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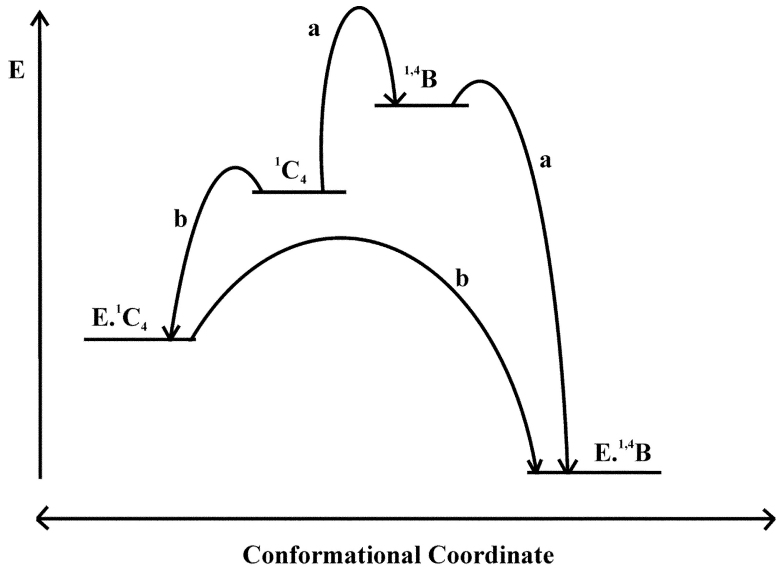

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Selection of a High-Energy Bioactive Conformation of a Sulfonium-Ion Glycosidase Inhibitor by the Enzyme Glucoamylase G2

Margaret A. Johnson,† Morten T. Jensen,‡ Birte Svensson,‡ and B. Mario Pinto*,†

Contribution from the Departments of Chemistry and of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, B.C., Canada, V5A 1S6, and Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500, Valby, Copenhagen, Denmark

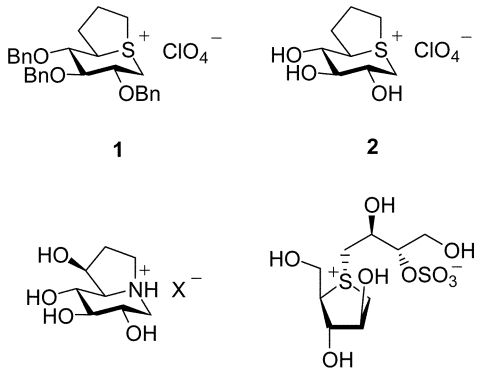
Received October 8, 2002; Revised Manuscript Received February 19, 2003; E-mail: bpinto@sfu.ca

Abstract: Transferred nuclear Overhauser effect and rotating-frame Overhauser enhancement NMR spectroscopies are used to probe the conformation of a bicyclic sulfonium ion, which is an analogue of the naturally occurring glycosidase inhibitor castanospermine, bound to the enzyme glucoamylase G2. Enzyme inhibition assays indicate that the bicyclic sulfonium ion is a slightly better inhibitor (K_i = 1.32 mM) of glucoamylase G2 than the naturally occurring sulfonium-ion glycosidase inhibitor, salacinol, with a K_i value of 1.7 mM. The NMR results are interpreted in terms of the selection by the enzyme of a high-energy conformation of the ligand that is already represented in the ensemble of free-ligand conformations.

Introduction

The selection by a protein receptor of a particular conformation of a ligand poses an interesting question. Thus, it is not readily obvious whether a high-energy conformation, detectable on the potential energy surface of the ligand, is selected directly from an ensemble of ligand conformations present in solution or whether that conformation arises upon distortion by the receptor after binding of a more-populated, lower-energy ligand conformation. The former situation may be viewed as the thermodynamic equivalent of the Curtin−Hammett/Winstein−Holness principle for kinetic schemes.[1] According to this principle, a particular conformation present in a ground-state ensemble reacts preferentially, because of a lower activation energy, even though it might be present as a minor component in an equilibrium mixture. Similarly, a protein receptor may select a bioactive conformation already existing in such a ground-state ensemble. The choice might involve the binding of a conformation near the global minimum or a local minimum or high-energy conformation that is scarcely populated; in the latter case, the binding energy for complex formation compensates for the conformational distortion. We have provided one example of the former type, namely the binding of two diheteromaltoside glycosidase inhibitors to the enzyme glucoamylase G2.[2] In each of these cases, the enzyme selected a conformation near the global minimum from two populated conformational families, about the global minimum and a local minimum; NMR evidence supported the existence of both these families. We have also reported two examples of the latter type,

Chart 1



1 **2** **3** **4**

namely the binding of a trisaccharide corresponding to the cell-wall polysaccharide of the Group A *Streptococcus* by a complementary monoclonal antibody,[3] and the binding of the glycosidase inhibitor, D-*gluco*-dihydroacarbose[4] by glucoamylase G2.[5] In the first case, a local minimum conformation on the potential energy surface, identified only *computationally* (ΔE = 2.3 kcal mol^{-1}, relative to the global minimum), was found to be bound by the antibody. In the second case, a local minimum conformation, identified *experimentally* by NMR spectroscopy of the ligand solution, and not the global minimum energy conformation, was bound by the protein receptor.

We recently reported, based on analysis of coupling constant data, an unusual preference of the bicyclic sulfonium ions **1** and **2** (Chart 1) for conformation **B** in the conformational

* Author to whom correspondence should be addressed.
† Simon Fraser University.
‡ Carlsberg Laboratory.

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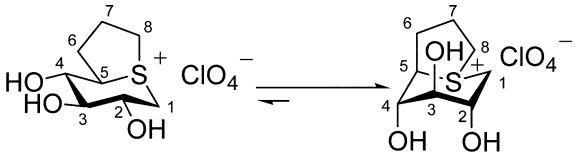
Scheme 1



A **B**

equilibrium in solution (Scheme 1).[6] In addition, compound **2** existed in this conformation in the solid state. We estimated the energy difference between conformations **A** and **B** in solution to be >3 kcal mol^{-1} and attributed the preference to the dominance of stabilizing electrostatic interactions between the oxygen atoms and the sulfonium center in conformation **B**. Compound **2** is an analogue of the naturally occurring glycosidase inhibitor castanospermine (**3**, Chart 1) and was designed[6] based on the principle that its permanent positive charge would mimic that of castanospermine **3** when protonated within the active sites of glycosidase enzymes. The charge on protonated castanospermine and other alkaloid glycosidase inhibitors provides stabilizing electrostatic interactions with active site carboxylate residues,[7] and might serve to mimic the charge in the oxacarbenium ion-like transition state in glycosidase-catalyzed hydrolysis reactions.[8] It was of interest to pose the following questions: (1) would compound **2**, although it existed preponderantly in conformation **B** and not conformation **A**, be an effective glycosidase inhibitor? and (2) if it were an inhibitor, would the bioactive conformation be **A** or **B**? It is interesting to note that, unlike the sulfonium ion **2**, protonated castanospermine (**3**) exists predominantly in conformation **A**, as does the neutral form.[9,10] Presumably, this difference is due to the greater steric effect in **3**, resulting from the shorter C−N$^+$ (vs. C−S$^+$) bonds, the presence of an axially oriented N−H, and the presence of an additional hydroxyl group on C-6; all three effects would destabilize conformation **B**.

We report herein that compound **2** is a competitive inhibitor of glucoamylase G2. We report also the determination of the bioactive conformation of the sulfonium ion **2** when bound to this enzyme by transferred nuclear Overhauser effect (trNOE) NMR experiments.[11] We show that compound **2** is bound in a high-energy boat conformation that resembles neither conformation **A** nor **B**.

Results and Discussion

Enzyme Inhibition Assays. Compound **2** was tested for its inhibition of three glycosidase enzymes, namely glucoamylase G2,[12,13] porcine pancreatic α-amylase (PPA), and barley α-amylase (AMY1).[14] The effects were compared to those of the known sulfonium-ion glycosidase inhibitor, salacinol (**4**).[15] Glucoamylase G2 was inhibited by salacinol (**4**) with a K_i value of 1.7 mM,[16] whereas compound **2** inhibited this enzyme with a K_i value of 1.32 mM. Salacinol (**4**) inhibited AMY1 and PPA with K_i values of 15 ± 1 and 10 ± 2 μM, respectively. However, compound **2** showed no significant inhibition (K_i > 5 mM) of either AMY1 or PPA.

NMR Analysis. We chose to probe the bioactive conformation of **2** when bound to glucoamylase G2 by trNOE NMR spectroscopy.[11] This effect occurs in rapidly exchanging protein−ligand complexes and has been proven to be effective in the determination of bound ligand conformations.[17] Nuclear Overhauser effect spectroscopy (NOESY) and transferred nuclear Overhauser effect spectroscopy (trNOESY) spectra of **2** in the absence and presence of glucoamylase G2, respectively, were recorded at 800 MHz. A spectrum was also recorded in the presence of bovine serum albumin (BSA) to check whether the altered viscosity of the sample would affect the correlation time and, therefore, the signs of the NOEs. In the absence of protein, or in the presence of BSA, compound **2** displayed the positive NOE effects, which is characteristic of a small molecule tumbling rapidly. In the presence of glucoamylase, negative NOEs were observed, indicating that **2** was binding to the enzyme; these are trNOE effects[11] and are characteristic of the bound conformation (Figure 1). Strong trNOEs between H4 and H1/1′ (Figure 2a) indicated an unusually close distance between these protons on opposite sides of the six-membered ring, and suggested the population of an unusual bound conformation, such as a boat or skew conformation.

A major concern in the interpretation of trNOE data is whether the observed effects result from direct cross-relaxation or from indirect pathways (spin diffusion). Transferred rotating-frame Overhauser enhancement spectroscopy (trROESY) experiments may be used to distinguish between direct and indirect dipolar relaxation pathways.[4,5,18,19] In the present study, trROESY experiments with the complex of glucoamylase G2 and

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(10) A ^{1}H NMR spectrum (600 MHz) of castanospermine (**3**) (2.0 mg) in D_2O (0.2 mL) containing CF_3CO_2H (5.0 μL) revealed that **3** existed preferentially in a conformation in which the six-membered ring resembled that of conformation **A**, as judged by the vicinal coupling constants (see Supporting Information). There were, however, changes in some of the coupling constants in the five-membered ring, indicating a change of conformation in this portion of the molecule. Similar *J* values were obtained when **3** was treated with DCl or CF_3SO_3H; Johnston, B. D.; Pinto, B. M., unpublished data.
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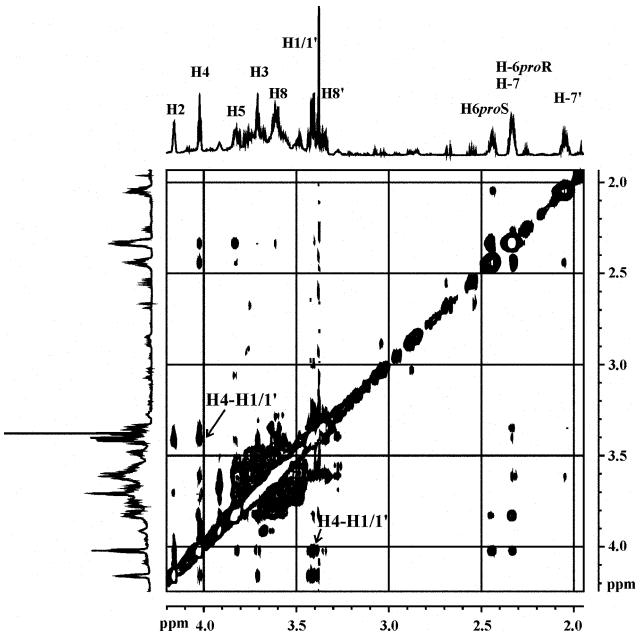

Figure 1. TrNOESY spectrum of **2** in the presence of glucoamylase G2, recorded at 800 MHz with a mixing time of 250 ms. The key contact between H4 and H1/1′ is labeled. Only negative contours are plotted.

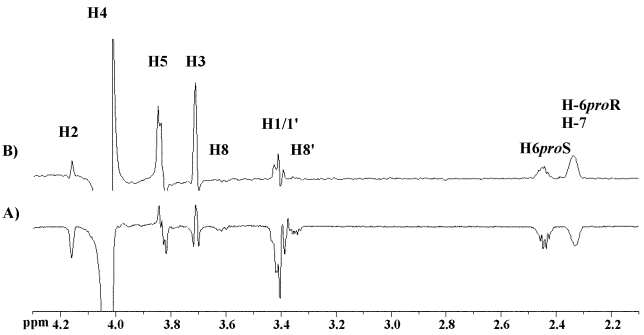

Figure 2. One-dimensional (1D) rows extracted from 2D spectra of **2** at the F_1 frequency of H4 (4.02 ppm) ((A) trNOESY spectrum of **2** in the presence of glucoamylase G2, with a mixing time of 250 ms; (B) NOESY spectrum of **2** in the absence of protein, with a mixing time of 600 ms). In each row, the H4 diagonal resonance is presented with negative phase; thus, NOE effects appear with positive phase, whereas trNOE effects appear with negative phase.

2 indicated effects (Figure S1, Supporting Information; e.g., Figure 3) similar to those observed in the trNOE experiments, except for the weak H3−H6*pro*S cross peak, which was absent in the trROESY spectra (Figure 3). One concludes that the remaining effects indeed arise from direct relaxation pathways.

In order to define the bound conformation of **2**, several possible conformations were subjected to refinement against the observed NOE effects, based on a relaxation matrix calculation. A starting conformation was subjected to simulated annealing with the experimental trNOEs included as restraints. The restraints did not include the H4−H1/1′ contact, as the H1 and H1′ resonances were not resolved, nor did it include the H3−H6*pro*S contact. Rather, a set of eight other contacts (see Experimental Section) were sufficient to define the conformation. The NOE buildup curves expected for the conformation, at each step in the calculation, were calculated, and the difference between the expected NOE effects and the experimentally observed NOE effects (NMR R-factor) was minimized,

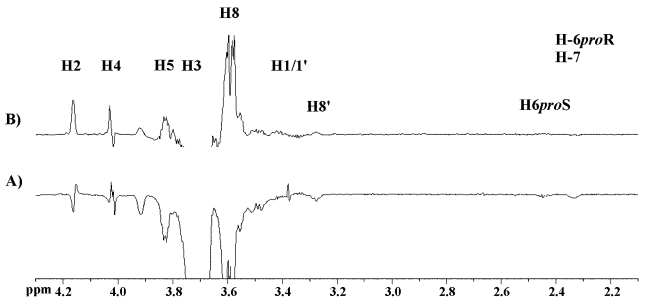

Figure 3. One-dimensional (1D) rows extracted from 2D spectra of **2** at the F_1 frequency of H3 (3.70 ppm) ((A) trNOESY spectrum of **2** in the presence of glucoamylase G2, with a mixing time of 250 ms; (B) trROESY spectrum of **2** in the presence of glucoamylase G2, with a mixing time of 250 ms). In each row, the H3 diagonal resonance is presented with negative phase; thus, trROE effects appear with positive phase, whereas trNOE effects appear with negative phase.

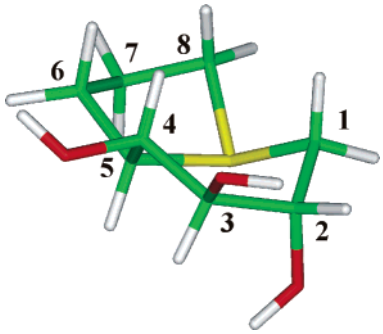

Figure 4. Refined ^{1,4}B conformation of **2** bound to glucoamylase G2, determined by trNOE NMR spectroscopy.

Table 1. τ_c Values Obtained from Refinement against Experimental NOE Buildup Curves

conformation	optimal τ_c (ns)	R factor at optimal τ_c
$^1C_4$	>290	0.10
$^4C_1$	31.7	0.10
$^1S_3$	11.0	0.09
^{1,4}B	7.8	0.09

as part of the energy function. This refinement resulted in a boat conformation (^{1,4}B) for the six-membered ring (Figure 4). The result was independent of the conformation used as input for the refinement; thus, input of the global minimum $^1C_4$ (**B**) conformation, or other possible $^4C_1$ (**A**), boat, and skew conformations, all led to the same ^{1,4}B conformation after refinement. The five-membered ring adopted an $^8T_7$ conformation in the final refined structure (Figure 4).

Refinement against the experimental buildup curves also allowed the calculation of an optimal τ_c value. The τ_c value may be estimated from the equation $\tau_c = V_h\eta/(kT)$, assuming isotropic motion.[20] Using typical values for a protein at 20 °C,[20] the τ_c of glucoamylase G2 (MW = 72 000) is estimated to be 30 ns, while that of **2** is estimated to be 120 ps. The average value, assuming 4% bound **2** (20:1 ligand:protein ratio), is 1.3 ns. Input of the $^4C_1$ (**A**) and $^1C_4$ (**B**) chair conformations resulted in very large τ_c values (Table 1), indicating poor agreement with the experimental data, whereas the skew and boat

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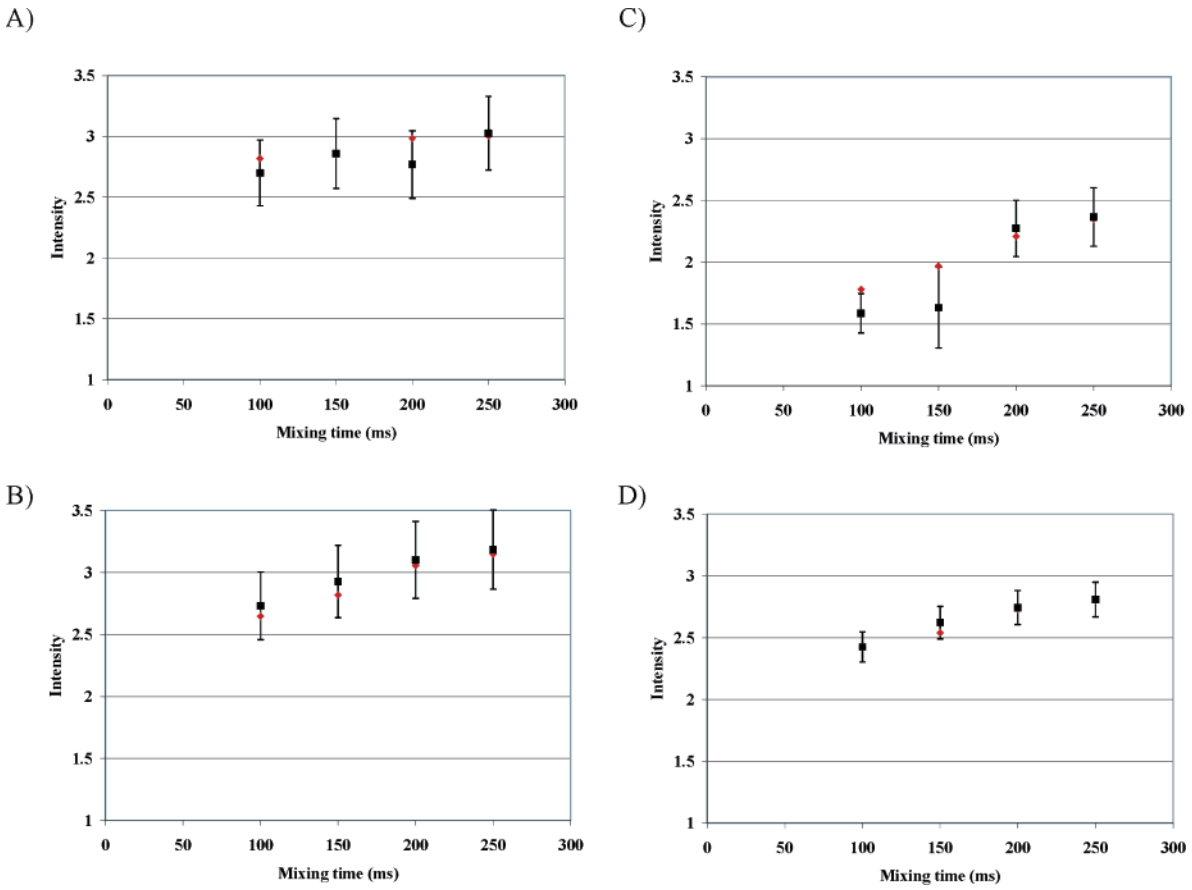

Figure 5. Representative trNOE buildup curves for **2** bound to glucoamylase G2. The observed buildup curves are shown as black squares, and the calculated buildup curves are shown as red diamonds. ((A) H8−H8′, (B) H3−H5, (C) H2−H4, and (D) H4−H6*pro*S.) Error bars in panels (A), (B), and (C) are ±10%, except for one point in panel (C), which was assigned an error of ±20%, because of severe baseline distortion. Error bars in panel (D) are ±5%.

conformations had more reasonable τ_c values (11.0 and 7.8 ns, respectively). The calculated NOE buildup curves after refinement gave good correlations with the experimentally observed buildup curves (Figure 5). These correlations are very good, especially for a trNOE study, and compare favorably to those obtained in state-of-the-art studies.[21]

In studies of this type, there is a question of whether the calculated NOE curves should include the kinetic matrix corresponding to the exchange between free and bound states of the ligand. However, if a system is in fast exchange, the relaxation matrix is equal to the weighted average of the relaxation matrices for the free and bound species in these systems,[18,22] as assumed in our calculations. We claim that in this case, the system is in fast exchange. Thus, we have performed similar studies with this enzyme and three other ligands, namely D-*gluco*-dihydroacarbose **6**, and the diheteromaltosides **7** and **8** (Chart 2). The K_i values for binding to the enzyme were 10^{-7}, 10^{-6}, and 10^{-3} M, respectively.[5,23] In the cases of **6** and **7**, as suggested by the greater inhibitory potencies, tighter binding leads to line broadening effects; we concluded that these systems were in the intermediate exchange regime.[5,23] In the case of **8**, however, a millimolar K_i value dictates a faster off-rate for the ligand from the complex and a fast exchange regime; no significant line broadening effects were observed. In our present system of glucoamylase G2 and **2** (with a millimolar K_i value), the trNOESY and trROESY spectra also do not show significant line broadening.

An estimate of the off-rate may be obtained from knowledge of the apparent K_i, the K_{eq} between the $^1C_4$ and $^{1,4}B$ conformations in the free ligand conformational equilibrium, and an assumed diffusion-controlled on-rate (see Supporting Information). The presence of the important H1/1′−H4 NOE contact in the NOESY spectra of **2** in the absence of the enzyme (Figure S2, Supporting Information; Figure 2b) indicates that the $^{1,4}B$ conformation is present at least to some extent, although coupling constant data suggest that the $^1C_4$ conformation predominates. Even if the $^{1,4}B$ conformation were present to the extent of only 1%, one estimates an off-rate for dissociation of the enzyme/**2** complex of $>10^3$ s^{-1}; a 10% population of this conformation would yield an off-rate of $>10^4$ s^{-1} (see Supporting Information).

We conclude, therefore, that the glucoamylase G2/**2** system is in the fast exchange regime, and that calculations of NOE curves based on the weighted average of the relaxation matrices for free and bound ligands (Figure 5), are valid.

Computational Analysis. The unusual $^{1,4}B$ bound conformation differs from the $^1C_4$ conformation observed in solution, and is higher in energy by approximately 6 kcal mol^{-1}, according to estimates based on the Tripos force field (Table 2). This value is clearly an overestimate owing to the approximations of the force field, since a difference in free energy of 6 kcal mol^{-1} above the global minimum would lead to the presence of only 0.003% of the boat conformation in the conformational ensemble

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Table 2. Energy Components for the Different Conformations of **2**, Based on the Tripos Force Field

conformation	vdW energy (kcal mol^{-1})	electrostatic energy (kcal mol^{-1})	total energy[a] (kcal mol^{-1})	Δ (total energy) from $^1C_4$ (kcal mol^{-1})
$^1C_4$	−0.30	−2.57	9.10	0.00
$^4C_1$	−1.12	6.59	19.09	9.99
$^1S_3$	−0.60	7.65	19.78	10.68
$^{1,4}B$	1.08	−0.68	15.35	6.25

[a] Includes terms not shown (angle bending, bond stretching, and torsional energy terms).

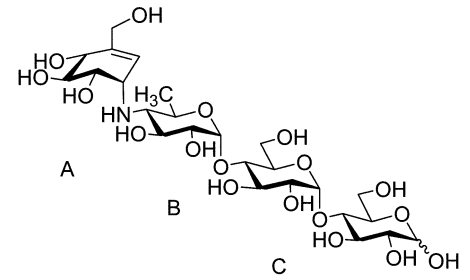
at 20 °C; yet, the important H1/1′−H4 NOE contact, indicative of this conformation, is observed in NOESY spectra of **2** in the absence of the enzyme (Figure 2b). Nevertheless, a component analysis of the total energy should give some indication of the relative importance of the steric and electrostatic contributions. The data in Table 2 indicate that the $^1C_4$ conformation is lowest in energy, due to stabilizing electrostatic interactions between the trans-1,2-oriented hydroxyl groups and between these groups and the positively charged sulfonium-ion center, as predicted in our previous work. The unusual $^{1,4}B$ conformation also derives some stabilization from intramolecular dipole−dipole interactions, between the pseudoaxially oriented 2-OH group and the sulfonium-ion center (Figure 4).

The binding of a high-energy conformation of **2** must result from specific interactions within the enzyme active site. The energy provided by formation of a neutral donor−acceptor hydrogen bond has been estimated to be 0.5−1 kcal mol^{-1},[24] so that the three hydroxyl groups would provide 1.5−3 kcal mol^{-1} if each were to form at least one hydrogen bond. Additional binding energy is likely provided by a strong electrostatic interaction between the S^+ center and a carboxylate group in the enzyme active site.

Interactions within the Binding Site. The $^{1,4}B$ conformation may be required for complementarity with the binding site. Crystal structures of glucoamylase from *Aspergillus awamori* with other inhibitors have shown that the base of the site requires a relatively flat molecule. Thus, it may accommodate a six-membered ring in the $^4C_1$ conformation, as seen in crystal structures of the bound inhibitors D-*gluco*-dihydroacarbose **6**[25] and 1-deoxynojirimycin **9**[26] (Chart 2). In addition, docking of the refined boat conformation of **2** to the crystallographically determined structure of *A. awamori* glucoamylase[25] revealed a possible binding mode within subsite 1 similar to that of 1-deoxynojirimycin **9**. In this binding mode, the S^+ center is positioned for a close electrostatic interaction with the carboxylate group of Glu 400, the catalytic base. In addition, the 3- and 4-OH groups form hydrogen bonds similar to those of the 4- and 3-OH groups of 1-deoxynojirimycin **9**, to Asp 55 and Leu 177, respectively, while hydrophobic contacts are made to Tyr 48, Trp 52, and Trp 178 (Figure 6).

A change in the conformation of the pyranose ring at the nonreducing end of a poly- or oligosaccharide substrate is likely to occur on binding to the enzyme and may be important in assisting catalysis.[27] The putative structure of the transition state

Chart 2



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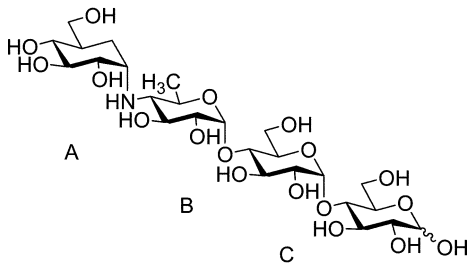
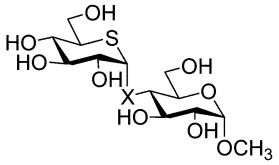
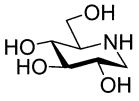


6 D



7 X = NH
8 X = S



9

is distorted due to the development of double-bond character in the C1−O5 bond, probably to a form resembling half-chair, boat, or skew conformations.[27] Evidence for the binding of high-energy conformations of substrates in enzyme-catalyzed glycosyl transfer reactions has been obtained from kinetic,[27] crystallographic,[28] and, very recently, trNOE NMR[29] studies.

Crystallographic studies of glycosidase enzyme−inhibitor complexes have also shown the binding of distorted six-membered ring conformations. Thus, for example, minor distortion of the $^4C_1$ chair of ring **A** in D-*gluco*-dihydroacarbose **6** in complex with glucoamylase has been observed.[25] In addition, acarbose **5** (Chart 2), in which ring **A** exists in a half-chair conformation, has a binding constant ∼3000 times greater than that of D-*gluco*-dihydroacarbose **6**, corresponding to a $\Delta\Delta G$

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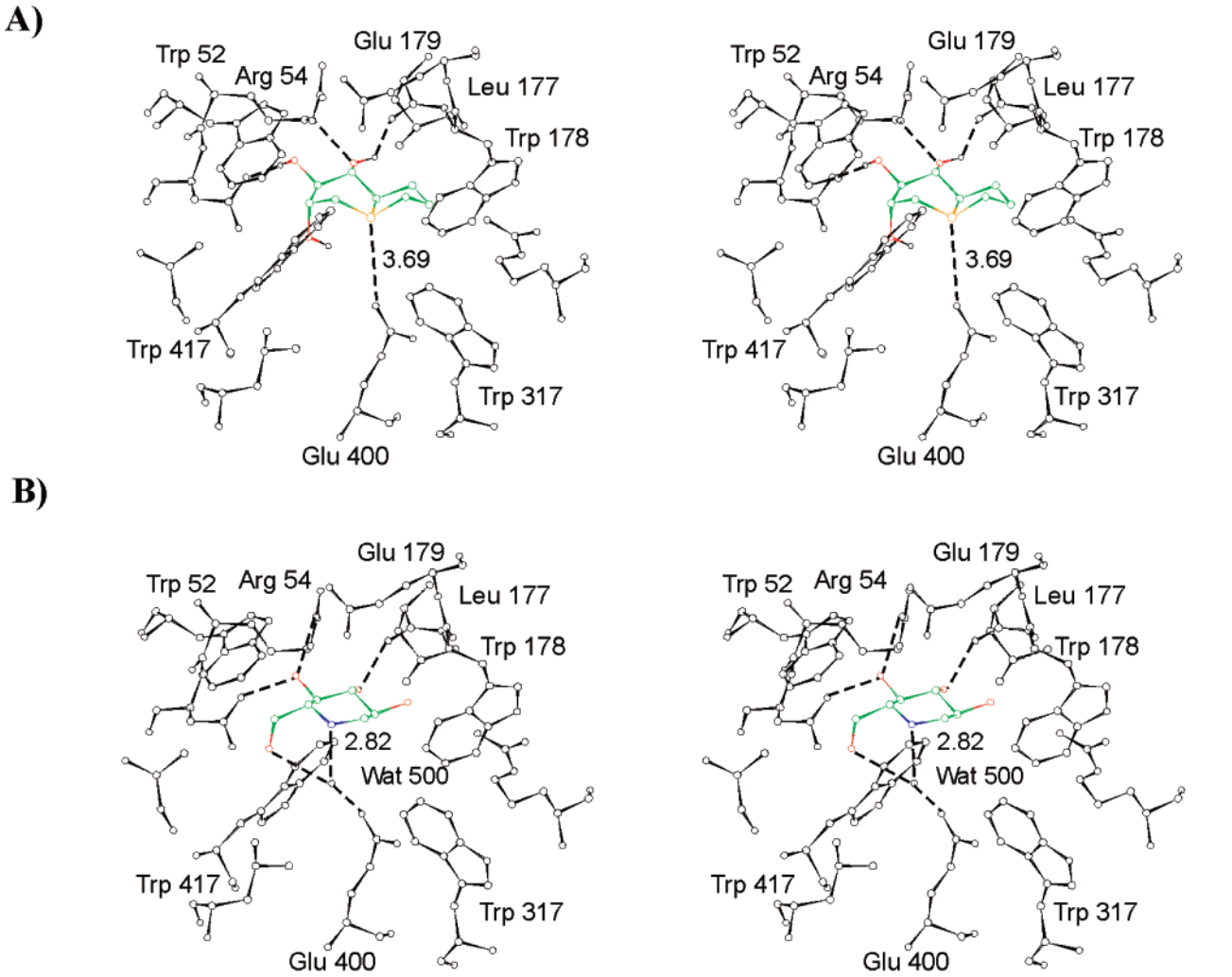
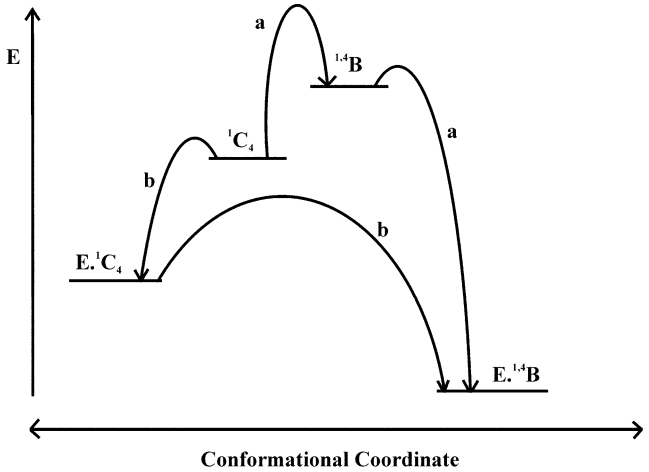


Figure 6. Putative binding modes of inhibitors **2** and **9** to glucoamylase G2. Hydrogen bonds and important electrostatic interactions are shown as dashed black lines. Residues of the binding site are shown in black. (A) Predicted binding mode of **2** in the ^{1,4}B conformation determined by trNOE NMR spectroscopy; an electrostatic interaction between S^+ and the catalytic base Glu 400 is predicted. (B) Binding mode of 1-deoxynojirimycin **9**, determined by X-ray crystallography (from ref 26).

of ~5 kcal mol^{-1}. Thus, inhibitors designed to mimic the half-chair shape of the transition state may be especially potent for this enzyme, and the same principle may be applied to other enzymes in which distortion of the substrate has been shown to be important in catalysis.[27] However, the question of the timing of the conformational change is still open. Enzyme kinetic studies may reveal a rate-limiting step that follows binding and precedes catalysis[27] but do not necessarily reveal the nature of this step (e.g., conformational change).

In the case of **2**, the observation of the important H1/1′−H4 NOE contact in NOESY spectra of **2** in the absence of the enzyme suggests that the ^{1,4}B conformation has a presence, albeit minor, in the ground-state conformational ensemble. The conformational itinerary of **2** can thus be represented by Scheme 2, assuming that the on-rates for binding of the different conformations to the enzyme are diffusion-controlled. The free ligand conformational equilibrium is given by the interconversion of $^1C_4$ and ^{1,4}B forms. In the case of glucoamylase binding of **2**, the enzyme could select the high-energy ^{1,4}B conformation

Scheme 2



(28) For example: Sidhu, G.; Withers, S. G.; Nguyen, N. T.; McIntosh, L. P.; Ziser, L.; Brayer, G. D. *Biochemistry* **1999**, *38*, 5346−5354. Davies, G. J.; Mackenzie, L.; Varrot, A.; Dauter, M.; Brzozowski, A. M.; Schülein, M.; Withers, S. G. *Biochemistry* **1998**, *37*, 11707−11713. Sulzenbacher, G.; Driguez, H.; Henrissat, B.; Schülein, M.; Davies, G. J. *Biochemistry* **1996**, *35*, 15280−15287. Tews, I.; Perrakis, A.; Oppenheim, A.; Dauter, Z.; Wilson, K. S.; Vorgias, C. E. *Nature Struct. Biol.* **1996**, *3*, 638−648. Strynadka, N. C. J.; James, M. N. G. *J. Mol. Biol.* **1991**, *220*, 401−424. Mark, B. L.; James, M. N. G. *Can. J. Chem.* **2002**, *80*, 1064−1074.

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that is already present in solution to give the enzyme complex, E·^{1,4}B (Scheme 2, path a). Alternatively, the enzyme could bind the lower-energy $^1C_4$ conformation (E·$^1C_4$), and then effect a conformational change of the ligand to the ^{1,4}B form in the enzyme-bound state to give E·^{1,4}B (Scheme 2, path b). We suggest that the presence of the ^{1,4}B form in the free ligand conformational equilibrium, albeit minor, does not require the imposition of path b. Binding of the ^{1,4}B conformation by the enzyme will shift the ground-state equilibrium, according to Le Chatelier's principle, thereby producing more of this conformation.

As a final point of interest, we comment on the conformational preferences of castanospermine **3**, the inspiration for the design of **2**. Castanospermine provides an example of a compound that exists at the opposite end of the conformational itinerary to **2**, because it exists preponderantly in the $^4C_1$ chair conformation, both in its unprotonated and protonated forms.[9,10] Interestingly, **3** exists in a $^1S_3$ skew conformation when bound to the exo-β-(1→3)-glucanase from *Candida albicans*.[30] The energy of this conformation is ~6 kcal mol^{-1} higher than that of the $^4C_1$ conformation, as calculated using the Tripos force field. The bound conformation of **3** in complex with glucoamylase is not known, but the K_i value with this enzyme is 1.5 μM.[31] Thus, replacement of NH^+ by the cognate S^+ atom has striking effects on the binding affinity to the protein receptor. If glucoamylase also binds **3** in the $^1S_3$ conformation, then the increased potency of **3** relative to **2** is likely due to greater complementarity within the active site.

Conclusions

Transferred nuclear Overhauser effect NMR measurements of a rapidly exchanging system consisting of the sulfonium-ion inhibitor **2** and the glucoamylase G2 enzyme indicate that the bound, bioactive conformation of **2** is one in which the six-membered ring exists in a ^{1,4}B conformation. The results suggest that this high-energy conformation is selected directly from a ground-state conformational ensemble, thereby shifting the equilibrium population of conformers.

Experimental Section

Enzyme Inhibition Assays. Glucoamylase G2 from *Aspergillus niger* was purified from a commercial enzyme (Novo Nordisk, Bagsvaerd, Denmark) as described.[12,13] The initial rates of glucoamylase G2-catalyzed hydrolysis of maltose were tested with 1 mM maltose as the substrate, in 0.1 M sodium acetate, pH 4.5, at 45 °C. The enzyme concentration was 7.0×10^{-8} M, and five inhibitor concentrations in the range from 1 μM to 5 mM were tested. The glucose released was analyzed in aliquots removed at appropriate time intervals, using a glucose oxidase assay adapted to microtiter plate reading, and with a total reaction volume for the enzyme reaction mixtures of 150 or 300 μL.[32] The K_i values were calculated assuming competitive inhibition, from $1/v = (1/V_{max}) + [(K_m)/(V_{max}[S]K_i)][I]$, where v is the rate measured in the presence or absence of inhibitor, [I] and [S] are the concentrations of inhibitor and substrate, $K_m = 1.6$ mM, and $k_{cat} = 11.3$ s^{-1} (k_{cat} is the catalytic constant), using ENZFITTER.[33]

Porcine pancreatic α-amylase (PPA) and bovine serum albumin (BSA) were purchased from Sigma. Amylose EX-1 (DP17; average degree of polymerization 17) was purchased from Hayashibara Chemical Laboratories (Okayama, Japan). Recombinant barley α-amylase isozyme 1 (AMY1) was produced and purified as described.[14] An aliquot of the PPA crystalline suspension (in ammonium sulfate) was dialyzed extensively against the assay buffer without BSA. The enzyme concentration was determined with the aid of amino acid analysis, as determined using an LKB model Alpha Plus amino acid analyzer. The inhibition of AMY1 (3×10^{-9} M) and PPA (9×10^{-9} M) activity toward DP17 amylose was measured at 37 °C in 20 mM sodium acetate, pH 5.5, 5 mM $CaCl_2$, 0.005% BSA (for AMY1) and 20 mM sodium phosphate, pH 6.9, 10 mM NaCl, 0.1 mM $CaCl_2$, 0.005% BSA (for PPA). Six different final inhibitor concentrations were used, in the range of 1 μM to 5 mM. The inhibitors were preincubated with enzyme for 5 min at 37 °C before the addition of substrate. Initial rates were determined by measurement of reducing sugar, using the copper-bicinchoninate method, as described.[14,34] The K_i values were calculated assuming competitive inhibition, as described above for the case of glucoamylase, with $K_m = 0.57$ mg/mL and $k_{cat} = 165$ s^{-1} for AMY1, and $K_m = 1$ mg/mL and $k_{cat} = 1200$ s^{-1} for PPA, as determined in the substrate concentration range 0.03−10 mg/mL using ENZFITTER.[33] For the K_i determinations, [S] = 0.7 mg/mL amylose DP17 for the AMY1 binding and [S] = 2.5 mg/mL amylose DP17 for the PPA binding.

NMR Spectroscopy. NOESY and trNOESY spectra were recorded on a Varian 800 MHz spectrometer (Canadian National High Field NMR Centre (NANUC), Edmonton, Alberta, Canada). NMR samples of **2** in the absence of any protein and in the presence of BSA were prepared by lyophilizing solid **2** from D_2O twice, followed by dissolution in CD_3COO^-/CD_3COOD buffer (50 mM acetate, pH 4.5). In each case, the concentration of **2** was 6 mM, whereas the concentration of BSA was 21.4 mg/mL, and that of glucoamylase G2 (GA) was 21.5 mg/mL (0.3 mM), for a **2**:GA ratio of 20:1. BSA and GA were also lyophilized from D_2O twice prior to preparation of the samples. One-dimensional (1D) spectra were recorded at 298 K with 48K points, followed by zero-filling to 64K points, exponential multiplication, and Fourier transformation. NOESY and trNOESY spectra were recorded at 298 K in phase-sensitive mode using the States (hypercomplex) method, with 4K points and 512 t_1 increments, 16 scans per increment, a sweep width of 15 ppm, a relaxation delay of 1.0 s, and mixing times of 50, 100, 150, 200, and 250 ms. A spin-lock pulse of 30 ms at 30 dB was applied after the first 90° pulse of the NOESY pulse sequence to reduce the intensity of protein resonances and ensure a uniform baseline.[35] Data acquired at NANUC were converted to Bruker format by XWIN-NMR (Bruker), and all further processing was performed within XWIN-NMR. TrROESY spectra were recorded with a composite spin-lock pulse at 4 kHz power and mixing times of 125 and 250 ms. The two-dimensional (2D) NOESY and trNOESY data (4K × 512) were processed for maximum resolution to better resolve cross peaks near the diagonal. The data were subjected to linear prediction to 2048 points in F_1, followed by zero-filling to 4K points and multiplication by a squared sine function; in F_2, the data were multiplied by a Gaussian function. Fourier transformation was then performed on a section of the spectrum (from 0.8 to 4.5 ppm), with a final data matrix of 4K × 4K. All 2D spectra were baseline-corrected automatically prior to integration of cross-peak volumes. Errors in NOEs were estimated to be approximately ±5% of the signal, except in the cases of cross peaks near the diagonal, where errors of ±10% were estimated, and except in the case of one point (Figure 1c), where an error of ±20% was assigned, because of severe baseline distortion.

Computational Analysis. Refinement against the observed NOE intensities was performed within the program XPLOR,[36] using a

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protocol described in ref 37. A set of eight NOE contacts was chosen for the refinement; thus, the list of trNOE contacts used for the calculation was H2−H4, H2−H3, H3−H4, H3−H5, H4−H5, H4−H6*pro*S, H5−H6*pro*S, and H8−H8′. Contacts to the H-1/1′ protons were not included, because these resonances were not resolved, and contacts to the H8 and H8′ protons were not included, because these resonances had not been stereospecifically assigned. However, the H8−H8′ trNOE contact was included, as an important geminal distance. The H6*pro*S signal was assigned on the basis of its NOE contact to H2 in the spectra of free **2**. The mixing time of 50 ms was not included, because of interference by zero-quantum effects. The $^{1}C_4$, $^{4}C_1$, $^{1,4}B$, and $^{1}S_3$ conformations were used as input in the refinement, and the τ_c value of 7.8 ns calculated for the $^{1,4}B$ conformation was adopted. However, refinement with τ_c values of 1.3 or 3.7 ns also resulted in a $^{1,4}B$ conformation. The refinement was performed without consideration of exchange or of the local protein environment.

Energies of the various conformations were measured using the Tripos force field within Sybyl 6.6 (Tripos, Inc.) after energy minimization. Del Re charges[38] were assigned, and a distance-dependent dielectric function ($\epsilon = r_{ij}$) was employed.

Molecular docking simulations were performed with AutoDock 3.0,[39] which combines rapid search methods with a grid representation of the protein. The scoring function is an empirical binding free-energy force field that includes van der Waals, electrostatic, hydrogen bonding, torsional, and desolvation free-energy terms.[39] Compound **2** in various conformations was built within Sybyl 6.6 (Tripos, Inc.), using the Tripos force field, and assigned Del Re charges.[38] The crystallographic coordinates of *A. awamori* glucoamylase[25] were used to represent the protein, which was assigned Kollman all-atom potentials and charges within Sybyl 6.6. Grid maps representing the protein were calculated with a grid-point spacing of 0.375 Å and 70 × 70 × 70 points. A Lamarckian genetic algorithm (LGA)[39] was used to search for potential binding modes of **2**, and 500 LGA docking runs were performed. The binding modes were clustered using a rmsd (root-mean-square deviation) cutoff of 2.0 Å, with respect to the starting position, and the binding mode shown for the boat conformation was found within the best three clusters. The water molecule Wat 500[25] was removed from the protein, because it was found to prevent favorable placement of **2** within subsite 1.

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Supporting Information Available: ^{1}H NMR data for castanospermine **3** in neutral and protonated forms, a NOESY spectrum of the free ligand **2**, a trROESY spectrum of glucoamylase/**2**, and a calculation showing the estimation of the off-rate for dissociation of the enzyme/**2** complex (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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