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# Calorimetric Study on the Binding of β-Cyclodextrin to the Starch-binding Domain of *Aspergillus niger* Glucoamylase

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*Aspergillus niger* glucoamylase (GA) consists of a catalytic domain and a starch-binding domain (SBD). SBD has two starch-binding sites, Sites 1 and 2. SBD can be isolated as a fragment protein. This calorimetric study aimed to examine the possibility of interactions between the two domains in a GA molecule and between the two binding sites of SBD [*Biochemistry* **38**, 6300 (1999) and *Eur. J. Biochem.* **225**, 133 (1994)] upon binding of a substrate analog, β-cyclodextrin (β-CD). The thermodynamic parameters of the binding of β-CD to the SBD fragment were virtually the same as those to SBD in the GA molecule. The values of the dissociation constants were 12–14 μM for Site 1 and 2 μM for Site 2, irrespective of the SBD fragment and SBD in the GA molecule. This result suggested that there was no inter-domain interaction between SBD and the catalytic domain for the binding of β-CD. In addition, amino acid mutation at one binding site did not affect the thermodynamic parameters at the other site. It was found that there is no interaction between the two binding sites in SBD and β-CD binds to Sites 1 and 2 independently.

Keywords: inter-domain interaction, inter-site interaction, isothermal titration calorimetry, ligand binding, starch-binding domain

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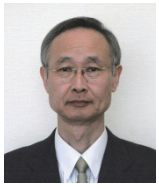

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## 1. Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) hydrolyzes starch from the non-reducing end to produce glucose. *Aspergillus niger* glucoamylase (GA) is an industrial enzyme used in the production of glucose. The GA molecule consists of 616 amino acid residues with a molecular weight of 82,500.[1] There are two domains in the molecule[2] [**Fig.1(a)**]: a catalytic domain with 440 residues at the N terminus and a starch-binding domain (SBD) with 110 residues at the C terminus. These two domains are connected by a highly *O*-glycosylated linker. The following results concerning the domain structure and ligand binding were obtained: (1) The two domains unfold independently.[3] (2) 1-deoxynojirimycin, an inhibitor of the GA molecule, and β-cyclodextrin (β-CD), a substrate analog, enhance the apparent thermal stability of each targeted domain independently. [3] β-CD binds to SBD but does not bind to the catalytic domain.[3,4] (3) SBD can be isolated as a fragment protein (SBDF), and its three-dimensional structure has been determined by NMR[5,6] and X-ray crystallography.[7] Detailed mechanisms of thermal unfolding and denaturant-induced unfolding of SBDF have been investigated.[8-12]

SBD and SBDF have two binding sites, Site 1 and Site 2 [**Fig.1(b)**].[5,6] The NMR solution structure of the complex of the SBDF with β-CD displayed that the hydrophobic residues W543 and W590 in Site 1 and Y527 and Y556 in Site 2 are important for stacking interactions with glucose residues of β-CD,[5,6] where the amino acid numbers in the residue correspond to those in the full-length enzyme molecule, *i.e.*, W543, W590, Y527, and Y556 correspond to W37, W84, Y21, and Y50 in SBDF, respectively.

SBD in the GA molecule enhances the activity of raw-starch granule degradation.[13] Sigurskjold *et al.* have investigated the binding mechanism of β-CD to wild-type SBD/SBDF and reported that the two sites of SBD in the GA molecule interact with each other and the interactions may affect the mechanism of raw-starch granule degradation.[14] Christensen *et al.* have compared the unfolding mechanism of the full-length enzyme and its shorter isozyme without SBD.[15] The two domains of the full-length enzyme unfold simultaneously whereas the isozyme unfolds with several unfolding units.[15] It was argued that the catalytic domain and SBD display inter-domain interactions, [15] which is inconsistent with the independent unfolding of the two domains reported in ref. 3.

In this study, we examined the possibility of interactions between the two domains of a GA molecule and between the two binding sites in SBD using thermodynamic parameters of the binding of β-CD as a probe. We tried to evaluate the thermodynamic parameters of the binding of β-CD at individual binding sites of SBD/SBDF, using GA, wild-type SBDF, and mutant SBDFs where amino acid residues essential for the binding were substituted.

## 2. Materials and Methods

### 2.1 Materials

A commercial preparation of *A. niger* GA was obtained from Sigma-Aldrich Co. LLC. (St. Louis, USA) and was further purified as reported previously[3] using HiTrapQ (GE Healthcare, Uppsala, Sweden) for ion exchange chromatography.

Wild-type SBDF was prepared as reported previously.[8,9] Five mutant forms of SBDFs were also prepared, where the hydrophobic amino acid residues in Site 1 and/or Site 2 were substituted with Ala or Leu: Y527A, Y556A, W543L, W590A, and Y556A/W590A. The mutations were introduced using the Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA). The primers used for the mutagenesis are listed in

**Table 1** Oligonucleotide sequences of the primers used for site-directed mutagenesis.

| Name | Sequence |
|---|---|
| Y527A-f | 5'-CTACCACCACCGCCGGCGAGAACATC-3' |
| Y527A-r | 5'-GATGTTCTCGCCGCCGGTGGTGGTAG-3' |
| Y556A-f | 5'-GTGCTGACAAGGCCACTTCCAGCG-3' |
| Y556A-r | 5'-CGCTGGAAGTGGCCTTGTCAGCAC-3' |
| W543L-f | 5'-CAGCTGGGTGACTTGGAAACCAGCGAC-3' |
| W543L-r | 5'-GTCGCTGGTTTCCAAGTCACCCAGCTG-3' |
| W590A-f | 5'-CTCCGTGGAGGCGGAGAGTGATC-3' |
| W590A-r | 5'-GATCACTCTCCGCCTCCACGGAG-3' |

The mutated nucleotides are underlined.

**Table 1**. Mutant SBDFs were purified by the same procedures as those used for wild-type SBDF.

The protein concentration was spectrophotometrically determined[16] at 280 nm with a molar absorption coefficient at 280 nm ($\varepsilon_{280}$) of 137,000 $cm^{-1}$ $M^{-1}$ for GA; 30,500 $cm^{-1}$ $M^{-1}$ for wild-type SBDF; 29,000 $cm^{-1}$ $M^{-1}$ for Y527A and Y556A; 25,000 $cm^{-1}$ $M^{-1}$ for W543L and W590A; and 23,500 $cm^{-1}$ $M^{-1}$ for Y556A/W590A.

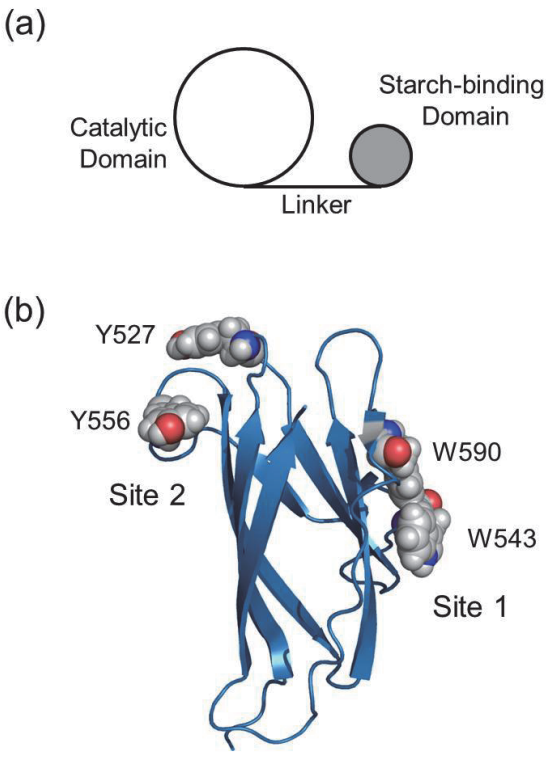


**Fig.1** Schematic representation of the domain structure of *Aspergillus niger* glucoamylase (a) and three dimensional structure of the starch-binding domain fragment (b). In the structure of SBDF, hydrophobic residues in the two binding sites, Sites 1 and 2, are shown by a space filling model. This figure was generated by PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.5 Schrödinger, LLC.) using the Protein Data Bank file 1AC0.

### 2.2 Isothermal titration calorimetry (ITC)

The binding of β-CD to the GA molecule and to wild-type and mutant SBDFs was observed by an isothermal titration

**Table 2** Thermodynamic parameters for binding of β-cyclodextrin to SBDF and GA (the full-length enzyme), assuming two independent sites (pH 7.0 and 10°C).

| Protein | Binding site of β-CD [a] | *n* | $K_d$ /μM [b] | $\Delta H$ /kJ mol$^{-1}$ [b] | $\Delta G^0$ /kJ mol$^{-1}$ | $T\Delta S^0$ /kJ mol$^{-1}$ |
|---|---|---|---|---|---|---|
| SBDF | Site 1 | 1 | 13.6 ± 0.7 | −46.0 ± 0.8 | −26.4 | −19.6 |
| | Site 2 | 1 | 1.0 ± 0.2 | −45.0 ± 0.5 | −32.6 | −12.4 |
| GA | Site 1 | 1 | 11.8 ± 0.3 | −46.0 ± 0.6 | −26.7 | −19.3 |
| | Site 2 | 1 | 1.0 ± 0.1 | −49.0 ± 0.5 | −32.5 | −16.5 |

The parameters were obtained by the two sets of sites model, which assumes two independent sites with different values of $K_d$ and/or $\Delta H$. The value of *n* for each site was fixed at unity in the analysis.
[a] The site was deduced by comparison with **Table 3** (See Results and Discussion).
[b] Values are given with estimated standard errors from the fit.

calorimeter, VP-ITC (MicroCal, Northampton, USA), at pH 7.0 and 10°C. β-CD was purchased from Nacalai Tesque (Kyoto, Japan). Sodium phosphate buffer at pH 7.0 was used at a concentration of 20 mM. Twenty-nine injections (10 μL) of β-CD solution (0.3–1 mM) were made into the protein solution (20–40 μM). The dilution heat resulting from the injection of the β-CD solution was negligible. The data were analyzed by the Origin 7.0 software supplied with the calorimeter using one set of sites model or two sets of sites model. The one set of sites model assumes that the affinities of the binding sites in SBD are the same. On the other hand, the two sets of sites model assumes two types of sites with different ligand affinities.

## 3. Results and Discussion

The binding of β-CD to SBDF and to the full-length enzyme GA was observed by ITC. **Fig.2** shows a typical binding isotherm for the binding of β-CD to wild-type SBDF (pH 7.0 and 10°C). The thermodynamic parameters were evaluated by the two sets of sites model*, which assumes two independent sites with different ligand affinities. The parameters obtained from the ITC data were the dissociation constant $K_d$ and the enthalpy change for binding $\Delta H$. The Gibbs energy change $\Delta G^0$ and the entropy change $\Delta S^0$ for binding were obtained from the equations $\Delta G^0 = -RT\ln(1/K_d)$ and $T\Delta S^0 = \Delta H - \Delta G^0$, respectively, where *T* and *R* are the absolute temperature and the gas constant, respectively. In this analysis, the value of *n*, the number of β-CD molecules bound to the protein, was fixed at unity. The obtained values are listed in **Table 2**. As shown in the table, the binding of β-CD to SBDF and to the full-length enzyme molecule (shown as GA in the table) was an enthalpy-driven reaction with an unfavorable entropy change. There was no essential difference in the parameters of SBDF and SBD of the GA molecule, suggesting that the catalytic domain of the GA molecule did not affect the binding of β-CD to SBD. In other words, there was no interaction between the two domains for binding, which is consistent with previous differential scanning calorimetric data.[3)]

There was a 10-fold difference in the values of $K_d$ of the two binding sites, Sites 1 and 2. This analysis, however, did not identify which site has a higher or lower binding affinity. To clarify this, the values of the thermodynamic parameters for the binding of β-CD to the five mutant SBDFs (Y527A, Y556A, W543L, W590A, and Y556A/W590A) were evaluated by ITC. The obtained values are listed in **Table 3**. Among all the mutants, Y527A, Y556A, W543L, and W590A bound to β-CD

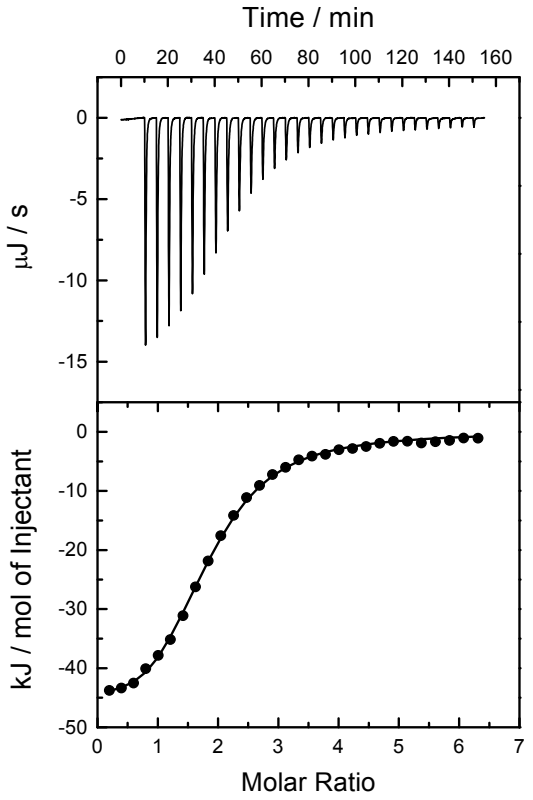


**Fig.2** Isothermal titration calorimetry of the binding of β-CD to wild-type SBDF at pH 7.0 and 10°C. The thermogram (top) and binding isotherm (bottom) are shown. In total, 10 microliters of the β-CD solution (1 mM) was injected 29 times into the wild-type SBDF solution (40 μM). In the binding isotherm, solid circles show experimental values and the solid line shows a theoretical curve fitted to the two sets of sites model.

with stoichiometry of approximately unity, *i.e.*, one β-CD molecule bound to each mutant. No binding heat was observed for Y556A/W590A, suggesting that β-CD molecules did not bind to this double mutant. These results indicate that the hydrophobic residues Y527, Y556, W543, and W590 are essential for the binding of β-CD at each site. The value of $K_d$ for the Site 2-mutated SBDFs Y527A and Y556A was approximately 15 μM and that for the Site-1 mutated SBDFs W543L and W590A was approximately 2 μM (**Table 3**). Judging from the site of mutation, each $K_d$ value, 15 and 2 μM, corresponds to that of Site 1 and Site 2 of these mutants, respectively. These values are consistent with those obtained by UV difference spectroscopy (17–28 μM for Site 1 and 1–6 μM for Site 2).[17,18)] In addition, it is noted that these values are comparable with those of wild-type SBDF and the GA molecule

* Differential scanning calorimetry of wild-type SBDF in the presence of β-CD showed that unfolding temperature $T_{1/2}$ increased with increasing concentration of β-CD (data not shown). Based on an ln[β-CD] vs. 1/ $T_{1/2}$ plot[3)], the number of β-CD molecules that dissociates from wild-type SBDF upon unfolding was evaluated to be 1.6±0.1. This value is comparable with the assumption described in the text.

**Table 3** Thermodynamic parameters for binding of β-cyclodextrin to mutant SBDFs at pH 7.0 and 10°C.

| Protein | Mutation site | Possible binding site of β-CD [a] | $n$ [b] | $K_d$ /μM [b] | $\Delta H$ /kJ mol$^{-1}$ [b] | $\Delta G^0$ /kJ mol$^{-1}$ | $T\Delta S^0$ /kJ mol$^{-1}$ |
|---|---|---|---|---|---|---|---|
| Y527A | Site 2 | Site 1 | 0.91 ± 0.02 | 15.8 ± 0.8 | −53.4 ± 1.5 | −26.0 | −27.4 |
| Y556A | Site 2 | Site 1 | 0.88 ± 0.01 | 13.6 ± 0.6 | −55.0 ± 1.0 | −26.4 | −28.6 |
| W543L | Site 1 | Site 2 | 0.74 ± 0.00 | 1.9 ± 0.1 | −52.4 ± 0.4 | −30.9 | −21.5 |
| W590A | Site 1 | Site 2 | 0.93 ± 0.01 | 2.0 ± 0.1 | −44.8 ± 0.3 | −30.9 | −13.9 |
| Y556A/W590A [c] | Sites1, 2 | none | - | - | - | - | - |

The parameters were obtained by a simple model, the one set of sites model that contains identical binding sites.
[a] β-CD was considered to bind to Site 1 of the Site 2-mutated protein and *vice versa*.
[b] Values are given with estimated standard errors from the fit.
[c] In the case of Y556A/W590A, no binding heat was observed by ITC.

(**Table 2**). Hence, the larger $K_d$ value (15 μM) can be allocated to Site 1 and the smaller value (2 μM) to Site 2, irrespective of wild-type SBDF and SBD in the GA molecule.

In conclusion, we observed no sign of inter-domain and inter-site interactions by the thermodynamic parameters of the binding of β-CD, which was used as a probe. This is not consistent with previous reports that have suggested interactions between the two domains and between the two binding sites of SBD.[14,15] The reason for this apparent discrepancy remains unknown. To clarify this, it is necessary to investigate the binding thermodynamics of the individual sites of SBD in the GA molecule. A calorimetric analysis using the GA mutants whose residues in Sites 1 and 2 of SBD are substituted has been planned.

## References


1) B. Svensson, K. Larsen, I. B. Svendsen, and E. Boel, *Carlsberg Res. Commun.* **48**, 529-544 (1983).
2) J. Lee, and M. Paetzel, *Acta Cryst.* **F67**, 188-192 (2011).
3) A. Tanaka, H. Fukada, and K. Takahashi, *J. Biochem*. **117**, 1024-1028 (1995).
4) A. Tanaka, *Biosci. Biotech. Biochem.* **60**, 2055‐2058 (1996).
5) K. Sorimachi, A. J. Jacks, M. F. Le Gal Coëffet, G. Williamson, D. B. Archer, and M. P. Williamson, *J. Mol. Biol.* **259**, 970-987 (1996).
6) K. Sorimachi, M. F. Le Gal Coëffet, G. Williamson, D. B. Archer, and M. P. Williamson, *Structure* **5**, 647‐661 (1997).
7) Y. Suyama, N. Muraki, M. Kusunoki, and H. Miyake, *Acta Cryst*. **F73**, 550–554 (2017).
8) A. Tanaka, S. Karita, Y. Kosuge, K. Senoo, H. Obata, and N. Kitamoto, *Biosci. Biotech. Biochem.* **62**, 2127-2132 (1998).
9) H. Sugimoto, M. Nakaura, Y. Kosuge, K. Imai, H. Miyake, S. Karita, and A. Tanaka, *Biosci. Biotech. Biochem.* **71**, 1535-1541 (2007).
10) H. Sugimoto, M. Nakaura, S. Nishimura, S. Karita, H. Miyake, and A. Tanaka, *Protein Sci.* **18**, 1715‐1723 (2009).
11) H. Sugimoto, Y. Noda, and S. Segawa, *J. Mol. Biol.* **412**, 304-315 (2011).
12) C. Ota, M. Ikeguchi, A. Tanaka, and D. Hamada, *Biochim. Biophys. Acta* **1864**, 1464-1472 (2016).
13) C. Christiansen, M. A. Hachem, Š. Janeček, A. Vikso-Nielsen, A. Blennow, and B. Svensson, *FEBS J.* **276**, 5006-5029 (2009).
14) B. W. Sigurskjold, B. Svensson, G. Williamson, and H. Driguez, *Eur. J. Biochem*. **225**, 133-141 (1994).
15) T. Christensen, B. Svensson, and B. W. Sigurskjold, *Biochemistry* **38**, 6300-6310 (1999).
16) C. N. Pace, F. Vajdos, L. Fee, G. Grimsley, and T. Gray, *Protein Sci.* **4**, 2411-2423 (1995).
17) M. P. Williamson, M. F. Le Gal-Coëffet, K. Sorimachi, C. S. M. Furniss, D. B. Archer, and G. Williamson, *Biochemistry* **36**, 7535-7539 (1997).
18) T. Giardina, A. P. Gunning, N. Juge, C. B. Faulds, C. S. M. Furniss, B. Svensson, V. J. Morris, and G. Williamson, *J. Mol. Biol.* **313**, 1149-1159 (2001).


## 要　旨


黒コウジカビのグルコアミラーゼは触媒ドメインとデンプン結合ドメイン（SBD）から構成される。SBD には 2 つの基質結合部位がある。本論文では，リガンド結合に関して 2 つのドメイン間および 2 か所の結合部位間に相互作用[*Biochemistry* **38**, 6300 (1999)，*Eur. J. Biochem.* **225**, 133 (1994)] があるかどうかを，基質アナログであるβ-シクロデキストリン（β-CD）との結合の熱力学量を指標に検討を行った。全長酵素および SBD フラグメント（SBDF）において，β-CD との結合の熱力学量に差は見られなかった。また SBDF について，1 つの結合部位の疎水性アミノ酸残基を置換しても，他方の部位の結合の熱力学量への影響はなかった。これらの結果から，リガンド結合に関して 2 つのドメイン間および 2 つの結合部位間に相互作用はないと考えられた。