Refined structure for the complex of D-*gluco*-dihydroacarbose with glucoamylase from *Aspergillus awamori* var. *X100* to 2.2 Å resolution: dual conformations for extended inhibitors bound to the active site of glucoamylase

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Abstract **The crystal structure at pH 4 of the complex of glucoamylase II(471) from *Aspergillus awamori* var. *X100* with the pseudotetrasaccharide D-*gluco*-dihydroacarbose has been refined to an R-factor of 0.125 against data to 2.2 Å resolution. The first two residues of the inhibitor bind at a position nearly identical to those of the closely related inhibitor acarbose in its complex with glucoamylase at pH 6. However, the electron density bifurcates beyond the second residue of the D-*gluco*-dihydroacarbose molecule, placing the third and fourth residues together at two positions in the active site. The position of relatively low density (estimated occupancy of 35%) corresponds to the location of the third and fourth residues of acarbose in its complex with glucoamylase at pH 6. The position of high density (65% occupancy) corresponds to a new binding mode of an extended inhibitor to the active site of glucoamylase. Presented are possible causes for the binding of D-*gluco*-dihydroacarbose in two conformations at the active site of glucoamylase at pH 4.**

Key words: X-Ray crystallography; Glucoamylase structure; Enzyme mechanism; Oligosaccharide hydrolysis; Carbohydrate-protein interaction

1. Introduction

Glucoamylase (α-1,4-D-glucan glucohydrolase, EC 3.2.1.3) hydrolyzes β-D-glucose from the non-reducing ends of starch and other related poly- and oligosaccharides [1–4]. The enzyme is used in the industrial production of glucose syrups, fructose sweeteners and ethanol [5,6]. Crystallographic studies have focused heretofore on the unligated enzyme at pH 4 and 6 [6], and the inhibitor complexes of 1-deoxynojirimycin [7] and acarbose [8] at pH 6. The pseudotetrasaccharide D-*gluco*-dihydroacarbose is chemically similar to acarbose, differing only in that it lacks the double bond of the valienamine moiety (Fig. 1). Thus, a $^4C_1$ chair conformation is anticipated for the first residue of D-*gluco*-dihydroacarbose, rather than the half-chair conformation for the first residue of acarbose [9]. Although very similar in structure, the dissociation constants of acarbose (10^{-12} M) and D-*gluco*-dihydroacarbose (10^{-8} M) differ significantly [10,11]. In an attempt to gain a more complete understanding of how extended substrate analogues bind to glucoamylase II(471), and in order to correlate the binding affinities to the structural differences of the pseudotetrasaccharide inhibitors, we have determined the structure of the complex of glucoamylase with D-*gluco*-dihydroacarbose at pH 4 (the pH of optimal activity for the enzyme). The results demonstrate that extended substrate analogues can bind to glucoamylase in at least two distinct conformations.

2. Materials and methods

Glucoamylase-II(471) was prepared from *A. awamori* var. *X100* as described by Neustroyev and Firsov [12]. Conditions for growth of the fungus were adjusted so that the glucoamylase I (M_r = 100,000 by SDS-PAGE), produced initially, was reduced in size by the action of fungal acid proteases to a mixture of glucoamylases of average M_r = 72,000. This mixture of proteins migrated as two partially resolved bands on SDS-PAGE.

The apparent heterogeneity of the protein did not prevent the reproducible growth of crystals of glucoamylase-II(471) by vapor diffusion. A solution consisting of 15 mg/ml protein, 50 mM potassium phosphate, pH 5.95, and 13% (w/v) polyethylene glycol 6000 (Fluka) was equilibrated against a solution of 50 mM phosphate, pH 5.95, and 20–30% (w/v) polyethylene glycol 6000. Crystals of space group $P2_12_12_1$ appeared in 1–2 weeks and grew slowly to a size of 0.2–0.3 mm. Crystals of glucoamylase-II(471) are stable in 50 mM potassium phosphate, pH 5.95, and 28% (w/v) polyethylene glycol 6000. The protein used in the crystallization was not exposed to the action of α-mannosidase, as suggested by Golubev et al. [13]. Crystals of glucoamylase have the same chemical composition regardless of the α-mannosidase treatment [6]. The D-*gluco*-dihydroacarbose complex was formed by soaking crystals for 2–3 days in a solution containing 1 mM D-*gluco*-dihydroacarbose (kindly provided by Professor K. Bock and Dr. M. Meldal), 33% (w/v) polyethylene glycol 6000, 50 mM potassium phosphate, pH 4.

Data collection was carried out at Iowa State University on a Siemens area detector. A total of 79,704 reflections were recorded from a single crystal, the unit cell parameters for which were a = 116.9, b = 104.3, and c = 48.48. Of the 30,813 possible unique reflections to 2.2 Å resolution, 29,937 were actually collected with an R-merge of 0.037. Of those, 28,934 had $|\mathbf{F}| > 2\sigma(|\mathbf{F}|)$. The data were 97% complete to 2.2 Å resolution. Data from the crystal of the complex were scaled against native data collected previously [5], by a process similar to that of Matthews and Czerwinski [14].

A model for D-*gluco*-dihydroacarbose (Fig. 1) was generated using software from Molecular Simulations Inc. The model was developed directly from the structure of four, α1–4-linked D-glucopyranose molecules, replacing or deleting the appropriate atoms. The initial model for

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D-*gluco*-dihydroacarbose was optimized by energy minimization using CHARMm. The model of acarbose, used in the refinement of the pH 6 complex of acarbose with glucoamylase [8], was used here without modification, as were standard groups for *N*- and *O*-linked glycans.

Initial phases for the complex of D-*gluco*-dihydroacarbose with glucoamylase-II(471) were calculated from the model of the native enzyme at pH 6.0 [5]. The model for the free enzyme at pH 6.0 also represented the initial conformation of glucoamylase-II(471) in the inhibitor complex. A Silicon Graphics 4D25 and the program TOM [15] were used for model building. Initially a single D-*gluco*-dihydroacarbose molecule in its α-conformation was built into the strongest electron density and refined by restrained least squares [16]. Refinement proceeded over the course of 64 cycles and involved the interpretation and building of the solvent structure and the modeling of specific side chains in multiple conformations. The coordinates (x, y and z) and an isotropic thermal parameter were adjusted for each atom of the model. Subsequent interpretations of the electron density at the active site had either a single molecule of D-*gluco*-dihydroacarbose in two conformations, a molecule of acarbose in two conformations, or a molecule of acarbose (occupancy of 0.35) and a molecule of D-*gluco*-dihydroacarbose (occupancy 0.65).

Water molecules were added to the model provided that (i) electron density was present at a level of at least 3σ in maps based on the Fourier coefficients $(|\mathbf{F}_{obs}|-|\mathbf{F}_{calc}|)e^{i\alpha_{calc}}$ and 3σ in maps based on Fourier coefficients $(2|\mathbf{F}_{obs}|-|\mathbf{F}_{calc}|)e^{i\alpha_{calc}}$, (ii) at least one hydrogen bond with an associated donor-acceptor distance of 2.2–3.2 Å could be made between the new site and an existing atom of the model, (iii) no close non-bonded contacts (less than 3.0 Å) could be made between the new site and existing atoms of the model, and (iv) the appearance of the electron density was approximately spherical. As a final criterion, sites for waters were omitted if their associated B parameters exceeded 80 Å^2 after refinement. Water occupancies were not refined, because of the high correlation between occupancy and thermal parameters for data of a nominal resolution of 2.2 Å. Thus solvent sites with B values between 50 Å^2 and 80 Å^2 probably represent water molecules with occupancy parameters below 1.0 and true thermal parameters substantially lower than those reported from the refinement.

Side chains were screened for multiple conformations on the basis of the following criteria: (i) electron density must be present at a level of at least 3σ in maps based on the Fourier coefficients $(|\mathbf{F}_{obs}|-|\mathbf{F}_{calc}|)e^{i\alpha_{calc}}$ and $(2|\mathbf{F}_{obs}|-|\mathbf{F}_{calc}|)e^{i\alpha_{calc}}$; (ii) alternative conformers must have reasonable contacts with nearby atoms; (iii) each conformer must adopt or be close to a conformation with staggered χ angles.

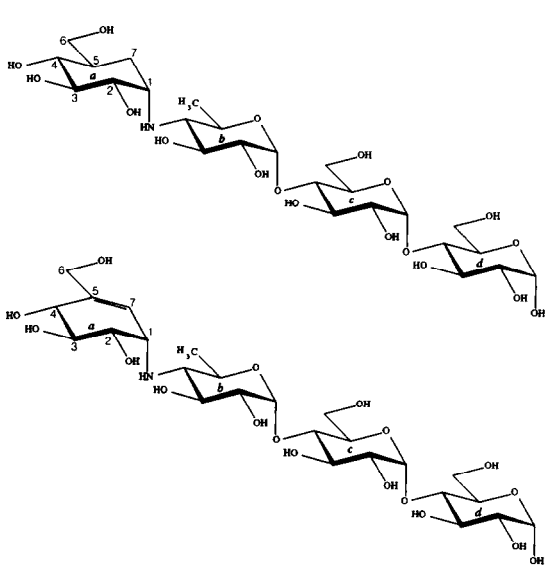

Fig. 1. D-*gluco*-Dihydroacarbose (top) and acarbose with associated labels for residues and atoms.

Table 1
Agreement between target and observed stereochemistry for the complex of D-*gluco*-dihydroacarbose with glucoamylase II(471)

Total number of atoms	4,543
Total number of solvent sites	629
Number of structure factors used in refinement[a]	29,411
R-factor[b]	0.125
Mean B (Å^2) for protein	10.7
D-*gluco*-dihydroacarbose	
ring A	6.4
ring B	7.0
ring C	12.1 (8.2)[c]
ring D	17.4 (13.0)[c]
RMS[d] deviation (in Å) for distances	
Two-center	0.013 (0.02)
Three-center	0.028 (0.30)
Four-center	0.039 (0.05)
RMS[d] deviation (in Å) for planar groups	0.013 (0.02)
RMS[d] deviation (in Å^3) for chiral volumes	0.137 (0.15)
RMS[d] deviation (in Å) for nonbonded contacts	
Determined by single torsion angles	0.222 (0.25)
Determined by multiple torsion angles	0.147 (0.25)
Hydrogen bond	0.179 (0.25)
RMS[d] deviation (in degrees) for torsion angles	
Planar peptides (ω = 180°)	2.3 (3)
Staggered side chains	13.5 (15)
Orthonormal	27.6 (20)
RMS[d] ΔB (in Å^2)	
Main chain bond	0.618 (1.0)
Main chain angle	1.022 (1.5)
Side chain bond	2.004 (2.0)
Side chain angle	2.912 (3.0)

Target root-mean-square deviations used in refinement are in parentheses.
[a] Comprises all observed moduli from 10 to 2.2 Å resolution, with $|\mathbf{F}| \geq \sigma(|\mathbf{F}|)$ and $|\mathbf{F}| \geq 3.0$.
[b] R-factor = $\Sigma||\mathbf{F}_{obs}|-|\mathbf{F}_{calc}||\backslash\Sigma|\mathbf{F}_{obs}|$.
[c] Value for the low weight conformer of D-*gluco*-dihydroacarbose.
[d] Root-mean-square.

3. Results and discussion

Statistics for the refined model appear in Table 1. In general, glucoamylase undergoes little conformational change in response to the binding of the inhibitor, consistent with results of the complex of 1-deoxynojirimycin and acarbose with the enzyme [7,8]. Electron density associated with the first two residues of the bound inhibitor is at the 16σ level (Fig. 2). The electron density branches, however, beyond residue B of the inhibitor, placing residues C and D in alternative locations in the active site with approximate weights of 0.65 and 0.35. The weights of the alternative locations for residues C and D were determined by manual adjustment of the occupancy factors so that the lower and higher weight alternatives gave average thermal parameters of approximately equal value. The electron density associated with residues C and D of the high occupancy conformer is at a level of 6σ and that for the low occupancy conformer is at a 3σ level. The conformer of weight 0.65 represents a new binding mode for an extended inhibitor bound to the active site of glucoamylase, whereas the lower weight conformer corresponds to the conformation of bound acarbose at pH 6.0 [8].

Given that D-*gluco*-dihydroacarbose was prepared from acarbose [9] and that acarbose has a dissociation constant 10^{-4}-fold lower than that of D-*gluco*-dihydroacarbose, the appearance of electron density at two locations may reflect a small

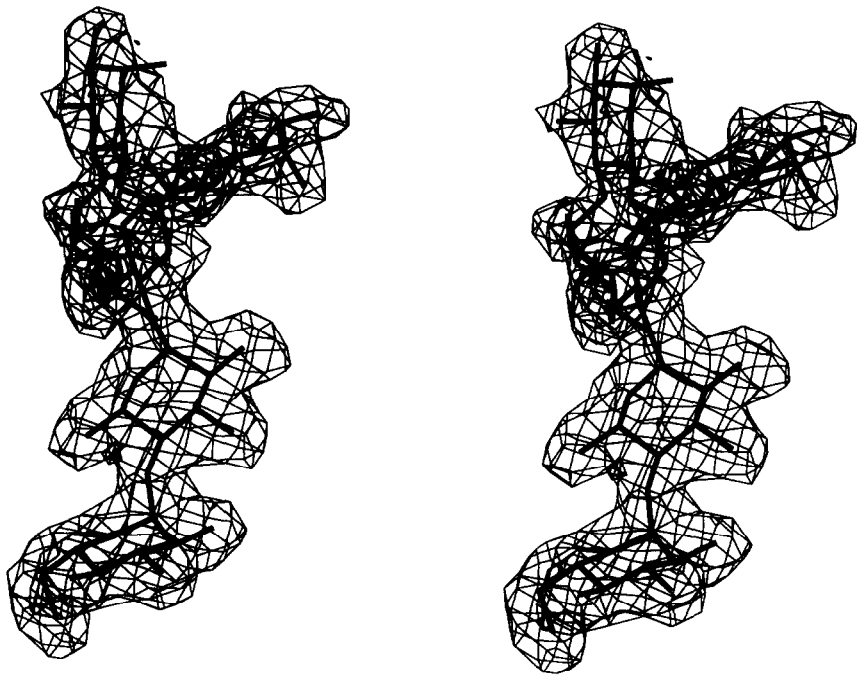

Fig. 2. Stereoview of the D-*gluco*-dihydroacarbose molecule with associated electron density. Residue A is at the bottom.

contaminant of acarbose in the D-*gluco*-dihydroacarbose sample. An electron density map at 2.2 Å resolution is not sufficient to directly observe the superposition of a $^4C_1$ chair onto a half-chair conformation of residue A. The major difference in the two conformations is the diheral angle defined by atoms C4, C5, C7 and C1 of residue A, which in acarbose is restrained to 0° and in D-*gluco*-dihydroacarbose to 49°. Refinement of acarbose at the active site results in a significant distortion of this torsion angle to a value of 40°; the electron density is sufficiently deterministic to offset the least squares penalty due to a serious violation of a geometric restraint. Refinement of D-*gluco*-dihydroacarbose, on the other hand, results in a torsion angle of 47°, an insignificant deviation from the target angle of 49°. The above implies that the electron density of the active site largely represents a molecule of D-*gluco*-dihydroacarbose, inferring that any impurity of acarbose must be no greater than one part in 100,000. Therefore, we have placed and refined a single molecule of D-*gluco*-dihydroacarbose at the active site with different locations for residues C and D. That coordinate set has been submitted to the Protein Data Bank at Brookhaven.

As the present evidence indicates that our sample of D-*gluco*-dihydroacarbose is pure, we are then challenged to find the underlying cause for the alternative conformations of the bound inhibitor. A small difference (not more than 0.2 Å) in the way rings A and B of D-*gluco*-dihydroacarbose bind to the active site relative to acarbose may lead to the observed conformational differences of residues C and D. Aleshin et al. [8]

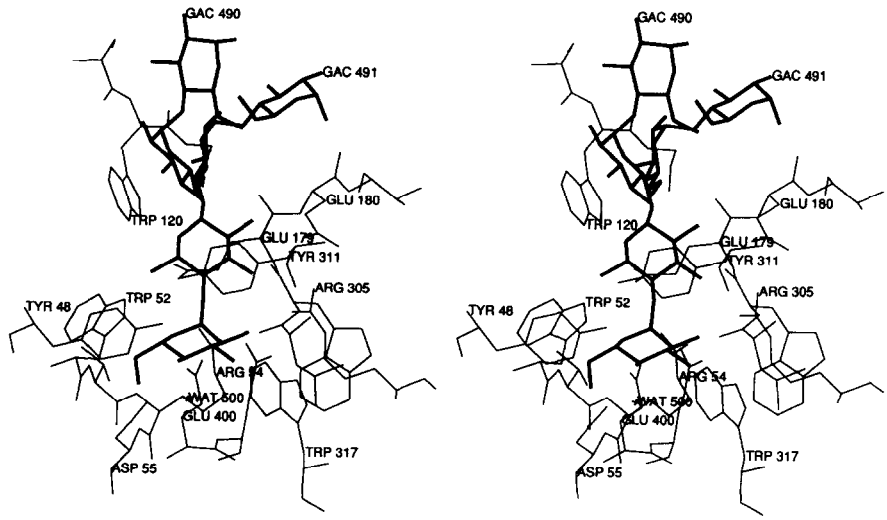

Fig. 3. Stereoview of the interaction of bound D-*gluco*-dihydroacarbose with glucoamylase. The D-*gluco*-dihydroacarbose molecule is represented in bold lines. Residue A of the inhibitor is at the bottom.

Table 2
Non-bonded contacts between glucoamylase II(471) and D-*gluco*-dihydroacarbose

Atom of the inhibitor	Atom of the protein	Contact distance (Å) high/low weight conformer
O6A	Water[500]	2.92
	Asp[55] OD1	2.72
C7A	Water[500]	3.29
	Tyr[48] OH	3.43
O2A	Arg[305] NH1	3.04
	Trp[178] CE3	3.36
	GAC[a] O3B	3.11
O3A	Carbonyl[177]	2.60
	Arg[54] NE	3.44
	Trp[417] CZ3	3.45
O4A	Asp[55] OD2	2.70
	Arg[54] NE	3.03
	Arg[54] NH2	2.85
	Arg[54] CZ	3.36
C1A	Water[500]	3.79
C2A	Water[500]	3.51
C3A	carbonyl[177]	3.42
C4A	Water[500]	3.59
C5A	Water[500]	3.76
	Glu[179]	3.47
C6A	Asp[55] OD1	3.48
O2B	Glu[180] OE2	2.73
	Glu[180] OE1	3.46
O3B	Carbonyl[178]	2.70
	Arg[305] NH1	2.83
	GAC[a] O2A	3.11
	Glu[180] OE2	3.26
N4B	Glu[179] OE1	2.59
C3B	Carbonyl[178]	3.22
C4B	Glu[179] OE1	3.37
C5B	Glu[179] OE1	3.18
C6B	Glu[179] OE1	3.40
O2C	GAC[a] O3D	2.71/3.47
	Thr[148] OG1[b]	2.57/3.59
	Thr[148] CG2[b]	3.23/4.28
	Thr[148] CB[b]	3.39/3.64
O3C	Thr[148] OG1[b]	3.47/4.23
O5C	Trp[120] CE3	4.84/3.17
	Trp[120] CD2	4.94/3.37
	Trp[120] CZ3	5.01/3.35
O6C	Carbonyl[179]	2.79/2.75
	Amide[121]	2.78/2.89
	Gly[121] CA	3.26/3.37
	Glu[179] CB	3.45/3.40
	GAC[a] O6D	4.27/2.95
C2C	Trp[120] CZ3	5.92/3.39
	Thr[148] OG1[b]	3.38/4.47
C6C	Carbonyl[179]	3.33/3.23
	Glu[179] CB	3.60/3.37
O1D	Carbonyl[180]	2.73/7.92
O2D	Carbonyl[205,b]	2.86/7.51
O3D	GAC[a] O2C	2.71/3.47
	Ser[119] OG	8.80/3.06
O5D	Ser-mannose[455] C6[b]	6.37/3.24
	Carbonyl[180]	3.41/7.35
O6D	Carbonyl[179]	4.96/3.35
	GAC[a] O6C	4.27/2.95
C1D	Carbonyl[180]	3.45/8.30
C6D	Tyr[175] CE2	3.44/4.97
	Amide[121]	4.12/3.25
	Gly[121] CA	3.86/3.62

Only distances less than or equal to 3.5 Å in the complex are listed as well as interactions between the attacking nucleophile, Water[500], and atoms of residue A of the inhibitor.
[a] GAC is D-*gluco*-dihydroacarbose.
[b] Crystal lattice contact.

observed that the glucosidic linkage between rings B and C is under strain in the acarbose complex. A very sensitive balance may exist between the strength of hydrogen bonds at ring A and the distortion of the glucosidic linkage between residues B and C. A chair conformer for ring A may not permit optimal hydrogen bonding to the active site of glucoamylase. Thus, excess binding energy is unavailable to distort the linkage between residues B and C. Alternatively, the acarbose and the D-*gluco*-dihydroacarbose complexes exist at different pH levels. The conformational differences may reflect the ionization of a specific group on the enzyme or even the imino linkage of the inhibitor. Bock et al. [9] find, for instance, that both acarbose and D-*gluco*-dihydroacarbose in their unligated states adopt different conformations in acid and alkaline solutions. The conformational differences in solution were localized to the imino linkage between residues A and B, but in the context of the rigid active site of glucoamylase, ionization of the imino linkage could result instead in a conformational change involving the linkage between residues B and C. We note, however, that the salt links between Glu[179] and the imino linkage of D-*gluco*-dihydroacarbose at pH 4 (Table 2) and Glu[179] and the imino linkage of acarbose at pH 6 [8] are identical to within experimental error of the X-ray structures. Thus, if the difference in pH is responsible for the conformational change in the inhibitor, then some ionizable group belonging to the enzyme is most likely responsible.

Further study is clearly required to satisfactorily explain the binding properties of extended inhibitors to glucoamylase. We are now in the process of investigating the structure of a D-*gluco*-dihydroacarbose complex with glucoamylase at 1.8 Å resolution, using a sample of D-*gluco*-dihydroacarbose that can be no more than 1 part-per-million in acarbose concentration. The preliminary results of the 1.8 Å resolution structure are consistent with those reported here, where a single molecule of D-*gluco*-dihydroacarbose binds in two discrete conformations (Fig. 3). Whether the conformational disorder is due to a pH effect or to the small conformational differences at residue A can only be addressed by structural investigations at high resolution and under different conditions of pH.

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