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Crystal Structure of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus* sp. 1011 Complexed with 1-Deoxynojirimycin at 2.0 Å Resolution[1]

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1-Deoxynojirimycin, a pseudo-monosaccharide, is a strong inhibitor of glucoamylase but a relatively weak inhibitor of cyclodextrin glucanotransferase (CGTase). To elucidate this difference, the crystal structure of the CGTase from alkalophilic *Bacillus* sp. 1011 complexed with 1-deoxynojirimycin was determined at 2.0 Å resolution with the crystallographic R value of 0.154 (R_{free} = 0.214). The asymmetric unit of the crystal contains two CGTase molecules and each molecule binds two 1-deoxynojirimycins. One 1-deoxynojirimycin molecule is bound to the active center by hydrogen bonds with catalytic residues and water molecules, but its binding mode differs from that expected in the substrate binding. Another 1-deoxynojirimycin found at the maltose-binding site 1 is bound to Asn-667 with a hydrogen bond and by stacking interaction with the indole moiety of Trp-662 of molecule 1 or Trp-616 of molecule 2. Comparison of this structure with that of the acarbose-CGTase complex suggested that the lack of stacking interaction with the aromatic side chain of Tyr-100 is responsible for the weak inhibition by 1-deoxynojirimycin of the enzymatic action of CGTase.

Key words: crystal structure, cyclodextrin glucanotransferase, 1-deoxynojirimycin, protein-sugar complex, inhibitor.

Cyclodextrin glucanotransferase (EC 2.4.1.19, CGTase), a member of the α-amylase family [glycosyl hydrolases family 13 (*1*)], catalyzes cyclization, coupling, disproportionation and hydrolysis of starch and mainly produces cyclic α-1,4–linked oligosaccharides called cyclodextrins (CD) as well as linear saccharides. In general, CDs consist of six, seven, or eight α-1,4–linked glucosyl units and are referred to respectively as α-, β-, γ-CDs. Since CDs form inclusion complexes with a variety of guest molecules and change their physicochemical properties (*2*), they have found applications in the pharmaceutical, chemical and food industries. In the study of CGTase, the most interest has focused on the mechanism of CD production. The X-ray structure of CGTase from *Bacillus stearothermophilus* was determined in 1991 (*3*), and the structures of CGTases from *Bacillus circulans* strain 8 (*4*), strain 251 (*5*), alkalophilic *Bacillus* sp. 1011 (*6*), and *Thermoanaerobacterium thermosulfurigenes* EM1 (*7*) have subsequently been reported. The crystal structures of CGTase-maltononaose complex (*8*) and CGTase-γ-CD complex (9) have revealed the presence of nine subsites from –7 to +2 for the binding of oligosaccharides and subsites –4c, +4c, and +3c for CD binding in the catalytic active site. These structures suggest that the interaction of the substrate with multiple subsites is essential for the cyclization reaction.

Inhibitors provide important clues to the elucidation of enzymatic reaction, such as information about enzymatic properties, especially recognition and binding to their substrates. Acarbose and 1-deoxynojirimycin have been used as inhibitors for sugar-catalyzing enzymes (Fig. 1). 1-Deoxynojirimycin is a strong inhibitor of exo-type glycosyl hydrolases like glucoamylase [IC_{50} for starch-degrading activity = 0.096 μM (*10*)], which hydrolyzes α-1,4–linked glucan and produces only β-glucose, but a weak inhibitor of endo-type glycosyl hydrolases like CGTase (IC_{50} for starch-degrading activity = 480 μM). In contrast, acarbose is a strong inhibitor of most glycosyl hydrolases [glucoamylase, IC_{50} = 0.0065 μM (*10*); CGTase, IC_{50} = 0.6 μM]. The crystal structure of acarbose-CGTase complex has been reported (*11*, *12*) and shows that acarbose is located at the subsites from –2 to +2 and tightly bound by hydrogen bonds and stacking interaction with the aromatic side chains. To elucidate the difference in their inhibition of CGTase between 1-deoxynojirimycin and acarbose in relation to the structural difference of sugar recognition and interaction, we determined the crystal structure of CGTase from alkalophilic *Bacillus* sp. 1011 complexed with 1-deoxynojirimycin. In this paper, we discuss the inhibition by 1-deoxynojirimycin of CGTase by comparison of this structure with that of acarbose-CGTase complex (*11*, *12*).

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Abbreviations: CD, cyclodextrin; CGTase, cyclodextrin glucanotransferase; MBS, maltose binding site.

MATERIALS AND METHODS

Preparation and Crystallization—Alkalophilic CGTase was expressed in *Escherichia coli* cells and purified by the reported procedure (*13*). The CGTase complex with 1-deoxynojirimycin was co-crystallized by hanging drop vapor diffusion. To a CGTase (20 mg/ml) solution containing 10 mM 1-deoxynojirimycin, an equiamount of the reservoir solution containing 20% (w/v) PEG3000, 20% (v/v) 2-propanol, 100 mM sodium citrate (pH 5.6), and 0.5 mM calcium chloride was added, and a droplet of the solution was equilibrated over the reservoir solution at room temperature. Rod-like crystals with the approximate dimensions of 1.0 mm × 0.3 mm × 0.2 mm were obtained in 1 or 2 weeks.

Data Collection—X-ray diffraction data of the CGTase complex with 1-deoxynojirimycin were collected to 1.8 Å resolution on an Enraf-Nonius FAST diffractometer equipped with an FR571 generator (40 kV, 50 mA, focal spot size 0.2 mm) at 286 K. Eight data sets of independent reflections obtained from two crystals were merged to give a set of 100,410 unique reflections with the $R_{merge}(I)$ value of 7.6% and completeness of 81.5%. Results are summarized in Table I.

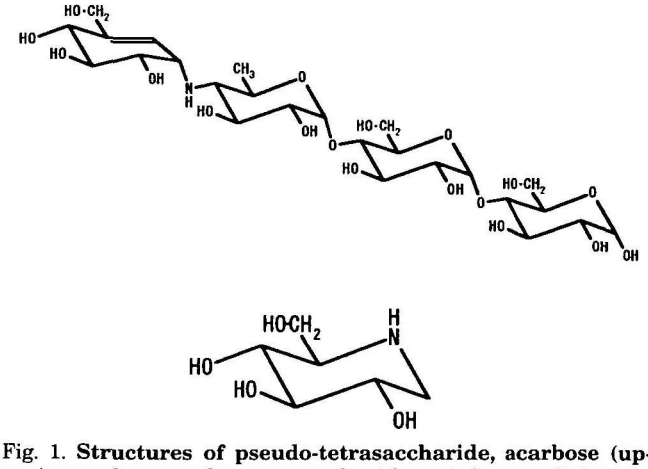


Fig. 1. **Structures of pseudo-tetrasaccharide, acarbose (upper) and pseudo-monosaccharide, 1-deoxynojirimycin (lower).**

Structure Determination and Refinement—The crystal structure was determined by molecular replacement method using the coordinates of native CGTase (*6*). 1-Deoxynojirimycin was found on $3F_o$–$2F_c$ and F_o–F_c electron density maps. The structure was refined at 2.0 Å resolution by using *X-PLOR* (*14*). Difference electron density peaks above 3σ in F_o–F_c map and in intermolecular contacts in the range of 2.5–3.3 Å with protein were considered as water, but those with the *B* value larger than 60 Å^2 were omitted during the refinement. The refinement converged at the *R* value of 0.154 (R_{free} = 0.214) for 2.0–10.0 Å resolution data (Table I). The final structure model contained two protein molecules, four calcium ions, four 1-deoxynojirimycin molecules, and 590 water molecules. The stereochemical quality of the structure was checked by program PROCHECK (*15*). Atomic coordinates have been deposited

TABLE I. **Summary of data collection and refinement statistics of alkalophilic *Bacillus* sp. 1011 CGTase-1-deoxynojirimycin complex.**

Data collection	
Space group	*P*1
Cell dimensions (*a*, *b*, *c*, α, β, γ)	64.78, 74.24, 79.03 Å, 85.03, 104.88, 101.02°
V_m (solvent content)	2.40 Å^3/Da (48.8%)
Resolution range	27.89–1.84
No. of unique reflections	100,410
Redundancy	3.0
Completeness	81.5%
R_{merge}	7.6%
Structure refinement	
No. of unique reflections used	86,468 ($F > 2\sigma$, resolution range 10.0–2.0 Å)
Completeness	96.3%
No. of CGTase/1-deoxynojirimycin/Ca^{2+}/water	2/4/4/590
Final *R* value	0.154
Final R_{free} value for 6% data	0.214
Mean B-factor in molecule 1/2	
(all atoms)	12.20/16.00 Å^2
(main-chain atoms)	11.48/15.38 Å^2
(side-chain atoms)	12.97/16.67 Å^2
Root mean square deviation from ideality	
bond length	0.013 Å
bond angle	2.70°
Average distance between equivalent Cα atoms of molecule 1 and 2	0.267 Å

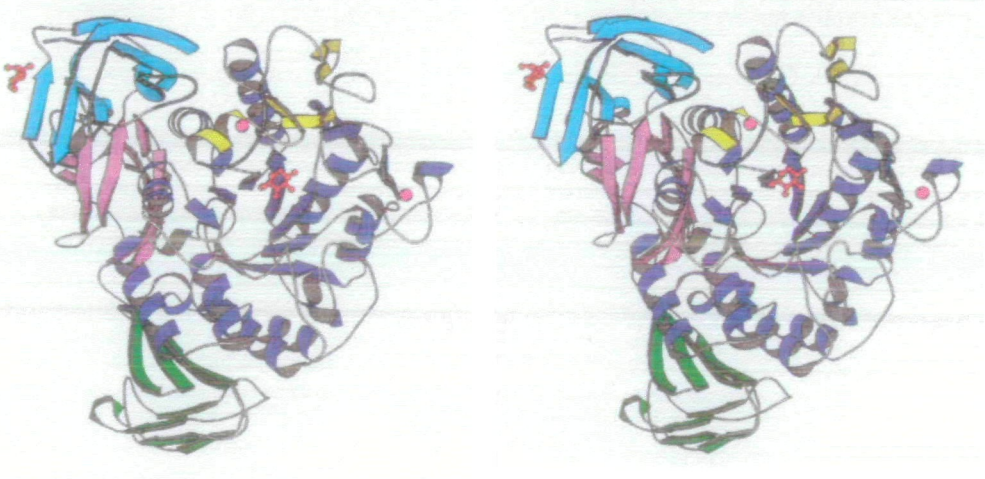


Fig. 2. **Stereoview of the structure of alkalophilic CGTase from *Bacillus* sp. 1011 complexed with 1-deoxynojirimycin.** CGTase consists of five domains: A (1–406, blue), B (139–203, yellow), C (407–496, green), D (497–584, purple), E (585–686, lightblue). Calcium ions are shown by magenta circles. Two 1-deoxynojirimycins (red) are located at the catalytic active site in domain A and MBS1 in domain E. The structure was drawn using the program MOLSCRIPT (*19*).

with Protein Data Bank (1I75).

RESULTS

Crystal Structure—Data collection and refinement are summarized in Table I. The crystal isomorphous with that of native CGTase belongs to the triclinic space group *P*1 with the unit-cell dimensions, a = 64.78 Å, b = 74.24 Å, c = 79.03 Å, α = 85.03°, β = 104.88°, γ = 101.02°. An asymmetric unit contains two independent CGTase molecules. According to Ramachandran plot (*16*), the (φ, ψ) angles fall in the normal range except those of Ala-152 and Tyr-195 ($\varphi \approx 55°$, $\psi \approx -130°$). These crystallographic properties (Table I) are the same as those observed in the native CGTase structure (*6*). The coordinate error was estimated to be less than 0.2 Å from Luzzati plot (*17*). Two 1-deoxynojirimycin molecules are located at the active center of domain A and the maltose-binding site 1 (MBS1) of domain E (Fig. 2).

TABLE II. **Average C^{α} distance (Å) after superposition between CGTase-1-deoxynojirimycin complex and native CGTase.**

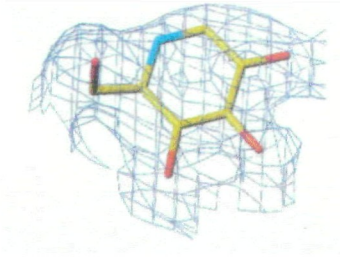
Domain	Molecule 1	Molecule 2
A	0.13 (0.12)	0.13 (0.11)
B	0.14 (0.11)	0.12 (0.11)
C	0.20 (0.10)	0.12 (0.11)
D	0.11 (0.10)	0.16 (0.09)
E	0.23 (0.09)	0.24 (0.14)
Total ave.	0.15	0.15

The values in parentheses are average C^{α} distances after superposition of each domain.

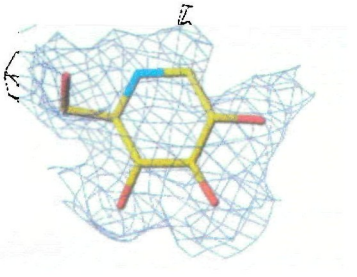
Comparison with Native CGTase—There are no significant conformational change of the side chain groups in the catalytic active site between the structure of the 1-deoxynojirimycin complex and the native structure. The difference of equivalent C^{α} positions between native CGTase (*6*) and the 1-deoxynojirimycin complex was calculated after the least-squares superposition (Table II). When the whole structure is superimposed, the domain E shows a larger C^{α} difference, 0.23 Å in molecule 1 and 0.24 Å in molecule 2, than the other domains (0.11–0.20 Å). The superposition of each individual domain gives a smaller C^{α} difference (0.09–0.14 Å), and the domain E shows no prominent deviation. This implies that the conformational difference of each domain is rather small and the relatively large C^{α} difference in domain E is caused by the movement of the domain.

Structure of 1-Deoxynojirimycin—The electron density map indicates that 1-deoxynojirimycins are located at the catalytic active site in domain A and in the maltose-binding site MBS1 in domain E of both independent molecules (Fig. 3). The pyranose ring of the four independent 1-deoxynojirimycin molecules has the $^4C_1$ chair conformation, and three secondary hydroxyl groups are in equatorial conformation. The structural difference is observed in the conformation of the primary hydroxyl group. 1-Deoxynojirimicyn bound to the active site of molecule 2 has the primary hydroxyl group with (+)-*gauche* conformation to the C5-N5 bond. On the other hand, the primary hydroxyl group of the other three 1-deoxynojirimycin molecules is in (–)-*gauche* conformation.

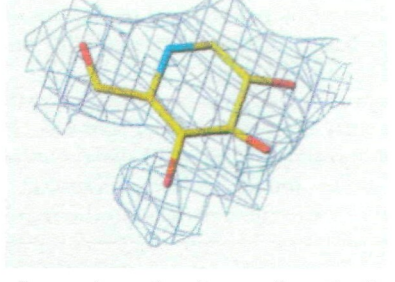
Interaction of CGTase with 1-Deoxynojirimycin—1-Deoxynojirimycin is located at the subsite –1 in the active site



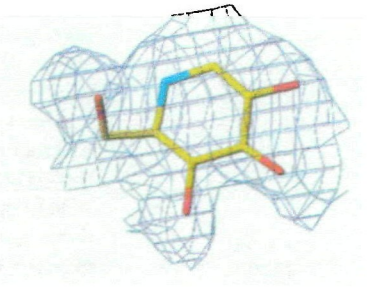
Catalytic active site in molecule 1



MBS1 in molecule 1

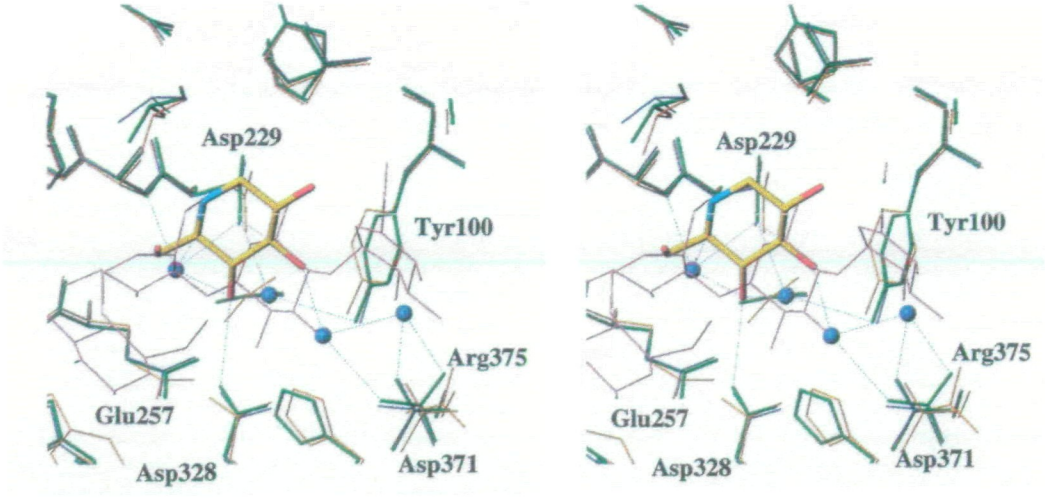


Catalytic active site in molecule 2

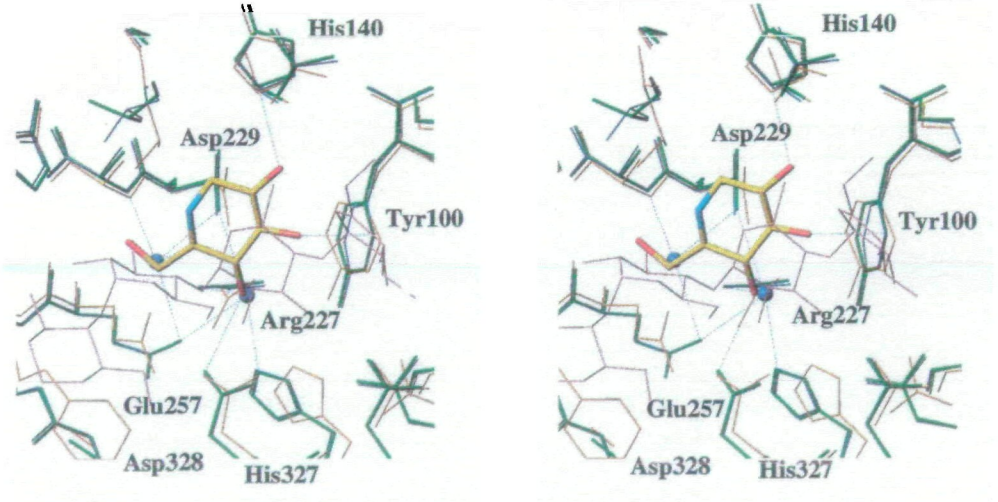


MBS1 in molecule 2

Fig. 3. **Electron density of 1-deoxynojirimycin in F_o–F_c omit map contoured at 2.7σ at the catalytic active site in domain A and MBS1 in domain E of molecules 1 and 2.**



Molecule 1



Molecule 2

Fig. 4. **Stereoviews of 1-deoxynojirimycins located at the catalytic active site in domain A of molecules 1 and 2 in comparison with native CGTase (*6*) and acarbose complex (*20*).** 1-Deoxynojirimycin complex, native CGTase, and acarbose complex are each shown by thick lines, blue thin lines, and magenta (acarbose) and orange (CGTase) thin lines. Water molecules contacting with 1-deoxynojirimycins are represented by large circles. The hydrogen bonds (≤3.3 Å) of 1-deoxynojirimycins to molecule 1, 2, or water are shown by broken lines.

TABLE III. **Hydrogen bond (Å) of 1-deoxynojirimycins at subsite-1 of catalytic active site in CGTase and at its corresponding site in glucoamylase (*18*).**

1-Deoxynojirimycin	Molecule 1		Molecule 2		Glucoamylase	
O2					Arg 305 NH1	3.22
					NOJ 481 O3	2.57
					water 1202	3.06
O3	water 972	2.67			Arg 54 NE	3.27
					Leu 177 O	2.65
O4	water 894	2.81	water 824	2.79	Arg 54 NH2	2.92
	Asp 328 OD2	3.12	Asp 328 OD1	3.18	Asp 55 OD1	2.73
	water 1015	2.85				
N5					water 501	2.82
					water 634	2.82
O6					Asp 55 OD2	2.57
					water 501	2.97

cleft and bound by hydrogen bonds with the catalytic residues Asp-229, Glu-257, or Asp-328 in the two independent molecules (Fig. 4 and Table III). In sugar complexes of CGTase, the pyranose ring of a glucosyl unit located at this subsite is in stacking interaction with the aromatic ring of Tyr-100, as observed in the crystal structure of CGTase from *Bacillus circulans* 251 complexed with acarbose (*11*, *12*). In contrast, the pyranose ring of 1-deoxynojirimycin in both molecules 1 and 2 is not stacked on the aromatic side chain of Tyr-100 but is rather perpendicular to the hydroxyphenyl group (Fig. 4).

The 1-deoxynojirimycin molecules located at MBS1 form hydrogen bonds with the side chain of Asn-667 (Fig. 5). In molecule 1, the pyranose ring of 1-deoxynojirimycin is nearly parallel to the indole moiety of Trp-662, while 1-deoxynojirimycin in molecule 2 is faced to the indole moiety of Trp-616.

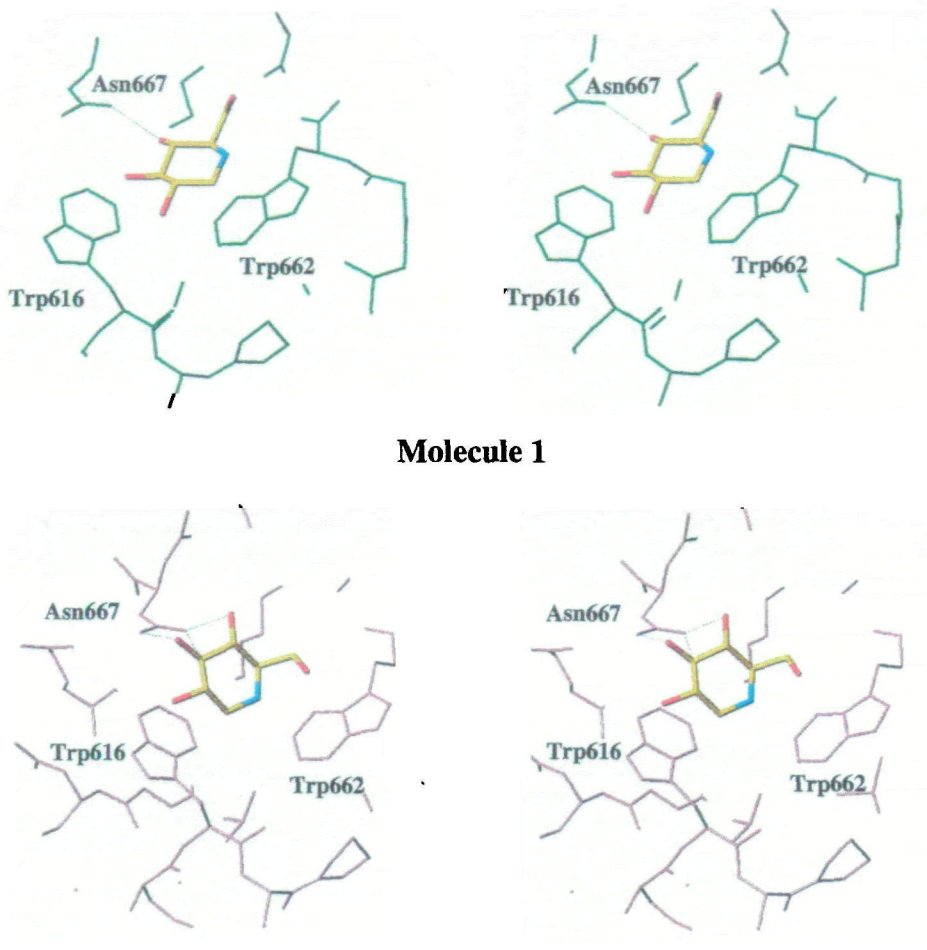


Fig. 5. **Stereoview of 1-deoxynojirimycin located at MBS1 in domain E of molecules 1 and 2.** The hydrogen bonds (≤3.3 Å) are shown by broken lines.

DISCUSSION

Inhibition of 1-Deoxynojirimycin for CGTase—The active center of CGTase has three catalytic residues, Asp-229, Glu-257, and Asp-328. In the enzyme reaction, Glu-257 donates a proton, and Asp-229 and Asp-328 stabilize the intermediate state. The present structure suggest that the inhibition is achieved by the block of active center with 1-deoxynojirimycin.

1-Deoxynojirimycin is a strong inhibitor of exo-type glycosyl hydrolases. Kinetic experiment showed the weak inhibition by 1-deoxynojirimycin of CGTase (IC_{50} for starch-degrading activity = 480 μM). Comparison (Table III) of the present structure with that of the 1-deoxynojirimycin-glucoamylase complex (*18*) suggests the origin of the weak inhibition by 1-deoxynojirimycin of CGTase. 1-Deoxynojirimycin in the glucoamylase complex forms direct six hydrogen bonds to the side chains; but in the CGTase complex, only two or three direct hydrogen bonds are observed. Therefore, CGTase binds 1-deoxynojirimycin more weakly than does glucoamylase. Moreover, in the CGTase complexes with oligosaccharides, a glucosyl unit bound to subsite −1 is always stacked on the planar side chain of Tyr-100, as observed in the structure of the acarbose complex (*11*, *12*). In contrast, 1-deoxynojirimycin does not have such stacking interaction with the hydroxyphenyl group of Tyr-100 (Fig. 4).

1-Deoxynojirimycins have one direct hydrogen bond with a catalytic residue Asp-328 in both molecule 1 and molecule 2, which is essential for substrate bonding and stabilization of the intermediate (*21*). The hydrogen binding with Asp-328 may cause a "miss" orientation, different from the normal geometry observed in other oligosaccharide complexes (*11*, *12*, *20*, *21*). The nucleophile Asp-229 in molecule 2 forms a water mediated hydrogen bond with 1-deoxynojirimycin. These hydrogen-bond structures are different from those observed in the other oligosaccharide complexes (*11*, *12*, *20*, *21*). The fact that 1-deoxynojirimycin has only one binding unit to the multiple subsites and has no stacking contact may account for its weaker inhibition of CGTase than acarbose.

Why Is 1-Deoxynojrimycin Found only at the Subsite-1 in Any Subsites of the Catalytic Active Site?—The active site cleft of CGTase has several subsites for sugar binding, but 1-deoxynojirimycin is bound only to subsite −1. CGTase mainly produces CDs, but also forms a variety of oligomers of sugar units as subproducts. In the catalytic reaction, one glucosyl unit necessarily locates at subsite −1 or its equivalent position, and after cleavage, the reducing end of the

substrate binds to the non-reducing end of the other glucosyl unit or water as acceptor. Since the stable binding of the glucosyl unit to the subsite −1 is essential for the reaction process, subsite −1 is expected to have higher affinity for a glucosyl unit than the other subsites. This may be why the pseudo-monosaccharide 1-deoxynojirimycin binds to only the subsite −1. Uitdehaag *et al.* have also suggested that subsite −1 tightly binds the glucosyl unit in the structure of CGTase complexes with γ-CD and maltononaose complexes (*8*, *9*).

Why Does the Pyranose Ring of 1-Deoxynojirimycin Not Stack on the Aromatic Ring of the Tyr-100?—In the CGTase complexes with oligosaccharides, the glucosyl unit located at subsite −1 is in a stacking interaction with the aromatic ring of the Tyr-100 (*8*, *9*, *11*, *12*, *20*, *21*). This is a common structure in the recognition and binding of substrates, but in the present structure, 1-deoxynojirimycin at subsite −1 has no such stacking interaction (Fig. 4). The present structure suggests two reasons for this. The first is that the active site cleft stably binds the substrate only if the substrate is an oligo-saccharide. In other words, the stacking interaction of the glucosyl unit is supported by hydrogen bonds and stacking interaction of the other glucosyl units. The second reason is that the interaction of 1-deoxynojirimycin differs from the glucosyl unit since the charge distribution in the pyranose ring is different from that of native substrate because of the replacement of O5 with N5 (Fig. 1). Therefore, this may prevent 1-deoxynojirimycin from forming a stacking contact with Tyr-100.

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