Glycoconj J (2012) 29:77–85
DOI 10.1007/s10719-011-9366-x

Critical roles of Asp270 and Trp273 in the α-repeat of the carbohydrate-binding module of endo-1,3-β-glucanase for laminarin-binding avidity

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Received: 14 September 2011 / Revised: 9 December 2011 / Accepted: 15 December 2011 / Published online: 27 December 2011

Abstract A carbohydrate-binding module from family 13 (CBM13), appended to the catalytic domain of endo-1,3-β-glucanase from *Cellulosimicrobium cellulans*, was overexpressed in *E. coli*, and its interactions with β-glucans, laminarin and laminarioligosaccharides, were analyzed using surface plasmon resonance biosensor and isothermal titration calorimetry. The association constants for laminarin and laminarioligosaccharides were determined to be approximately 10^6 M^{-1} and 10^4 M^{-1}, respectively, indicating that 2 or 3 binding sites in the α-, β-, and γ-repeats of CBM13 are involved in laminarin binding in a cooperative manner. The binding avidity is approximately 2-orders higher than the monovalent binding affinity. Mutational analysis of the conserved Asp residues in the respective repeats showed that the α-repeat primarily contributes to β-glucan binding. A Trp residue is predicted to be exposed to the solvent only in the α-repeat and would contribute to β-glucan binding. The α-repeat bound β-glucan with an affinity of approximately 10^4 M^{-1}, and the other repeats additionally bound laminarin, resulting in the increased binding avidity. This binding is unique compared to the recognition mode of another CBM13 from *Streptomyces lividans* xylanase.

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Keywords Carbohydrate-binding module · β-glucan · Affinity and avidity · Site-directed mutagenesis · Biomolecular interaction

Abbreviations

CBM	Carbohydrate-binding module
SPR	Surface plasmon resonance
ITC	Isothermal titration calorimetry
His-tag	Polyhistidine-tag
PCR	Polymerase chain reaction
Ni-NTA	Ni-nitrilotriacetic acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
CD	Circular dichroism
DLS	Dynamic light scattering

Introduction

The carbohydrate-binding module (CBM) is defined as a contiguous amino-acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate-binding activity [1–3]. To date, CBMs have been classified into 64 different families based on amino-acid sequence, binding specificity, and ternary structure [3]. They are considered to have a critical function in localizing the connected enzymes to their substrates [4]. In addition to their biological function, CBMs are good targets for the analysis of carbohydrate recognition mechanisms. We recently cloned a gene encoding the endo-1,3-β-glucanase from the genomic DNA of *Cellulosimicrobium cellulans* DK-1 and found that the enzyme has a catalytic domain and a CBM family 13 (CBM13), named CBM-DK, connected by a Gly/Ser-rich linker region [5]. Compared with the structures of other

CBM13s from *Streptomyces lividans* xylanase 10A and *Streptomyces olivaceoviridis* E-86 xylanase, whose crystal structures are available [6–8], 6 Cys residues are conserved and are predicted to form disulfide bonds (Fig. 1). CBM13s have a β-trefoil fold displaying the pseudo-3-fold symmetry arising from imperfect tandem α-, β-, and γ-repeats. Previous studies of CBM13 from *Streptomyces lividans* xylanase 10A have shown that each repeat has a xylan binding site, and that the 3 binding sites interact with xylan in an additive and cooperative manner [10]. It has also been shown that the conserved Asp residues, Asp19, Asp61, and Asp102 (Fig. 1), located in the α-, β-, and γ-repeats, respectively, participate in xylan binding through hydrogen bonding [6, 11]. In CBM-DK, Asp270, Asp311, and Asp355 correspond to the conserved Asp residues (Fig. 1), and are predicted to participate in β-glucan binding. In addition, it seems that a unique hydrophobic residue, Trp273, located in the α-repeat, would be exposed to the solvent and interact with β-glucan,

(a)

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                260              270              280              290              300
CBM-DK      GTGA VRAA=NGMCVD VPWADPTDGN PVQIVTCSGN AAQTWTRGSD
                 8               19               29               39               49
CBM-SlXy    DG-Q IKGVGS-R-L- --D-STS--T QL-LWD-HSG TN-Q-AATDA
               314              325              335              345              355
CBM-SoXy    -G-Q IKGVGS-R-L- --N-ST---T Q--LYD-HSA TN-Q--YTDA

                        310              320              330              340
CBM-DK       GTVRALG=KCL DVRDGSTTRG AAVQVWTCNG TGAQLWAYDA GS
                         60               68               78               88
CBM-Xy       -EL-VY-D--- -A==AG-SN- SK--IYS-W- GDN-K-RLNS DG
                        366              374              384              394
CBM-SoXy     -EL-VY-D--- -A==AG-GN- TK--IYS-W- GDN-K-RLNS DG

                   350              360              370              380
CBM-DK      KALRNPQS GLCLDATGGA PLHDGQRLQT WTCNGTTAQQ WTL
                    97              107              116              126
CBM-Xy      SVV=GV-- --|---V-NG TA=N-TLI-L Y-|SNGSN-R --RT
                   403              413              422              433
CBM-SoXy    SIV=GV-- --|---V--G TA=N-TLI-L YS-SNGSN-R --RT
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(b)

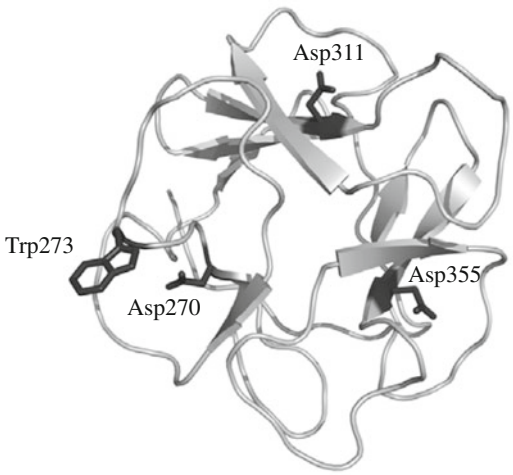

Fig. 1 Structural comparison and model of CBM-DK. **a** Comparison of the amino acid sequences of CBM-DK with CBM13s from *Streptomyces lividans* xylanase 10A (CBM-SlXy) and *Streptomyces olivaceoviridis* E-86 xylanase 10A (CBM-SoXy). Single dashes denote identity to CBM-DK, and double dashes indicate no amino acid residue at that position. The conserved Cys residues are boxed, and the substituted residues, Asp270, Trp273, Asp311, and Asp355, are in italic. The homologies of CBM-DK with CBM-SlXy and CBM-SoXy are 35% and 37%, respectively. **b** The 3D structural model of CBM-DK. The model was created with Swiss-Model [9], using the X-ray crystal structure of CBM-SoXy (PDB ID; 1V6W) [8]. The four mutated residues, Asp270, Trp273, Asp311, and Asp355, are explicitly shown

based on the previously reported crystal structures of CBM13s [6–8]. The corresponding residues in the β- and γ-repeats are Asp314 and Gly358, respectively (Fig. 1).

In this study, we overexpressed CBM-DK, corresponding to Gly257-Leu383 of endo-1,3-β-glucanase from *Cellulosimicrobium cellulans* DK-1, in *E. coli* with an N-terminal polyhistidine-tag (His-tag). In order to determine the quantitative effect of additive and cooperative interaction with β-(1-3)-glucans, the binding of CBM-DK to both laminarin and laminarioligosaccharides was analyzed using surface plasmon resonance (SPR) biosensor and isothermal titration calorimetry (ITC). Because the laminarin from *Laminaria digitata* used in this study has an average molecular weight of 6 k and the ratio of β-(1-3) to β-(1-6) linkages is 7:1 [12], it can interact with CBM-DK in a multivalent manner. Although the multivalent binding is considered to increase the binding strength due to the avidity effect [13], the quantitative correlation between affinity and avidity is poorly understood, particularly for carbohydrate binding. The second purpose of this study was to elucidate the role of each repeat in CBM-DK in β-glucan binding. In order to determine whether or not the contribution of each repeat is equal, the conserved Asp residues, Asp270, Asp311, and Asp355, located in the α-, β-, and γ-repeats were mutated to Ala, individually or in combination, and the binding affinities of the mutants for laminarin and laminarioligosaccharides were compared. Together with the results with the W273S mutant, we found that the α-repeat primarily contributes to β-glucan binding, which is different from the case of CBM13 of xylanase.

Materials and methods

Materials

Laminarin from *Laminaria digitata* and laminarioligosaccharides from *Poria cocos* were purchased from Sigma-Aldrich Co. (USA) and Seikagaku Kogyo Co. (Japan), respectively. The plasmid vector, pET28-a (Novagen, USA), was used for expression of proteins.

Expression and purification of CBM-DK

The DNA region encoding CBM-DK was first amplified as *Nde*I/*Eco*RI fragments by polymerase chain reaction (PCR) using the primer pairs F1 and R1 (F1; 5′-GTCGGCCCATA TGGGGACCGGCGCGGTG-3′, R1; 5′-GCGAATT CTCAGAGCGTCCACTGCTGGGC-3′) and the genomic DNA from *Cellulosimicrobium cellulans* DK-1 as the template [5]. The resultant PCR products were then cloned into the pET28-a expression vector that encodes an N-terminal His-tag. Site-directed mutagenesis was performed by overlap extension PCR methods [14]. In this study, CBM-DK wild-type with an N-terminal His-tag is simply referred to as CBM-DK, and its mutant is referred to as the substituted residue name (*e.g.*, D270A stands for the mutant in which Asp270 was substituted with Ala).

Recombinant *E. coli* BL21(DE3) harboring the expression vector containing either CBM-DK or its mutants were grown at 37°C in Luria-Bertani medium to mid-log phase, and isopropyl-β-thiogalactoside was added to a final concentration of 0.1 mM to induce protein expression. After an additional incubation of 4 h at 37°C, the bacteria were harvested, resuspended in 20 mM potassium phosphate buffer (pH 8.0) containing 300 mM NaCl, and sonicated. Because the overexpressed CBM-DK protein was insoluble, it was solubilized in 20 mM potassium phosphate buffer (pH 8.0) containing 8 M urea, and refolded by stepwise dialysis in 0.2 mM oxidized glutathione, 2 mM reduced glutathione, and 20 mM potassium phosphate buffer (pH 8.0) containing 2 M, 1 M, and 0 M urea, in succession, at 4°C. The refolded protein was loaded onto a Ni-nitrilotriacetic acid (Ni-NTA) column and eluted using 20 mM potassium phosphate buffer (pH 8.0) containing 300 mM NaCl and 500 mM imidazole. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out using a 15% separating gel, and the resultant protein bands were stained with Coomassie brilliant blue. The purified protein concentrations were calculated from absorbance values recorded at 280 nm, using the extinction coefficient of 2.05 ml mg^{-1} cm^{-1}.

Circular dichroism (CD) spectrometry

The purified proteins were dialyzed against 40 mM potassium phosphate buffer (pH 7.0) at 4°C. The far-UV CD spectra were measured on JASCO J-720 spectrometer, using a scan speed of 20 nm/min, a time response of 1 s, a bandwidth of 1 nm, an average over 8 scans, and a cell with 0.2 cm optical path length.

Dynamic light scattering (DLS) measurement

Zetasizer μV (Malvern Instruments Ltd.) was used for measuring DLS to analyze the oligomeric state of CBM-DK in 40 mM potassium phosphate buffer (pH 7.0) at 25°C. The measurement was performed using a laser beam of 830 nm at an angle of 90°.

SPR measurement

The Biacore biosensor system, Biacore 2000 (GE Healthcare Bioscience, Uppsala, Sweden), was used to measure real-time β-glucan interactions. CBM-DK and its mutants were covalently linked to sensor chip, CM5, and β-glucans

with various concentrations in 10 mM phosphate buffer (pH 7.3) containing 0.14 M NaCl and 0.005% Tween20 were applied over the sensor chip at a rate of 20 μl/min during 3 min. The surface was regenerated with one 15 μl injection of a solution of 10 mM acetic acid buffer (pH 4.0). All experiments were performed at 25°C.

The sensorgrams for β-glucan and CBM-DK interactions were examined by first adjusting for background changes reflected by the bulk refractive indices, and were next analyzed using BIA evaluation 3.2 software. The equilibrium association constant (K_a) values were determined by Scatchard analysis using Eq. 1:

$$RU_{eq}/C = K_a \times RU_{max} - K_a \times RU_{eq} \quad (1)$$

where C is the free analyte concentration, RU_{eq} is the steady-state response, and RU_{max} is the total surface binding capacity.

ITC measurement

ITC experiments were carried out on an iTC200 calorimeter interfaced with a microcomputer (GE Healthcare Bioscience, Uppsala, Sweden). All samples were in 40 mM phosphate buffer (pH 7.0), and all solutions were thoroughly degassed. The solution of laminarin or laminarioligosaccharides was titrated into the CBM-DK solution using a 40-μl syringe. Each titration consisted of a preliminary 0.3-μl injection followed by 29 subsequent 1.3-μl additions. The heat for each injection was subtracted from the dilution heat of the titrant, measured by injecting the β-glucan solution into buffer alone. Each corrected heat was divided by the moles of β-glucan injected. Data were analyzed using the Origin software supplied by GE Healthcare Bioscience.

Results

The cDNA encoding CBM-DK was successfully amplified by PCR, using genomic DNA from *Cellulosimicrobium cellulans* as the template, and cloned into the pET28-a vector. The plasmids encoding CBM-DK mutants were constructed by site-directed mutagenesis. CBM-DK and its mutants with an N-terminal His-tag were overexpressed in *E. coli*, and most of the protein was in the insoluble fraction (Fig. 2a). The protein was solubilized by the addition of urea and refolded by the step-wise dilution of urea. The solubilized and refolded protein was purified using a Ni-NTA affinity column (Fig. 2a). The far-UV CD spectrum of purified CBM-DK indicated that it had folded correctly (Fig. 2b) and agreed with the postulated β-strand-rich structure, as observed in the crystal structures of CBM13s at the C-terminal modules of xylanases [6–8]. In the DLS analysis, one size distribution

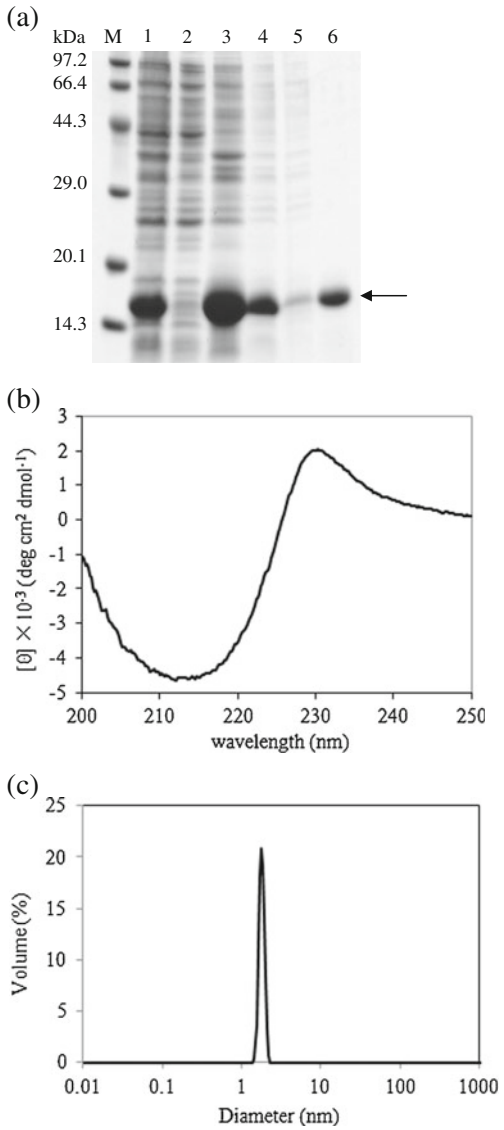

Fig. 2 Purification and structural analysis of CBM-DK. **a** SDS-PAGE analysis after each purification step. Lanes: M, standard molecular weight marker; 1, crude extract; 2, soluble fraction after sonication; 3, solubilized fraction using 8 M urea; 4, refolded fraction after dialysis; 5, flow through fraction from Ni-NTA affinity column; 6, the eluted protein from Ni-NTA affinity column. The arrow indicates the band of CBM-DK (15. 4 kDa). **b** Far-UV CD spectrum of 13 μM CBM-DK. **c** Size distribution by mass of 20 μM CBM-DK analyzed using DLS

by mass was observed for CBM-DK, in which the molecular mass was estimated to be 13.9±1.1 kDa (Fig. 2c), indicating that CBM-DK exists as a monomer. This is also supported by the results of gel-filtration HPLC analysis (data not shown).

Binding of laminarin was analyzed by SPR using a sensor chip on which CBM-DK was immobilized. When a protein is immobilized by amine coupling, the amino group exposed to the solvent becomes immobilized. To eliminate the possibility that the binding surface of CBM-DK was masked and interfering with β-glucan binding when

immobilized, another CBM-DK with an additional Lys at the N-terminus was immobilized on the sensor chip, and its binding to β-glucans was analyzed. The results showed that the binding sensorgrams and affinity to laminarin and laminarioligosaccharides were similar to those of immobilized CBM-DK. Figure 3a shows typical sensorgrams for the binding of laminarin to CBM-DK. In Scatchard plots (Fig. 3b), a gradual increase in the slope at a lower occupancy was observed, indicating the existence of different binding modes. The K_a values determined from the linear fit in the range of 5–40 μM and 0.625–5 μM of laminarin are 1.1×10^6 M^{-1} and 8.0×10^6 M^{-1}, respectively (Table 1). Because CBM-DK has 3 binding sites and was immobilized on the sensor chip, the multivalent binding to laminarin should have occurred on not only a CBM-DK molecule but simultaneously on multiple CBM-DK molecules in addition to the monovalent binding that took place. In order to distinguish the avidity effect derived from immobilization of multiple CBM-DK molecules, ITC measurements were carried out. Figure 4 shows a typical ITC profile for the

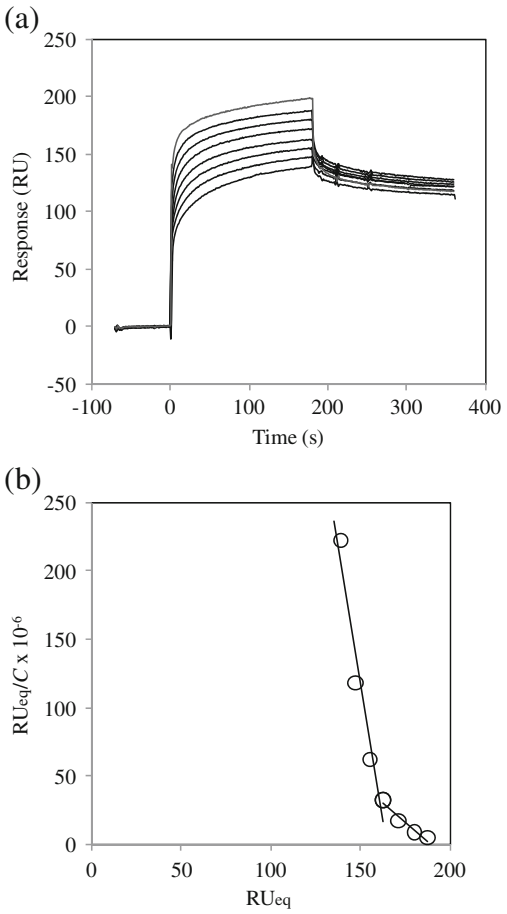

Fig. 3 SPR analysis of interactions between laminarin and immobilized CBM-DK. **a** Sensorgrams for the binding of laminarin (0.625–40 μM). **b** Scatchard plots for the binding of laminarin. The K_a values determined from the linear fitting to the laminarin in the range of 5–40 μM and 0.625–5 μM, respectively

binding of laminarin to CBM-DK. The thermodynamic parameters obtained are summarized in Table 2. From the assumption that each CBM-DK binds to laminarin independently, the K_a value 1.32×10^6 M^{-1} corresponds to the binding strength derived from a single CBM-DK molecule. A binding stoichiometry (n) of 0.12 indicates that approximately 8 CBM molecules bind to a single laminarin molecule. The binding reactions are enthalpy-driven.

The binding of laminarioligosaccharides was analyzed by both SPR and ITC. Scatchard plots for the laminarioligosaccharides generated straight lines, in which K_a values for the binding of laminaritetraose and laminarihexaose obtained from the slopes were 1.5×10^4 M^{-1} and 2.8×10^4 M^{-1}, respectively (Table 1), which were comparable to those obtained by ITC (Table 2). The observed binding is expected to be monovalent, and the K_a values are about 2-orders lower than that for laminarin binding. The n value of ~1 indicates that the laminarioligosaccharide binds to one of the 3 sites on CBM-DK, with the observed heat.

In order to determine the role of each repeat of CBM-DK in β-glucan binding, 3 Asp residues, Asp270, Asp311, and Asp355, were mutated to Ala, individually or in combination, and the β-glucan binding of each mutant was analyzed by SPR. The K_a values obtained are summarized in Table 1. In both binding to laminarioligosaccharides and binding to laminarin, no increase in RU was observed for the mutants D270A, D270A/D311A, or D270A/D355A, while the K_a values for the mutants D311A, D355A, and D311A/D355A were similar to those for CBM-DK. The results clearly indicate that the α-repeat is more critical than the other 2 repeats for β-glucan binding. In addition, the decreased K_a value for laminarin binding of W273S supports the proposed role of the α-repeat. While little increase in RU was observed for D270A with the laminarin concentrations analyzed, an increase in RU was observed for W273S, indicating that the contribution of Asp270 to β-glucan binding is larger than that of Trp273. In order to analyze the laminarin binding in a single D311A/D355A molecule, the ITC measurement was carried out. The K_a value determined was lower than that of CBM-DK wild-type (Table 2), which would be due to the decreased contribution of β- and/or γ-repeats.

Discussion

Several binding mechanisms should be considered for the interactions of CBM-DK with laminarin and laminarioligosaccharides (Fig. 5). In the interaction between laminarin and CBM-DK immobilized on the flexible dextran surface of SPR sensor chip, multiple CBM-DK molecules could be involved. One of the advantages of using this system is that the molecular interaction with a low binding affinity could be observed, due to the avidity effects. In the low

Table 1 K_a values (M^{-1}) for interactions of β-glucans with immobilized CBM-DK and its mutants on the sensor chip

CBM	Laminaritetraose[a]	Laminarihexaose[b]	Laminarin[c]	Laminarin[d]
CBM-DK	1.5×10^4	2.8×10^4	8.0×10^6	1.1×10^6
D270A	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]
W273S	n.d.[e]	n.d.[e]	7.9×10^5	1.8×10^5
D311A	3.3×10^3	2.1×10^4	1.3×10^7	1.6×10^6
D355A	2.6×10^3	1.7×10^4	7.4×10^6	1.4×10^6
D270A/D311A	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]
D270A/D355A	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]
D311A/D355A	5.2×10^3	3.8×10^4	2.4×10^7	–
D270A/D311A/D355A	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]

[a] Determined from a Scatchard analysis using laminaritetraose concentrations of 0.16–1.28 mM

[b] Determined from a Scatchard analysis using laminarihexaose concentrations of 0.08–0.64 mM

[c] Determined from a Scatchard analysis using laminarin concentrations of 0.625–5 μM

[d] Determined from a Scatchard analysis using laminarin concentrations of 5–40 μM

[e] Not determined from the sensorgram because little increase of RU was observed

concentration of laminarin, 3 or more binding sites in multiple CBM-DK molecules could be involved in laminarin binding (Fig. 5e), resulting in a higher binding strength than that observed by ITC. In the high concentration of laminarin, 2 or 3 binding sites in CBM-DK molecule(s) are predicted to be involved (Fig. 5d). In contrast, the K_a value obtained by ITC is expected to be derived from the interaction of a single CBM-DK molecule because ITC detects the heats of interactions in solution. The K_a value for laminarin binding was determined to be 1.32×10^6 M^{-1}, which is comparable to the K_a value determined using laminarin at concentrations of 5–40 μM in the SPR experiments, and is about 2-orders higher than the binding affinity for laminarioligosaccharides (Tables 1 and 2). Taken together, the results indicate that CBM-DK interacts with laminarioligosaccharides in a monovalent manner, while it interacts with laminarin in a multivalent manner. Possibly due to the flexibility and/or the branch of the β-(1-6) linkage, a laminarin molecule could simultaneously bind to 2 or 3 binding sites within a single CBM-DK molecule (Fig. 5b).

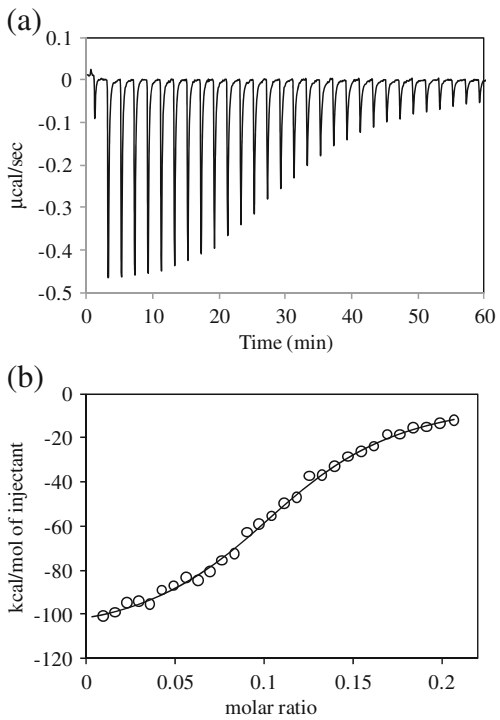

Fig. 4 ITC analysis of interactions between laminarin and CBM-DK. **a** The 60 μM laminarin solution was injected 30 times in 1.3-μl increments into the 60 μM CBM-DK solution. Data were obtained at 25°C. **b** The data points were obtained by integration of the peaks in (**a**), corrected for the dilution heat, and plotted against the molar ratio, laminarin/CBM-DK. The data were fitted using a nonlinear least-squares method

The present mutational analysis clearly showed that the contribution of each binding site of the α-, β-, and γ-repeats to β-glucan binding is nonequivalent, which is not the case with CBM13 of *Streptomyces lividans* xylanase 10A [10]. No increase in RU was observed for the binding of Asp270 mutants to laminarin and laminarioligosaccharides (Table 1), indicating that the α-repeat primarily contributes to β-glucan binding. In the mutant of D311A/D355A, because laminarin is long enough to interact with multiple CBM molecules as described below, 3 or more α-repeats of adjacent D311A/D355A molecules immobilized on the SPR sensor chip would be involved in laminarin binding, resulting in the K_a value similar to that of CBM-DK wild-type (Table 1). The decreased binding affinity of the W273S mutant also supports the critical role of the α-repeat. In the amino-acid sequence, there is a unique hydrophobic residue, Trp273, within the α-repeat. The corresponding residue in the CBM13 α-repeat of *Streptomyces lividans* xylanase 10A is Asp22 (Fig. 1a), and it contributes to carbohydrate binding [6]. Taken together, Trp273 could participate in binding to

Table 2 Thermodynamic parameters of β-glucan binding to CBM-DK and D311A/D355A

β-glucan	*n*	K_a (M^{-1})	$\Delta G°$	ΔH (kcal·mol^{-1})	$T\Delta S°$
CBM-DK					
Laminarin	0.12	1.32 (±0.08)×10^6	−8.35	−112 (±2)	−103
Laminaritetraose	0.99	1.63 (±0.28)×10^4	−5.74	−11.2 (±3.6)	−5.46
Laminarihexaose	0.95	3.46 (±0.18)×10^4	−6.19	−11.9 (±0.7)	−5.71
D311A/D355A					
Laminarin	0.10	4.25 (±0.82)×10^5	−7.67	−89.1 (±8.2)	−81.4

The S.D. were based upon fitting errors

laminarin and laminarioligosaccharides through a hydrophobic interaction, as observed with the carbohydrate recognition by CBMs [15, 16]. In CBM13 of *Streptomyces lividans* xylanase 10A, hydrophobic ring stacking interactions are observed at conserved residues, Trp34 in the α-repeat, Tyr74 in the β-repeat, and Tyr117 in the γ-repeat [6]. Similar hydrophobic interactions are also observed in CBM13 of *Streptomyces olivaceoviridis* E-86 xylanase [7, 8]. The corresponding residues in CBM-DK are Val285, Trp326, and Trp371, respectively (Fig. 1a). In our experiments using the mutants V285A, W326A, and W371A, the strength in binding to laminarin was similar to that of CBM-DK wild-type (data not shown). In this CBM, Trp273 in the α-repeat could contribute to

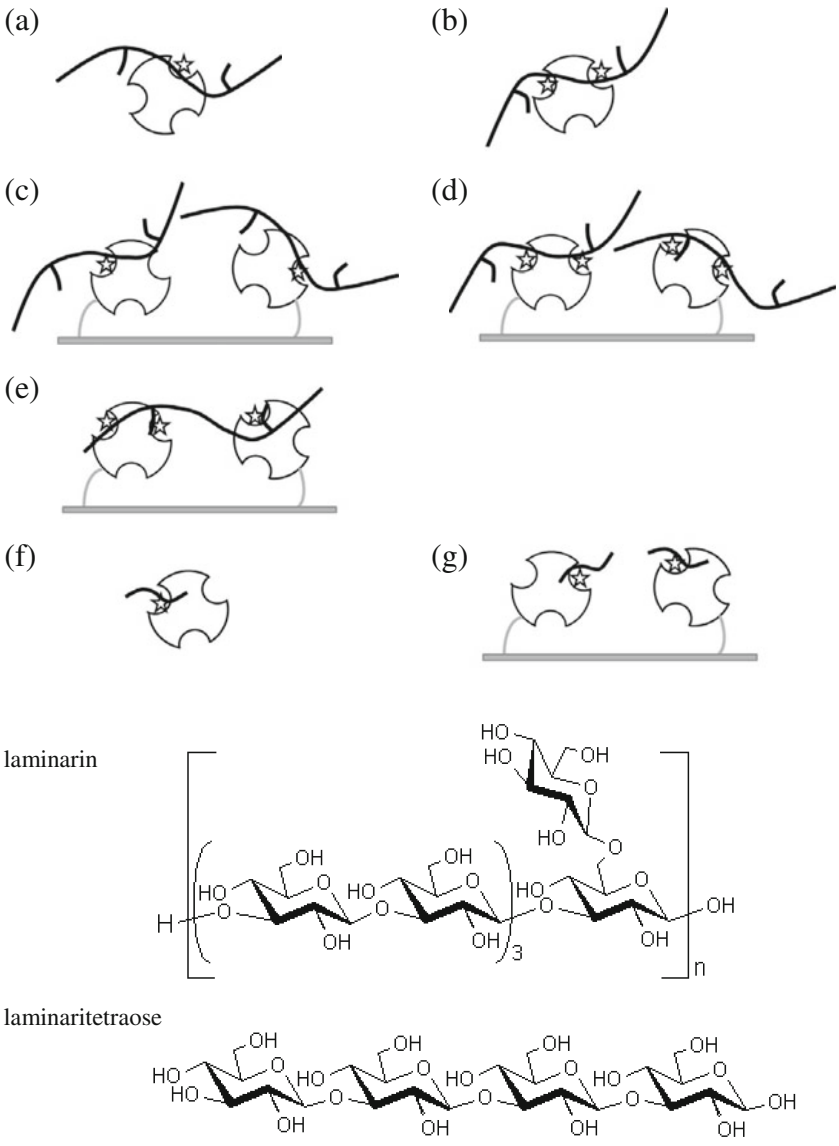

Fig. 5 Schematic representation of CBM-DK binding to laminarin and laminarioligosaccharide. **a** Monovalent binding to laminarin in solution. **b** Multivalent binding to laminarin within a single CBM-DK molecule in solution. **c** Monovalent binding to laminarin within a single CBM-DK molecule immobilized. **d** Multivalent binding to laminarin within a single CBM-DK molecule immobilized. **e** Multivalent binding to laminarin within multiple CBM-DK molecules immobilized. **f** Monovalent binding to laminarioligosaccharide in solution. **g** Monovalent binding to laminarioligosaccharide within a single CBM-DK molecule immobilized. The curved long bars with branches, curved short bars, and circles with hollows represent laminarins, laminarioligosaccharides, and CBM-DKs, respectively. The stars refer to the binding of CBM-DK to glucose unit of laminarin and laminarioligosaccharide. The chemical structures of laminarin and laminaritetraose are also indicated. The branched position of β-(1-6) linkage in laminarin is heterogeneous, and the ratio of β-(1-3) to β-(1-6) linkages is 7:1 [12]

CBM-DK binding of β-glucan. Because the residues corresponding to Trp273 in the β- and γ-repeats are Asp314 and Gly358, respectively, the additional binding effects would be much less than that of Trp273.

The ITC experiments showed that the binding stoichiometry for laminarioligosaccharides was approximately 1. Considering that the α-repeat primarily contributes to β-glucan binding, the laminarioligosaccharide would bind to the site on the α-repeat with the heat observed. Because the binding strength of laminarin to CBM-DK in solution is about 2-orders higher than those of laminarioligosaccharides, the β- and/or γ-repeats would be involved in the glucan binding. While laminarin is long enough to interact with multiple sites in the 2 or 3 repeats, laminarioligosaccharides are not (Fig. 5). In laminarin binding, the site on the α-repeat would first bind and localize it, and then other site(s) on the β- and/or γ-repeats could bind it in a cooperative manner, resulting in a binding strength to laminarin that is higher than those to laminarioligosaccharides. The binding stoichiometry for laminarin, 0.12, indicates that approximately 8 molecules of CBM-DK are involved in the complex formation with 1 molecule of laminarin. Assuming that the 8 CBM-DK molecules bind to laminarin independently, the binding enthalpy change (ΔH) derived from 1 CBM-DK molecule can be estimated to be approximately −14 kcal/mol. This is lower than those for the binding to laminarioligosaccharides (Table 2), which is expected to be due to the multivalent binding of laminarin to the β- and/or γ-repeats in addition to the α-repeat.

In our analysis to determine the role of CBM13 in the enzymatic function of the appended catalytic domains, we and others have shown that CBM13 contributes to the hydrolytic activity for insoluble carbohydrates such as curdlan and yeast-glucan, but not for soluble carbohydrates such as laminarin and laminarioligosaccharides [4, 17–19]. Assuming that the insoluble carbohydrates are in the aggregated form and long enough to simultaneously bind to both the catalytic domain and the CBM13 of an endo-1,3-β-glucanase molecule, the CBM13 could contribute to localization of the catalytic domain on condensed carbohydrates, which could accelerate hydrolysis. For efficient catalysis in general, the enzyme should rapidly bind and release the substrate. Considering that the catalytic domain of endo-1,3-β-glucanase from *Cellulosimicrobium cellulans* DK-1 itself has the ability to bind to the substrates with a K_a value around 10^6 M^{-1} [19], the binding affinities of CBM-DK for the substrates determined in this study seem to be quite reasonable. The full-length endo-1,3-β-glucanase could bind to insoluble glucans tightly via CBM and release the shorter glucans as products.

Acknowledgments The authors thank Mr. Takahiro Maruno of Osaka University and Prof. Yuji Kobayashi of Osaka University of Pharmaceutical Sciences for technical support and helpful discussion. This work was partly performed under the Joint Usage/Research Program of Medical Research Institute, Tokyo Medical and Dental University.

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