Insights into the Mechanism of Drosophila melanogaster Golgi α-Mannosidase II through the Structural Analysis of Covalent Reaction Intermediates*

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The family 38 golgi α-mannosidase II, thought to cleave mannosidic bonds through a double displacement mechanism involving a reaction intermediate, is a clinically important enzyme involved in glycoprotein processing. The structure of three different covalent glycosyl-enzyme intermediates have been determined to 1.2-Å resolution for the Golgi α-mannosidase II from Drosophila melanogaster by use of fluorinated sugar analogues, both with the wild-type enzyme and a mutant enzyme in which the acid/base catalyst has been removed. All these structures reveal sugar intermediates bound in a distorted $S_2$ skew boat conformation. The similarity of this conformation with that of the substrate in the recently determined structure of the Michaelis complex of a β-mannanase (Ducros, V. M. A., Zechel, D. L., Murshudov, G. N., Gilbert, H. J., Szabo, L., Stoll, D., Withers, S. G., and Davies, G. J. (2002) Angew. Chem. Int. Ed. Engl. 41, 2824–2827) suggests that these disparate enzymes have recruited common stereo electronic features in evolving their catalytic mechanisms.

N-Linked glycosylation is a post-translational modification found in eukaryotes whereby a complex oligosaccharide is attached to a surface asparagine residue within the sequence Asn-X(Ser/Thr) through an N-glycosidic linkage. The initial part of the pathway, occurring in the endoplasmic reticulum, is highly conserved and has been well characterized in various organisms (1–3). The latter Golgi part is species-, tissue-, and cell-dependent and is the source of the diverse nature of glycosylation.

In various tumor cell lines such as those from breast, colon, and skin cancer, the distribution of the cell surface N-linked sugars is altered (4). Because N-linked glycosylation is associated with cell-cell interactions, this alteration correlates with the progression of tumor metastasis (5, 6). Enzymes of this glycosylation pathway are therefore potential targets for the development of inhibitors as cancer treatments. Golgi α-mannosidase II (GMII; 1,3,1,6 α-mannosidase II; EC 3.2.1.114), a glycosidase involved in trimming of two mannose residues in the Golgi, has been the target of one such drug candidate. In early clinical trials, the compound swainsonine, a well known GMII inhibitor, was found to reduce tumor growth and metastasis when taken orally (7, 8). Cross-reactivity of swainsonine with lysosomal mannosidase limits its clinical usefulness, and, therefore, a detailed understanding of the mechanism of GMII is necessary to develop drug candidates that specifically target this glycosidase.

From the primary structure, GMII has been classified into the glycosyl hydrolase family 38 (9–11). Members of this family of glycosidases hydrolyze α-mannosides with net retention of configuration at the anomeric center (12). By analogy with other retaining glycosidases, these enzymes are postulated to catalyze reactions by a double displacement mechanism (13–15). In this mechanism, a covalent glycosyl-enzyme intermediate is first formed via an oxocarbenium ion-like transition state (glycosylation). A carboxylic acid in the active site acts as the catalytic nucleophile in the formation of this intermediate, assisted by a second carboxylic acid that acts as a general acid catalyst. Hydrolysis of the glycosyl-enzyme intermediate (deglycosylation) occurs in a second step, facilitated by general base catalysis provided by the conjugate base of the acid catalyst from the first step. By using a mechanism-based inactivator, 5-fluoro-β-1-gulosyl fluoride (5FGuFl), the covalent glycosyl-enzyme intermediate formed on two representative members of this family has been trapped, and the residue acting as the catalytic nucleophile was identified in each case (16, 17).

The structure of the catalytic portion of the GMII from Drosophila melanogaster (dGMII) was determined by x-ray crystallography along with structures of complexes with several ligands, namely swainsonine and deoxymannojirimycin (18). The ligand binding site features coordination to a zinc ion and includes a conserved aspartate residue, Asp-204 (dGMII numbering), analogous to the residue identified in the previous trapping studies as the catalytic nucleophile. These results suggested that the acid/base catalyst is most likely Asp-341. These structures, however, only give a first insight into the structural aspects of the catalytic mechanism. A key

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The atomic coordinates and structure factors (codes 1QWNN, 1QX1, 1QWU) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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** The abbreviations used are: GMII, Golgi α-mannosidase II; DNP-Man, 2,4-dinitrophenyl α-D-mannoside; DNP, 2,4-dinitrophenol; 5FGuFl, 5-fluoro-β-1-gulosyl fluoride; dGMII, Drosophila melanogaster GMII; 2FMaNFl, 2-deoxy-2-fluoro-α-D-mannoside fluoride; wt, wild-type; MES, 4-morpholineethanesulfonic acid; r.m.s.d., root mean square deviation; CGTase, cyclodextrin glucanotransferase.
element of the catalytic mechanism is the glycosyl-enzyme intermediate formed during catalysis. Determination of the structure of such intermediates has provided more detailed insights, not only into the residues involved in catalysis, but also into the possible role of saccharide distortion along the reaction pathway, as discussed recently (19), primarily for β-glycosidases. Recent studies of β-glycosidases have revealed saccharide distortion in Michaelis (enzyme-substrate) complexes but not in α-glycosyl-enzyme intermediates (with the exception of the family 11 xylanase (20) and of the family 26 β-mannanase (21)). Until now, no structure of trapped intermediates on a true α-glycosidase has yet been published, though the structure of the β-glycosyl-enzyme intermediate on a family 13 transglycosidase, the cyclodextrin glucanotransferase from Bacillus circulans was reported (22).

In this paper we have used both 5FGuIF and 2-deoxy-2-fluoro-α-D-mannosyl fluoride (2FManF) to trap the glycosyl-enzyme intermediates formed on both the wild-type (wt) and D341N mutant of dGMII and examined the crystallographic structures of these intermediates. These are the first structures of covalent intermediates determined for any α-mannosidase. Furthermore, this is the first true α-glycoside hydrolase for which the structure of the covalent intermediate has been determined. As such, these studies add to our understanding of how α-glycosidases catalyze hydrolysis of glycosidic linkages.

**EXPERIMENTAL PROCEDURES**

**Materials**

The 2,4-dinitrophenyl α-D-mannoside (DNS-Man) (23), 5FGuIF (16), and 2FManF (24) were synthesized according to published procedures. All chemicals and buffer salts were obtained from Sigma unless noted otherwise.

**Creation of the D341N Mutant dGMII**

To facilitate mutagenesis, the expression plasmid for dGMII (18), which contains a metallothionein promoter and Bip-secretion sequence, was first reduced in size from 6642 to 4610 bp by Sall digestion followed by re-ligation. The single codon mutation was introduced to this plasmid using the QuikChange site-directed mutagenesis kit (Stratagene). The forward primer had the sequence CTG CTG ATT CCG TTG GAC AAC TTC CGC TTC AAG C. The presence of the mutation was confirmed by sequencing. A 238-bp A del/partial SalI fragment, which contained the mutated sequence, was then isolated from the plasmid and inserted into an A del/partial SalI-digested native expression plasmid. The presence of the mutation and the absence of any frameshifts or secondary mutations were confirmed by sequencing the final construct.

This D341N construct and a bacteriostatic resistance plasmid were then used to co-transfect Drosophila cells. The presence of the secreted, full-length protein in the medium from transfected cells induced with 10 μM cadmium was confirmed by Western blot analysis. Anti-pentaHis antibody (Qiagen) was used as the primary antibody whereas an alkaline phosphatase-conjugated anti-mouse antibody was used as the secondary antibody. Alkaline phosphatase activity was monitored with NBT/BCIP reagent (Sigma). Stable populations of transfectants were selected using blasticidin (16 NBT/BCIP reagent (Sigma). Stable populations of transfectants were selected using blasticidin (16 μg/ml) and then screened for the presence of the secreted, full-length protein in the medium from transfected cells induced with 10 μM cadmium using an LC-Packings high pressure liquid chromatography system. Briefly, the intact protein (10–20 μg) was pre-incubated for 5 min with either 4 mM 5FGuIF (wt or D341N mutant dGMII) or 40 mM 2FManF (D341N mutant dGMII). The inactivation of the wt dGMII by 5FGuIF was observed in the same manner except that the enzyme concentration was lower (0.22 mg ml⁻¹), and the temperature of the experiment was reduced to 8 °C.

**Mass Spectrometric Analysis of Intermediate Formed on dGMII**

The analyses of protein samples were carried out using a Sciex API-300 mass spectrometer interfaced with an LC-Packings high pressure liquid chromatography system. Briefly, the intact protein (10–20 μg) was pre-incubated for 5 min with either 4 mM 5FGuIF (wt or D341N mutant dGMII) or 40 mM 2FManF (D341N mutant dGMII). The inactivation of the wt dGMII by 5FGuIF was observed in the same manner except that the enzyme concentration was lower (0.22 mg ml⁻¹), and the temperature of the experiment was reduced to 8 °C.

**Crystallization and Data Collection**

Crystallization of dGMII was carried out using hanging drop vapor diffusion as described previously (18). Because of a relatively rapid loss of diffusion quality with time, crystals were less than 24 h old at the time of crystal evaluation and freezing. For the 2FManF and native protein complexes, the crystals were grown in the absence of inhibitor and then soaked with the compound for ~30 min. Prior to freezing, the crystals were passed through drops containing 10, 15, 20, and 25% methyl-pteridinol in crystallization buffer and 5 μl of appropriate compound. The crystals were mounted in nylon Cryoloops (Hampton Research) and frozen directly in a liquid nitrogen cryostream.

All data were collected at 100 K. Data were collected either at the Ontario Cancer Institute on a MAR Research 2300 image plate detector mounted on a rotating anode generator with copper target, operated at 50 kV and 100 mA with both focusing using Osmic optics, or at the Cornell High Energy Synchrotron Source, beamline F1, using an ADSD Quantum 4 CCD detector in the rapid readout mode. Typically 300–400 frames of 0.5° oscillation were collected for each data set. Data reduction and scaling were carried out using Denzo and Scalepack, respectively (26). Data collection statistics are provided in Table I.

**Structure Determination**

The software program CNS (27, 28) was used for the refinement of the structures of the complexes. For the calculation of R_sym, a test set comprising ~2000 reflections was removed from the data set. The structures of the complexes were solved by molecular replacement. Briefly, rigid body refinement was carried out against the published...
structure of native dGMII (Protein Data Bank code 1HTY) with Tris and water molecules in the region of the active site removed (18). Asp-204 was changed to alanine in the Protein Data Bank file to remove bias around the region of formation of the covalent bond. Rigid body refinement was followed by simulated annealing to 5500 K. group B-factor refinement, and individual B-factor refinement, prior to generation of electron density maps. At this initial stage R-factors were typically in the range of 22%, and the \( F_o - F_c \) density maps clearly showed the presence of bound compound and unassigned waters. Refinement of the model involving manually fitting the compounds into the density, fitting waters, and checking side-chains for proper fit to the structure was carried out using ProFit (www.bioinf.org.uk/software/profit). Graphics were generated using PyMol (30). Protein Data Bank (31) codes for the deposited coordinates and diffraction data are given in Table I.

### RESULTS

**Kinetic Analysis**—The kinetic parameters for the activated substrate, DNP-Man, were determined for both the \( \text{wt} \) and D341N mutant enzyme. As might be expected from removal of the acid/base catalyst, the \( k_{\text{cat}} \) value for the D341N mutant dGMII was lower by roughly 200-fold compared with that of the \( \text{wt} \) enzyme (\( k_{\text{cat}} \) values are 0.048 and 8.6 s\(^{-1}\), respectively). The \( K_m \) value for the mutant enzyme was also 2 orders of magnitude lower compared with the \( K_m \) value of the \( \text{wt} \) enzyme (\( K_m \) value is 0.05 and 5.5 mM, respectively). This lowering of the \( K_m \) value is unlikely to be because of a tightening of the true binding, but is more likely because of an accumulation of the intermediate species as a result of the deglycosylation step becoming rate-limiting. This behavior is also reflected in the fact that the \( k_{\text{cat}}/K_m \) value, which reflects the first irreversible step, most likely glycosylation, is largely unaltered. This is reasonable, because a good leaving group such as DNP requires very little proton assistance for departure, thus removal of the acid catalyst will not significantly affect the glycosylation step. However, removal of general base catalysis clearly slows down the second step, deglycosylation. Thus the intermediate accumulates. Nonetheless, turnover of the intermediate species was unfortunately sufficiently rapid to prevent us from observing the intermediate in crystals of the D341N mutant dGMII soaked with DNP-Man.

When 5FGulF was incubated with the D341N mutant dGMII at 37 °C, the compound was found to act as an apparent reversible inhibitor with an approximate \( K_i \) value of 0.6 mM. Considering that the \( \text{wt} \) enzyme has a \( K_m \) value of 5 mM for DNP-Man, 5FGulF binds considerably more tightly than might be expected for a very simple substrate analogue, especially considering its minimal aglycone (fluoride) and the inverted configuration at C-5. This result is suggestive, however, of 5FGulF acting as a slow substrate with a rate-limiting deglycosylation step, as seen with some other 5-fluorosugars and their corresponding glycosidases (16, 17, 32). When 5FGulF was assayed as an inactivator, no inactivation was observed, presumably because the deglycosylation step is still fast relative to the assay time. However, when the mutant enzyme was incubated with 5FGulF, and the residual activity was assayed at 16 °C, time-dependent inactivation was indeed observed with a second order rate constant, \( k/K_i \) value of 33 s\(^{-1}\) M\(^{-1}\) (Fig. 1, \( a \) and \( b \)). When excess 5FGulF was removed the inactivated enzyme was found to reactivate over time, demonstrating the catalytic competence of the intermediate formed upon hydrolysis of 5FGulF by the D341N mutant dGMII.

The above results suggest that 5FGulF is acting as a slow substrate for the D341N mutant dGMII, with deglycosylation as the rate-limiting step. Inactivation is only observed when assayed at low temperatures such that deglycosylation is slow relative to the assay time. To confirm whether this also applied to the \( \text{wt} \) enzyme, 5FGulF was assayed as a substrate using the chloride ion electrode. At 37 °C, 5FGulF was found to be a substrate of \( \text{wt} \) dGMII with a \( k_{\text{cat}} \) value of 5.1 \( \times \) 10\(^{-3}\) s\(^{-1}\) and a \( K_m \) value of 0.2 mM. As in the case of the mutant enzyme, the relatively low \( K_m \) value is likely because of the accumulation of the intermediate species as a result of the deglycosylation step becoming rate-limiting. Consistent with this hypothesis, when the \( \text{wt} \) dGMII was incubated with 5FGulF and the residual

### Table I

| Protein Data Bank code | wt-5FGulF | D341N-2FManF | D341N-5FGulF |
|------------------------|-----------|-------------|-------------|
| Crystal                | (P2\(_1\),2\(_1\),2\(_1\)) | (P2\(_1\),2\(_1\),2\(_1\)) | (P2\(_1\),2\(_1\),2\(_1\)) |
| Cell dimensions (Å)    | 69.02/110.02/138.96 | 69.05/109.83/138.91 | 68.85/109.89/138.77 |
| Mosaicity              | 0.125     | 0.43        | 0.398       |
| Data collection        |           |             |             |
| X-ray source           | CHESS-F1  | CHESS-F1    | OCI/rotating anode |
| Wavelength (Å)         | 0.9504    | 0.9504      | 1.54        |
| Data processing (bi res shell) |           |             |             |
| Reflections (total/unique) | 2,449,245/324,605 | 1,589,410/246,051 | 476,418/68,989 |
| I/σ                    | 23.7 (3.4) | 14.7 (2.9)  | 16.8 (2.9)  |
| % completeness         | 96.8 (91.4) | 95.3 (88.7) | 99.9 (98.7) |
| R<sub>free</sub>        | 0.075 (0.49) | 0.106 (0.556) | 0.11 (0.45) |
| R<sub>free</sub>/R<sub>free</sub> (reflections for R<sub>free</sub>) | 0.175/0.192 (2225) | 0.169/0.188 (2180) | 0.151/0.189 (2336) |
| Amino acids            | 1014      | 1014        | 1014        |
| Alternate conformations | 40        | 30          | 30          |
| Water molecules        | 1035      | 1042        | 1025        |
| r.m.s.d. bonds (Å)     | 0.02      | 0.02        | 0.02        |
| r.m.s.d. angles (°)    | 1.97      | 2.02        | 1.72        |
| Average B-factor (Å<sup>2</sup>) |           |             |             |
| Overall                | 12.84     | 16.02       | 16.92       |
| Protein main chain     | 10.23     | 13.02       | 14.78       |
| Protein side chain     | 12.20     | 16.37       | 16.69       |
| Water                  | 22.19     | 25.90       | 25.97       |
| Covalent ligand        | 7.78      | 14.33       | 10.35       |
activity assayed at 8 °C, time-dependent inactivation was observed. The reactivation, however, was too rapid for the determination of any reliable kinetic parameters.

Although the 2-deoxy-2-fluorosugars have been successfully used to label β-retaining glycosidases, they have proven quite ineffective with α-retaining glycosidases. Therefore, it was not surprising that all attempts at trying to inactivate either wt or D341N mutant dGMII with 2FManF were unsuccessful. Instead, the 2FManF was found to be a substrate of wt dGMII with a pseudo-second order rate constant, $k_{\text{cat}}/K_m$, of $8.5 \times 10^{-5} \text{ s}^{-1} \text{ M}^{-1}$ (where the $K_m > 25 \text{ mM}$). This high $K_m$ value is most likely a reflection of the 2-hydroxyl group being important to substrate binding, which would not be surprising given the previously observed role of the 2- and 3-hydroxyl groups in binding to the active site zinc (18). Unfortunately, because of the extremely low $k_{\text{cat}}$ value, it was not possible to determine any kinetic parameters for the hydrolysis of 2FManF by the D341N mutant dGMII. However, when tested as a reversible inhibitor, a $K_i$ value of 7.5 mM was determined for this compound with the D341N mutant dGMII. Once again, this value is considerably lower than the $K_m$ value for 2FManF hydrolysis with wt dGMII, suggesting that 2FManF is a very slow substrate of the mutant enzyme with a rate-limiting deglycosylation step.

Mass Spectrometric Analysis—The kinetic analysis clearly indicates that there is accumulation of an intermediate species in the case of the hydrolysis of 5FGulF by either the wt or D341N mutant dGMII. The results also suggest that the deglycosylation step is rate-limiting for hydrolysis of 2FManF by D341N mutant dGMII, likely resulting in an accumulation of an intermediate species. To determine the nature of these intermediate species, each was analyzed using electrospray ionization-mass spectrometry.

Compared with the free D341N mutant enzyme (120,502 ± 20 Da), the mass of the inactivated D341N mutant enzyme (120,672 ± 20 Da) was greater by 170 Da (see Fig. 1c for representative data). This value corresponds, within experimental error, to the expected increase of 181 Da for the addition of a covalently bonded 5-fluoro-gulosyl moiety. A similar increase in mass was observed when comparing the mass of the free wt dGMII with that of the inactivated wt enzyme. This demonstrates that a covalent intermediate is also formed during the hydrolysis of 5FGulF by wt dGMII.

Incubation of the D341N mutant dGMII with 2FManF yielded a species with a mass (120,634 ± 20 Da) higher by 166 than that of the free enzyme (120,468 ± 20 Da). This corresponds to the covalent addition of a 2-fluoro-mannosyl moiety to the enzyme (theoretical change, 165 Da). Only ~50% of the enzyme was labeled under these conditions, most likely because of the rapid turnover of the intermediate species at steady state. This is consistent with the failure of 2FManF to inactivate dGMII. However, the observation of a covalent species clearly shows that the hydrolysis of 2FManF by the mutant enzyme goes via a covalent glycosyl-enzyme intermediate and that this intermediate accumulates during steady state hydrolysis.

Structure of the Intermediate Formed from wt dGMII Reaction of 5FGulF—We have been able to obtain a very high resolution structure (1.20 Å) structure of wt dGMII reacted with 5FGulF. Inspection of this three-dimensional structure revealed very little change in the overall backbone structure in comparison with the free enzyme (18) (r.m.s.d. = 0.132 Å for α carbon atoms versus 1HTY). Very clear electron density corresponding to a complexed sugar was found in the active site identified from previous studies (18). This density corresponds to a 5-fluoro-gulosyl moiety in a $\beta$-linkage between the two atoms. In fact, this conserved aspartate has been identified as the catalytic nucleophile in other family 38 α-mannosidases (16, 17). Thus, the crystallographic data, taken together with the kinetic and mass spectrometric data, confirm that a covalent glycosyl-enzyme intermediate is formed during the hydrolysis of 5FGulF by wt dGMII.

Comparing this structure with that of the complex with deoxymannojirimycin (18) reveals some interesting features...
In both structures, the 3-hydroxyl group hydrogen bonds to Asp-472 and Asp-92. In addition, this functional group interacts with the active site zinc. These interactions seem to fix the 3-hydroxyl in the same position for both structures. In both complexes, the sugar 4-hydroxyl groups interact with the same residues (Asp-472 and Tyr-727), although there is a slight shift in the exact spatial positioning of the functional group. In contrast, by virtue of the covalent bond, the anomeric carbon is significantly closer to Asp-204 in the intermediate structure compared with the deoxymannojirimycin structure. Although there is some movement of Asp-204, this change in distance is mainly accommodated by the movement of the sugar ring and its change in conformation. The covalently bonded sugar adopts a 1S skew boat conformation whereas the deoxymannojirimycin adopts a 1C chair conformation. This change in conformation places the 2-hydroxyl group in a pseudo-equatorial position in the intermediate structure, thus avoiding a steric clash that could otherwise exist between this group and OD1 of Asp-204. Upon formation of the covalent bond with the sugar, the catalytic nucleophile moves away from the active site zinc. This is a common feature of all of the covalent intermediates described in the paper with the average inter-atomic distance between the zinc and the OD1 of Asp-204 increasing almost 0.5 Å, to 2.64 ± 0.06 Å in the three complexes, whereas in 34 other complexes that we have studied, this distance is 2.18 ± 0.05 Å.2 This movement may come about, because the attractive force of the Asp-204 OD1 toward the zinc is lessened because of the formation of the covalent intermediate.

The OD2 oxygen of Asp-204 is positioned 2.88 Å away from the ring oxygen of the covalently bound sugar. A similar interaction was seen in the structure of the covalent intermediate formed on a family 13 enzyme, cyclodextrin glucanotransferase (CGTase) (22). This oxygen-oxygen interaction is thought to

\[ \text{Covalent Intermediates of Golgi } \alpha\text{-Mannosidase II} \]

Fig. 2. a, stereo diagrams for the electron density map around the region of Asp-204 and the covalently bound sugar formed during the hydrolysis of 5FGulF by wt dGMII (covalent bond is not depicted). b, stereo diagrams for the structure of the covalent intermediate (green) formed during the hydrolysis of 5FGulF by the wt dGMII in relation to selected active site residues (yellow). For clarity, only those parts of the side chain from the C-atom onwards are shown. In the case of Arg-876, only the backbone atoms are shown. The covalent intermediate is shown in green with the arrow indicating the position of the new covalent bond.

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possibly stabilize positive charge development on the ring oxygen during the transition states. This may, in part, explain why the α-retaining glycosidases seem to have more charge build-up on the ring oxygen whereas the β-retaining glycosidases, where this interaction is not possible, seem to have charge build-up on the anomeric carbon during the transition state (15). Alternatively, but mechanistically equivalent, the interaction may be destabilizing in the intermediate, but this destabilization is relieved at the transition state, thereby constituting an example of ground state strain. Functionally equivalent interactions and arguments were made about a tyrosine interaction with the ring oxygen in family 11 xylanases (20).

In the complex of dGMII with the potential cancer therapeutic agent, swainsonine (Fig. 3c) (18), the five-membered ring of swainsonine mimics a flattened hexose ring with the two hydroxyl groups positioned in the roughly the same orientation as the 2- and 3-hydroxyl groups of the covalent intermediate. This
allows these functional groups to interact optimally with Asp-92 and the active site zinc, positioning the ring nitrogen roughly half-way between the location of the ring oxygen and anomeric carbon of the covalent intermediate sugar. Indeed, the ring nitrogen develops an interaction (2.88 Å) with the catalytic nucleophile, Asp-204. However, in contrast to the covalent intermediate and deoxymannojirimycin, only the OD1 of Asp-204, and not the OD2, is involved in the interaction. The hydroxyl group on the six-membered ring of swainsonine is well positioned to interact with Asp-472 and Tyr-727, residues that interact with the 4-hydroxyl group in the intermediate. The rest of the swainsonine six-membered ring fits into the hydrophobic pocket, which, in the intermediate, is occupied by the hydroxymethyl group of the mannoside.

Interestingly, the average interaction distance for interactions with the 2-, 3-, and 4-hydroxyl groups for deoxymannojirimycin is 2.68 Å, whereas the average for the covalent intermediate is 2.57 Å and for swainsonine is 2.50 Å. Thus, the swainsonine structure seems to more closely mimic that of the covalent intermediate than does the deoxymannojirimycin structure, both in terms of distances and in terms of geometry, as alluded to previously. This is consistent with swainsonine more closely mimicking a transition state structure, given that the reactive intermediate should itself more closely resemble the transition state according to the Hammond postulate (33).

Intermediate Structure Formed from D341N dGMII Hydrolisis of 2FManF—one concern that may be raised about the value of these structures is that gulosides are C-5 epimers of the natural substrates, mannosides, and furthermore that the 5FGulF inactivator has an extra fluorine atom attached to the C-5 position. Therefore, it may be argued that intermediate structure formed from the hydrolysis of 5FGulF does not reflect the structure of the intermediate formed during hydrolysis of the natural substrate. This is particularly important when considering the conformation of the trapped species, because the skew boat conformation might be an inherent consequence of the 5-fluoro-gulo sugar structure rather than arising from active site constraints.

To this end, a structure was determined to 1.30-Å resolution of the covalent intermediate formed during the hydrolysis by D341N mutant dGMII of a manno-configured inactivator, 2FManF. Because this compound has the natural C-5 configuration, it is likely that the intermediate formed during its hydrolysis would better reflect the conformation of the inter-

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**Fig. 4.** Electron density (omit) map around the region of Asp-204 and the covalently bound sugar formed during the hydrolysis of 2FManF by D341N mutant dGMII. The close (green) and product (cyan) configurations of the 2FMan sugar are shown.

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**Fig. 5.** a, overlays of the structures of the sugar in the covalent intermediate during 2FManF (cyan) hydrolysis with that of 5FGulF (green). b, the same overlay, this time showing the sugar in respect to selected active site residues. The side chains from the 2FManF complex are in cyan, and those from the 5FGulF complex are in green.

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As in the case of the structure of wt dGMII complexed with 5FGulF, the backbone structure of the D341N mutant dGMII complexed with 2FManF did not change significantly (r.m.s.d. = 0.137 ± 0.06 Å for a carbon atoms versus 1HTY, 0.069 ± 0.06 Å versus the 5FGulF complex). Once again, electron density corresponding to a sugar was found in the active site (Fig. 4). In this structure, the apparent distance from the anomeric carbon to Asp-204 OD1 was 1.79 Å. This distance is not consistent with any one type of bond or interaction and most likely represents an average structure between the cova-
lent intermediate and another bound species such as the product. To model these two species a “close” covalent species was introduced with a 204 OD1 to C-1 distance of 1.5 Å and 50% occupancy. A “product” species with a 204 OD1 to C-1 distance of 2.17 Å could then be modeled into the residual density. Individual B-factor refinement of the partially occupied positions resulted in appropriate values. This interpretation is consistent with the mass spectrometry results, which suggested that there is less than 100% accumulation of the intermediate at the steady state.

The bound 2-fluoro-mannosyl moiety adopts a conformation similar to that of the 5-fluoro-gulosyl moiety, namely a 1S5 skew boat conformation (Fig. 5). This is despite the fact that the configuration at C-5 is inverted from that of 5FGulF compound so that the bulky substituent is pseudo-equatorial as opposed to axial. The major difference seen in comparing these structures (Fig. 5a) is the C-6 hydroxymethyl group, which is equatorial in the intermediate for 2FManF hydrolysis whereas it is pseudo-axial in the intermediate formed during 5FGulF hydrolysis. Despite this difference, the 6-hydroxyl group occupies a similar position in both structures because of the difference in rotational angles around C-5 and C-6 and slight differences in the sugar positioning. This common positioning of the 6-hydroxyl group allows maintenance of the hydrogen bonding to the backbone carbonyl of Arg-876 whereas avoiding the positioning of the 6-hydroxyl group of the gulosyl moiety toward the hydrophobic pocket on the β-face of the sugar formed by Phe-206, Trp-415, and Tyr-727.

To demonstrate that the mutation of Asp-341 does not lead to changes in the conformation of the intermediate, the structure of the intermediate formed during 5FGulF hydrolysis by D341N mutant dGMII was also determined. Comparison of this structure with that of the intermediate formed during wt dGMII-catalyzed hydrolysis of 5FGulF reveals no significant changes. These three separate structures suggest that a 1S5 skew boat conformation would also be adopted by the covalent intermediate formed during hydrolysis of the natural substrate by dGMII.

**DISCUSSION**

**Implications of the 1S5 Skew Boat Conformation in the Intermediate Structure**—From a catalytic standpoint there are several possible advantages for the glycosyl moiety in the glycosyl-enzyme intermediate adopting a 1S5 skew boat conformation. In the deglycosylation step, a nucleophilic water molecule attacks the anomeric carbon, displacing Asp-204. By adopting this conformation steric clashes between the syn-hydrogens at C-3 and C-5 and the attacking water are minimized. Kinetic

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**Fig. 6.** a, comparison of the structure of the covalent intermediate formed during 2FManF hydrolysis (right) with that of the –1 subsite sugar in the Michaelis complex of β-mannanase (left) (21). b, comparison of the conformations adopted by the sugar in the glycosylation of a β-retaining mannosidase (top) and deglycosylation steps of an α-retaining mannosidase (bottom).
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isotope effect analyses conducted on other glycosidases have suggested that the sugar ring passes through a transition state conformation in which C-2, C-1, O-5, and C-5 are in a plane (14, 15). The 1S₂ skew boat conformation positions these atoms so that they are closer to the position required for a planar transition state. The skew boat conformation also brings the 2-hydroxyl group to a pseudo-equatorial position, further decreasing the energy barrier to the transition state species. Interestingly, this conformation also places the leaving group anti-periplanar to the lone pair of the ring oxygen, a requirement for departure of the leaving group according to Deslongchamps’ anti-periplanar lone-pair hypothesis (34).

Structural Commonalities of α- and β-Glycosidases—Recently, the three-dimensional structure of a β-mannanase from Pseudomonas cellulosa (endo-β-mannosidase) was determined at various points along its reaction pathway (21). Interestingly, a comparison of the structure of the sugar in the Michaelis complex of the β-mannanase structure with that of the covalent intermediate formed during 2FManF hydrolysis by D341N mutant dGMII shows that the conformations adopted by these two structures are essentially identical (Fig. 6a). In this “β-mannosidase” structure, the substrate bound is 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-mannosidose. In this structure, two histidines provide hydrogen bonds to the 2- and 3-hydroxyl groups, which may help to distort the −1 subsite to the skew boat conformation. Although the aglycon does not sterically clash with the 2-hydroxyl group, it forces the axial directionality for the scissile bond as it binds to the +1 subsite. It is interesting that seemingly quite different active sites can result in the sugar adopting similar conformations.

This commonality in the conformation of the bound sugar in the α- and β-mannosidases makes mechanistic sense. During the glycosylation step, the β-mannanase transforms a β-mannosyl species into an α-mannoside, whereas in the deglycosylation step of dGMII a β-mannoside is transformed into an α-mannose (Fig. 6b). Because the two steps involve similar transformations, it may be that a common conformational itinerary is followed, presumably through similar transition states. From the results reported herein coupled with suggestions from other inhibitor-dGMII complexes under refinement in our laboratory (33), an itinerary involving an axial transition state would be predicted for the deglycosylation step of dGMII. This is in accord with the discussion above suggesting similarity to the β-mannanase mechanism, which follows a 1S₂ → B₂,₅ → 0S₂ itinerary (19, 21).

Most α- and β-retaining glycosidases are thought to go through a double displacement mechanism in which transition states have considerable oxocarbenium ion character. In this mechanism, the transition state must adopt a conformation in which C-2, C-1, O-5, and C-5 are in a plane. In a pyranose ring, the conformations for which this is the case are the 2B and B₂,₅ boat conformations and the H₄ and H₅ half-chair conformations (14). Therefore, as pointed out in Refs. 21 and 35, there are only a limited number of pseudo-rotational itineraries that glycosidases can take to accommodate this restriction. This is seen from the Michaelis complex and covalent intermediates structure of a number of α-glycosidase (36). It is therefore likely that, analogous to the mannosidases, the sugar in the Michaelis complexes of β-retaining glycosidases will adopt similar conformation as the sugar in the covalent intermediate of α-retaining glycosidases with the same glycon specificity.

Based upon observed 1S₂ skew boat conformation (or a conformationally very similar 1₄-B boat conformation) in the Michaelis complexes (or in some cases, the product complex) of a number of β-glucosidases, along with C₁ chair conformations for the covalent intermediates, a pseudo-rotational itinerary of 1S₂ → H₄ → C₁ seems likely for the deglycosylation step for this group of enzymes. By analogy, from the results reported herein, an itinerary of C₁ → H₄ → 1S₂ would be predicted for the glycosylation step of α-glucosidases.

The only structure to date of a covalent intermediate on an α-glucosidase is that of the family 13 transglycosylase cycloheximide glucosyltransferase, which clearly shows the intermediate sugar to be bound in a C₁ chair conformation in apparent conflict with this prediction. However, because CGTase is not a true glycosidase and instead carries out a transglycosylation reaction, it is possible that CGTase has evolved a mechanism for minimizing nucleophilic attack on the glycosyl-enzyme intermediate by an incoming water molecule. This may well involve a conformational change of the glucose moiety in the −1 subsites followed by the binding of the acceptor sugar to the +1 subsite. If this is the case, then it is possible that the active conformation of the −1 subsite sugar in the covalent intermediate of CGTase is a skew boat conformation. An answer to this question will therefore require further structural studies.

CONCLUSIONS

Although substrate distortion has been seen in β-retaining glycosidases, no significant substrate or intermediate distortion has been seen previously in α-retaining glycosidases. The three structures presented in this paper clearly point to a covalent intermediate that is distorted to a 1S₂ skew boat conformation during hydrolysis of α-mannosides by dGMII. The conformation adopted by the covalently bound sugar is similar to that of the sugar bound in the Michaelis complex of a β-retaining mannosidase, suggesting that there are common structural elements in the reaction pathways of α- and β-retaining mannosidases and, presumably, other retaining glycosidases. In considering the α- and β-retaining glycosidases in general, although they operate by a common overall mechanism via a covalent glycosyl-enzyme intermediate, the details of the intermediate conformations, in particular with respect to saccharide ring distortions, are distinct. Although the covalent intermediate is seen in a low energy sugar conformation in a number of β-retaining glycosidases, the results presented here suggest sugar ring distortion in the covalent intermediate as a feature of the α-retaining enzymes.

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