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Determination of the solution conformation of D-*gluco*-dihydroacarbose, a high-affinity inhibitor bound to glucoamylase by transferred NOE NMR spectroscopy

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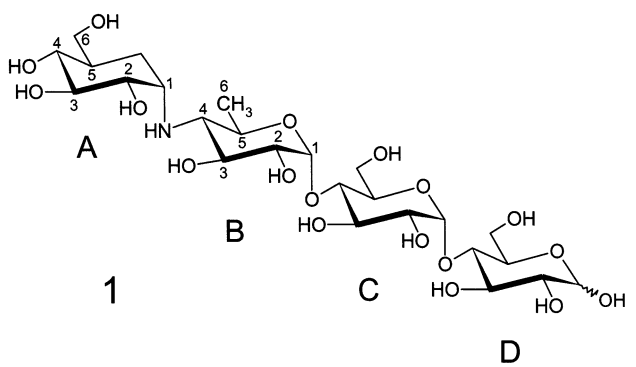
Abstract

The determination of the bound solution conformation of D-*gluco*-dihydroacarbose (GAC), a tight-binding inhibitor of several glycosidase and amylase enzymes, by glucoamylase is described. Transferred NOE NMR experiments and line-broadening effects indicate that GAC is bound in a conformation resembling that observed in the crystal structure. This contrasts with the predominant conformation of GAC when free in solution. The NMR results also suggest regions on the carbohydrate that are in close contact with the protein. The determination of the bound solution conformation of GAC by glucoamylase using transferred NOE (trNOE) measurements is a significant achievement given the high affinity constant ($K_a = 3 \times 10^7$ M^{-1}) for this receptor–ligand pair. It is striking that the off-rate for complexation is still sufficiently high to permit observation of trNOEs.

Keywords: Glucoamylase; D-*Gluco*-dihydroacarbose; Glycosidase inhibitor; Bound conformation; Transferred NOE NMR spectroscopy

1. Introduction

The conformational analysis of D-*gluco*-dihydroacarbose (GAC **1**), a tight-binding inhibitor of *Aspergillus awamori* var. *X100* glucoamylase [1], has shown that the conformational preferences about the *N*-glycosidic linkage are strongly dependent on pH [2].



Thus, whereas predominantly one conformational family about the minimum **A** (ϕ_H, ψ_H

Abbreviations: G1, glucoamylase 1; G2, glucoamylase 1 without the starch-binding domain; GAC, D-*gluco*-dihydroacarbose; CD, catalytic domain; τ_c, rotational correlation time; NOE, nuclear Overhauser effect; trNOE, transferred NOE; ROE, rotating-frame Overhauser effect; trROE, transferred ROE.

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−44, −27) was populated in basic aqueous solution (pH 9.0), another conformational family about the minimum **B** (ϕ_H, ψ_H −22, −179) predominated at acidic pH (pH 3.0). In the latter 'inverted' conformation **B**, the interglycosidic nitrogen atom should be protonated at pH 3.0. Although it is conformation **B** that is favored at low pH in solution, at a pH of 4.0 for crystal growth, it is conformation **A** that is bound by the enzyme in the solid-state structure. Thus, the crystal structure of the complex of GAC with the catalytic domain of glucoamylase, refined to 1.7 Å resolution, pH 4.0 [3,4], indicates that **1** is bound in a conformation (ϕ_H, ψ_H −17, 5) that more closely resembles conformation **A**, and that conformation **B** (see Fig. 1) could not be readily accommodated in the enzyme active site without major alterations in the enzyme conformation to relieve adverse van der Waals contacts. It is of interest, therefore, to examine the bound conformation in solution to verify that crystal packing forces are not the cause for the chosen conformation and to pose the question, "can the inverted conformation **B** (with respect to rings A and B of the pseudotetrasaccharide **1**) be accommodated in the active site of the enzyme?" The study is of general interest since it provides a check on conclusions drawn from X-ray crystal structures of enzyme–GAC complexes for the design of more potent inhibitors or for the engineering of enzymes with modified hydrolytic properties.

We describe here the study of the conformation of GAC **1** when bound to glucoamylase G2 [5] in acetate buffer (pH 4.5, the approximate pH optimum for the enzyme) by use of transferred nuclear Overhauser effect (trNOE) NMR experiments and line-broadening effects. The results show that it is indeed a conformation that resembles **A** that is selected from an equilibrium mixture of **A** and **B** and is bound by the enzyme in solution.

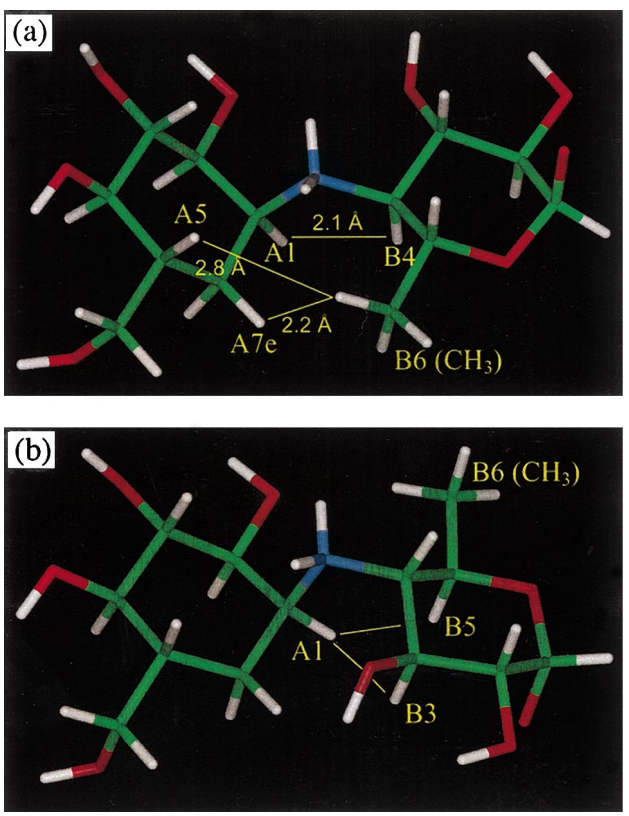

Fig. 1. Conformations of GAC **1** at the glycosidic linkage between rings A and B: (a) shows the model of the inhibitor from the complex [3] and (b) shows the model of the 'inverted' conformation defined by Bock et al. [2]. Distances less than 3 Å are indicated in (a).

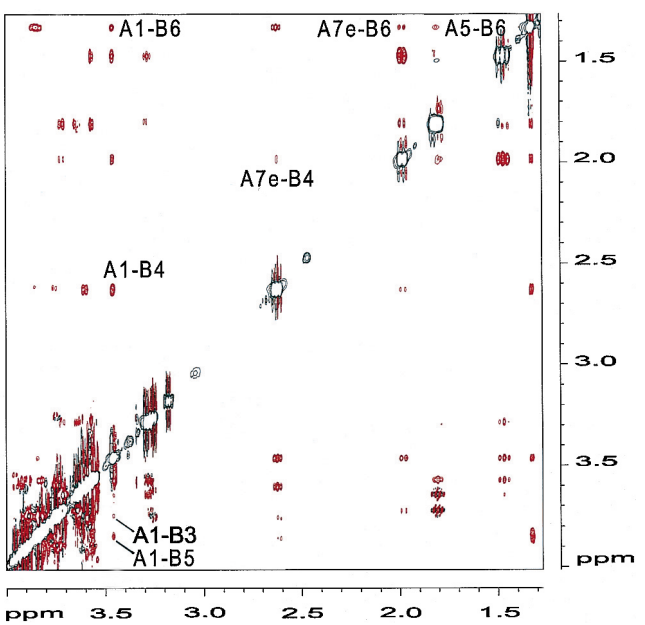

Fig. 2. Section of the NOESY spectrum of GAC at 318 K in acetate buffer. Positive (red) and negative (black) levels are plotted. The molecule displays small positive NOEs at 318 K. Interglycosidic NOEs between the rings A and B are labeled. The observed NOEs cannot be interpreted in terms of a single conformational family (see Table 1).

2. Experimental

NMR experiments were performed on an AMX 600 spectrometer (Bruker) with an inverse triple probe equipped with a *z*-gradient. Spectra were recorded and analyzed with UXNMR or XWINNMR programs (Bruker). For the NMR experiments, a solution of glucoamylase (M_w 72,000 Da), purified as described previously [6]) in deuterated acetate buffer (50 mM CD_3CO_2D–CD_3CO_2Na in D_2O, pD 4.5, not corrected for isotope effect), was concentrated five times in centriprep concentrators (Amicon) and transferred to an NMR tube. The sample contained 13 mg of glucoamylase (181 nmol in 500 μL). This sample was titrated with a stock solution of GAC (MW 647) in deuterated acetate buffer, while monitoring the magnitudes of the trNOEs by selective transient NOE experiments, as previously described [7]. The final sample contained a protein:ligand molar ratio of 1:30 (sample volume ~590 μL). The temperature was then adjusted to optimize the magnitudes of the observed trNOEs. This resulted in a temperature of 295 K at which all trNOE and trROE experiments were performed. For the trNOE experiment, a modified NOESY pulse sequence, incorporating a $T_{1\rho}$ filter [8] and a purge gradient during the mixing time, and for the trROE experiments, the standard ROESY pulse sequence were used. Residual water was suppressed with presaturation. TrNOE spectra were recorded with five mixing times, and trROE spectra were recorded with five spin-lock times (30–200 ms). The time dependence of the trNOEs was similar to that observed in our previous work [9]. The NMR sample of GAC **1** contained 6.3 mg (9.7 μmol) of the pseudotetrasaccharide in 500 μL of acetate buffer. A NOESY spectrum was recorded with a mixing time of 600 ms at 318 K. The chemical shifts (ppm relative to external DSS) of the ^{1}H NMR resonances of rings A and B of **1** in acetate buffer in the absence (318 K) and presence (295 K) of G2 are given in Table 2. The assignments were made with the aid of DQF–COSY and TOCSY experiments.

Molecular models of the catalytic domain of glucoamylase in complex with GAC **1** from the Brookhaven data base (pdb code 1gai) and the different conformations of GAC [2] were displayed and analyzed with Insight II (Molecular Simulations Inc.).

3. Results and discussion

The NOESY spectrum of GAC **1** in acetate buffer (pH 4.5) (Fig. 2) shows many NOE contacts across the glycosidic linkage between the rings A and B. These NOEs cannot be rationalized in terms of a single conformational family and suggest the presence of a mixture of two conformational families about the minima **A** and **B** at this glycosidic linkage, at this pH (see Table 1). A qualitative comparison of the magnitudes of these interglycosidic NOEs yields an estimate of ~20–30% of conformations in the region of minimum **B**.

A sample of glucoamylase in acetate buffer was titrated at 318 K with GAC **1**. At a ligand:protein ratio of 6:1, no ligand resonances could be detected because of line-broadening effects. These observations suggest the presence of a slow or intermediate kinetic exchange process on the chemical-shift time scale in which a high-affinity interaction between ligand and protein results in broadening of the ligand resonances due to the long correlation time of the complex. At a ligand:protein ratio of 10:1, ligand resonances were

Table 1
Observed interglycosidic NOEs for **1** indicative of the two conformational families populated in aqueous solution, and observed trNOEs for **1** in the complex with glucoamylase

	Observed NOEs	Observed NOEs	Observed trNOEs
	A	**B**	**1**
A1–B3		+	
A1–B4	+		+ [a]
A1–B5		+	
A1–B6	+		+
A2/A3–B6		+ [a]	
A5–B6	+		+
A7e–B6	+		+
A7e–B4	+		+ [a]

[a] The effects are very weak.

detected. Similar results were obtained in the case of the binding of a disaccharide inhibitor to glucoamylase [9,10]. During the titration, trNOEs were monitored with selective 1D NOE experiments, and the titration was stopped at a ligand:protein ratio of 30:1, which gave high-intensity NOEs. The sample showed negative NOEs, whereas the free ligand sample showed positive NOEs. The fact that the change in sign of the NOEs was not caused by the change in viscosity of solution was confirmed by performing an experiment in which glucoamylase was replaced by bovine serum albumin (BSA); in this case, positive NOEs were observed. This difference in the sign of the observed NOEs between the free inhibitor **1** and the inhibitor in the presence of the enzyme reflects the longer correlation time of the inhibitor when it is bound to the protein. Therefore, these negative NOEs are trNOEs that are indicative of the bound conformation of **1** [11–15]. Thus, although the exchange rate appears to be in the intermediate regime on the chemical-shift time scale, it is fast on the T_1-relaxation time scale. A further argument to support ligand binding derives from the observed line broadening and shift in resonance frequencies of some resonances, especially of those hydrogens in the vicinity of the glycosidic linkage. These effects are reminiscent of those of similar magnitudes observed with heteromaltoside analogues when bound to glucoamylase [9,10]; in these cases, the resonances A1, A2 and B4 were broadened significantly. The extreme line-broadening and chemical-shift changes upon binding suggest that a special effect, superimposed on the normal exchange process, is operating here. We have shown in our previous work [9] that this effect derives from the close contacts to protein side chains that result in a fast T_2- or $T_{1\rho}$-relaxation for these protons.

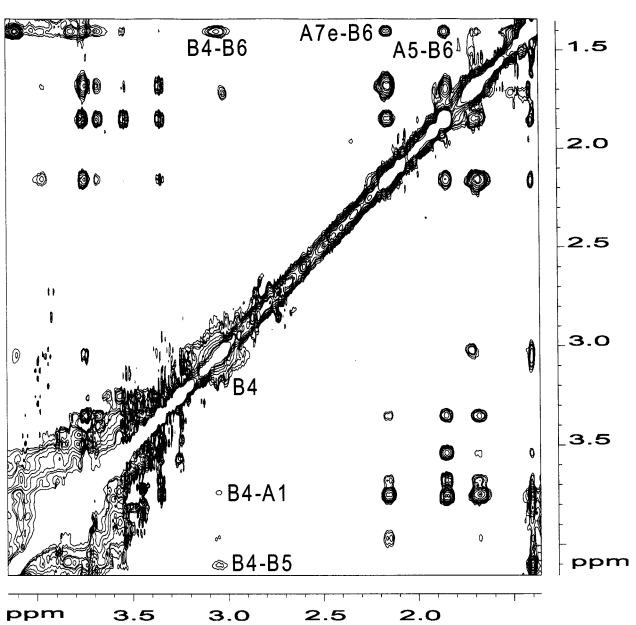

Fig. 3. Section of the trNOE spectrum (mixing time 200 ms) of GAC **1** and glucoamylase at 295 K in acetate buffer. Only negative levels are shown. The interglycosidic trNOEs between rings A and B are labeled (see Table 1).

Further optimization of the trNOEs was performed by variation of the temperature over the range 290–318 K, while monitoring the effects by 1D selective trNOE experiments. Greatest negative trNOEs were observed at 295 K, at which temperature the subsequent 2D experiments were performed.

The NOESY spectrum of the mixture of GAC **1** and glucoamylase in acetate buffer at 295 K (Fig. 3) shows negative NOEs that are indicative of the bound conformation of **1**. Further evidence for ligand binding comes from the pronounced chemical shift changes of several resonances of the rings A and B in the trNOE as compared with the NOE spectrum of **1** (see Table 2). The largest shifts are found for protons about the A–B glycosidic linkage. Of special note, the resonance of proton B4 is shifted by 0.4 ppm to a lower field. Chemical-shift changes of up to 0.2 ppm have been observed previously for carbohydrate–protein binding [16]. The shift of the B4 signal is accompanied by a large line-broadening effect ($W_{1/2} = 56$ Hz), which suggests a special environment for this proton in the complex (see above).

In the trNOE spectra, the interglycosidic NOEs A1–B3, A1–B5 and A2/A3–B6, which define the 'inverted' conformation **B**, are missing (see Table 1). All observable trNOE effects are consistent with conformation **A** being selected by the enzyme. The cross-peaks involving B4 are weak, and an explanation will be given below. As shown in Fig. 4, comparison of the results from trNOE and trROE

Table 2
^{1}H NMR chemical shifts (ppm) for rings A and B of **1** in acetate buffer in the absence (318 K) and presence (295 K) of glucoamylase [a]

	A1	A2	A3	A4	A5	A6a [b]	A6b [b]	A7e [c]	A7a [c]	B1	B2	B3	B4	B5	B6
Free **1**	3.46	3.57	3.57	3.29	1.82	3.64	3.73	1.47	1.99	5.29	3.60	3.76	2.63	3.86	1.33
1 and GA	3.75	3.73	3.54	3.33	1.85	3.67	3.75	1.69	2.16	5.35	3.59	3.97	3.04	4.10	1.40

[a] Most of the resonances of rings C and D in the mixture with G2 cannot readily be assigned and, therefore, the shifts of these protons are not given.
[b] These assignments may be interchanged.
[c] a, Axial position; e, equatorial position.

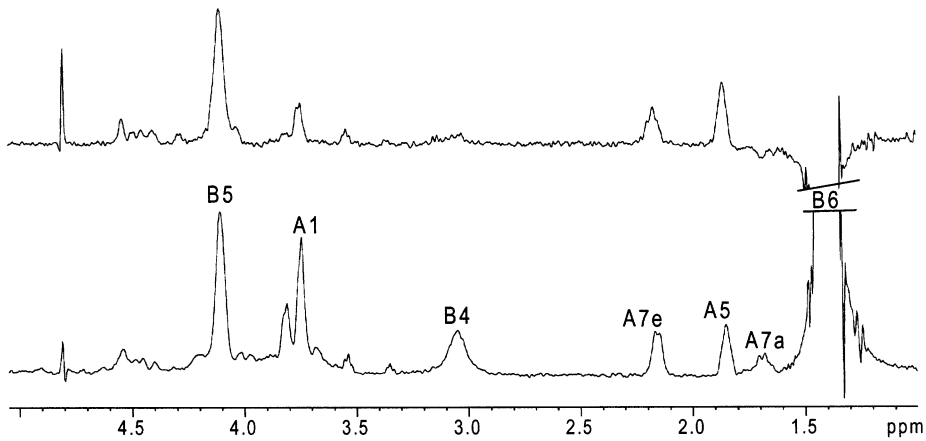

Fig. 4. Cross sections of trNOE (lower trace, mixing time 200 ms) and trROE (upper trace, spin lock time 200 ms) spectra at the position of the resonance of the methyl group B6. The reduction of the peak intensity for the A1 resonance and change in sign of the A7a resonance in the trROE trace indicate spin-diffusion effects for the resonances A7a and A1. Note also the line broadening of the resonance B4 in the trNOE trace. In the trROE trace, this peak has completely relaxed during the spin-lock time in the ROESY experiment. The A1 resonance also shows line broadening, although it is not as pronounced as in the B4 resonance.

[7,17,18] experiments indicates that some spin-diffusion effects interfere with the trNOEs. Spin diffusion is not distinguishable from real trNOEs in normal trNOE spectra, but can be detected with ROE experiments in which spin diffusion gives peaks of different sign than direct trNOE effects [7,17,18]. As seen in Fig. 4, the trROE effect A7a–B6 shows a change of sign, indicating a spin-diffusion pathway that is most likely mediated by the trNOE A7e–B6. In this regard, the trNOE A1–B6 is also influenced by spin diffusion, since the cross-peak intensity is reduced significantly in the trROE spectrum. Knowledge of these effects also supports **A** as being the bound conformation, since the distance between the protons A7a and B6 in that conformation is 3.7 Å, hence too long to observe a trNOE effect.

Additional support for the trNOE-derived bound conformation **A** comes from the inspection of the crystal structure of the complex of the catalytic domain of glucoamylase with GAC **1** [3,4]. In this structure, a short distance between the proton B4 and $H\varepsilon_1$ of Tyr311 of the protein is found; this leads to a very effective $T_{1\rho}$- or T_2-relaxation and gives rise to line broadening of the B4 resonance. The fast relaxation also explains the reduced intensities of the cross-peaks involving B4, for example, A1–B4 and A7e–B4.

4. Conclusions

In summary, NMR experiments involving trNOE measurements and line-broadening effects have shown that the solution conformation of GAC **1** bound by glucoamylase G2 is in the region of the potential-energy surface populated by minimum **A**, and resembles the

bound conformation of GAC in the solid state. Our experiments give no indication that the 'inverted' conformation **B** is accommodated in the enzyme active site. The determination of the bound solution conformation of GAC **1** by glucoamylase G2 using transferred NOE measurements is a significant achievement given the high affinity constant ($K_a = 3 \times 10^7$ M^{-1})[19] for this receptor–ligand pair. It is striking that the off-rate for complexation is still sufficiently high to permit observation of trNOEs.

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