Thermodynamics of Binding of Heterobidentate Ligands Consisting of Spacer-Connected Acarbose and β-Cyclodextrin to the Catalytic and Starch-Binding Domains of Glucoamylase from *Aspergillus niger* Shows That the Catalytic and Starch-Binding Sites Are in Close Proximity in Space†

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ABSTRACT: The binding to glucoamylase 1 from *Aspergillus niger* (GA1) of a series of four synthetic heterobidentate ligands of acarbose and β-cyclodextrin (β-CD) linked together has been studied by isothermal titration calorimetry. GA1 consists of a catalytic and a starch-binding domain (SBD) connected by a heavily *O*-glycosylated linker region. Acarbose is a strong inhibitor of glucoamylase and binds exclusively in the catalytic site, while the cyclic starch mimic β-CD binds exclusively to the two sites of SBD. No spacer or spacer arms of 14, 36, and 73 Å in their extended conformations connect acarbose and β-CD. These compounds were used as probes for bidentate ligand binding to both domains in order to estimate the distance between the catalytic site and the SBD binding site in solution. ΔH of binding of the four heterobidentate ligands is within experimental uncertainty equal to the sum of ΔH of binding of free acarbose and β-CD, indicating ligand binding to both domains. However, the binding constants are 4−5 orders of magnitude smaller than for the binding of acarbose ($K \approx 10^{12}$ M^{-1}), increasing with spacer length from 2×10^7 M^{-1} for no spacer to 1×10^8 M^{-1} for the 73 Å spacer. Subsequent titrations with β-CD of the glucoamylase−bidentate ligand complexes revealed that only one of the two binding sites of SBD was vacant. Further titrations with acarbose to these mixtures showed complete displacement of the acarbose moiety of the bidentate ligands from the catalytic sites. These experiments show that the bidentate ligands bind to both the catalytic domain and SBD. The weakening of the bidentate ligand binding compared to acarbose is a purely entropic effect point to steric hindrance between SBD and the β-CD moiety. To test this, titrations of glucoamylase 2, a form containing the catalytic domain and the linker region but lacking SBD, with the bidentate ligands were carried out. The results were indistinguishable from the binding of free acarbose. Thus, the reduced affinity of the bidentate ligands observed with GA1 stems from interactions due to SBD. The results show that the catalytic and starch-binding sites are in close proximity in solution and thus indicate considerable flexibility of the linker region.

Many polysaccharide hydrolyzing enzymes consist of a catalytic domain and a binding domain that enables the enzyme to attach to the polysaccharides, for example, starch, cellulose, chitin, xylan, and others (*1*−*9*). However, very little is known about how these domains interact in the process of hydrolyzing a polysaccharide. Glucoamylase 1 from *Aspergillus niger* (GA1[1]) is an enzyme of this type. It is an exo-glycosidase that catalyzes the hydrolysis of α-1 → 4-glycosidic and α-1 → 6-glycosidic linkages of oligo- and polysaccharides of glucose to produce β-glucose as the primary product (*10*). The enzyme is produced by the fungus in two forms, glucoamylase 1 (GA1) and glucoamylase 2 (GA2). GA1 contains 616 amino acids forming a catalytic domain from amino acid residue 1 to 440, a linker region from amino acid 441 to 508 rich in Ser and Thr which are *O*-glycosylated mainly by mono-, di-, and trisaccharides of mannosyl units (*11*), and a starch-binding domain (SBD) from amino acid 509 to 616 (Figure 1). The other form (GA2) only contains the catalytic domain and the linker region from amino acid 1 to 512. This form readily degrades soluble starch but not raw starch or starch granules (*12*−

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[1] Abbreviations: GA1, glucoamylase 1 from *Aspergillus niger*; GA2, glucoamylase 2, a truncated form of glucoamylase without the starch-binding domain; ITC, isothermal titration calorimetry; SBD, starch-binding domain; β-CD, β-cyclodextrin (cyclomaltoheptaose); NOJ, 1-deoxynojirimycin; L0, L14, L36, L73, heterobidentate ligands of covalently linked acarbose and β-cyclodextrin without spacer (L0) and with spacers of 14, 36, and 73 Å in length, respectively.

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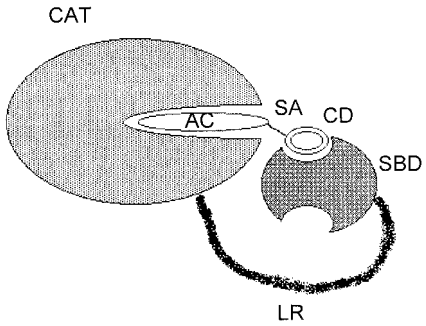

FIGURE 1: Schematic structure of glucoamylase 1 from *A. niger*. The enzyme consists of a catalytic domain (CAT), a starch-binding domain (SBD), and an *O*-glycosylated linker region (LR) joining the two domains. Also shown is the possibility of heterobidentate binding of ligands consisting of acarbose (AC) and β-cyclodextrin (CD) moieties covalently coupled by a poly(ethylene glycol) spacer arm (SA). Acarbose binds in the active site, and β-cyclodextrin binds in one of the two starch-binding sites of the starch-binding domain.

14). Crystal structures of the $(\alpha/\alpha)_6$-barrel catalytic domain including part of the linker region of a closely related GA from *Aspergillus awamori* var. *X100* without ligand (*15*, *16*) and with the bound inhibitors 1-deoxynojirimycin (NOJ) (*17*), acarbose (*18*, *19*), or D-*gluco*-dihydroacarbose (*19*, *20*) are available. Furthermore, solution NMR structures of the SBD without ligand (*21*) and with bound β-cyclodextrin (β-CD) (*22*) have also been determined. The only structural information available about the whole enzyme is scanning tunneling microscopic images, which suggest that the two domains are spatially separated with the linker region in an extended conformation (*23*).

Differential scanning calorimetric investigations on different proteolytically truncated forms produced from GA1 have shown that the linker region stabilizes the structure of the catalytic domain (*24*). The same conclusion was reached by sequence alignments of different glucoamylases (*25*). These studies showed a correlation between longer linker region and increased stability. A study on linker region deletion mutants also showed reduced catalytic domain stability, while activity toward both soluble and insoluble starch remained unchanged (*26*). Moreover, deglycosylation of the linker region of GA led to thermal destabilization with unchanged overall conformation as probed by circular dichroism and UV spectroscopy (*27*). Deglycosylation also leads to less reversibility of thermal unfolding, suggesting that the linker region may primarily destabilize unfolded GA1 (*27*).

Acarbose is a strong inhibitor of glucoamylase and binds in the active site with an affinity of $K = 7.7 \times 10^{11}$ M^{-1} (*28*), while β-CD binds exclusively to the two sites of SBD with an affinity of 4.8×10^4 M^{-1} for both sites (*29*−*31*). Four heterobidentate ligands with acarbose and β-CD linked together have been synthesized. One has no spacer, while in other three ligands spacer arms consisting of poly(ethylene glycol) moieties are attached to the reducing end of acarbose by a β-thio glycosidic linkage and to one of the primary hydroxyl groups of β-CD by a thioether bond (Figure 2). The lengths of the spacers in their extended conformation are 14, 36, and 73 Å, respectively. We have used these compounds as probes for bidentate ligand binding to both domains in order to estimate the distance for GA1 in solution between the catalytic site and the SBD binding site using isothermal titration calorimetry (ITC). This technique enables the determination of free energy, enthalpy, entropy, and stoichiometry of an association reaction in a single experiment and is thus ideally suited to provide thermodynamic descriptions of protein−ligand interactions.

The thermodynamics of binding of acarbose to a number of mutant variants of GA1 has been reported previously (*32*). One of these mutants is Arg54 → Leu in which a critical hydrogen bond in the active site between the 4-OH group of acarbose and the side chain of Arg54 is abolished. This leads to a highly reduced affinity for acarbose, $K = 3.9 \times 10^6$ M^{-1} (*32*). To investigate whether changes in the binding interactions of the heterobidentate ligands compared to free acarbose and β-CD take place in the active site or on the SBD sites, ITC measurements of binding to this mutant of the heterobidentate ligands were also carried out.

MATERIALS AND METHODS

Materials. GA1 and GA2 were purified from the commercial preparation (AMG 200L) from Novo Nordisk A/S essentially as described previously (*12*). The gene construction and production of the mutant variant Arg54 → Leu has also been described previously (*33*). Acarbose was a generous gift from Bayer AG (Wuppertal, Germany). β-CD and NOJ were purchased from Sigma. The syntheses of the heterobidentate ligands will be described elsewhere (*34*).

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) measurements (*35*, *36*) were performed on an MCS isothermal titration microcalorimeter from MicroCal, Inc. (Northampton, MA). All titrations were carried out at 27 °C in 50 mM sodium acetate buffer, pH 4.5. In each ITC experiment, solutions of 17−25 μM GA were titrated with 21 portions of 13 μL of ligand in the same buffer at 3 min intervals. The concentrations of the ligands in the injection syringe were 177 μM for acarbose and the heterobidentate ligands and 400 μM for β-CD. The instrument was calibrated using electrical heat pulses. Acquisition of the titration thermograms was controlled by the Observer software from MicroCal, Inc., and integration of the heat signals was carried out using the Origin software from the same manufacturer. The binding isotherms were corrected for heat of dilution of the ligands from blank titrations of ligand into buffer. Nonlinear regression analysis of binding isotherms was performed as described previously (*36*) and outlined briefly below. In the analysis of the binding isotherms for the reaction $P + nL \rightleftharpoons PL_n$, the site binding constant K for the ith site is given by

$$\frac{[PL]}{[P_{i-1}][L]} = K \tag{1}$$

assuming that the n sites are identical and independent. If the stoichiometric concentrations of protein and ligand are called $[P]_0$ and $[L]_0$, respectively, then the concentration of complex is given by

$$[PL_n] = {}^1/_2(n[P]_0 + [L]_0 + 1/K - \sqrt{(n[P]_0 + [L]_0 + 1/K)^2 - 4n[P]_0[L]_0}) \tag{2}$$

The heat evolved after one addition of ligand, ΔQ, is proportional to the calorimeter cell volume, V_0, the molar

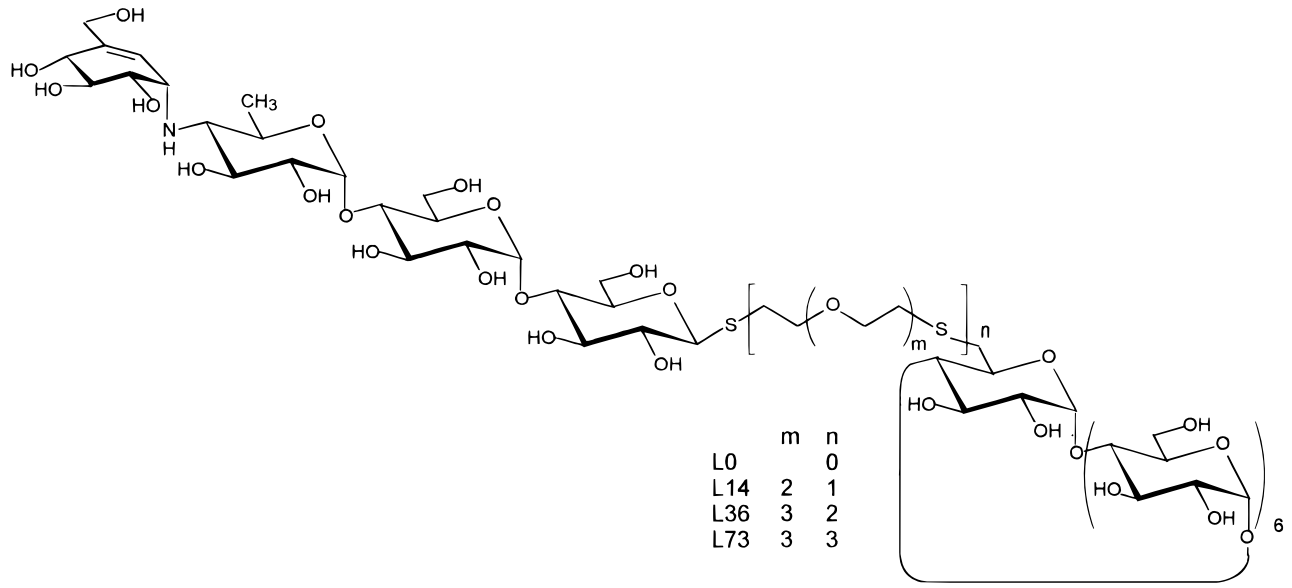

FIGURE 2: Structures of the heterobidentate ligands formed by acarbose and β-cyclodextrin joined by spacer arms of poly(ethylene glycol) of varying lengths attached to the reducing end of acarbose by a β-thio glycosidic linkage and to one of the primary hydroxyl groups of β-CD by a thioether bond. The four ligands used have either no spacer (0 Å, L0) or spacer arms of 14, 36, and 73 Å (L14, L36, and L73), respectively.

enthalpy of binding, ΔH, and the change in complex concentration:

$$\Delta Q = V_0 \Delta H \Delta[\mathrm{PL}_n] \tag{3}$$

where n is the stoichiometry of the reaction. By fitting the binding isotherm to the above expressions by nonlinear regression, the binding constant, enthalpy, and stoichiometry can be determined, and subsequently standard free energy, $\Delta G°$, and standard entropy, $\Delta S°$, can be calculated from

$$\Delta G° = -RT \ln K = \Delta H - T\Delta S° \tag{4}$$

where R is the universal gas constant, T is the absolute temperature, and the standard state is defined as the fictitious state in which all components of the system have concentrations of 1 M.

RESULTS

GA1 was titrated with each of the four heterobidentate ligands. The four compounds, whose structures are outlined in Figure 2, either lack a spacer or have spacer arms of 14, 36, and 73 Å, respectively, in their extended conformations. Hence, they are dubbed L0, L14, L36, and L73, respectively. It is anticipated that only if the spacer arm is long enough compared to the distance between the catalytic site and one of the starch-binding sites of SBD will bidentate binding be observed. The results are summarized in Table 1, and a typical thermogram and binding isotherm for L36 are shown in Figure 3. The binding thermodynamics of acarbose to GA1 and β-CD to SBD determined previously (*28*, *30*) is also listed in Table 1 together with the results of β-CD binding to GA1. There is no significant difference between β-CD binding to GA1 and SBD. The sum of ΔH for acarbose and β-CD binding to GA1 is -86.7 ± 1.7 kJ mol^{-1}, and this is very close to ΔH for the binding of the heterobidentate ligands. L73 seems to be an exception, approximately 19 kJ mol^{-1} short of showing additivity with respect to ΔH. This enthalpy additivity suggests that both ends of the heterobidentate molecules bind to GA1. However, the bindings are 4−5 orders of magnitude weaker than the binding of acarbose which corresponds to reductions in negative free energy of more than 23 kJ mol^{-1}. This suggests the possibility that binding of the heterobidentate ligands takes place only in one site by either the acarbose moiety or the β-CD moiety. This would most likely be binding to the catalytic site by the acarbose moiety, since the bidentate ligands bind 3 orders of magnitude stronger than β-CD.

Since SBD has two β-CD binding sites, one and only one site must be vacant after binding of one equivalent of a heterobidentate ligand. That this is indeed the case is shown by subsequent titrations with β-CD (Figure 4 and Table 1). The apparent stoichiometries of β-CD binding after binding of heterobidentate ligands are all somewhat below one. The apparent stoichiometries of the bidentate ligands are, on the other hand, a little larger than one. This happened because the heterobidentate ligand titrations were carried out to an extent that exceeded an equimolar amount to approach saturation. Hence, after the catalytic site and one of the SBD sites have been filled by bidentate ligand, some of the excess ligand can bind to the vacant SBD binding site by its β-CD moiety.

Although binding of the heterobidentate ligands is substantially stronger than that of β-CD, it cannot immediately be ruled out that binding takes place only at an SBD site. A proof of the binding of the acarbose moiety of the heterobidentate ligands in the catalytic site was provided by further titrations with acarbose, a representative thermogram of which is shown in Figure 5a. The very small heat signals are of the same size as heat of dilution signals. This shows that either acarbose does not bind at all or it displaces the acarbose moiety of heterobidentate ligands with an exact cancellation of the endothermic ΔH of dissociation with exothermic ΔH of association. Since acarbose has a much higher affinity for GA than that of any of the heterobidentate

Table 1: Binding Constant, Free Energy, Enthalpy, Entropy, and Stoichiometry of Binding of Heterobidentate Ligands to Glucoamylase 1 and 2 Determined by Isothermal Titration Calorimetry at 27 °C, pH 4.5[a]

cell content	ligand	K, M^{-1}	$-\Delta G°$, kJ mol^{-1}	$-\Delta H$, kJ mol^{-1}	$T\Delta S°$, kJ mol^{-1}	apparent stoichiometry
GA1[b,c] + NOJ	acarbose	$(7.7 \pm 5.5) \times 10^{11}$	68.9 ± 1.6	32.8 ± 1.4	$+36.1 \pm 2.1$	0.95 ± 0.01
GA1	β-CD	$(4.8 \pm 1.1) \times 10^{4}$	26.9 ± 1.3	53.9 ± 0.9	-27.0 ± 3.9	1.76 ± 0.09
SBD[d]	β-CD	$(5.2 \pm 0.3) \times 10^{4}$	27.1 ± 0.2	55.5 ± 0.7	-28.4 ± 0.7	1.99 ± 0.01
GA1	L0	$(1.9 \pm 0.8) \times 10^{7}$	41.8 ± 1.1	84.3 ± 3.6	-42.5 ± 3.9	1.08 ± 0.27
GA1[e]	L0	$(1.0 \pm 1.9) \times 10^{7}$	40.3 ± 4.5	86.0 ± 4.1	-45.7 ± 6.1	1.11 ± 0.33
GA1[f]	L0	$(8.4 \pm 0.7) \times 10^{6}$	39.8 ± 0.2	88.2 ± 0.3	-48.4 ± 0.4	1.06 ± 0.10
GA1	L14	$(2.5 \pm 1.7) \times 10^{7}$	42.5 ± 0.7	89.4 ± 0.9	-46.9 ± 1.2	1.19 ± 0.11
GA1	L36	$(6.6 \pm 1.1) \times 10^{7}$	44.9 ± 1.8	94.3 ± 1.7	-49.4 ± 2.5	1.07 ± 0.12
GA1	L73	$(9.6 \pm 1.2) \times 10^{7}$	45.9 ± 0.8	69.2 ± 0.6	-23.3 ± 1.0	1.25 ± 0.08
GA1 + L0	β-CD	$(5.4 \pm 2.2) \times 10^{4}$	27.2 ± 1.0	53.5 ± 3.1	-26.3 ± 3.3	0.82 ± 0.07
GA1 + L14	β-CD	$(7.5 \pm 1.6) \times 10^{4}$	28.0 ± 0.5	55.9 ± 7.4	-27.9 ± 7.4	0.84 ± 0.12
GA1 + L36	β-CD	$(5.8 \pm 0.4) \times 10^{4}$	27.4 ± 0.2	56.9 ± 1.7	-29.5 ± 1.7	0.81 ± 0.01
GA1 + L73	β-CD	$(6.6 \pm 0.5) \times 10^{4}$	27.7 ± 0.2	53.1 ± 1.5	-25.4 ± 1.5	0.87 ± 0.01
GA2[b]	NOJ	$(3.3 \pm 0.2) \times 10^{4}$	25.8 ± 0.1	11.2 ± 0.1	$+14.5 \pm 0.2$	1.00 ± 0.07
GA2	L0	$>10^{8}$	>46.0	33.6 ± 0.6	>12.4	0.78 ± 0.01
GA2	L14	$>10^{8}$	>46.0	31.8 ± 0.6	>14.2	0.68 ± 0.02
GA2	L36	$>7 \times 10^{7}$	>45.1	27.0 ± 0.8	>18.1	0.63 ± 0.02
GA2	L73	$>10^{8}$	>46.0	41.4 ± 0.1	>4.6	0.79 ± 0.01
GA2[c] + NOJ	L0	$(1.6 \pm 0.9) \times 10^{12}$	70.2 ± 1.5	32.1 ± 2.2	$+38.1 \pm 2.7$	0.78 ± 0.01
GA2[c] + NOJ	L73	$(2.2 \pm 0.9) \times 10^{12}$	70.9 ± 1.5	37.7 ± 3.5	$+33.2 \pm 3.8$	0.71 ± 0.01
GA2[b,c] + NOJ	acarbose	$(8.8 \pm 3.2) \times 10^{11}$	68.2 ± 0.9	40.6 ± 0.1	$+27.6 \pm 0.9$	1.01 ± 0.01

[a] The concentrations used were 17−25 μM glucoamylase, 177 μM acarbose or heterobidentate ligands, and 400 μM β-cyclodextrin unless otherwise stated in footnote *e* or *f*. [b] Data from ref *28*. [c] Displacement experiment of 1-deoxynojirimycin. [d] Data from ref *30*. [e] [GA1] = 12.5 μM; [L0] = 88 μM. [f] [GA1] = 50 μM; [L0] = 320 μM.

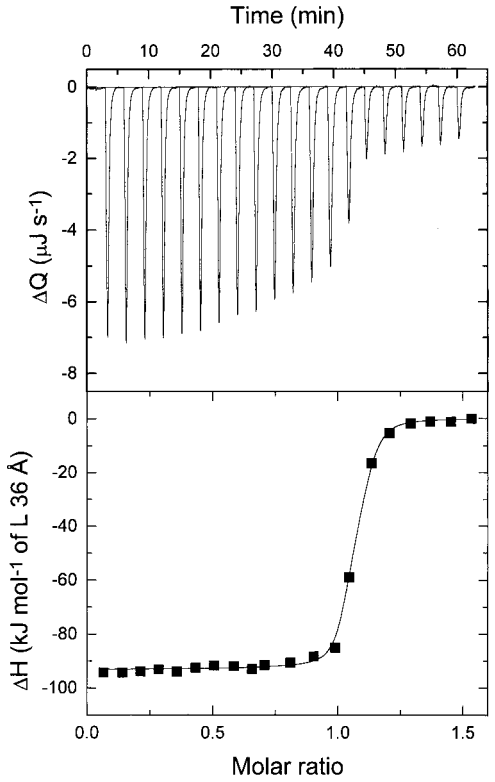

FIGURE 3: Thermogram (top) and binding isotherm (bottom) of the binding of the heterobidentate ligand L36 to glucoamylase 1 obtained by isothermal titration calorimetry.

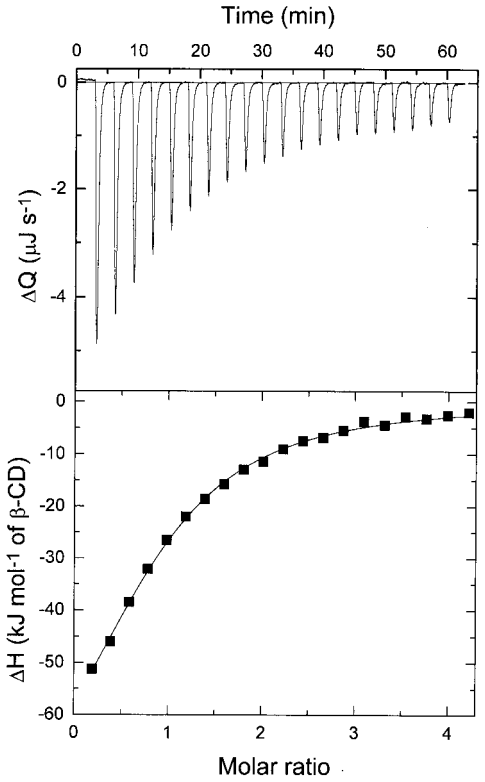

FIGURE 4: Thermogram (top) and binding isotherm (bottom) of the binding of β-cyclodextrin binding to the starch-binding domain of glucoamylase 1 which has already been bound to the heterobidentate ligand L36.

ligands, it must displace the acarbose moiety of these. Thus, ΔH for binding of acarbose in the catalytic site is the same whether it is free or part of a heterobidentate ligand. It is therefore concluded that the observed enthalpy additivity is a result of true additivity and not the result of a fortuitous cancellation of opposite changes in ΔH of interactions in the two different binding sites. A control experiment with the same batch of GA1 titrated with acarbose reproduced the previously published values (*28*) (Table 1, top entry). These experiments clearly show that the heterobidentate ligands indeed bind to both the catalytic domain and SBD.

At this point, there is still the possibility that the bidentate binding occurs between different GA1 molecules with the formation of higher-order complexes. Since the apparent stoichiometry is clearly 1:1 (Table 1), these complexes must

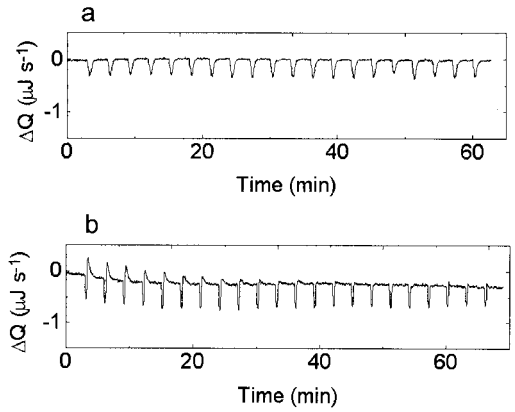

FIGURE 5: (a) Thermogram of the binding of acarbose to a mixture of glucoamylase 1, the heterobidentate ligand L36, and β-cyclodextrin. Acarbose displaces the acarbose moiety of the heterobidentate ligand. (b) Thermogram of the binding of acarbose to a mixture of the glucoamylase 1 active site mutant Arg54 → Leu, the heterobidentate ligand L36, and β-cyclodextrin.

be formed after the equation $nP + nL \rightleftharpoons P_nL_n$. The equilibrium constant of this reaction is given by

$$K = \frac{[P_nL_n]}{([P]_0 - n[P_nL_n])^n([L]_0 - n[P_nL_n])^n} \quad (5)$$

However, the binding isotherms have been fitted according to a model in which only binary complexes are formed according to eqs 2 and 3. The heat signals are then proportional to the apparent concentration of binary complex, $[PL]_{app}$, which will be equal to $n[P_nL_n]$ for the formation of n-mers. Hence, the observed binding constant is

$$K_{app} = \frac{n[P_nL_n]}{([P]_0 - [P_nL_n])([L]_0 - [P_nL_n])} \quad (6)$$

By dividing this equation with eq 5 one obtains

$$K_{app} = Kn([P]_0 - n[P_nL_n])^{n-1}([L]_0 - n[P_nL_n])^{n-1} \quad (7)$$

From eq 7 it is clear that if quaternary or higher-order complexes were formed, then a strong dependence on the concentrations of the interacting species should be observed for K_{app}. Two experiments using 12.5 and 50 μM GA1 titrated with 88 and 320 μM L0, respectively, were carried out, and the results are listed in Table 1. No concentration dependence can be observed within experimental uncertainty. In addition, native polyacrylamide gel electrophoresis of the titration mixtures only showed one band corresponding to GA1 (data not shown). Further evidence was given by dynamic light-scattering experiments in which no defined complex was observed. Hence, we are confident that only binary binding is occurring.

The values of the thermodynamic functions $\Delta G°$, ΔH, and $T\Delta S°$ are also listed in Table 1. The reduced affinity of the heterobidentate ligands compared to acarbose is clearly a pure entropy effect. This points to a steric clash between the spacer and/or β-CD of the ligands and the SBD of the protein. In GA2, which lacks the SBD, this proposed clash should not appear. This is confirmed by titrations of GA2. The results of both direct titrations with the heterobidentate ligands and titrations in which the ligands displace the weak competitive inhibitor NOJ (*28*) were indistinguishable from the binding behavior of acarbose (Table 1). Thus, the adverse effect characteristic of GA1 seems to stem from interactions due to the SBD. Our results therefore indicate that the catalytic and starch-binding sites are in close proximity in solution and suggest considerable flexibility of the highly *O*-glycosylated linker region.

Control experiments in which GA1 was titrated with free acarbose after binding to free β-CD and vice versa revealed no influence on the binding of either ligand by the other. Acarbose binding to GA2 is not affected by the presence of β-CD, either (data not shown). The effects observed for the heterobidentate ligands are thus caused by the covalent connection between the binding moieties.

There is a small but significant increase in K with increasing length of the spacer from 2×10^7 M^{-1} for L0 to 1×10^8 M^{-1} for L73. As far as the ligands are concerned, longer spacer arms allow for conformational flexibility (increased entropy) and hence stronger binding. This effect is very small though, with the ligands used in this study, and it is probably not possible to obtain full free-energy additivity just by prolonging the spacer chain.

The side chain of Arg54 hydrogen bonds to the critical OH-4 group of the pseudosugar ring at the nonreducing end of acarbose in the catalytic site (*16*). It has already been shown that changing this residue to Leu (or Lys) greatly reduces the affinity for acarbose (*36*). We have conducted the same type of ITC measurements using bidentate ligands with this mutant, and the results are summarized in Table 2. Subsequent titrations were conducted with β-CD after binding of L36 and L73 both failed due to instrument malfunction and could not be repeated because of lack of material. The affinities for the heterobidentate ligands are approximately 1 order of magnitude smaller than for the wild type, but the same increase in affinity with increasing spacer chain length is observed for Arg54 → Leu as for the wild type. However, there is no longer additivity for the two binding components, acarbose and β-CD, with respect to ΔH. In fact, acarbose can displace the acarbose moiety of the heterobidentate ligands as shown in Figure 5b for L0. This means that the acarbose part contributes very little to the binding to Arg54 → Leu GA1 and that the interactions in the catalytic site change when acarbose is connected to the spacer and β-CD. The displacement titration with acarbose shown in Figure 5b shows heat signals with small endothermic components in the beginning of the thermogram. This confirms that ΔH does not cancel out but is more exothermic for binding of the heterobidentate ligands than for acarbose to this mutant.

DISCUSSION

The strongly reduced affinity of the heterobidentate ligands for GA1 compared to acarbose and β-CD is purely an entropic effect. If the free energies were additive, then $\Delta G°$ values close to -100 kJ mol^{-1} would have been expected, corresponding to a binding constant of 10^{17} M^{-1}. The additivity of ΔH clearly indicates that the molecular interactions between the heterobidentate ligands and protein in the two binding sites are identical to the interactions of acarbose and β-CD. Furthermore, if ternary complexes of GA1, acarbose, and β-CD were formed, this would carry a cratic

Table 2: Binding Constant, Free Energy, Enthalpy, Entropy, and Stoichiometry of Binding of Heterobidentate Ligands to Arg54 → Leu Glucoamylase 1 Determined by Isothermal Titration Calorimetry at 27 °C, pH 4.5[a]

cell content	ligand	K, M^{-1}	$-\Delta G°$, kJ mol^{-1}	$-\Delta H$, kJ mol^{-1}	$T\Delta S°$, kJ mol^{-1}	apparent stoichiometry
R54L	acarbose	$(2.3 \pm 0.1) \times 10^6$	36.4 ± 0.2	41.5 ± 0.3	-5.2 ± 0.3	1.06 ± 0.01
R54L	β-CD	$(9.0 \pm 0.8) \times 10^4$	28.5 ± 0.2	53.7 ± 1.6	-25.2 ± 1.6	1.80 ± 0.03
R54L	L0	$(4.1 \pm 0.6) \times 10^6$	38.0 ± 0.3	68.2 ± 0.8	-30.2 ± 0.9	1.08 ± 0.11
R54L	L14	$(4.4 \pm 0.7) \times 10^6$	38.1 ± 0.4	69.8 ± 1.0	-31.6 ± 1.1	1.21 ± 0.13
R54L	L36	$(6.7 \pm 1.1) \times 10^6$	39.2 ± 0.4	62.4 ± 2.3	-23.2 ± 2.3	1.10 ± 0.09
R54L	L73	$(1.3 \pm 0.3) \times 10^7$	40.9 ± 0.5	69.9 ± 3.0	-29.0 ± 3.0	1.10 ± 0.10
R54L + L0	β-CD	$(3.1 \pm 0.5) \times 10^4$	25.8 ± 0.4	51.4 ± 2.8	-25.6 ± 2.8	0.89 ± 0.07
R54L + L14	β-CD	$(3.5 \times 0.3) \times 10^4$	26.1 ± 0.2	52.0 ± 2.9	-25.9 ± 2.9	0.88 ± 0.10

[a] The concentrations used were 19 μM glucoamylase Arg54 → Leu, 177 μM acarbose or heterobidentate ligands, and 400 μM β-cyclodextrin.

entropy penalty that is 10 kJ mol^{-1} ($-T\Delta S°$) larger than forming a binary complex between GA1 and a heterobidentate ligand (*37*). The total amount of adverse entropy then comes to 60 kJ mol^{-1}. Some of this entropy reduction may come from strongly reduced conformational freedom of the ligand spacer arms and/or the linker region of GA1. One might expect that the heterobidentate ligands could bind by the acarbose moiety alone and leave the β-CD out of the SBD binding site, thereby obviating the heavy entropy penalty. This is clearly not the case, even for L0. The most probable explanation for this is that SBD of GA1 is close in space to the catalytic site and that a steric contact between this domain and β-CD of the ligand is unavoidable when the acarbose moiety is bound in the active site. This strongly reduces the affinity by an adverse entropy effect.

The thermodynamic parameters for one β-CD molecule binding to the complex of GA1 and a heterobidentate ligand (Table 1) are the same as that of β-CD binding to unliganded GA1 (*30*). This confirms the previously reported result that the two binding sites of SBD seem to be virtually thermodynamically identical, at least toward binding of β-CD. ITC measurements of β-CD binding to GA1 under varying conditions of temperature and pH also show very little variation, and no differences between the two sites could be observed (T. Christensen, B. Svensson, and B. W. Sigurskjold, unpublished experiments). This is very surprising since the two binding sites are structurally very different and interact differently with β-CD as shown by NMR (*22*). It is not clear what the mechanistic significance of the thermodynamically identical binding sites of SBD is. Neither is it clear how thermodynamic identity is achieved by two structurally different sites. Williamson and co-workers (*38*) have studied the binding of β-CD by two GA1 mutants with altered tryptophan residues in SBD, Trp563 → Lys, and Trp590 → Lys, by NMR and UV difference spectroscopy. They found that each of these mutants abolished binding to one of the binding sites. They also found slightly different binding constants for the two sites: $K = 3.6 \times 10^4$ M^{-1} for site 1 (site 2 knocked out by W590K) and $K = 1.6 \times 10^5$ M^{-1} for site 2 (site 1 knocked out by W563K). The wild-type affinity was 6.9×10^4 M^{-1} which to a reasonable approximation is the average of the two unchanged sites of the mutants. However, as the authors point out, the sum of the maximum UV absorbance changes for β-CD binding to the mutants does not add up to the maximum change for the wild type, indicating that mutation in one site of SBD might affect the other. Fitting ITC isotherms of β-CD binding to GA1 using a model containing two independent sites with the K values reported by Williamson et al. (*38*) gives the curve shown in Figure 6. Clearly, the two binding sites

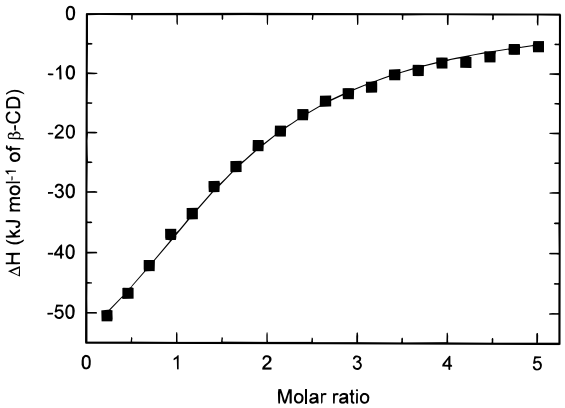

FIGURE 6: Binding isotherm of the binding of β-cyclodextrin to the starch-binding domain of glucoamylase 1. The line has been generated by nonlinear regression with two sites and fixed values of binding constants: $K = 3.6 \times 10^4$ and 1.6×10^5 M^{-1}. ΔH values obtained for the two sites are -52.9 ± 0.9 and -54.9 ± 0.9 kJ mol^{-1}, respectively.

cannot be resolved in this fit and result in identical ΔH values. It is thus entirely likely that the two sites indeed are somewhat different in affinity.

Scanning tunneling microscopic studies on the architecture of GA1 suggest that the linker region is in an extended and rigid conformation and that the catalytic domain and SBD have no contact (*23*). These studies, however, have been carried out on dry samples of unliganded GA1, and it is not known how the dryness of the sample might influence the protein structure. In the crystal structure the first approximately 30 of the 70 amino acid residues of the linker region are closely curled around the catalytic domain (*15*−*24*). If one imagines the end of this part of the linker region being prolonged by the 40 highly *O*-glycosylated part of the linker and the C-terminal part of SBD, the SBD would indeed get close to the catalytic site.

The mechanism of binding of acarbose to GA1 wild type contains at least two intermediary states as shown by stopped-flow kinetic studies (*39*, *40*). The additive enthalpies of the heterobidentate ligands indicate that their acarbose moiety binds to wild-type GA1 the same way and, hence, goes through the same intermediates. However, if the last step in acarbose binding has a $\Delta H = 0$, then it is not possible to rule out that the heterobidentate ligands can be caught in a kinetic trap. The last step in free acarbose binding has a rate constant $k \approx 0.6$ s^{-1} at 8 °C (*39*, *40*). This corresponds to $t_{1/2} \approx 1$ s and about 10 s to reach equilibrium. ITC is a slow technique; the apparatus constant is approximately 9 s for the decay of the heat signals. A reaction with the above-mentioned rate constant would not give rise to significant

line broadening of the heat signals. The injections were carried out at 3 min intervals, and the thermograms show good baseline separation with no indication of a very slow step (Figures 3−5). If the last step is slow with an enthalpy significantly different from 0, then $t_{1/2}$ must be in the magnitude of hours.

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REFERENCES

1. Nanmori, T., Shinke, R., Aoki, K., and Nishira, H. (1983) *Agric. Biol. Chem. 47*, 941−947.
2. O'Neill, G., Goh, S. H., Warren, R. A. J., Kilburn, D. G., and Miller, R. C., Jr. (1986) *Gene 44*, 325−330.
3. Kitamoto, N., Yamagata, H., Kato, T., Tsukagoshi, N., and Udaka, S. (1988) *J. Bacteriol. 170*, 5848−5854.
4. Nitschke, L., Heeger, K., Bender, H., and Schultz, G. E. (1990) *Appl. Microbiol. Biotechnol. 33*, 542−546.
5. Itkor, P., Tsukagoshi, N., and Udaka, S. (1990) *Biochem. Biophys. Res. Commun. 166*, 630−636.
6. Svensson, B., and Søgaard, M. (1993) *J. Biotechnol. 29*, 1−37.
7. Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S., and Tanaka, H. (1994) *J. Bacteriol. 176*, 4465−4472.
8. Lawson, C. L., van Montfort, R., Strokopytov, B., Rozeboom, H. J., Kalk, K. H., de Vries, G. E., Penninga, D., Dijkhuizen, L., and Dijkstra, B. W. (1994) *J. Mol. Biol. 236*, 590−600.
9. Millward-Sadler, S. J., Poole, D. M., Henrissat, B., Hazlewood, G. P., Clarke, J. H., and Gilbert, H. J. (1994) *Mol. Microbiol. 11*, 375−382.
10. Hiromi, K., Kawai, M., and Ono, S. (1966) *J. Biochem. 59*, 476−480.
11. Gunnarsson, A., Svensson, B., Nilsson, B., and Svensson, S. (1984) *Eur. J. Biochem. 145*, 463−467.
12. Svensson, B., Pedersen, T. G., Svendsen, I., Sakai, T., and Ottesen, M. (1982) *Carlsberg Res. Commun. 47*, 55−69.
13. Svensson, B., Larsen, K., Svendsen, I., and Boel, E. (1983) *Carlsberg Res. Commun. 48*, 529−544.
14. Svensson, B., Larsen, K., and Gunnarsson, A. (1986) *Eur. J. Biochem. 154*, 497−502.
15. Aleshin, A. E., Golubev, A., Firsov, L. M., and Honzatko, R. B. (1992) *J. Biol. Chem. 267*, 19291−19298.
16. Aleshin, A. E., Firsov, L. M., and Honzatko, R. B. (1994) *J. Biol. Chem. 269*, 15631−15639.
17. Harris, E. M. S., Aleshin, A. E., Firsov, L. M., and Honzatko, R. B. (1993) *Biochemistry 32*, 1618−1626.
18. Aleshin, A. E., Hoffman, C., Firsov, L. M., and Honzatko, R. B. (1994) *J. Mol. Biol. 238*, 575−591.
19. Aleshin, A. E., Stoffer, B., Firsov, L. M., Svensson, B., and Honzatko, R. B. (1996) *Biochemistry 35*, 8319−8328.
20. Stoffer, B., Aleshin, A. E., Firsov, L. M., Svensson, B., and Honzatko, R. B. (1995) *FEBS Lett. 358*, 57−61.
21. Sorimachi, K., Jacks, A. J., Le Gal-Coëffet, M.-F., Williamson, G., Archer, D. B., and Williamson, M. P. (1996) *J. Mol. Biol. 259*, 970−987.
22. Sorimachi, K., Le Gal-Coëffet, M.-F., Williamson, G., Archer, D. B., and Williamson, M. P. (1997) *Structure 5*, 647−661.
23. Kramer, G. F. H., Gunning, A. P., Morris, V. J., Belshaw, N. J., and Williamson, G. (1993) *J. Chem. Soc., Faraday Trans. 89*, 2595−2602.
24. Williamson, G., Belshaw, N. J., Noel, T. R., Ring, S. G., and Williamson, M. P. (1992) *Eur. J. Biochem. 207*, 661−670.
25. Coutinho, P. M., and Reilly, P. J. (1997) *Proteins: Struct., Funct., Genet. 29*, 334−347.
26. Libby, C. B., Cornett, C. A. G., Reilly, P. J., and Ford, C. (1994) *Protein Eng. 7*, 1109−1114.
27. Wang, C., Eufemi, M., Turano, C., and Giartosio, A. (1996) *Biochemistry 35*, 7299−7307.
28. Sigurskjold, B. W., Berland, C. R., and Svensson, B. (1994) *Biochemistry 33*, 10191−10199.
29. Belshaw, N. J., and Williamson, G. (1993) *Eur. J. Biochem. 211*, 717−724.
30. Sigurskjold, B. W., Svensson, B., Williamson, G., and Driguez, H. (1994) *Eur. J. Biochem. 225*, 133−141.
31. Tanaka, A. (1996) *Biosci. Biotechnol. Biochem. 60*, 2055−2058.
32. Berland, C. R., Sigurskjold, B. W., Stoffer, B. B., Frandsen, T. P., and Svensson, B. (1995) *Biochemistry 34*, 10513−10161.
33. Frandsen, T. P., Christensen, T., Stoffer, B., Lehmbeck, J., Dupont, C., Honzatko, R. B., and Svensson, B. (1995) *Biochemistry 34*, 10162−10169.
34. Payre, N. (1996) Ph.D. Dissertation, University of Grenoble, France.
35. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L.-N. (1989) *Anal. Biochem. 179*, 131−137.
36. Bundle, D. R., and Sigurskjold, B. W. (1994) *Methods Enzymol. 247*, 288−305.
37. Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1−63.
38. Williamson, M. P., Le Gal-Coëffet, M.-F., Sorimachi, K., Furniss, C. S. M., Archer, D. B., and Williamson, G. (1997) *Biochemistry 36*, 7535−7539.
39. Olsen, K., Svensson, B., and Christensen, U. (1992) *Eur. J. Biochem. 209*, 777−784.
40. Olsen, K., Christensen, U., Sierks, M. R., and Svensson, B. (1993) *Biochemistry 32*, 9686−9693.
41. Christensen, U., Olsen, K., Stoffer, B. B., and Svensson, B. (1996) *Biochemistry 35*, 15009−15018.

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