Thermodynamics of Reversible and Irreversible Unfolding and Domain Interactions of Glucoamylase from *Aspergillus niger* Studied by Differential Scanning and Isothermal Titration Calorimetry†

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ABSTRACT: The stability of three forms of glucoamylase from *Aspergillus niger* has been investigated by differential scanning and isothermal titration calorimetry: Glucoamylase 1 (GA1), which consists of a catalytic domain and a starch-binding domain (SBD) connected by a heavily O-glycosylated linker region; glucoamylase 2 (GA2), which lacks SBD; and a proteolytically cleaved glucoamylase (GACD), which contains the catalytic domain and part of the linker region. The structures of the catalytic domain with part of the linker region and of SBD are known from crystallography and NMR, respectively, but the precise spatial arrangement of the two domains in GA1 is unknown. To investigate the stability of the three glucoamylase forms, we unfolded the enzymes thermally by differential scanning calorimetry (DSC). Aggregation occurs upon heating GA1 and GA2 at pH values between 2.5 and 5.0, whereas no aggregation is observed at higher pH (5.5−7.5). At all pH values, the catalytic domain of GA1 and GA2 unfolds irreversibly, while SBD unfolds reversibly in the pH range 5.5−7.5 where aggregation does not occur. The unfolding of the catalytic domain of all glucoamylase forms seems to follow an irreversible one-step mechanism with no observable reversible intermediates on the experimental time scale. SBD of GA1 unfolds reversibly, and the ratio between the van't Hoff and calorimetric enthalpies is 1.4 ± 0.1. Assignment of peaks of the DSC profile to the domains at pH 7.5 is achieved by using two different ligands: Acarbose, a very strong inhibitor that binds exclusively to the catalytic domain, and β-cyclodextrin, a small starch analogue of which 2 molecules bind solely to the two binding sites present in SBD. Differences are seen in the unfolding processes of GA1 and GA2 since the former unfolds with one peak at all pH values, while the calorimetric trace of the latter can be resolved into more peaks depending on pH and the chemical composition of the buffers. In general, peaks corresponding to unfolding of GA2 are more complex than the peaks of GA1 and GACD. Some part of GA2 unfolds before the rest of the molecule which may correspond to the linker region or a particular early unfolding part of the catalytic domain. This leads to the conclusion that the structure of the GA2 molecule has a larger cooperative unfolding unit and is less stable than the structures of GA1 and GACD and that the C-terminal part of the linker region has a destabilizing effect on the catalytic domain.

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) is a multidomain exo-glycosidase that cleaves α(1 → 4)- and, with less efficiency, α(1 → 6)-glucosidic linkages of starch and related oligo- and polysaccharides from the nonreducing ends releasing β-D-glucose (*1*). Glucoamylase from *Aspergillus niger* occurs naturally in two forms, glucoamylase 1 (GA1[1]) and glucoamylase 2 (GA2), where GA1 has a total of 616 amino acids of which residues 1−440 correspond to the catalytic domain and residues 509−616 to the starch-binding domain (SBD). A linker region (residues 441−508) rich in glycosylated Ser and Thr residues connects the two domains. GA2 has a total of 512 amino acids and contains the catalytic domain and the linker region, but lacks SBD, resulting in an enzyme with the ability to degrade soluble starches, but not raw starch granules (*2*, *3*). The frequency of glycosylation is higher in the C-terminal part of the linker region than in the N-terminal part. By proteolytic cleavage of GA1 it is possible to obtain a modified glucoamylase (GACD, glucoamylase catalytic domain) which contains residues 1−470 and is composed of the entire catalytic

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[1] Abbreviations: GA1, glucoamylase 1 from *Aspergillus niger*; GA2, glucoamylase 2, a form lacking the starch-binding domain; SBD, starch-binding domain; GACD, a proteolytically cleaved form of glucoamylase containing the catalytic domain and the first 30 amino acid residues of the linker region; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; T_d, denaturation temperature; T_u, unity temperature, the temperature at which the rate constant k equals 1 min^{-1}; ΔH_{cal}, calorimetric enthalpy; ΔH_{vH}, van't Hoff enthalpy; $\langle C_p \rangle$, molar excess heat capacity; $C_{p,f}$, molar heat capacity of the folded state; $C_{p,u}$, molar heat capacity of the unfolded state; E_a, activation energy; β-CD, β-cyclodextrin.

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domain and the first 30 residues of the N-terminal part of the linker region (*4*).

The crystal structure of the catalytic domain and part of the linker region has been solved for a closely related glucoamylase from *Aspergillus awamori* var. *X100* without inhibitors (*5*, *6*) and complexed to the active site inhibitors 1-deoxynojirimycin (*7*), acarbose (*8*), and D-*gluco*-dihydroacarbose (*9*), respectively. The catalytic domain folds into an $(\alpha/\alpha)_6$-barrel, and the part of the linker region present in the structure is wrapped around the catalytic domain. The glucoamylases from *Aspergillus niger* and *Aspergillus awamori* var. *X100* are 95% identical in their amino acid sequences. An NMR structure of SBD of glucoamylase from *Aspergillus niger* is available with and without bound β-cyclodextrin (*10*, *11*), which is a cyclic analogue of starch. SBD consists of 8 β-strands forming two β-sheets, resulting in an open-sided distorted β-barrel. SBD has two independent binding sites for β-cyclodextrin, one on each side of the domain. The structure of the complete GA1 molecule is not available; thus the spatial arrangement of the two domains and the linker region is not known. Scanning tunneling microscopic images suggest that the catalytic domain and SBD are separated in space by the linker region in an extended conformation (*12*). In contrast, recent work using isothermal titration calorimetry (ITC) in which GA1 was titrated with heterobidentate ligands of covalently connected acarbose and β-cyclodextrin moieties (*13*) indicated that the catalytic domain and SBD are in close proximity (*14*).

In this paper, differential scanning calorimetry (DSC) has been used to further investigate the relationship between the domains of glucoamylase. Previously, DSC has shown that unfolding of the structure of the catalytic domain is irreversible, whereas unfolding of the structure of SBD is reversible (*15*). The thermal unfolding of glucoamylase is highly pH dependent. The enzyme aggregates at lower pH values, resulting in irreversible unfolding also of SBD. The two ligands acarbose and β-cyclodextrin have been used to assign the observed peaks of the DSC experiments to the different structural regions of the glucoamylase molecule, and in this way, the roles of the linker region and SBD in stability have been established.

MATERIALS AND METHODS

Materials. Acarbose was a generous gift from Bayer AG (Wuppertal, Germany), and β-cyclodextrin was purchased from Sigma. A commercial preparation containing glucoamylase 1 (GA1) and glucoamylase 2 (GA2) was obtained from Novo Nordisk A/S (Bagsværd, Denmark), and the two forms were separated and purified essentially as described previously (*2*, *4*).

The catalytic domain (GACD) was obtained by proteolytic cleavage by subtilisin Novo and purified as described in an earlier paper (*4*). Further purification of this GACD preparation was carried out using affinity chromatography with a β-cyclodextrin column prepared from epoxy-activated Sepharose 6B from Pharmacia Biotech (Sweden). The sample containing GACD was loaded onto the column in 50 mM sodium acetate, pH 4.5 (5−6 mL h^{-1}), and GACD appeared in the pass-through. GA1 and SBD adsorbed to β-cyclodextrin-Sepharose and were subsequently eluted with 1.28 mM β-cyclodextrin in 50 mM sodium acetate, pH 5.5. The purity of GACD was tested on Tricine 12% SDS−PAGE gels (*4*). A protein band corresponding to GA1 is seen in the GACD preparation before the last purification step, but this component was absent after the β-cyclodextrin-Sepharose column and only GACD was present. None of the protein-stained bands were sharp due to the heterogeneity of the glycosylations in the linker region. To further evaluate the purity of the GACD fraction, we performed an ITC experiment on the purified GACD using β-cyclodextrin as the ligand (data not shown). From determination of the apparent stoichiometry of binding, it is clear that less than 1% of SBD is present on a molar basis. It is not apparent on the Tricine SDS−PAGE gel whether the contaminant was free SBD, GA1, or both, but the concentration of the contaminant is definitely too small to adversely influence the DSC experiments.

Protein concentrations were determined by absorbance measurements at 280 nm using a Hitachi U-1100 spectrophotometer and molar extinction coefficients for GA1, GA2, and GACD of 1.37×10^5, 1.09×10^5, and 1.09×10^5 M^{-1} cm^{-1}, respectively (*4*, *16*).

Differential Scanning Calorimetry. DSC experiments were performed on an MCS differential scanning calorimeter from MicroCal, Inc. (Northampton, MA). This instrument has a cell volume of 1.1934 mL. Temperature calibration was done using two hydrocarbons with known melting points sealed in steel capillary tubes supplied by MicroCal, Inc. Heat calibration was done using electrical pulses. The scan rate was 60 °C h^{-1} unless otherwise specified, and an external pressure of 3 bar was applied over the cells. All protein solutions were dialyzed against the desired buffer, and the dialyzate was used as reference. All solutions were degassed by stirring under vacuum prior to scanning. The reversibility of the unfolding of the enzymes was controlled in all experiments by rescanning the samples after cooling. DSC investigations of the pH dependence of GA1 (24.0−38.8 μM) and GA2 (20.9−37.8 μM) were performed in a 50 mM sodium acetate−phosphate buffer in the pH range 2.5−7.5. To obtain constant ionic strength in all pH dependence experiments, we added NaCl to a final ionic strength of 0.1 M. Scans on GA1 in 50 mM sodium acetate, pH 4.5, with and without 3 M guanidinium chloride were performed using GA1 concentrations of 19.3 and 16.1 μM, respectively, and a scan rate of 120 °C h^{-1}. Scans on GA2 with and without 32.5 μM acarbose were performed in 50 mM sodium acetate buffer, pH 4.5, using GA2 in concentrations of 21.6 and 23.1 μM, respectively. Additional DSC experiments were performed on GA1, GA2, and GACD in 50 mM phosphate buffer, pH 7.5, using protein concentrations of 9.2−33.8, 24.0−29.1, and 22.1−22.4 μM, respectively. In some experiments, ligands were added to the enzymes prior to scanning. In these experiments the concentration of acarbose in the cell was in 1.2−2.4 times molar excess and the concentration of β-cyclodextrin was in 10 times excess. Scan rate dependence experiments (scan rates 15, 30, 45, 60, 90, and 120 °C h^{-1}) were performed on G1 (24.1 μM) and G2 (15.1−28.0 μM) in 50 mM MES buffer at pH 6.0. To analyze the DSC data, we used the Origin software from MicroCal, Inc. supplied with the calorimeter. In all DSC experiments buffer scans were subtracted from protein scans. The molar heat capacity (C_p) was obtained by normalizing with the protein concentrations and the known volume of the cell of the calorimeter. The molar heat capacity of the native, folded

protein ($C_{p,f}$) was fit to a straight line and then subtracted from the experimentally obtained C_p leading to the molar excess heat capacity function, $\langle C_p \rangle$ (*17*). A baseline was fit to $\langle C_p \rangle$ and in scans with two distinct peaks a baseline fitting both peaks was determined. The molar heat capacity change of the unfolding process is the difference between the heat capacity of the unfolded and folded forms, $\Delta C_p = C_{p,u} - C_{p,f}$. ΔC_p values from both reversible and irreversible unfolding were determined by the extrapolation of $C_{p,u}$, and the heat capacity differences between the folded and unfolded forms were determined at the maximum temperature of $\langle C_p \rangle$ in certain experiments. In scans containing two distinct peaks, ΔC_p was determined at maximum temperature of the major peak. Both the reversible and irreversible peaks were analyzed using the Origin software. To analyze the reversible peaks, we subtracted the baseline in order to fit the obtained curve to a reversible non-two-state model from which the thermodynamic parameters were calculated. The baseline was also subtracted from the irreversible peaks, and the curves were analyzed according to procedures used for irreversible unfolding (*18*). All uncertainties represent three standard deviations of the regression analyses.

Isothermal Titration Calorimetry. The ITC experiments were performed using an MCS isothermal titration calorimeter from MicroCal, Inc. (Northampton, MA) (*19*). The reference cell was filled with water, and calibration of the calorimeter was done using electrical heat pulses. Solutions were degassed by stirring under vacuum before the experiments were performed. All titrations were done at 27 °C, and ligands in the syringe were injected in 20 equal volumes of 13 μL into the glucoamylase solution in the sample cell of a volume of 1.3187 mL. GA1 (19.6 μM) (heated to 70 °C prior to titration) was titrated with 144 μM β-cyclodextrin. In subsequent titrations by acarbose, the concentrations of GA1 and acarbose were 16.1 and 150 μM, respectively. For the titrations of GA2 (heated to 47 °C before titration), the enzyme concentration was 28.5 μM and the concentration of acarbose was 209 μM. In the titration of GACD by acarbose, the enzyme and acarbose concentrations were 22.4 and 177 μM, respectively. All titrations with GA1, GA2, and GACD were performed in 50 mM sodium phosphate buffer at pH 7.5. Integration of heat signals from the ITC experiments was done using the Origin software from MicroCal, Inc. For fitting the binding isotherms both the Origin software from MicroCal, Inc. and software previously described (*20*) were used. All uncertainties represent three standard deviations of the nonlinear fits.

RESULTS

Scan Rate Dependence and Irreversible Unfolding of the Catalytic Domain of Glucoamylase. Irreversible denaturation may proceed from the folded form, F, through one or more reversibly unfolded form(s), U, to an irreversibly unfolded form, D, according to the Lumry-Eyring model (*21*):

$$\mathrm{F} \underset{\Delta H_U}{\overset{K}{\rightleftharpoons}} \mathrm{U} \xrightarrow[\Delta H_i]{k} \mathrm{D}$$

where K and ΔH_U are the equilibrium constant and enthalpy of unfolding associated with the reversible transition, and k and ΔH_i are the temperature-dependent rate constant and enthalpy associated by the irreversible step (*18*, *22*). If the irreversible process is significant only late in the DSC experiment, that is, when almost all of the enthalpy of unfolding is due to the reversible part of the reaction, then it is reasonable to analyze the peak according to a reversible model (*18*). In an alternative model, unfolding may proceed in one step on the DSC time scale without any observable reversible intermediates.

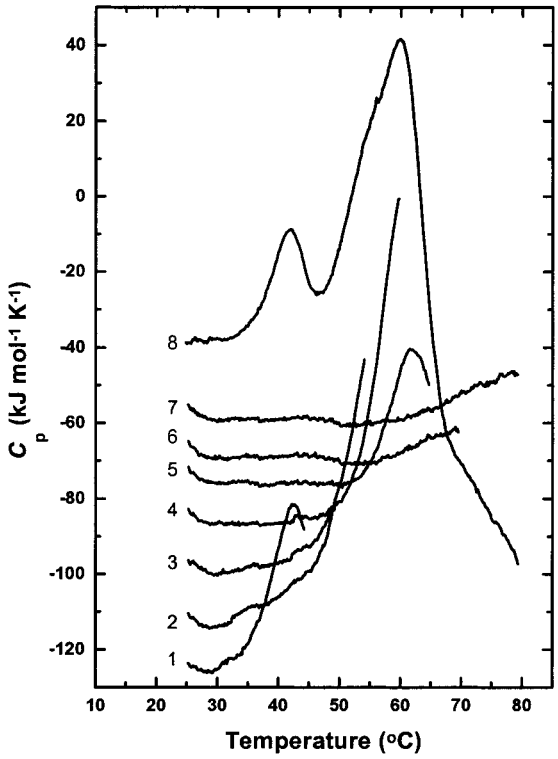

FIGURE 1: Unfolding of 29.0 μM GA2 in 50 mM phosphate buffer at pH 7.5 in a series of repeated heating and subsequent cooling in 5 °C intervals to determine the temperature range of the irreversible step. It is obvious that both the minor and the major peaks unfold irreversibly, and the irreversibility is prominent early in the unfolding process so the calorimetric traces of glucoamylases have to be analyzed according to an irreversible model. The scans were stopped at 45 (1), 50 (2), 55 (3), 60 (4), 65 (5), 70 (6), and 80 °C (7). Curve (8) is a full scan.

$$\mathrm{F} \xrightarrow[\Delta H_i]{k} \mathrm{D}$$

Here, ΔH_i is the enthalpy of both the unfolding and the irreversible processes. In this case, unfolding is completely under kinetic control. Therefore, it is very important to establish when the irreversible process becomes dominant and whether unfolding goes through reversible steps.

At pH 7.5, GA2 unfolds irreversibly, whereas in the rescan of GA1 a small peak appears originating from SBD (see later). The unfolding of GACD is also completely irreversible at this pH. To determine in which temperature range the unfolding of the catalytic domain of glucoamylase is predominantly irreversible, we exposed the three glucoamylase forms to repeated heating and subsequent cooling in 5 °C intervals at pH 7.5 until the domains, except for SBD, were unfolded irreversibly (Figure 1). The observed changes in heat capacity of the assumed native form in Figure 1 are due to a slight increase in pressure during the repeated scans, which shifts the observed position of the curves. Buffer scans show the same effect. The same buffer scan has been

Table 1. Activation Energy, Unity Temperature, and Enthalpy of the Irreversible Unfolding of GA1 and GA2 at Different Scan Rates in MES Buffer at pH 6.0

	GA1			GA2					
	peak 1			peak 1			peak 2		
scan rate (°C h^{-1})	E_a (kJ mol^{-1})	T_u (°C)	ΔH (kJ mol^{-1})	E_a (kJ mol^{-1})	T_u (°C)	ΔH (kJ mol^{-1})	E_a (kJ mol^{-1})	T_u (°C)	ΔH (kJ mol^{-1})
15	236 ± 5	76.2 ± 0.3	2326 ± 41	180 ± 5	78.9 ± 0.8	1269 ± 45	322 ± 37	81.1 ± 1.0	361 ± 41
30	245 ± 5	74.4 ± 0.2	2172 ± 33	198 ± 2	75.1 ± 0.2	1085 ± 13	295 ± 12	81.6 ± 0.3	355 ± 12
45	262 ± 5	73.0 ± 0.2	2047 ± 31	211 ± 3	73.7 ± 0.2	1489 ± 39	302 ± 20	79.1 ± 0.4	493 ± 39
60	266 ± 5	72.4 ± 0.2	1997 ± 33	240 ± 7	71.2 ± 0.4	1231 ± 105	249 ± 31	79.7 ± 0.7	699 ± 105
90	256 ± 5	72.2 ± 0.2	2171 ± 36	220 ± 3	71.9 ± 0.2	1528 ± 55	277 ± 21	78.7 ± 0.2	607 ± 53
120	253 ± 7	71.8 ± 0.2	2098 ± 49	231 ± 4	71.2 ± 0.2	1325 ± 59	269 ± 24	78.7 ± 0.2	580 ± 58
Average	253 ± 2	73.0 ± 0.1	2122 ± 15	208 ± 2	73.0 ± 0.2	1160 ! 24	289 ± 10	79.3 ± 0.2	387 ± 24

subtracted from each of the protein scans. The traces make it possible to examine at which temperature the heating of the catalytic domain results in complete unfolding and if the irreversible step occurs early or late in the transition. All glucoamylase forms show that the irreversible unfolding process of the catalytic domain occurs over a broad temperature range and dominates the unfolding very early in the DSC scans. No major differences are seen in the temperature ranges for the irreversible processes of the three forms of glucoamylase. Thus the irreversible process seems not to be dependent on whether the linker region or SBD is present. The presence of the active site inhibitor acarbose, however, results in a delay of the irreversible process(es) (see later).

When the unfolding is irreversible, but goes through reversible steps, the excess heat capacity curve can be fit to the following equation:

$$\langle C_{p,exc}\rangle = \left[\frac{K\Delta H_U}{(K+1)^2}\left(\frac{k}{\nu}+\frac{\Delta H_U}{RT^2}\right)+\Delta H_i\frac{1}{\nu}\frac{kK}{K+1}\right]\exp\left(-\frac{1}{\nu}\int_{T_0}^{T}\frac{kK}{K+1}\,dT\right)$$

where ΔH_U is the enthalpy of unfolding and ΔH_i is the enthalpy arising from the irreversible processes, whatever the chemical nature of these may be. $\langle C_{p,exc}\rangle$ is the experimentally obtained curve, K is the equilibrium constant for the reversible process, k is the rate constant for the irreversible process, ν is the scan rate of the particular experiment, R is the universal gas constant, and T is the absolute temperature (*22*). The rate constant k is given by the Arrhenius expression:

$$k = \exp\left(-\frac{E_a}{R}\left[\frac{1}{T}-\frac{1}{T_u}\right]\right)$$

where E_a is the energy of activation and T_u is the unity temperature which is defined as the temperature at which $k = 1$ min^{-1}. T_u is related to the traditional pre-exponential factor of the Arrhenius equation by $A = \exp(E_a/R/T_u)$ and is hence a measure of the activation entropy. ΔH_U and ΔH_i can be resolved only if the unfolding follows the Lumry-Eyring model. In a one-step irreversible unfolding the expression for the excess heat capacity reduces to ($K \rightarrow \infty$):

$$\langle C_{p,exc}\rangle = \Delta H_i\frac{k}{\nu}\exp\left(-\frac{1}{\nu}\int_{T_0}^{T}k\,dT\right)$$

Here ΔH_i is the enthalpy of both the unfolding process itself and the irreversible reaction. This equation has been used to fit the irreversible unfolding scans. For the validity of the one-step irreversible model to be tested, the activation energy may be estimated in three other ways (*23*, *24*). First, from the equation

$$k = \frac{\nu\langle C_{p,exc}\rangle}{\Delta H_{cal}-\Delta H(T)}$$

where ΔH_{cal} is the total area under the peak, $\Delta H(T) = \int_{T_0}^{T}\langle C_{p,exc}\rangle\,dT$, and E_a can be derived from an Arrhenius plot. Second, E_a can be calculated directly from the ratio between the maximum excess heat capacity $\langle C_{p,exc}\rangle^{max}$ and ΔH_{cal}

$$E_a = eRT_d^2\frac{\langle C_{p,exc}\rangle_{max}}{\Delta H_{cal}}$$

Here, the "denaturation" temperature, T_d, is the temperature of $\langle C_{p,exc}\rangle^{max}$. Third, from the scan rate dependence of T_d

$$\ln\left(\frac{\nu}{T_d^2}\right) = \text{constant} - \frac{E_a}{RT_d}$$

E_a is determined by the slope in a plot of $\ln(\nu/T_d^2)$ vs $1/T_d$.

If the activation energies calculated according to these three equations comply with the activation energy obtained from the curve fits of the scan rate experiments, the one-step mechanism will be verified. The test can only be done with confidence when the calorimetric traces contain a single peak. In this series of experiments, the DSC profiles of GA1 and GA2 consist of one and two peaks, respectively. Therefore, only GA1 is tested in the following. From the three methods the activation energies are 283 ± 32, 263 ± 9, and 537 ± 29 kJ mol^{-1}, respectively. Two of the three procedures of confirming the one-step mechanism agree reasonably well with the activation energy of 253.0 ± 2.0 kJ mol^{-1} calculated from the curve fits (Table 1). In the last method to justify the one-step model, the activation energy is twice the expected value. This is a quite large deviation, but since this method relies entirely on the temperature of maximum C_p and the exact enthalpy contribution from SBD unfolding is not known, T_m may have a considerable uncertainty. With this reservation we assume that GA1 unfolds according to an irreversible one-step mechanism. The unity temperature, T_u, is calculated from the first method using all scan rate experiments, and the resulting value is

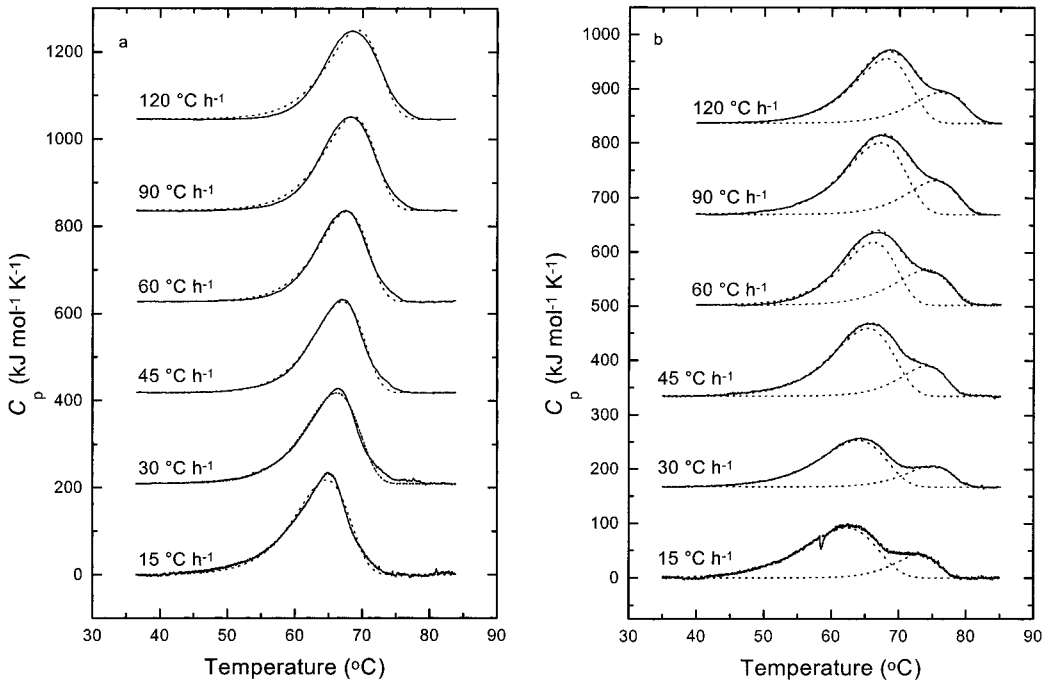

FIGURE 2: (a) Scan rate dependency of unfolding of 24.1 μM GA1 in 50 mM MES buffer pH 6.0. The solid lines are experimentally obtained DSC curves, and the dotted lines are fits to a one-step irreversible model. This glucoamylase unfolds in an irreversible one-step process with one peak for all scan rates (Table 1). (b) Scan rate dependency of unfolding of 15.1−28.0 μM GA2 in 50 mM MES buffer pH 6.0. The solid lines are experimentally obtained DSC curves, and the dotted lines are fits to a one-step irreversible model. GA2 has a more complex pattern of peaks compared to GA1, and the calorimetric trace can be resolved into two irreversible one-step peaks (Table 1). The lines have been shifted vertically for clarity.

72.6 ± 2.2 °C which agrees very well with the T_u of 73.0 ± 0.1 °C obtained from the curve fits (Table 1). The DSC trace of GACD also shows a single peak, but because of lack of enzyme, scan rate dependence experiments have not been performed. The second transition peak of GA2 and that of GACD resemble that of GA1, and since the unfolding process of GA1 is approximately concordant with the one-step mechanism, it is assumed that the unfolding processes of GACD and GA2 follow the same mechanism.

In the scan rate dependence experiments at pH 6.0, the calorimetric traces of GA1 and GA2 can be resolved into one and two irreversible one-step peaks, respectively (Table 1, Figure 2). All glucoamylase forms, except a specific domain (SBD of GA1, see later), unfold in a one-step irreversible way, and no reversible intermediates can be detected. The average kinetic parameters for the large peak of GA2 and the peak of GA1 are very similar, but ΔH_i differs. When the enthalpies of the two peaks of GA2 are added, the total enthalpy is still smaller than the enthalpy of GA1 since the two enzymes differ in size. The T_u values of GA1 and the first peak of GA2 seem to decrease with increasing scan rate. This is not observed to the same extent with the second peak of GA2. The second peak of the calorimetric trace of GA2 shows quite large discrepancies in the enthalpy of the different scan rates (Table 1). One explanation may be that this peak does not correspond to a true all-or-none irreversible unfolding.

pH Dependence of Glucoamylase Unfolding. DSC scans and rescans of GA1 and GA2 were recorded at varying pH in the range 2.5−7.5 (DSC traces not shown). At pH values between 2.5 and 5.0, aggregation occurs when heating GA1 or GA2, and since the enthalpy of aggregation is negative, the heat capacity change for the unfolding process becomes negative which complicates the interpretation. This means that it is very difficult to analyze completely the unfolding reaction of glucoamylase at lower pH values, since the enthalpy of aggregation and the enthalpy of unfolding cannot be resolved. At higher pH values (5.5−7.5), no aggregation is observed for either GA1 or GA2, and part of the structure of the GA1 molecule unfolds reversibly (Tables 2 and 3).

The calorimetric trace of unfolding of GA1 is almost symmetrical at all pH values in contrast to that of GA2. The trace of GA2 can be resolved into two or three irreversible one-step peaks depending on pH, whereas the trace of unfolding of GA1 is well-described by a single irreversible one-step peak. The trace of GA1 is more irregular at lower pH values, but it is still well fit to only one irreversible one-step peak (Table 1 in Supporting Information). The second peak of the resolution of the calorimetric trace of GA2 resembles the peak of GA1. At lower pH values, GA2 has a minor peak at higher temperatures, but at higher pH values GA2 has a minor peak at lower temperatures (Table 2 in Supporting Information). Therefore, it seems as if the structure of GA2 has a larger cooperative unit than GA1, indicating that the linker region has some destabilizing effect on some parts of the GA2 molecule since the thermodynamic parameters of the major peak of GA2 correspond to the parameters of GA1. The linker region shows different effects on the GA2 molecule depending on pH. In GA1, the linker region is probably kept in a specific position by SBD, resulting in a smaller cooperative unfolding unit. This corroborates the results from a recent paper of ours (*14*) that the catalytic domain and SBD are in close proximity in space. From the analysis of the calorimetric trace of GA1 it is not possible to see the contribution from SBD because the two domains unfold at the same temperature.

Table 2. Activation Energy, Unity Temperature, and Enthalpy of Unfolding of the Irreversible Unfolding of GA1, GA2, and GACD in Phosphate Buffer at pH 7.5 (Figure 3)

	peak 1			peak 2			peak 3		
cell content	E_a (kJ mol^{-1})	T_u (°C)	ΔH (kJ mol^{-1})	E_a (kJ mol^{-1})	T_u (°C)	ΔH (kJ mol^{-1})	E_a (kJ mol^{-1})	T_u (°C)	ΔH (kJ mol^{-1})
GA1							228 ± 6	64.6 ± 0.5	1759 ± 279
GA1 + acarbose[a]	315 ± 40	61.0 ± 0.6	382 ! 57				238 ± 11	74.4 ± 0.4	1503 ± 56
GA1 + β-CD							224 ± 9	64.9 ± 0.4	1896 ± 56
GA2	350 ± 27	45.0 ± 0.4	183 ± 15	273 ± 57	56.1 ± 1.5	147 ± 54	219 ± 9	66.0 ± 0.3	1148 ± 50
GA2 + acarbose	286 ± 25	45.9 ± 0.5	243 ± 24				202 ± 3	77.3 ± 0.2	1935 ± 23
GA2[b]				348 ± 66	55.0 ± 1.0	82 ± 28	215 ± 6	66.3 ± 0.2	1085 ± 31
GACD							206 ± 8	64.4 ± 0.4	1460 ± 64
GACD + acarbose							218 ± 3	74.9 ± 0.1	1984 ± 21

[a] The first peak corresponds to the starch-binding domain and is reversible. [b] Heated to 47 °C and cooled before scanning.

Table 3. Denaturation Temperature and Calorimetric and Van't Hoff Enthalpies for the Reversible Unfolding of theSBD of GA1 with and without Binding of the Ligands Acarbose and β-Cyclodextrin at pH 7.5

cell content	T_d (°C)	ΔH_{cal} (kJ mol^{-1})	ΔH_{vH} (kJ mol^{-1})
SBD (rescan of GA1)	56.7 ± 0.2	311 ± 12	434 ± 21
SBD + β-cyclodextrin (rescan of GA1)	59.8 ± 0.2	336 ± 5	565 ± 10
SBD + acarbose (rescan of GA1)	56.7 ± 0.1	202 ± 7	517 ± 23
GA1[a]	57.0 ± 0.1	198 ± 6	527 ± 19

[a] Heated to 70 °C before scanning.

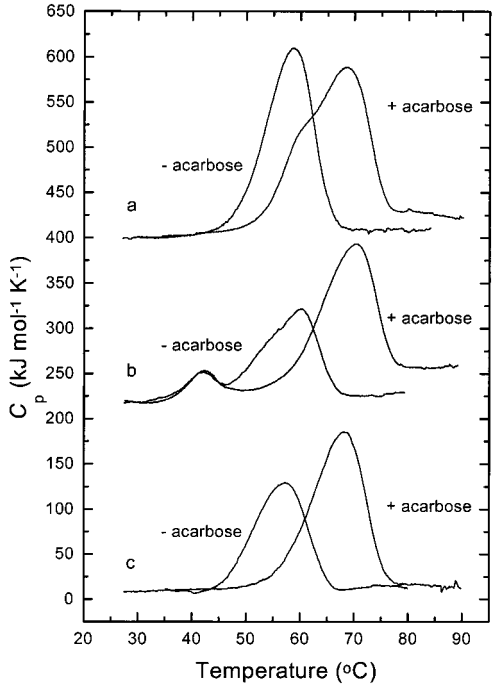

FIGURE 3: Unfolding of glucoamylase forms in 50 mM phosphate buffer pH 7.5: (a) 14.8 and 16.3 μM GA1 in the absence and presence of 24.3 μM acarbose, respectively; (b) 29.0 and 25.5 μM GA2 in the absence and presence of 29.7 μM acarbose, respectively; and (c) 22.4 and 22.1 μM GACD in the absence and presence of 29.1 μM acarbose, respectively (Table 2). The lines have been shifted vertically for clarity.

Both GA1 and GA2 are most stable, that is, show highest denaturation temperature (where $\langle C_{p,exc} \rangle$ has its maximum) and highest activation energy, at pH 4.0−4.5. There is a tendency of increasing enthalpy as the denaturation temperature is decreasing. Usually, the enthalpy of unfolding increases with increasing temperature because ΔC_p is positive for protein unfolding. However, for unfolding reactions involving irreversible steps the opposite effects have been observed (*25*). A small reversible peak appears in the rescan of GA1 at pH 5.5−7.5, and this peak has been assigned to SBD (see later). The T_d of SBD decreases with increasing pH (Table 1 in Supporting Information), and the enthalpy of unfolding is supposed to increase as well due to the positive ΔC_p. In this instance the enthalpy does not show the expected pattern. Since the van't Hoff enthalpy is larger than the calorimetric enthalpy in all cases, the reason for the above observation is probably that some degree of reversible oligomerization occurs concomitantly with the unfolding process. The observed enthalpy is the sum of the enthalpies of oligomerization and unfolding. Preliminary experiments on proteolytically degraded SBD show a negative ΔC_p, even though the domain unfolds reversibly. This effect often arises from oligomerization processes, which have favorable enthalpy contributions. This is consistent with the presence of an oligomerization step in the unfolding of SBD.

To investigate if it is possible to prevent aggregation of GA1 at pH 4.5, we added 3 M guanidinium chloride to a sample prior to scanning and compared it with a sample without guanidinium chloride. Aggregation is prevented in the sample containing guanidinium chloride, and a small peak from reversible unfolding appears in the rescan corresponding to SBD (data not shown). The denaturation temperature decreases by adding guanidinium chloride to the sample, but whether it is the decreased denaturation temperature or the presence of guanidinium chloride (or both) that prevents aggregation is not known.

Since the unfolding of the structure of glucoamylase proceeds without directly observable aggregation at higher pH values and part of the GA1 molecule unfolds reversibly, a series of scans on the three glucoamylase forms, GA1, GA2, and GACD, were performed at pH 7.5. The forms all show an endothermic peak upon unfolding with a positive ΔC_p (Figure 3), but it is difficult to accurately determine the heat capacity change of unfolding for all of the enzymes. From the molar heat capacity curves in Figure 3, ΔC_p for GA1, GA2, and GACD is estimated to be approximately 15, 15, and 3 kJ mol^{-1} K^{-1}, respectively, and somewhat larger for GA2 with bound acarbose. A positive ΔC_p is always observed for unfolding of proteins except when other processes such as aggregation are also occurring (*26*, *27*). GA1 and GACD both show a regular peak in the first scan

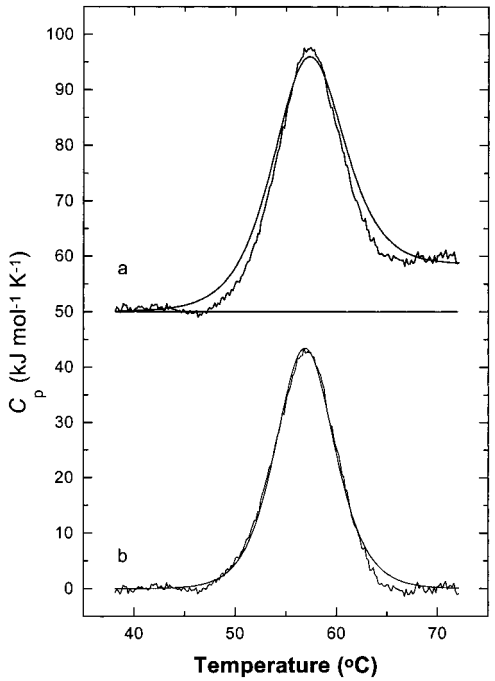

FIGURE 4: Reversible analysis of 14.8 μM SBD in 50 mM phosphate buffer pH 7.5 obtained from the rescan of GA1. The solid lines are experimentally obtained DSC curves: (a) a reversible two-state model, the dotted line is the calculated curve; and (b) a reversible non-two-state model, the dotted line is the calculated curve. The ratio between the calorimetric and the van't Hoff enthalpies is 1.4, so the unfolding process does not conform to a true two-state transition but seems to contain some intermediate folding units (see Table 3). The lines have been shifted vertically for clarity.

at pH 7.5 (Figure 3a,c), and GA1 shows a small peak from reversible unfolding in the rescan (Figure 4), whereas GACD is completely irreversible. GA2 shows a more complex pattern of peaks with a minor peak at lower temperature and a major, irregular peak at higher temperature with a shoulder at the low-temperature side (Figure 3b). Like GACD, GA2 is completely irreversible upon rescanning.

GA1 and GACD unfold via an irreversible one-step mechanism at pH 7.5, and the calorimetric trace can be resolved into one peak where the activation energy and the unity temperature are almost the same for the two glucoamylase forms, while the enthalpy of unfolding is much larger for GA1 (Table 2). The calorimetric trace of GA2 can be resolved into three irreversible one-step peaks where the largest has values similar to those of GA1 and GACD, except for the enthalpy. The enthalpies for GA2 and GACD are similar, which reflects similar stability of these forms that are nearly of the same size. GA2 unfolds with a characteristic minor peak before the larger peak. The larger peak of GA2 is more irregular than the peaks of GA1 and GACD. This means that SBD has some stabilizing effects on the GA1 molecule and the linker region has a destabilizing effect on parts of the GA2 molecule. Although GACD does contain part of the linker region, the heavily O-glycosylated C-terminal part is absent in GACD (*4*). Therefore, it seems that the C-terminal part of the linker region has the destabilizing effect.

The thermodynamic parameters determined for GA1 in different buffers do not vary significantly, and in all buffers, this enzyme shows a DCS profile that can be resolved in a one-step irreversible mechanism of one peak (Tables 1, 2, and 4). This is not the case for GA2. At pH 6, the calorimetric trace can be resolved into two one-step irreversible transitions, but all of the thermodynamic parameters determined in either MES buffer or phosphate/acetate buffer differ significantly (compare Tables 1, 3, and 4). The calorimetric trace using phosphate/acetate buffer pH 7.5 can be resolved into two peaks, whereas it can be resolved into three peaks in phosphate buffer (Tables 3 and 4). This means that GA2 is sensitive to the environment in the sense that its thermal stability is dependent on which particular buffer is used.

The Influence of Ligands on Glucoamylase Unfolding and Assignment of Peaks. Others have suggested previously that the reversible peak of GA1 is due to SBD (*15*). To investigate this further, we added different ligands to the protein solutions before scanning. First β-cyclodextrin is examined. It has been established earlier that two molecules of this ligand bind to SBD of glucoamylase (but not to the catalytic domain) (*15*, *28*, *29*). Compared to free GA1, the peak of the first scan of the GA1-β-cyclodextrin complex changes shape somewhat, but the thermodynamic parameters of the unfolding of GA1 do not change significantly in the absence of the ligand (Table 2). In the rescan, T_d of the small peak increases by 3 °C (Table 3), confirming that the reversible peak corresponds to SBD. Second, acarbose, a strong competitive inhibitor of glucoamylase (*16*, *30*, *31*), is added to GA1 prior to scanning. Upon scanning the peak splits into two: a major peak with a T_u at 74.4 °C and a minor peak with a T_u at 61.0 °C (Figure 3a, Table 2). In the rescan of GA1 and acarbose, the minor peak does not change its position and resembles the rescan peak of GA1 in the absence of acarbose (Table 3). Since acarbose binds exclusively to the active site of the catalytic domain and only the part of the molecule corresponding to the major peak is affected by the ligand, it is reasonable to conclude that the major peak corresponds to the catalytic domain and the minor peak to SBD. A final set of experiments was performed to confirm that SBD is responsible for the reversible peak; a sample of GA1 is heated to 70 °C on a water bath to unfold the large irreversible part of the peak in the first scan. Half of the heated sample was tested by DSC where only the reversible peak is left (data not shown). The rest of the heated sample was used for ITC measurements using the two ligands β-cyclodextrin and acarbose. First, a titration of the preheated sample by β-cyclodextrin was performed to test the specific binding ability of the part of the molecule responsible for the reversible peak. Second, a subsequent titration by acarbose was performed. The same DSC and ITC experiments were carried out on a control sample that was not heated in advance. Both the heated and the control sample bind 2 molecules of β-cyclodextrin, and hardly any differences are seen in the values of Gibbs free energy and stoichiometry while very minor differences are seen in enthalpy and entropy between the two samples (Table 4). Only the control sample that was not heated in advance can bind acarbose (Table 4). It is thus possible to assign the reversible peak to SBD and the irreversible part of the first peak to the catalytic domain. It cannot be determined from the scans of GA1 when the linker region unfolds.

To assign parts of the GA2 molecule to each of the peaks, we performed a scan in the presence of acarbose. The minor peak is unaffected, while the major peak is clearly stabilized

Table 4. Binding Constant, Gibbs Free Energy, Enthalpy, and Stoichiometry of Binding the Ligands β-Cyclodextrin and/Or Acarbose to GA1, GA2, and GACD at 27 °C, pH 7.5

cell content	ligand	K (M^{-1})	$-\Delta G°$ (kJ mol^{-1})	$-\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1} K^{-1})	apparent stoichiometry
GA1	acarbose	$> 10^8$	> 45.8	49.4 ± 2.6	>_- 3.5	1.0 ± 0.01
GA1	β-cyclodextrin	$(5.6 \pm 1.2) \times 10^4$	27.4 ± 0.5	47.7 ± 0.6	- 20.4 ± 5.9	2.1 ± 0.2
GA1 + β-cyclodextrin	acarbose	$> 10^8$	> 46.4	49.1 ± 3.9	>_- 2.7	1.2 ± 0.1
GA1 (heated to 70 °C)	β-cyclodextrin	$(4.4 \pm 1.3) \times 10^4$	26.7 ± 0.7	35.4 ± 8.9	- 8.7 ± 8.9	2.2 ± 0.4
GA1 (heated to 70 °C) + β-cyclodextrin	acarbose	nb[a]	nb	nb	nb	nb
GA2	acarbose	$> 10^8$	> 46.6	51.5 ± 0.9	>_- 4.9	0.75 ± 0.01
GA2 (heated to 47 °C)	acarbose	$> 10^8$	> 46.2	50.8 ± 0.7	>_- 4.7	0.7 ± 0.01
GACD	acarbose	$> 10^8$	> 45.4	55.0 ± 1.6	>_- 9.6	1.0 ± 0.01

[a] No observable binding; GA1 preheated to 70 °C does not bind acarbose. The association constants of binding acarbose to GA1 and GA2 have been determined previously by displacement experiments and are $(9.4 \pm 6.2) \times 10^{11}$ and $(8.8 \pm 3.2) \times 10^{11}$ M^{-1}, respectively (*30*).

by acarbose (Table 2, Figure 3b). Since acarbose only binds to the catalytic domain it is reasonable to conclude that the major peak originates from that domain. To study this into greater detail, we heated a sample of GA2 on a water bath to 47 °C to knock out the minor peak in the same way as described above. One-half of the preheated sample was scanned in DSC to examine whether the minor peak is absent and the rest of the preheated sample was used for ITC with acarbose. A control experiment was performed in which GA2 was not heated in advance. The minor peak is absent in the DSC scanning of the preheated sample, showing that the part of the protein corresponding to the minor peak unfolds irreversibly. If the sample was allowed to refold for a longer period of time (one week at room temperature), the minor peak is still absent so the irreversibility is not due to very slow refolding (*35*). No significant differences are seen in the binding of acarbose between the heated and the control sample. The part of the molecule corresponding to the minor peak is probably not involved in binding of acarbose, and the active site of the catalytic domain is not affected by heating to 47 °C (Table 4). The peaks of GA2 are composed of contributions from the linker region, and the catalytic domain and the minor peak might therefore be the linker region or some part of the catalytic domain far away from the active site that unfold irreversibly before the rest of the GA2 molecule. The minor peak of GA2 is only present at higher pH values (pH 7.0−7.5) (Figure 2b), so the unfolding of this particular structure element has a stronger pH dependence than the rest of the molecule. A minor peak at higher temperatures is observed when GA2 is unfolded at lower pH values. This peak probably corresponds to part of the catalytic domain close to the active site since it is stabilized by acarbose as well as the rest of the GA2 molecule (data not shown).

The minor peak of the GA2 scan at pH 7.5 is not present in the first scan of GA1, even though this form of glucoamylase also contains the linker region (Figure 3a). A plausible explanation is that the linker region is wrapped around the catalytic domain and kept in position by SBD, which probably makes domain−domain contacts with the catalytic domain. In the first scan, some degree of cooperativity between the catalytic domain and SBD is seen. The unfolding process of the catalytic domain becomes more continuous when it is kept together by the linker region and SBD, which is not the case for GA2. On the other hand, when the catalytic domain is unfolded, SBD refolds into a form that is able to bind β-cyclodextrin. Therefore, unfolding of the catalytic domain depends on the presence of the linker region and SBD, whereas SBD acts as an independent folding unit. This has also been confirmed by preliminary examinations of free SBD. The linker region will not unfold before the rest of the molecule in GA1 when it is kept in position by SBD. In contrast, the linker region of GA2 is not kept in any particular conformation by SBD and has therefore a higher degree of freedom that allows it to unfold before the catalytic domain.

GACD has been titrated with acarbose and no major differences are seen compared to the other forms of glucoamylase (Table 4), so the lack of the densely O-glycosylated part of the linker region has no effect in binding this ligand.

Reversible Unfolding of SBD. The ΔH contribution of the rescan of GA1 at pH 7.5 is 14.5% of the first scan when the areas of the two peaks are compared. A second rescan shows almost 100% reversibility of the small peak (data not shown). This reversibility is also dependent on pH and it is not observed when the aggregation occurs. The aggregation can be prevented and the reversible peak regained at pH 4.5 by adding guanidinium chloride in a concentration of 3 M. This suggests that aggregation is responsible for the irreversibility of SBD unfolding at lower pH values.

Since the unfolding of SBD is reversible, it can be analyzed by reversible thermodynamics (Table 3, Figure 5). The observed heat capacity change between folded and unfolded SBD is about 10 kJ mol^{-1} K^{-1}. If reversible unfolding occurs via a two-state mechanism (all-or-none), the ratio between the van't Hoff enthalpy (ΔH_{vH}) and the calorimetric enthalpy (ΔH_{cal}) will be unity (*17*, *32*−*34*). The $\Delta H_{vH}/\Delta H_{cal}$ ratio for SBD is 1.4 ± 0.1, which means that on the time scale of the DSC experiment, the unfolding of the domain can be reasonably approximated with a two-state transition, but a small degree of oligomerization may occur as well.

DISCUSSION

In this study, an analysis has been performed at pH 6.0 on the irreversible thermal unfolding of GA1 and GA2 using different scan rates. The catalytic domain of the two glucoamylase forms unfolds in a one-step irreversible process, and the calorimetric traces can be resolved into one and two peaks for GA1 and GA2, respectively. Therefore, these proteins unfold in a process where no reversible intermediates are detectable. The first peak of GA2 resembles that of GA1 except for the enthalpy, which is due to the different sizes of the molecules. GA2 has at this pH a peak at higher temperature that is not present in GA1. This means

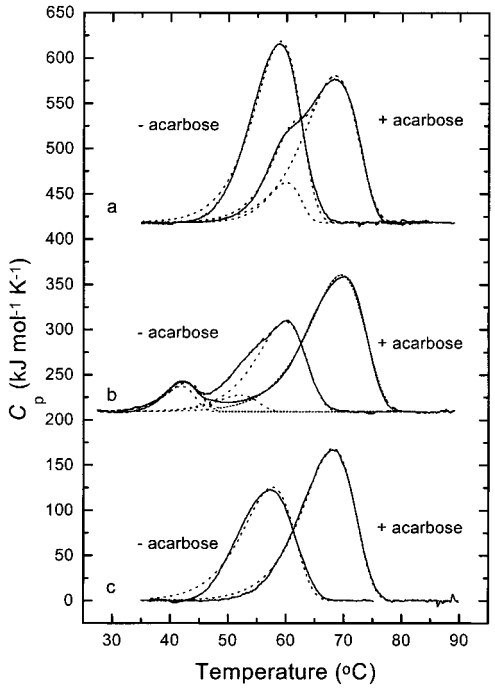

FIGURE 5: The calorimetric traces of Figure 3 are fitted to a one-step irreversible unfolding model in 50 mM phosphate buffer pH 7.5 without and with binding of acarbose. The solid lines are experimentally obtained DSC curves, and the dotted lines are the fits to a one-step irreversible model: (a) GA1, (b) GA2, and (c) GACD. Protein and ligand concentrations are the same as in Figure 3. The lines have been shifted vertically for clarity.

that most of the catalytic domain of GA2 probably unfolds as the catalytic domain of GA1. The second peak of GA2 corresponds to a part of the protein that unfolds differently. The enthalpies obtained by the scan rate experiments differ substantially which may indicate that this part of the catalytic domain does not unfold according to the one-step mechanism. To determine exactly the unfolding mechanism of this unfolding unit is not possible because the unfolding process of GA2 is so complex. Potekhin and Kovrigin (*35*) have observed that some reversible two-state processes can be misinterpreted as an irreversible one-step process if the refolding process is very slow at the refolding temperature. This is probably not the case for the glucoamylase molecules since they do not refold even if they cool down and are kept at room temperature for a longer period of up to two weeks (data not shown). However, refolding has not been attempted at temperatures a few degrees below the denaturation temperature as suggested by Potekhin and Kovrigin (*35*).

The catalytic domain of glucoamylase is a very large domain consisting of 440 amino acid residues. It is more difficult for larger domains to refold in vitro, and in the present study it has not been possible to find conditions where the catalytic domain refolds upon cooling. Proteins or protein domains with a molecular weight less than about 20 kDa often, but not always, unfold reversibly. It has been observed that larger molecules do refold reversibly, for example, pepsinogen with a molecular weight of almost 60 kDa (*36*). In the case of SBD of glucoamylase, this domain containing about 100 amino acids is much smaller than the catalytic domain. Therefore, SBD has an appropriate size to enable reversible unfolding, and even though the domain most probably is involved in interactions with the catalytic domain in the folded state, these apparently do not affect the reversibility of SBD unfolding. SBD has one disulfide bridge (Cys509−Cys604) (*11*) that results in fewer degrees of freedom upon unfolding of the 3D structure. This will probably facilitate the refolding process. The deviation from a reversible two-state mechanism observed for SBD is caused by oligomerization of the domain. It is possible to regain the binding ability of β-cyclodextrin after refolding, so the oligomerization process is also reversible (Table 4).

It has been suggested in the literature that GA1 and GA2 unfold with five and four two-state components, respectively, from analysis of DSC data using a reversible two-state model (*37*). It is not possible, however, to assign a peak from irreversible unfolding to a reversible model, unless the irreversible process is only significant very late in the transition (*18*). Only reversible peaks can be fit to two-state models obtaining both calorimetric and van't Hoff enthalpies. The irreversible processes of unfolding of the catalytic domain of glucoamylase take place very early in the unfolding process (Figure 1), and the resulting enthalpy is the sum of the contributions from the irreversible processes. Therefore, it is not possible to use a reversible two-state model to describe the unfolding of the catalytic domain of glucoamylase, and the postulate that the catalytic domain unfolds in four separate components is invalid. To investigate the irreversible steps in the unfolding of the catalytic domain in a proper way, one must perform scan rate-dependent experiments (see, e.g., refs *18*, *23*, *34*).

The differences in the shapes of the DSC profiles of GA1, GA2, and GACD at pH 7.5 are remarkable. Both GA1 and GACD unfold with one nearly symmetrical peak, whereas GA2 unfolds with more discernible peaks. The most likely explanation of this observation is that this glucoamylase has a somehow larger cooperative unfolding unit and that parts of the catalytic domain unfold in a broader temperature range. The catalytic domains of GA1, GA2, and GACD unfold in all cases in a one-step irreversible way, and the analysis shows that the calorimetric traces of unfolding can be resolved into one one-step irreversible peak for GA1 and GACD both in the absence and in the presence of acarbose (Figure 5a,c). The calorimetric trace of GA2 is different, and the analysis thereof reveals three and two one-step irreversible peaks without and with the presence of acarbose, respectively (Figure 5b). The major peak of GA2 is resolved into two peaks in the absence of acarbose and only one peak in the presence of the ligand. This means that acarbose can hold the structure together even in the presence of the destabilizing linker region. The T_u values increase about 10 °C after the addition of acarbose in all cases, which corresponds to a decrease in activation entropy after addition of the ligand. The acarbose is a strong active site inhibitor of glucoamylases, and it is evident that the ligand will decrease the degrees of freedom upon binding. The activation energy is the minimum energy that is necessary to reach the transition state for the unfolding process, and it is almost the same for all glucoamylases for the peaks corresponding to the catalytic domain both in the absence and in the presence of acarbose. This means that the structure of the transition state of unfolding probably is the same in all cases and that the dissociation of acarbose happens at the same time as the molecules get enough energy to reach the transition state. The enthalpy of acarbose release can be estimated from the heat capacity determined previously by

ITC (*31*). At the temperature corresponding to $\langle C_{p,exc}\rangle^{max}$, the enthalpy of acarbose release is about 80 kJ mol^{-1}, which is a small fraction of the total enthalpy, measured in the unfolding process. The smaller cooperative unit upon acarbose addition is responsible for the increase in total enthalpy for all glucoamylase forms.

According to transition-state theory, the activation enthalpy and entropy of the thermoinactivation process can be calculated from the rate constant (*38*)

$$k = \frac{k_B T}{h} \exp\left(-\frac{\Delta H^{\neq}}{RT}\right) \exp\left(\frac{\Delta S^{\neq}}{R}\right)$$

where k_B and h are Boltzmann's and Planck's constants, respectively, and $\Delta H^{\neq}$ and $\Delta S^{\neq}$ are the activation enthalpy and entropy, respectively. The activation enthalpy for a unimolecular reaction is

$$\Delta H^{\neq} = E_a - RT$$

and the activation entropy is

$$\Delta S^{\neq} = R\left(\frac{E_a}{RT_U} - \ln\frac{k_B T}{h} + 1\right)$$

$\Delta H^{\neq}$ and $\Delta S^{\neq}$ were determined at 65 °C from the above equations at pH 4.5 are 266 kJ mol^{-1} and 487 J mol^{-1} K^{-1}, respectively. These values compare with the activation enthalpy and entropy at pH 4.5 of 311 kJ mol^{-1} and 610 J mol^{-1} K^{-1}, respectively, determined on glucoamylase from *Aspergillus awamori* (*39*, *40*).

At first it seems as SBD has a stabilizing effect on the GA1 molecule, but this might be an indirect effect. We suggest that SBD keeps the linker region wrapped around the catalytic domain in a tight position which neutralizes the destabilizing effect of the linker region. The unfolding of GA1 does not show a minor peak as GA2; instead GA1 unfolds in a regular peak due to the presence of SBD. It has been shown previously by ITC experiments, using heterobidentate ligands of covalently connected acarbose and β-cyclodextrin moieties, that the catalytic domain and SBD are close together in space (*14*). In the GA1 form, the two domains may be held together by domain−domain interactions which will stabilize both the catalytic domain and the linker region, resulting in virtually simultaneous unfolding of the whole GA1 molecule. Unfolding of the catalytic domain of GA1 depends on the presence of the linker region and SBD, whereas the reversible unfolding/refolding processes of SBD occur independently of the catalytic domain. When acarbose is bound, SBD unfolds before the rest of the molecule, but the ligand holds the catalytic domain together and unfolding of this domain still occurs with one regular peak. Therefore, unfolding of the catalytic domain is dependent on the linker region and SBD or acarbose, whereas unfolding of SBD proceeds whether or not the catalytic domain is present. The difference between GA2 and GACD lies in the length of the linker regions of about 30 and 70 amino acids, respectively. GACD contains only the N-terminal and less densely glycosylated part of the linker region, and this is probably less flexible than the entire linker region. According to the DSC experiments, this short linker region does not destabilize the catalytic domain to the same extent as the full-length linker region present in GA2. Therefore, particularly the highly O-glycosylated C-terminal part of the linker region seems to be responsible for the larger cooperative unit of GA2. This suggests that the glycosylations play a significant role in destabilizing part of the catalytic domain of GA2 at this pH. Apparently, the linker region has to be full length to exert its destabilizing effect. Another interesting observation is that the linker region unfolds irreversibly. It has previously been assumed that the linker was flexible and part of the time wrapped around the catalytic domain and part of the time in an extended conformation (*12*, *15*, *41*). When the linker region is wrapped around the catalytic domain it will only cover some parts of the domain. It will be important to know if the length of the linker region plays a role in the stability of GA1. Work is in progress in our laboratories to study this point using glucoamylase mutants with alterations in the linker region.

The reversible transition has been assigned to SBD, since its position is affected only by binding of β-cyclodextrin and not by acarbose. Furthermore, the part of GA1 corresponding to the reversible peak maintains the ability to bind β-cyclodextrin after refolding (Table 4), so it is unequivocal that the reversible transition corresponds to SBD. In the study of Williamson et al. (*15*), they showed that isolated SBD containing the C-terminal part of the linker region is reversible by thermal unfolding. The denaturation temperature and calorimetric and van't Hoff enthalpies found for SBD containing part of the linker region in that work are in accordance with the values found in the present work. When adding β-cyclodextrin to the sample before scanning, Williamson et al. (*14*) found a somewhat larger increase in the denaturation temperature than in the present work. If the linker region is totally omitted from SBD in a form containing residues 509−616, the unfolding of SBD by heat is irreversible (*10*). Therefore, the linker has a stabilizing effect on SBD.

Unfolding of glucoamylases seems to be very dependent on pH, and the maximum stability for both GA1 and GA2 is at pH 4.0−4.5 where the enzyme also has its maximum activity. Both GA1 and GA2 aggregate upon heating at pH between 2.5 and 5.0, which are values close to the pI of 4.0 and 4.2 for GA1 and GA2, respectively (2). At higher pH, aggregation is not observed. It is desirable to find conditions where aggregation does not take place in order to determine the thermodynamic parameters for the unobscured irreversible process. Adding 3 M guanidinium chloride to the sodium acetate buffer at pH 4.5 can prevent the aggregation process, and the rescan shows the reversible peak from SBD. Therefore, the irreversibility of SBD at lower pH values is due entirely to the aggregation, but the irreversible unfolding of the catalytic domain and linker region is the result of other reactions as well, presumably covalent changes (*39*, *42*, *43*).

GA1 and GA2 unfold differently as a function of pH. The peaks of GA2 display a more complicated picture than GA1 with additional peaks and shoulders. At lower pH minor peaks appear at higher temperatures, but these are probably not due to the linker region since they are dependent on the presence of acarbose at pH 4.5; they arise from unfolding of the catalytic domain. At lower pH, the linker region of GA2 cannot be assigned to any particular peak, and it is either unfolded at all temperatures or unfolded concomitantly with the catalytic domain. The most common amino acids

in the linker region are Ser and Thr, which are all O-glycosylated. Otherwise, the linker region consists mostly of amino acid residues with aliphatic side chains. The linker region of GA2 also contains two Cys residues, one Tyr, and two Lys, and only the lysines will be charged at the pH values investigated here. Even though the pK_a values for the three amino acids mentioned above all are higher than 9, there will be a small charge difference between the higher and lower pH values. This may offer an explanation for the unfolding pattern of the linker region of GA2 at different pHs.

In conclusion, it has been possible to assign the peaks in DSC experiments of the different glucoamylase forms to the domains in the molecules. The catalytic domain always unfolds in a one-step irreversible way, whereas SBD of the GA1 unfolds reversibly at pH values where aggregation is prevented. In GA1 the linker region first of all helps to keep SBD in position for domain−domain interactions, making the molecular structure tighter than for the GA2 molecule. GA2 has a larger cooperative unfolding unit where part of the catalytic domain unfolds differently and part unfolds in a way resembling the unfolding of GA1. The linker region thus perturbs the structure of the catalytic domain during thermal unfolding. Further experimentation is needed to elucidate the role of the linker region in degradation of raw starch granules by glucoamylase, especially with respect to the influence of the glycosylation. Since the overall multi-domain architecture with catalytic and binding domains connected by glycosylated linker is common to many polysaccharide hydrolyzing enzymes, the results reported here are possibly also of relevance to these.

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SUPPORTING INFORMATION AVAILABLE

Two tables with thermodynamic data of the pH dependence of the unfolding of GA1 (Table 1) and GA2 (Table 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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