyl region of MU-glycosides (as described previously), one would expect that shifting the binding position of MUS-CB relative to that of MU-CB would have an effect larger than the observed 3-fold change in binding affinity. Although only the three-dimensional structure of a complex with Cel6Acd could absolutely prove the binding position of MUS-CB, these data show that MUS-CB can be used in place of MU-CB to study enzyme−substrate interactions in the Cel6A active-site cleft.

Currently in cellulase research, the ability of active-site loops to undergo large conformational changes and promote catalysis is a subject of much interest. The catalytic domains of exocellulases such as *T. reesei* Cel6A have large surface loops that enclose the active site in a tunnel (*29*), while those same loops in the homologous endocellulase *T. fusca* Cel6Acd are pulled back to form a more accessible active-site cleft (*28*). It has been suggested that these loops in *T. fusca* Cel6Acd are flexible and close over substrates during catalysis, a theory that is supported by data from site-directed mutagenesis experiments (*30*). The use of nonhydrolyzable

analogues such as MUS-CB may provide new insights into catalysis by *T. fusca* Cel6A, as their pre-steady-state binding interactions can be used in an attempt to identify conformational changes in the Cel6A active site.

For this reason, the pre-steady-state binding of MUS-CB by *T. fusca* Cel6Acd was examined in a stopped-flow spectrofluorimeter under pseudo-first-order conditions. The data (Figure 3) indicate that MUS-CB binds to Cel6Acd by a one-step, bimolecular association reaction with no observable conformational change. This result is consistent with the low steady-state activity of Cel6A on MU-CB, which is degraded to MUF + cellobiose with $k_{cat} = 2.3 \times 10^{-4}$ s^{-1} at 37 °C (*14*). Because Cel6A has been shown to have significantly (several orders of magnitude) more activity on cellotetraose than on cellotriose (*31*, *32*), we believe that MU-disaccharides are hydrolyzed poorly by Cel6A because the binding of their (short) cellulose chain is insufficient to trigger the proposed conformational change. We believe that MU-glycosides with longer cellulose chains should be able to trigger this conformational change in Cel6A, and the synthesis of MUS-cellotriose is currently in progress in our laboratory.

In conclusion, the data show that MUS-CB is unable to be cleaved by cellulases and that MUS-CB possesses useful fluorescence emission properties that permit the measurement of its binding interaction with cellulases. These interactions are only slightly perturbed relative to the those of the parent compound MU-CB, indicating that MUS-CB is a useful ground-state substrate analogue for MU-CB. As a result, MUS-CB is a useful tool for studies of cellulase pre-steady-state reaction kinetics (currently in progress). Besides their use with cellulases, compounds containing 4-methylumbelliferone have been used as substrates for many enzymes (such as proteases, phosphatases, lipases, and other carbohydrate hydrolases) (*33*). For this reason, compounds derived from 7-mercapto-4-methylumbelliferone would be expected to have applications in fluorescence-based mechanistic studies of a wide variety of enzyme systems.

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