Thermodynamics of Inhibitor Binding to the Catalytic Site of Glucoamylase from *Aspergillus niger* Determined by Displacement Titration Calorimetry

Bent W. Sigurskjold,* Carolyn R. Berland, and Birte Svensson

Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark

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ABSTRACT: The binding of different inhibitors to glucoamylase G2 from *Aspergillus niger* and its temperature and pH dependencies have been studied by titration calorimetry. The enzyme binds the inhibitors 1-deoxynojirimycin and the pseudo-tetrasaccharide acarbose with association constants of 3×10^4 and 9×10^{11} M^{-1}, respectively, at 27 °C. The binding free energy for both ligands is remarkably temperature-invariant in the interval from 9 to 54 °C as the result of large compensating changes in enthalpy and entropy. Acarbose and 1-deoxynojirimycin bound with slightly different free energy–pH profiles, with optima at 5.5 and 5.5–7.0, respectively. Variations in $\Delta H°$ and $T\Delta S°$ as a function of pH were substantially larger than variations in $\Delta G°$ in a partly compensatory manner. Two titratable groups at or near subsite 1 of the catalytic site were found to change their pK_a slightly upon binding. The hydrogenated forms of acarbose, D-*gluco*- and L-*ido*-dihydroacarbose, bind with greatly reduced association constants of 3×10^7 and 2×10^5 M^{-1}, respectively, and the pseudo-disaccharide methyl acarviosinide, lacking the two glucose units at the reducing end compared to acarbose, has a binding constant of 8×10^6 M^{-1}; these values all result from losses in both enthalpy and entropy compared to acarbose. Three thio analogues of the substrate maltose, methyl α- and β-4-thiomaltoside and methyl α-4,5′-dithiomaltoside, bind with affinities from 3×10^3 to 6×10^4 M^{-1}. All ligands bind with remarkably favorable contributions from entropy, indicating good structural complementarity with efficient solvent displacement in complex formation.

Glucoamylase is a very important industrial enzyme used to convert starch to glucose (Reilly, 1979). Glucoamylase produced by the fungus *Aspergillus niger* occurs in two forms, glucoamylases G1 and G2. The G1 form is a multidomain enzyme consisting of a catalytic domain and a granular starch binding domain that enables this form to hydrolyze raw starch; the G2 form contains only the catalytic domain (Svensson et al., 1983, 1986). Both forms of the enzyme have similar catalytic properties toward soluble substrates (Svensson et al., 1982). The enzyme catalyzes the hydrolysis of both α-(1→4)- and α(1→6)-glycosidic linkages, the former 30–50 times faster than the latter (Hiromi et al., 1966a). Because of the relative inefficiency by which glucoamylase hydrolyzes the α(1→6) branch points of starch, and because of the accumulation of isomaltose, isomaltotriose, and panose resulting from condensation reactions in concentrated industrial saccharification mixtures, the yield of the conversion of starch to glucose is only 96%. It would therefore be desirable to attempt to modify this enzyme to enhance the overall conversion efficiency. Toward this end, studies of the interactions between glucoamylase from *Aspergillus niger* and specific inhibitors provide a valuable tool for understanding the mechanism of the enzyme's action on starch and maltooligodextrins.

Glucoamylase is, like many other glycosylases, inhibited by sugar analogues with a basic nitrogen atom adjacent to C-1 [for a review, see Bock and Sigurskjold (1990)]. Two examples of this type of inhibitor are 1-deoxynojirimycin and the very strong inhibitor acarbose (Truscheit et al., 1981). Acarbose is a tetrasaccharide analogue whose nonreducing end contains a pseudosugar moiety with a double bond and a nitrogen atom instead of the glycosidic oxygen (Figure 1). 1-Deoxynojirimycin is a glucose analogue with a nitrogen replacing the endocyclic oxygen and is deoxygenated at the anomeric center. Recently, two crystal structures of the closely related glucoamylase from *Aspergillus awamori* var. *X100* uncomplexed (Aleshin et al., 1992) and in complex with 1-deoxynojirimycin (Harris et al., 1993) have been published. Furthermore, crystal structures of the complexes between this glucoamylase and acarbose (Aleshin et al., 1994) and of D-*gluco*-dihydroacarbose, a hydrogenated form of acarbose (Stoffer et al., unpublished results), have become available.

However, to fully appreciate the interactions between a protein binding site and its ligands, a detailed thermodynamic description is a highly desirable complement to structural data [see, for example, Hinz (1983) and Raffa and Porreca (1989)]. In the present paper, we report the results of a detailed study of the thermodynamics of acarbose and 1-deoxynojirimycin binding to the G2 form of glucoamylase from *Aspergillus niger* using titration microcalorimetry. In addition, the energetics of binding of G2 to a number of different inhibitors are described.

MATERIALS AND METHODS

Proteins and Inhibitors. Commercial glucoamylase (1,4-D-glucan glucohydrolase, EC 3.2.1.3) from *Aspergillus niger* (AMG 200L) was purchased from Novo Nordisk A/S (Bagsværd, Denmark), and the G1 and G2 forms were purified essentially as described previously (Svensson et al., 1982). The protein was then lyophilized and stored at 4 °C until it was used. Immediately before each experiment, the required amount of protein was weighed and dissolved in the appropriate buffer. Concentrations of glucoamylase G1 and G2 were determined spectrophotometrically at 280 nm using $\epsilon = 1.37 \times 10^5$ and 1.09×10^5 M^{-1} cm^{-1}, respectively (Clarke & Svensson, 1984). Acarbose and 1-deoxynojirimycin (moranoline) were gifts from E. Möller, and methyl α,β-acarviosinide was synthesized by F. Heiker, both at Bayer AG (Wuppertal,

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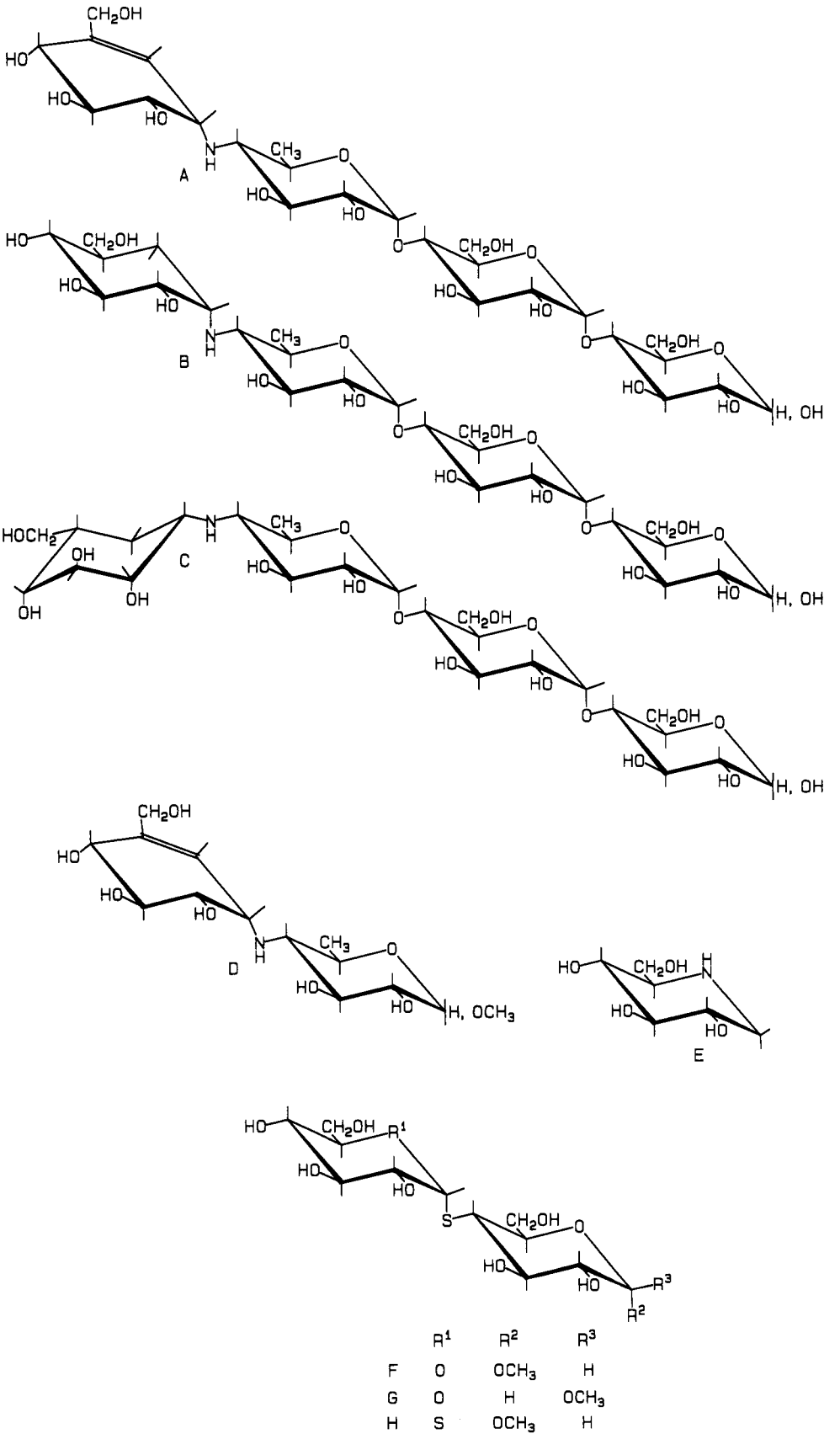

FIGURE 1: Structures of inhibitors of glucoamylase: acarbose (A), D-*gluco*-dihydroacarbose (B), L-*ido*-dihydroacarbose (C), methyl α,β-acarviosinide (D), 1-deoxynojirimycin (E), methyl α-4-thiomaltoside (F), methyl β-4-thiomaltoside (G), and methyl α-4,5′-dithiomaltoside (H).

Germany). D-*gluco*- and L-*ido*-dihydroacarbose (Bock et al., 1991) and methyl β-4-thiomaltoside (Bock et al., 1994) were gifts from K. Bock and M. Meldal (this laboratory). Methyl α-4-thiomaltoside was a gift from H. Driguez (Grenoble, France), and methyl α-4,5′-dithiomaltoside (Mehta et al., 1994) was a gift from B. M. Pinto (Burnaby, Canada).

Buffers. All experiments except those measuring the dependence of binding upon pH were performed in 0.05 M

sodium acetate buffer, pH 4.5. The pH dependence studies were performed at pH values between 3.4 and 6.5 using appropriate mixtures of 0.2 M Na_2HPO_4 and 0.1 M citric acid, as described by McIlvaine (1921). Studies at pH 4.5 were performed both in 0.05 M sodium acetate and in 0.05 M tetraethylenepentamine buffers, and studies at pH 7.5 were performed in both 0.05 M sodium phosphate and 0.05 M MOPS buffers.

Titration Calorimetry. Calorimetric measurements were carried out using an OMEGA titration microcalorimeter (MicroCal, Inc., Northampton, MA). This instrument has been described in detail by Wiseman et al. (1989). The reference cell was filled with water, and the instrument was calibrated using standard electrical pulses. All solutions were thoroughly degassed by stirring under vacuum before use. The thermodynamics of all ligands except acarbose were measured by direct titration. Solutions of glucoamylase G1 or G2 (0.05–0.1 mM) were titrated with 20 identical 13-μL injections at 3-min intervals. The injection syringe, on which a stirrer paddle is mounted, stirred the solutions at 400 rpm, ensuring immediate mixing. The concentration of the ligand solution was chosen to ensure that the protein would be close to saturation with ligand well before the final injection. In this way, both heats of binding and heats of dilution could be measured in a single experiment. The peaks of the obtained thermograms were integrated using the ORIGIN software (MicroCal, Inc.) supplied with the instrument. The isotherm obtained in this way was fitted using nonlinear regression, as described previously (Sigurskjold et al., 1991). The fitting procedure gives the binding constant of the ligand, K, the heat of binding, $\Delta H°$, the concentration of binding sites (stoichiometry), and the heat of dilution of the ligand. From these quantities, it is possible to calculate the free energy and the entropy of binding using standard thermodynamic relationships. From experiments carried out at different temperatures, heat capacity changes and van't Hoff enthalpies can be estimated.

Displacement Experiments. The thermodynamics of acarbose could not be measured by direct titration since the binding is too tight ($K > 10^{11}$ M^{-1}, Svensson & Sierks, 1992). In order to obtain a binding isotherm from which the binding constant is determinable, it is necessary that the binding constant is neither too small nor too large. Wiseman et al. (1989) have shown that the product of the binding constant and the protein concentration, K[protein], must lie in the interval from 1 to 1000, and preferably between 10 and 100. Because of the very strong binding of acarbose to glucoamylase, the protein concentrations that fall within this window are so low that the heat evolved during the reaction is well below the detection limit. Instead of a direct measurement, a displacement experiment can be carried out in which the protein is first saturated with a weaker binding ligand whose binding parameters can be measured directly, and this ligand in turn is displaced by titration with the stronger binding one. The first ligand will inhibit the binding of the second ligand and thereby reduce the apparent binding constant. If the binding of both ligands is exothermic, then the heat of binding for the second ligand will be reduced by the endothermic heat of dissociation of the first ligand. It is therefore necessary that the magnitudes of the molar enthalpies of binding for the two ligands are significantly different. General analysis methods for displacement binding isotherms with ligands that do not necessarily differ much in affinity have been described (Eatough, 1970; Biltonen & Langerman, 1979; Khalifah et al., 1993), but if the binding constants differ by several orders of magnitude, the analysis becomes straightforward using the following approximation: For each displacement experiment, a solution of glucoamylase G2 was first titrated to saturation with a relatively weak inhibitor, e.g., 1-deoxynojirimycin. The injection syringe was then cleaned and refilled with an acarbose solution for the second titration. The second experiment was fitted to the usual isotherm expression and gave apparent K_{app} and ΔH_{app}. These were then simply related to the values for acarbose (K_{ac} and $\Delta H°_{ac}$) by the following equations:

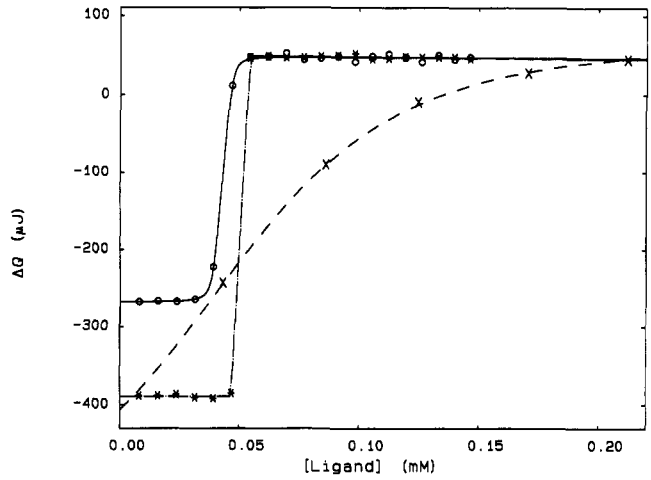

FIGURE 2: Binding isotherms of titrating 49 μM glucoamylase G2 with 4.53 mM 1-deoxynojirimycin (×, – –), 0.838 mM acarbose (*, – · –), and 0.838 mM acarbose inhibited with 1-deoxynojirimycin (○, —). The transition point in the displacement experiment shifts relative to that in the direct titration due to the dilution of the glucoamylase solution by the initial titration with 1-deoxynojirimycin. The difference in the level of initial heats in the displacement titration compared to the direct titration with acarbose is caused by the endothermic dissociation of 1-deoxynojirimyin. The shape of the fitted curve to the direct acarbose isotherm is equally good for any value of $K > 10^8$ M^{-1}.

$$\Delta H°_{ac} \cong \Delta H_{app} + \Delta H°_{in} \quad (1)$$

and

$$K_{ac} \cong K_{app} K_{in} \quad (2)$$

where $\Delta H°_{in}$ and K_{in} are the enthalpy and binding constant, respectively, for the inhibitor determined independently.

RESULTS AND DISCUSSION

Displacement Experiments. Figure 2 shows binding isotherms of the binding of 1-deoxynojirimycin and acarbose to glucoamylase G2 and the displacement isotherm of binding acarbose inhibited by 1-deoxynojirimycin. Clearly, the binding constant for acarbose cannot be determined directly because of the almost rectangular shape of the isotherm. Since the difference in $\Delta H°$ between 1-deoxynojirimycin and acarbose is 18 kJ mol^{-1}, and the binding constants differ by approximately 6 orders of magnitude, the binding thermodynamics of acarbose can be determined accurately using displacement experiments with 1-deoxynojirimycin as an inhibitor and eqs 1 and 2 for the analysis. It is also clear from Figure 2 that the inhibitor of acarbose binding should not have a smaller association constant than 1-deoxynojirimycin, since this would lead to the displacement isotherm being almost rectangular as well. The stoichiometry is 1:1 for both acarbose and 1-deoxynojirimycin, even though it is known that the latter compound also has a secondary binding site with a lower occupancy (affinity) (Harris et al., 1993). This weak binding

Table 1: Determination of the Thermodynamic Functions for the Binding of Strong Inhibitor Acarbose to Glucoamylase G1 and G2 by Displacement Titration Calorimetry at 27 °C, pH 4.5

enzyme	ligand	K (M^{-1})	$-\Delta G°$ (kJ mol^{-1})	$-\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})
G2	1-deoxynojirimycin	$(3.3 \pm 0.2)^a \times 10^4$	25.8 ± 0.1	11.2 ± 0.1	14.5 ± 0.2
	acarbose (apparent values)[b]	$(2.7 \pm 1.0) \times 10^7$	42.4 ± 0.9	29.3 ± 0.1	13.1 ± 0.9
	acarbose	$(8.8 \pm 3.2) \times 10^{11}$	68.2 ± 0.9	40.6 ± 0.1	27.6 ± 0.9
G1	1-deoxynojirimycin	$(4.7 \pm 1.7) \times 10^4$	26.9 ± 0.9	7.0 ± 0.9	19.9 ± 1.3
	acarbose (apparent values)[b]	$(2.0 \pm 1.1) \times 10^7$	42.0 ± 1.3	25.8 ± 1.1	16.2 ± 1.7
	acarbose	$(9.4 \pm 6.2) \times 10^{11}$	68.9 ± 1.6	32.8 ± 1.4	36.1 ± 2.1
G1	methyl α,β-acarviosinide	$(7.8 \pm 2.6) \times 10^6$	39.6 ± 0.8	31.0 ± 0.3	8.6 ± 0.9
	acarbose (apparent values)[c]	$(3.8 \pm 0.9) \times 10^4$	26.3 ± 0.6	7.1 ± 0.5	19.2 ± 0.8
	acarbose	$(2.9 \pm 1.2) \times 10^{11}$	65.9 ± 1.0	38.1 ± 0.6	27.8 ± 1.2

[a] Three standard deviations from regression analysis (3σ confidence level). [b] Inhibited by 1-deoxynojirimycin. [c] Inhibited by methyl α,β-acarviosinide.

cannot be observed by titration calorimetry with the concentration range of 1-deoxynojirimycin used here.

In Table 1, the values for the binding of 1-deoxynojirimycin and acarbose to glucoamylase G2 at 27 °C are shown. Throughout this paper, uncertainties are given as three standard deviations (3σ confidence level) obtained from nonlinear regression analysis. Although the binding constant between acarbose and G2 cannot be measured directly, it is possible to measure the enthalpy in the direct experiment. The direct titration of glucoamylase G2 with acarbose gives an enthalpy of $\Delta H° = -40.9 \pm 0.6$ kJ mol^{-1}, which is identical to the result from the displacement experiment. This agreement validates our use of displacement experiments and the approximate eqs 1 and 2.

The binding of 1-deoxynojirimycin and acarbose to glucoamylase G1, which contains a granular starch binding domain, shows essentially the same affinities as for G2, although small but significant mutually compensating changes in $\Delta H°$ and $-T\Delta S°$ can be detected (Table 1). Titration experiments of binding to a proteolytically cleaved and isolated fragment of the starch binding domain (Belshaw & Williamson, 1990) showed a weak affinity for acarbose (approximately 10^3 M^{-1}) and undetectable binding of 1-deoxynojirimycin (data not shown). The displacement technique thus allows studies of the catalytic site that will also be useful in *Aspergillus niger* glucoamylase mutants which are commonly available in the G1 form.

Acarviosine is closely related to acarbose, differing in the lack of the two glucose units at the reducing end (Figure 1). A ^{1}H NMR spectrum showed that the mixture of α- and β-methyl glycosides of acarviosine is 22/78 (α/β) (not shown). The methyl acarviosinides are 2 orders of magnitude stronger inhibitors than 1-deoxynojirimycin, and the results from a displacement experiment using this ligand mixture as the first inhibitor and acarbose as the second are also shown in Table 1. The results indicate that the binding constant, as well as the magnitude of the enthalpy of acarbose binding, is slightly underestimated in the displacement experiments using acarviosinide as the first ligand. This implies that the displacement of acarviosinide by acarbose was less complete than the displacement of 1-deoxynojirimycin by acarbose, even though the binding constant for acarviosinide is 5 orders of magnitude smaller than that of acarbose and that of 1-deoxynojirimycin is 7 orders smaller. Hence, all displacement experiments reported here were carried out using 1-deoxynojirimycin as the first, weaker inhibitor.

pH Dependence. We have determined the thermodynamics of binding for both acarbose and 1-deoxynojirimycin as a function of pH in the range 3.4–7.5 at 27 °C; the results are summarized in Figure 3. These values are not corrected for the heat of ionization of the buffers, but since the number of protons released is less than 1 (see below) and since the heat

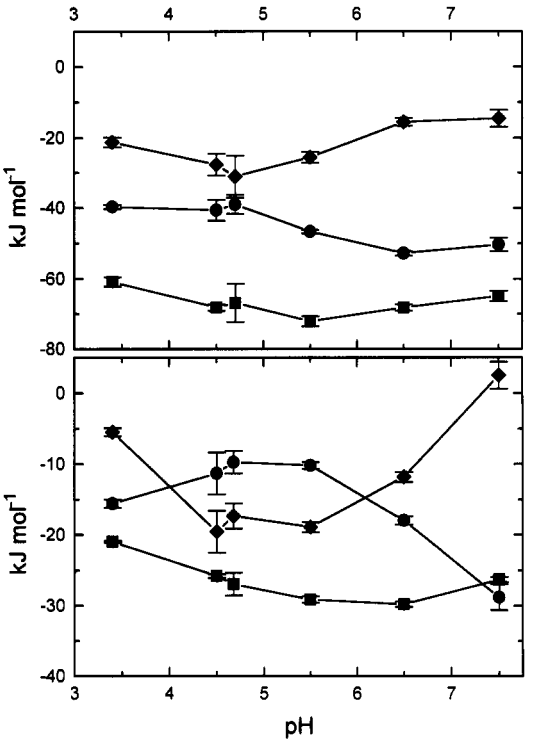

FIGURE 3: pH dependence of the binding of acarbose (top) and 1-deoxynojirimycin (bottom) to glucoamylase G2 at 27 °C. The curves represent $\Delta G°$ (■), $\Delta H°$ (●), and $-T\Delta S°$ (◆). Note that the energy scale in the acarbose diagram is twice as large as that of the 1-deoxynojirimycin diagram.

of ionization for acetate buffers is nearly 0, the correction for the heat of ionization is expected to make a less than 1 kJ mol^{-1} difference in the heats of reaction. The two inhibitors both show a rather strong pH dependence in their thermodynamics of binding. However, the optimal pH for binding is somewhat different: near 5.5 for acarbose and a broader optimum range around 5.5–7.0 for 1-deoxynojirimycin. Both of these optimal pH values are significantly different from the optimal pH for maltooligodextrin hydrolysis of 4.5 (Meagher & Reilly, 1989; Sierks et al., 1990). The pH optima seem, however, to be related to the pK_a values of the nitrogen moiety in each inhibitor: for acarbose $pK_a \cong 5.0$ (Truscheit et al., 1981), and for 1-deoxynojirimycin $pK_a = 6.6$ (Inouye et al., 1966, 1968). It seems reasonable to speculate that increasing the basicity of the solution increases the affinity of the enzyme for both ligands, but that both ligands are much less potent inhibitors in their deprotonated forms, causing decreased binding beyond the pK_a's of the ligands. This same phenomenon has been observed in the inhibition of a related starch-hydrolyzing enzyme, barley α-amylase, by acarbose (Søgaard et al., 1993): the strength of this inhibition falls off dramatically above pH 6.

Table 2: Temperature Dependence of the Thermodynamics of the Binding of Acarbose and 1-Deoxynojirimycin to Glucoamylase G2[a]

ligand	temp (°C)	K (M^{-1})	$-\Delta G°$ (kJ mol^{-1})	$-\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})
1-deoxynojirimycin	8.93	$(7.9 \pm 4.3)^b \times 10^4$	26.4 ± 1.3	7.8 ± 1.2	18.6 ± 1.7
acarbose	9.09	$(7.7 \pm 5.9) \times 10^{11}$	64.2 ± 1.8	31.1 ± 1.2	33.1 ± 2.2
1-deoxynojirimycin	17.94	$(3.6 \pm 0.9) \times 10^4$	25.4 ± 0.6	10.5 ± 1.0	14.9 ± 1.2
acarbose	18.03	$(5.5 \pm 5.7) \times 10^{11}$	65.4 ± 2.5	38.6 ± 1.1	26.8 ± 2.7
1-deoxynojirimycin	24.86	$(3.3 \pm 0.3) \times 10^4$	25.8 ± 0.3	11.2 ± 3.0	14.5 ± 3.0
acarbose	25.00	$(8.8 \pm 3.3) \times 10^{11}$	68.2 ± 0.9	40.6 ± 3.0	27.6 ± 3.1
1-deoxynojirimycin	36.22	$(3.5 \pm 1.6) \times 10^4$	26.9 ± 1.1	11.3 ± 2.0	15.7 ± 2.3
acarbose	36.29	$(9.8 \pm 6.2) \times 10^{11}$	71.0 ± 1.6	50.6 ± 2.1	20.5 ± 2.6
1-deoxynojirimycin	44.71	$(3.0 \pm 0.4) \times 10^4$	27.2 ± 0.4	12.9 ± 0.8	14.3 ± 0.9
acarbose	44.71	$(5.0 \pm 1.9) \times 10^{11}$	71.2 ± 1.0	57.3 ± 0.9	13.9 ± 1.3
1-deoxynojirimycin	53.80	$(4.3 \pm 1.8) \times 10^4$	29.0 ± 1.2	12.7 ± 2.3	16.3 ± 2.6
acarbose	53.75	$(2.2 \pm 1.0) \times 10^{11}$	70.9 ± 1.2	70.7 ± 2.3	0.2 ± 2.8

[a] All values for acarbose have been obtained by displacement experiments. [b] Three standard deviations from regression analysis (3σ confidence level).

As a related part of the series of pH measurements, we have determined the numbers of protons taken up or released into the solution upon binding of the inhibitor at both pH 4.5 and 7.5. These studies were performed by measuring the titration isotherm in two different buffers at the same pH. The buffers were chosen to have large differences in their heats of ionization. In this situation, since the buffer capacity of the buffers greatly exceeds that of the protein, the number of protons taken up, n, is readily determined from the following equation:

$$n = \frac{\Delta H_1 - \Delta H_2}{\Delta H_{1,\text{ion}} - \Delta H_{2,\text{ion}}} \quad (3)$$

where ΔH_j is the heat of binding in buffer j and $\Delta H_{j,\text{ion}}$ is the heat of ionization of buffer j. The heats of ionization for MOPS and phosphate buffers were taken as $\Delta H_{\text{ion}} = 22.1$ and 5.10 kJ mol^{-1}, respectively (Fukada et al., 1987), and for tetraethylenepentamine and acetate as $\Delta H_{\text{ion}} = 33.0$ and -0.08 kJ mol^{-1}, respectively (Kresheck, 1986). The values of K were not affected by the buffer changes, indicating that possible interactions between the buffer salts and the reactants are insignificant. For both ligands, 0.5 ± 0.1 proton is *released* by complex formation at pH 4.5 and 0.7 ± 0.1 proton is *taken up* at pH 7.5. The number of protons released at the given pH values is thus independent of the ligand. This fact strongly suggests that proton uptake and release are due to ionizable groups in the protein that change pK_a upon binding. Since 1-deoxynojirimycin is a monosaccharide that preferably binds to subsite 1 and to a somewhat smaller extent to subsite 2 (Harris et al., 1993), these groups should be located within the first subsite. Also, since the number of protons bound changes in opposite directions at the two different pH values, there must be at least two ionizable groups involved: one with a pK_a in the acidic range and another with a pK_a at or slightly above neutral pH. These groups may also be responsible for the pH dependence of the binding shown in Figure 3. This finding of two ionizable groups is in agreement with the findings of Hiromi et al. (1966b), who studied the kinetics of the related glucoamylase from *Rhizopus delemar*. They found that two groups in the catalytic center change their pK_a's in opposite directions when the trisaccharide panose is bound, but only the most acidic group changes when maltose is bound. It should be possible to determine likely candidates for the ionizable groups in G2 by examining the X-ray structure of the related glucoamylase complexed with 1-deoxynojirimycin (Harris et al., 1993), acarbose (Aleshin et al., 1994), and D-*gluco*-dihydroacarbose (Stoffer et al., unpublished results). The putative catalytic acid (Glu 179) is a likely candidate for one such group with a pK_a of 5.9 (Meagher & Reilly, 1989).

Acarbose adopts a conformation around the glycosidic linkages that is very similar to the conformation of maltotetraose (Bock & Pedersen, 1984) at basic pH, whereas at acidic pH, acarbose (and D-*gluco*-dihydroacarbose) populates a somewhat different conformation to a high degree due to protonation of the glycosidic nitrogen atom (Bock et al., 1991). Thus, pH-dependent conformational preferences may be at least partly responsible for the pH profiles of binding (Figure 3).

Finally, we have briefly investigated the effect of ionic strength on the binding of these two ligands. Addition of 200 mM NaCl to the acetate buffer used in the experiments did not significantly affect the thermodynamics of binding (results not shown). From this fact, we can conclude that long-range electrostatic attraction or repulsion is relatively unimportant for the binding of 1-deoxynojirimycin and acarbose to glucoamylase G2.

Temperature Dependence. We have studied the temperature dependence of the binding thermodynamics of 1-deoxynojirimycin and acarbose over the range 9–54 °C. The thermodynamic functions $\Delta H°$, $T\Delta S°$, and $\Delta G°$ as a function of temperature are shown in Table 2. It is clear from the table that both associations become increasingly enthalpically driven as the temperature increases. This pattern of temperature dependence is characteristic of hydrophobic association.

The data in Table 2 can be used to construct van't Hoff plots for 1-deoxynojirimycin and acarbose. One can estimate ΔH_{vH} from these plots using the linear van't Hoff equation:

$$\ln K = \ln A - \Delta H_{\text{vH}}/RT \quad (4)$$

where R is the universal gas constant, ΔH_{vH} is the van't Hoff enthalpy, and A is a factor related to the entropy. From the plots (Figure 4), we deduce that $\Delta H_{\text{vH}} = -15 \pm 30$ and -9 ± 20 kJ mol^{-1} for acarbose and 1-deoxynojirimycin, respectively. These very small values of ΔH_{vH} reflect the weak temperature dependence of K, and they are very different from the calorimetrically measured heats, ΔH_{cal}. A very similar difference between ΔH_{cal} and ΔH_{vH} for protein–sugar interactions has been observed before for an antibody–oligosaccharide system (Sigurskjold & Bundle, 1992). There are at least two possible explanations for this discrepancy. First, calorimetry measures both the enthalpy change of the binding reaction, ΔH_{int}, the intrinsic enthalpy change, and the heat of any concomitant reactions, ΔH_{con}, which accompany the binding reaction but do not directly influence the intrinsic binding constant K. Such reactions could, for example, involve changes in protonation, hydration, or conformation. To a first approximation, the van't Hoff enthalpy is equal to the

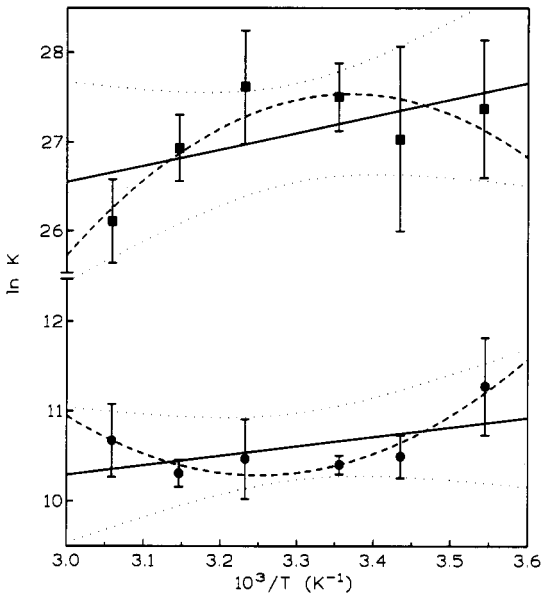

FIGURE 4: van't Hoff plots of the binding of 1-deoxynojirimycin (●) and acarbose (■) to glucoamylase G2. The solid straight lines have been obtained by linear regression using the linear van't Hoff equation. The dotted lines are 95% confidence intervals to the linear fit. The dashed lines represent parabolic fits by linear regression to the van't Hoff equation assuming a constant (non-zero) heat capacity change.

intrinsic enthalpy, and $\Delta H_{cal} \cong \Delta H_{vH} + \Delta H_{con}$. Therefore, a large difference between the van't Hoff and calorimetric enthalpies could be caused by a large, temperature-dependent value of ΔH_{con}. However, it is not clear what the sources of such a large concomitant heat are. Comparison of the X-ray structures of unliganded glucoamylase G2 (Aleshin et al., 1992) and glucoamylase G2 in complex with 1-deoxynojirimycin (Harris et al., 1993), acarbose (Aleshin et al., 1994), and D-*gluco*-dihydroacarbose (Stoffer et al., unpublished results) suggests that no large conformational changes occur upon binding. Heats of ionization of the buffer and the protein or ligand can contribute to ΔH_{con}. However, acetate and other carboxylic acids have very small heats of ionization (Kresheck, 1986). Since the heats of ionization of the buffers were found to originate from pK_a shifts of two groups on the protein and these groups are probably carboxyls, ΔH_{ion} is unlikely to contribute significantly to ΔH_{con}. Perhaps a more plausible source of concomitant enthalpy is that due to the change in hydration of both the ligands and the binding site of the protein. Solvent reorganization around hydrophobic groups is characterized by large enthalpy and entropy values that are strongly temperature-dependent [see, for example, Lee (1991)].

A second explanation for at least some of the discrepancy between the van't Hoff and calorimetric enthalpies is that the linear form of the van't Hoff equation is somewhat inappropriate in this case. The derivation of the linear equation assumes that $\Delta H(T)$ is a constant ($\Delta C_p = 0$) or only a very weak function of temperature over the temperature interval being studied. However, our calorimetric data suggest that $\Delta H(T)$ may be a strong function of temperature. The large uncertainties in our measurements of ΔH_{vH}—which occur despite the high accuracy of our data for K—together with the small correlation coefficients of the determination (r = 0.61 for acarbose and r = 0.55 for 1-deoxynojirimycin) support the hypothesis that the linear van't Hoff equation is inappropriate for this system. Moreover, the plots shown in Figure 4 show definite curvature.

The data in Table 2 can also be used to determine the heat capacity changes of the binding reactions. Since the heat capacity change is defined by $\Delta C_p = (\partial \Delta H°/\partial T)_p$, the slope of the line $\Delta H°$ vs T gives ΔC_p, which as a first approximation is assumed to be temperature-independent over this narrow range of temperatures. Linear regression of the data (not shown) gives the values for acarbose, $\Delta C_p = -0.84 \pm 0.22$ kJ mol^{-1} K^{-1}, and for 1-deoxynojirimycin, $\Delta C_p = -0.098 \pm 0.069$ kJ mol^{-1} K^{-1}. This analysis yields rather uncertain results, but clearly both heat capacities are negative and that of acarbose is much larger in magnitude than that of 1-deoxynojirimycin. The heat capacity changes of several reactions involving proteins in aqueous solution are correlated with the changes in solvent-accessible hydrophobic and hydrophilic surface areas during the reaction. This correlation has been well established for protein folding and for the transfer and dissolution of model compounds into water [for a recent review, see Murphy and Freire (1992)]. The hydrophobic contribution to the heat capacity is large, negative, and dominant at ambient temperatures, while the hydrophilic contribution to the heat capacity is smaller and positive. Interestingly, the same correlation seems to be valid for the dissolution of monosaccharides in water (Sigurskjold & Bundle, 1992). The magnitudes and the negative signs of the heat capacity changes for 1-deoxynojirimycin and acarbose then support the conclusion that hydrophobic interactions are important in these recognition processes, and as would be expected, the heat capacity change for acarbose is substantially larger than that of 1-deoxynojirimycin.

The heat capacity change can also be determined by nonlinear analysis of the van't Hoff plot. The dashed lines in Figure 4 represent fits to a parabolic van't Hoff equation assuming a temperature-independent heat capacity change. The heat capacity changes ($\Delta C_{p,vH}$) derived from these fits are $+1.9 \pm 0.5$ and -2.4 ± 1.2 kJ mol^{-1} K^{-1} for 1-deoxynojirimycin and acarbose, respectively. These are different from the calorimetric heat capacity changes and have opposite signs for the two ligands. Comparison with the calorimetric values suggests $\Delta C_{p,con} = -1.8 \pm 0.5$ and $+1.6 \pm 1.2$ kJ mol^{-1} K^{-1}. $\Delta C_{p,vH}$ and $\Delta C_{p,con}$ have opposite signs for the two inhibitors, indicating fundamentally different interactions with the protein. A negative value of $\Delta C_{p,vH}$ as for acarbose suggests that the binding tends to become relatively more exothermic with temperature, leading to a decreasing K. The reverse is true for the 1-deoxynojirimycin–glucoamylase G2 complex, in which most of the subsites have been left unoccupied. Furthermore, 1-deoxynojirimycin binds in subsite 1 (Harris et al., 1993), not in subsite 2, which supposedly has the largest free energy contribution according to subsite mapping (*vide infra*).

The numeric values of the heat capacity changes are much larger than the values of $\Delta S°$, which do not exceed 0.1 kJ mol^{-1} K^{-1} for any case. As Ha et al. (1989) pointed out, since the temperature dependencies of the enthalpy and entropy components of the free energy are given by $(\partial \Delta H°/\partial T)_p = \Delta C_p$ and $(\partial(T\Delta S°)/\partial T)_p = \Delta C_p + \Delta S°$, then, if $\Delta C_p \gg \Delta S°$, the changes in enthalpy and entropy with temperature will compensate each other, and therefore the large ΔC_p compared to $\Delta S°$ is the origin of the weak temperature dependence of $\Delta G°$.

The observed entropies in this study are calculated as $T\Delta S_{app} = \Delta H_{cal} - \Delta G_{cal} = T(\Delta S_{int} + \Delta S_{con}) + \Delta G_{con}$, and the observed entropies cannot, in principle, be directly compared to ΔH_{cal}. However, if ΔG_{con} stems mainly from hydration changes, this value is ostensibly quite small in magnitude compared to the enthalpy and entropy values because of the extensive enthalpy–entropy compensation of these processes (Lumry & Rajender,

Table 3: Thermodynamics of Binding of Various Inhibitors to Glucoamylase G2 at 27 °C

	K (M^{-1})	$-\Delta G°$ (kJ mol^{-1})	$-\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})
1-deoxynojirimycin	(3.3 ● 0.2)[a] × 10^4	25.8 ± 0.1	11.2 ± 0.1	14.5 ± 0.2
acarbose[b]	(8.8 ± 3.2) × 10^{11}	68.2 ± 0.9	40.6 ± 0.1	27.6 ± 0.9
D-*gluco*-dihydroacarbose	(3.2 ± 1.8) × 10^7	43.2 ± 1.4	29.8 ± 0.5	13.4 ± 1.4
L-*ido*-dihydroacarbose	(2.2 ± 1.1) × 10^5	30.7 ● 1.3	9.8 ● 1.3	20.9 ± 1.8
methyl α-acarviosinide[c]	(2.0 ± 1.6) × 10^7	41.9 ± 2.0	22.5 ± 1.8	19.4 ± 2.7
methyl β-acarviosinide[c]	(3.5 ± 1.7) × 10^6	37.6 ± 1.2	30.5 ± 0.5	7.1 ● 1.3
methyl α-4-thiomaltoside	(3.2 ± 1.4) × 10^4	25.9 ± 1.1	5.7 ± 0.7	20.2 ● 1.3
methyl β-4-thiomaltoside	(5.8 ± 4.4) × 10^3	21.6 ± 1.9	13.7 ± 2.2	7.9 ± 2.9
methyl α-4,5′-dithiomaltoside	(3.0 ± 1.6) × 10^3	20.0 ± 1.4	5.7 ± 1.7	14.3 ± 2.2

[a] Three standard deviations from regression analysis (3σ confidence level). [b] Displacement experiment. [c] Calculated from the mixture of methyl α,β-acarviosinide (Table 1); see text.

1970; Sigurskjold & Bundle, 1992; Lemieux, 1993), and $T\Delta S_{app}$ can, to a reasonable approximation, be compared to ΔH_{cal}.

The temperature dependence of binding for both acarbose and 1-deoxynojirimycin is very modest. At the highest temperature studied, 54 °C, glucoamylase starts to unfold (Williamson et al., 1992). This unfolding was detected in the calorimetric experiment since the calorimetrically measured concentration of binding sites corresponded to approximately 90% of the protein concentration determined by ultraviolet absorption. The folded fraction of glucoamylase thus retains its high affinity for the two inhibitors, even at a temperature where the protein structure is extremely marginally stable and starts to unfold.

The values of the kinetic parameters K_m and k_{cat} for a number of substrates vary considerably more with temperature than the binding constants for acarbose and 1-deoxynojirimycin (Meagher & Reilly, 1989; Olsen et al., 1992). The activation energy for the hydrolysis of maltose and maltotriose was 62.2 kJ mol^{-1}, and the binding energies estimated from K_m were −11.1 and −41.1 kJ mol^{-1}, respectively, determined by van't Hoff and Arrhenius analyses (Meagher & Reilly, 1989). This might indicate that the inhibitors are not adequately characterized as ground state (1-deoxynojirimycin) and transition state (acarbose) analogues, but that their binding mechanisms involve interactions that are not found in the binding of substrates (and vice versa). The kinetics of the binding of acarbose has at least three steps at 8 °C, pH 4.5 (Olsen et al., 1993). The initial two steps, with an association constant $K_1K_2 = 1.6 \times 10^7$ M^{-1}, resemble the binding of maltotetrose. The third step is a unique binding step, occurring instead of the catalytic step, and will have a binding constant $K_3 = (4.9 \pm 1.5) \times 10^4$ M^{-1}, if it is assumed that $K = K_1K_2K_3$. Olsen et al. (1993) commented that this is not behavior expected of a true transition state analogue, which one might expect to bind without initial ground state binding steps. However, if essential conformational changes were required to bind the transition state, then initial ground state-like binding may be required to arrive at the transition state and to obtain a fast on-rate. But, as mentioned earlier, no large conformational changes seem to accompany ligand binding (Aleshin et al., 1992, 1994; Harris et al., 1993; Stoffer et al., unpublished work). The activation energy for the acid (nonenzymatic) catalyzed hydrolysis of maltose is 136.8 kJ mol^{-1} (Capon, 1969). If it is assumed that glucoamylase obeys a general acid mechanism, and that the difference in activation energy between the acid- and enzyme-catalyzed processes corresponds to the stabilization of the transition state, then the binding energy of the transition state in the hydrolysis catalyzed by glucoamylase G2 would be 62.2 − 136.8 = −74.6 kJ mol^{-1}. This is close to the binding free energy of acarbose, and this compound thus seems to have a binding energy in the vicinity of what would be expected of an ideal transition state analogue.

Binding of Different Inhibitors. We have measured the thermodynamics of binding to glucoamylase G2 of a variety of substrate analogues; the results are summarized in Table 3. The values for methyl α- and β-acarviosinide in Table 3 have been calculated from the observed values for the 22%/78% (α/β) mixture of both anomers. We assumed that the anomeric dependence of the disaccharide is the same as that observed for methyl α- and β-4-thiomaltoside. The binding constants found for the dihydro derivatives of acarbose are in excellent agreement with the binding constants determined from enzyme kinetics (Svensson & Sierks, 1992). We note from the table that all association processes are both entropically and enthalpically favored. This is in contrast to many protein–protein and protein–ligand association reactions, which are usually enthalpically driven with unfavorable changes in entropy (Ross & Subramanian, 1981; Hinz, 1983; Wiesinger & Hinz, 1986), although other counterexamples are also known (e.g., Takahashi et al., 1981; Sigurskjold et al., 1991; Sigurskjold & Bundle, 1992). The fact that both the entropy and enthalpy changes are favorable results in relatively large changes in free energy upon binding. Three ligands, methyl α-4-thiomaltoside, methyl α-4,5′-dithiomaltoside, and L-*ido*-dihydroacarbose, are dominated by the entropic contribution to the binding, whereas methyl α-acarviosinide has a roughly equal distribution between $\Delta H°$, and $-T\Delta S°$. Upon complexation, both the protein and the ligand lose entropy from restrictions in translational, rotational, vibrational, and conformational freedom (Finkelstein & Janin, 1989). The only source of significant positive entropy is the release of water molecules to bulk solvent from the interacting surfaces. Thus, solvent displacement seems to be an important driving force for such recognition processes [see, for example, Lemieux (1993)]. Efficient solvent displacement as a result of good structural complementarity between the binding site and the ligand will display itself in such favorable entropy contributions to binding.

Measurements of the binding of a number of deoxy and deoxyhalo derivatives of methyl β-maltoside (Bock & Pedersen, 1987, 1988) were also attempted. However, even though these compounds are very poor substrates for glucoamylase, under the conditions used in titration calorimetry with high enzyme/substrate ratios, the turnover was still so fast that heats of hydrolysis dominated the recorded exotherms, obscuring the determination of heats of association.

1-Deoxynojirimycin is a monosaccharide and also does not possess an anomeric center. Its binding therefore is not directly comparable to the binding of the other ligands. It is also the only one of the ligands that has an endocyclic nitrogen atom, and presumably electrostatic interactions between this basic nitrogen and an acidic group in the binding site are responsible

for a major part of the binding energy.

Of all the ligands tested, acarbose has both the most favorable enthalpic and entropic interactions. Changes in the structure of acarbose lead to reduced affinity from losses in both enthalpy and entropy (D-*gluco*- and L-*ido*-dihydroacarbose and methyl acarviosinide). Hence, there is no enthalpy–entropy compensation in this case (Lumry & Rajender, 1970; Lemieux, 1993), and acarbose seems to be ideally composed for the interaction with the catalytic site of glucoamylase. The binding constant, close to 10^{12} M^{-1}, is by far the largest reported between a protein and a carbohydrate. Hydrogenation of the double bond of acarbose leads to two isomers with chair conformations for the pseudosugar moiety. The D-*gluco* form has the normal glucose configuration and conformation, but binds more than 4 orders of magnitude more weakly than acarbose. However, D-*gluco*-dihydroacarbose is still a strong inhibitor (Bock et al., 1991; Svensson & Sierks, 1992), showing that the more planar $^2H_5$ half-chair conformation of the valinamine unit of acarbose is important for strong binding, but that structural features of the rest of the molecule play important roles as well. These probably include electrostatic interactions with the basic glycosidic nitrogen atom and perhaps hydrophobic interactions with the 6-deoxy function of the second hexose unit. Recent crystallographic data for the complex between glucoamylase and D-*gluco*-dihydroacarbose show that this ligand, in contrast to acarbose, binds in two different modes—one similar to the binding of acarbose and the other a mode in which the two glucose units are in a different conformation (Aleshin et al., 1994; Stoffer et al., unpublished work). The much weaker binding of methyl acarviosinide, which lacks the two glucose units at the reducing end, clearly emphasizes that the glucose units of acarbose also interact very substantially with the protein or that they strongly influence the interactions with the first two rings.

At least three studies have been reported on the subsite mapping of glucoamylase using substrates of different chain lengths (Hiromi, 1970) for the enzyme from *Aspergillus niger* (Meagher et al., 1989; Sierks et al., 1989; Ermer et al., 1993). By this method, each subsite is assigned a certain free energy of binding, assuming that the overall binding is a simple algebraic sum of binding energies for each subsite. Seven putative subsites have been identified in this way, but the last three (at the reducing end) seem to contribute modestly to binding (–0.8 kJ mol^{-1}). Typical binding energies for subsites 1–4 are as follows: –1.1, –21.8, –7.3, and –3.5 kJ mol^{-1}, respectively (Ermer et al., 1993). The methyl α- and β-acarviosinides lose 26.3 ± 2.2 and 30.6 ± 1.5 kJ mol^{-1} of binding energy, respectively, compared to the binding energy of acarbose (Table 3). However, the expected loss from the subsite mapping data for subsites 3 and 4 would only amount to 10.8 kJ mol^{-1}. The acarbose data suggest that the binding of the glucose units in subsites 3 and 4 is thus dependent on the binding in subsites 1 and 2 as well. The overall binding seems to be a cooperative process of interactions involving several subsites that cannot be split up into distinct subsites, each giving an additive contribution to binding.

The L-*ido*-dihydro derivative of acarbose has an inverted $^1C_4$ chair with three axial hydroxyl groups as the preferred conformation of the ring at the nonreducing end compared to the $^4C_1$ conformation of D-*gluco*-dihydroacarbose (Bock et al., 1991; Figure 1). It is still a strong inhibitor of glucoamylase G2 (Table 3; Bock et al., 1991; Svensson & Sierks, 1992) even though the stereochemistry of the hydroxyl groups of L-*ido*-dihydroacarbose prevents appropriate hydrogen bond pairing, giving rise to the large loss in $\Delta H°$. The relatively large association constant suggests that the glycosidic NH, and perhaps the 6-deoxy function of the second hexose ring, provides large contributions to the binding. Maybe rather surprisingly, L-*ido*-dihydroacarbose shows a more favorable entropy of binding than D-*gluco*-dihydroacarbose, despite the expected steric misfit of the inverted ring. This could mean that the L-*ido* form is closer to the overall transition state structure than the D-*gluco* form, despite its inability to form the "right" hydrogen bonds, or perhaps that the three hexose rings at the reducing end interact more similarly to the acarbose rings than those of D-*gluco*-dihydroacarbose.

Methyl α-4-thiomaltoside and methyl α-4,5′-dithiomaltoside have similar $\Delta H°$ values, i.e., the difference in binding energy between them lies in the entropy. This would indicate some interaction with the endocyclic oxygen atom of the nonreducing end of maltose, or it could reflect a mere steric effect from the bulkier sulfur atom. The α-methyl disaccharides bind somewhat stronger than the corresponding β-anomers, but this is a result of relatively large and partly compensating changes in enthalpy and entropy. The α-anomers bind with the most favorable entropy, indicating a better complementarity between the ligands and the binding cavity. Maltose has a binding energy at 25 °C of –19 kJ mol^{-1} (Olsen et al., 1992), which is somewhat weaker than the $\Delta G°$ values for methyl α- and β-4-thiomaltosides. The origin of the increasing affinity for the maltose analogues with a sulfur atom in the glycosidic linkage is not clear. A sulfur atom in the ring of the nonreducing end clearly diminishes affinity (methyl α-4,5′-dithiomaltoside). Such an absence of additivity between congeners has been reported numerous times before for enzyme–ligand binding [see, for example, Kati et al. (1992)].

Methyl α-acarviosinide and D-*gluco*-dihydroacarbose have similar $\Delta G°$ values, but the first is significantly more entropy-driven than the latter. This again indicates that methyl α-acarviosinide fills the binding pocket with better complementarity, even though subsites 3 and 4 have been left unoccupied. The pairs of α- and β-anomers of acarviosinide and 4-thiomaltoside, respectively, have very similar $-T\Delta S°$ values. Their differences in $\Delta H°$ would then reflect the sum of the differences between the different ring conformations, S and NH in the glycosidic linkage, and the 6-deoxy function of the second unit. These differences are very similar: 16.8 ± 1.9 and 16.8 ± 2.3 kJ mol^{-1} for the α- and β-anomers, respectively. Since the difference in $\Delta H°$ between acarbose and D-*gluco*-dihydroacarbose would in turn give roughly the effect of the conformational change (=10.8 ± 0.5 kJ mol^{-1}), the residual value of 6.0 ± 1.5 kJ mol^{-1} represents the sum of effects from the S→NH and CH_2OH→CH_3 shifts. The S→NH difference probably contributes the most, since there is little difference in the hydrolysis of methyl β-maltoside and β-6-deoxymaltoside (Bock & Pedersen, 1987; Sierks & Svensson, 1992).

Conclusion. The binding of various inhibitors to glucoamylase G2 from *Aspergillus niger* exhibits several remarkable features. The enzyme binds a large number of chemically rather different sugar analogues, and many of them quite tightly. The binding is characterized by a weak temperature dependence, even up to temperatures where global unfolding commences, and a somewhat stronger pH dependence, both with large compensating changes in the entropy and enthalpy. The strong binding can be measured accurately by displacement titration calorimetry when two ligands have suitable differences in binding constants and enthalpies. The

strong inhibitor acarbose binds with large favorable contributions from both enthalpy and entropy, and changes in the acarbose structure lead to diminished interactions of both enthalpic and entropic origins. We propose that this is the hallmark of strong ligand (transition state analogue) binding.

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