Geometric Attributes of Retaining Glycosyltransferase Enzymes Favor an Orthogonal Mechanism

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Abstract

Retaining glycosyltransferase enzymes retain the stereochemistry of the donor glycosidic linkage after transfer to an acceptor molecule. The mechanism these enzymes utilize to achieve retention of the anomeric stereochemistry has been a matter of much debate. Re-analysis of previously released structural data from retaining and inverting glycosyltransferases allows competing mechanistic proposals to be evaluated. The binding of metal-nucleotide-sugars between inverting and retaining enzymes is conformationally unique and requires the donor substrate to occupy two different orientations in the two types of glycosyltransferases. The available structures of retaining glycosyltransferases lack appropriately positioned enzymatic dipolar residues to initiate or stabilize the intermediates of a dissociative mechanism. Further, available structures show that the acceptor nucleophile and anomeric carbon of the donor sugar are in close proximity. Structural features support orthogonal (front-side) attack from a position lying ≈90° from the C1-O phosphate bond for retaining enzymes. These structural conclusions are consistent with the geometric conclusions of recent kinetic and computational studies.

Introduction

Understanding the fundamental structure-function relationships of glycosyltransferase enzymes is an essential step in the directed development new drugable inhibitors. Glycosyltransferases synthesize biological oligo- and polysaccharides, many of which have been associated with disease processes. In addition to being the biochemistry i.e. an axial donor becomes equatorial in the product. Leloir glycosyltransferases donate a monosaccharide unit from a nucleotide-sugar (“glycosyl donor”) to a “glycosyl acceptor”, typically a hydroxyl group of an oligosaccharide [23]. Two stereochemical classes are known. Retaining glycosyltransferase enzymes preserve the stereochemistry about the anomeric carbon atom of the donor sugarin the new glycosidic linkage i.e. an axial donor stereochemistry results in an axial stereochemistry in the product. Inverting glycosyltransferases invert the anomeric stereochemistry i.e. an axial donor becomes equatorial in the product. The mechanism of the inverting reaction is widely accepted and is mechanistically straightforward; the acceptor hydroxyl acts as a nucleophile and approaches the anomeric carbon from the opposite side to the donor-nucleoside linkage eventually resulting in inversion of anomeric stereochemistry as the nucleoside leaves. The mechanism for retaining glycosyl transfer stereospecificity is more problematic and remains a matter of debate. Mechanisms can be broadly classified as proceeding with primarily dissociative (SA1) or primarily associative (S2) character. Postulate mechanisms are outlined in Figure 1.

The earliest mechanism proposed to explain retaining glycosyl-transfer was the double displacement mechanism (Fig. 1A): an initial nucleophilic substitution provided by an enzyme nucleophile forms an inverted covalent enzyme-carbohydrate intermediate which is in turn attacked by the acceptor molecule leading to a net retention of anomeric stereochemistry [24]. Each step of this process occurs as described above for inverting glycosyltransferases via backside attack through a single transition state resulting in inversion (Fig. 1B). Although there is mass spectrometric evidence that migh support the existence of a covalent glycosyl-enzyme intermediate in retaining glycosyltransferases [25,26], such an intermediate has not been detected structurally, kinetically or spectroscopically [27–33].

Retaining substitution with dissociative character (Fig. 1D,E) has been proposed as an alternative [27–33]. Nucleotide diphosphates (NDPs) are excellent leaving groups, and the resulting oxocarbenium cation could be stabilized by adjacent protein dipoles. Both of these factors would favour a dissociative process. However, unimolecular dissociation would result in the loss of stereochemical integrity. No partially inverted products have ever been described for a retaining glycosyltransferases. Enzymes that do involve dissociative character are hydrolases or transferases that do not transfer stereocenters (reviewed in [34]). Thus proposed dissociative pathways also require that steric hindrance is provided by the enzyme to force the generation of retained product (Fig. 1E).
Another dissociative variant is called $S_{N_i}$ (Nucleophilic Substitution with internal return; Fig. 1D). This mechanism involves partial nucleotide diphosphate dissociation and charge development within a polar active site cage prior to nucleophilic attack by the acceptor [35]. This is usually drawn as a dissociative transition state with a long distance interaction between the anomeric carbon atom and the incoming nucleophile and a shorter interaction with the departing leaving group. This leads to a short-lived intermediate ion pair which rapidly collapses in a second step. $S_{N_i}$ has previously been invoked to explain gas-phase chemical reactions, but its acceptance as a suitable pathway for retaining glycosyltransferases has met with resistance [25,26].

Recent kinetic investigation of trehalose-6-phosphate synthase, a metal-free retaining glycosyltransferase, concludes that the available evidence favors a “front-side $S_{N_i}$” intermediate having substantial dissociative character at the rate-limiting transition state [36]. The same conclusion is supported by kinetic and computational studies of the solvolysis of isotopically labeled $\alpha$-D-glycopyranosyl fluorides in hexafluoro-2-propanol in which the kinetic isotope effects are most consistent with a “front-face” geometry [37]; the authors favor a stepwise $S_{N_i}$ mechanism, although the data show a concerted transition-state with both the leaving group and the incoming nucleophile in close proximity to the anomeric carbon gives closely similar computed isotope effects. Finally, a computational study of lipopolysaccharide-$\alpha$-1,4-galactosyltransferase C, a Leloir retaining glycosyltransferase finds a front-side geometry at the transition state which is described as “$S_{N_i}$-like” with significant charge development in the donor sugar as the transition state is reached [38].

It has also been suggested that retaining transfer may contain both dissociative and associative elements [39], and the two are not mutually exclusive. Absolute distinction between associative...
and dissociative reaction pathways is not always possible; dissociative pathways progress into associative pathways as the transition state develops a less stable and shorter-lived oxocarbenium cation intermediate [40]; the mechanism illustrated as Fig. 1C reflects this continuum from the SN1 mechanism of Fig. 1D, involving a discrete if short-lived intermediate, to the SN2 case of Fig. 1B involving only a single transition state without an intermediate. The precise character of the Fig. 1C mechanism depends upon the intimate details of charge development and nucleophilic attack (vide infra). We use the term “orthogonal” for this mechanism to mean a process involving the nucleophile and the leaving group on the same side (a.k.a. “front-side” attack) where the approach of the nucleophile is approximately orthogonal to the breaking bond axis, and proceeding in a single step from reactants to products without an intermediate.

Knowledge of the mechanistic details of glycosyltransferases can be derived from a number of experimental approaches of which structural studies play a central role in providing starting points for computation, and geometrical constraints on the enzymatic groups required to interpret the kinetics. The modest degree of sequence homology among glycosyltransferase families has made the prediction of tertiary structures difficult. However, structural determinations in recent years have revealed that the catalytic domains of most glycosyltransferases display one of two fold types designated GT-A or GT-B [41,42]. With few exceptions, the donor binding Rossmann folds of glycosyltransferases contain a “DXD motif” that consists of an Asp-X-Asp amino acid triplet used to coordinate the phosphates of the donor molecule through a divalent cation with octahedral geometry. Some inverting enzymes do not require a divalent metal cofactor, though to date there is only one retaining Leloir-type enzyme that has been characterized as metal independent [43].

A neutron structure of the human retaining enzyme GTA at LANCE PCS (PDB 4DHH associated with [44]) has been reported. More detailed analysis of this structure has revealed an aprotic active site that appears to be incompatible with a dissociative mechanism. To examine the generality of this observation, we report a re-investigation of the published geometric presentation between donor and acceptor substrates in the enzymatic active sites of previously reported GT-A fold glycosyltransferases. The analysis of the structures, together with literature data from NMR, MS, kinetics, and computational studies, point to the orthogonal mechanism for retaining glycosyltransferases as both the simplest and the most consistent with the available data.

Methods

Deposited GT-A fold PDBs identified by CAZy were analyzed for geometric parameters using SetoRibbon, a continued development of SETOR [45] with adaptations for high throughput geometric analysis. Of the eleven families with deposited structures (Table 1), four were found which had unambiguous densities complete for donor (nucleotide and monosaccharide) and acceptor molecules (or analogs): GT-6 retaining enzyme human blood-
group A glycosyltransferase, GTA [L266M/G268A [46]]; GT-7 inverting enzyme 1,4-galactosyltransferase T1, GalT1 (wt [47]); GT-8 retaining enzyme lipooligosaccharide transferase C, LgtC (C128/174S [48]); and GT-43 inverting enzyme β-1,3-glucuronosyl transferase 1, GlcAT-1 (M344H [49,50]). Retaining enzymes GTA and LgtC were both crystallized with deoxy-acceptor analogs, which allowed confident modeling of their respective nucleophilic atoms. Inverting enzymes GlcAT-I and GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable. For example unambiguous density for the carbohydrate moiety of GalT1 required combining separate donor-bound and acceptor-bound structures for analysis, and the accuracy of the analyzed geometry is potentially reduced as the same steric constraints as the bisubstrate liganded active site are not necessarily applicable.

Figure 3. Reaction center dipoles. Opposed to the placement of the acceptor nucleophile (Green spheres), the closest polar residues to leaving group β-phosphate O3 and C1 lay acutely (67° and 75°, respectively) for inverting enzymes (A,B) and lie nearly in-line (171° and 155°, respectively) for retaining enzymes GTA (C,D). This may help to stabilize the associative intermediates without hindering the opposite angle of attack from the acceptor molecule nucleophile. Also, the O3-C1 vectors lay loosely perpendicular to the enzyme macrodipole vectors to stabilize the inverting transition states (green arrows) (A,B), and loosely parallel to stabilize the retaining transition states (C,D) (green ⦒, dipole oriented with the cationic end above the page and the anionic end in the page).

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Results and Discussion

Analysis of Available Structural Data

It is well accepted that dissociative (S1 or S8) mechanisms require activation and stabilization of the ions as they form [53–55]. In homogenous solution this is usually achieved by a polar protic solvent such as water. Within an enzyme, solvent molecules are thought to be excluded from the proximity to the donor sugar anomicer carbon (C1) electrophile of these enzymes to prevent destructive donor hydrolysis from nucleophilic attack by water. The neutron structure of GTA shows no water in proximity to the active site; indeed, the active site is aprotic to a distance of >4.5 Å from the reaction site. In the absence of solvent it falls upon the enzyme to provide correctly positioned and oriented dipoles with
which to stabilize anion pair intermediates. The closest observed enzymatic polar groups to donor sugar C1, β-phosphate nucleofuge (atom O3) and ring O5 for a number of retaining and inverting enzymes are outlined in Table 2. These centers would share the charge of an intermediate oxocarbeniumion as would develop in a SN1o rS Ni process. All lie too far away (~4.5 Å) to initiate a dissociative mechanism, but may extend the lifetime of a dipolar transition state.

For retaining enzymes GTA and ManT the closest nucleophiles to C1 (Glu303 and Asp167, respectively) could be considered candidate nucleophiles for a double displacement reaction, however structurally conserved nucleophiles are absent in many reported retaining enzymes including LgtC and Extl2 which have

### Table 1. GT-A fold glycosyltransferase families with deposited structures.

| Family   | Example enzyme | Stereo-specificity | Example Complex(es) |
|----------|----------------|-------------------|---------------------|
| GT-2     | SpsA           | Inverting         | UDP                |
| GT-6     | GTA            | Retaining         | UDP-Gal-Gal-Fuc    |
| GT-7     | GalT1          | Inverting         | UDP-Gal, GlcNAc-GlcNAc |
| GT-8     | LgtC           | Retaining         | UDP-Gal-Gal-Glc    |
| GT-13    | GntT           | Inverting         | UDP-GalNAc, UDP-Glc |
| GT-15    | Kre2           | Retaining         | GDP+Man+GlcNAc     |
| GT-27    | GNAC-Pep       | Retaining         | UDP+GlcNAc         |
| GT-43    | GlcAT-I        | Inverting         | UDP-GlcUA, UDP+Gal-Gal-Xyl |
| GT-55    | MpgS           | Retaining         | GDP-Man             |
| GT-64    | Extl2          | Retaining         | GDP+GalNAc         |
| GT-78    | MgS            | Retaining         | GDP                |
| GT-81    | ManT           | Retaining         | GDP                |

Bold underlined families were assessed to have unambiguous whole acceptor and donor molecule electron density for analysis; those in italics have donor density. doi:10.1371/journal.pone.0071077.t001

### Table 2. Active site residue identities and geometric values.

| Stereospecificity | Inverting | Retaining |
|-------------------|-----------|-----------|
| Example enzyme    | GlcAT-I   | GalT1     | GntT | LgtC | GTA | Extl2 | ManT |
| PDB(1)            | 1V84      | 1TVY      | 2AM3 | 1GA8 | 2RJ7 | 1OMZ  | 2WVL |
| PDB(2)            | 1KWS      | 1TW5      |      |      |      |       |
| Nu – C1 dist.     | 4.4 Å     | 4.2 Å     | 4.0 Å* | 2.2 Å | 2.5 Å |
| <Nu-C1-O3         | 160°      | 150°      | 151 °  | 90°  | 74°  | NA    | NA    |
| Nu – O3 dist.     | 5.8 Å     | 5.6 Å     | 5.4 Å* | 2.8 Å | 2.2 Å |
| O3– nearest polar X | H2Ob | K279      | Y184 | H78  | K346 | H2O  | Y268  |
| O3- X dist.       | 4.4 Å     | 4.4 Å     | 5.4 Å  | 4.7 Å | 5.6 Å | 3.8 Å | 4.4 Å |
| <X-O3-C1          | 91°       | 80°       | 87°   | 171° | 149° | 131°  | 59°   |
| C1 nearest polar Y | H308 | W314      | D211 | Q189 | E303 | R293  | D167  |
| C1-Y dist.        | 3.6 Å     | 4.5 Å     | 5.2 Å  | 3.5 Å | 4.8 Å | 3.7 Å | 3.5Å  |
| <Y-C1-O3          | 67 °      | 75°       | 71°   | 162° | 155° | 167°  | 142°  |
| O5 nearest polar Z | R156 | W314      | D291 | Q189 | R352 | R293  | D168  |
| O5-Z dist.        | 5.9 Å     | 3.4 Å     | 3.9 Å  | 4.2 Å | 5.8 Å | 3.2 Å | 3.8 Å |
| <Z-O5-C1          | 113°      | 123°      | 96°   | 82°  | 83°  | 97°   | 68°   |
| c                 | H2Ob      | H2Ob      | H2Ob  | D103 | D211 | H2Ob  | NA    |
| <b/Mc              | 89°       | 82°       | 87°   | 105° | 116° | 101°  | NA    |
| f                 | D196      | H347      | H2Ob  | D105 | D213 | H2Ob  | N313  |
| <b/Mf              | 114°      | 104°      | 95°   | 92°  | 90°  | 88°   | 82°   |

With the exception of the GTA neutron diffraction studies [44] hydrogen atoms are not directly observed, so distances are given between centers of non-hydrogen atoms. Enzyme names indicate the model did not contain an acceptor molecule.

*PDB 2AM3 has a glycerol molecule modeled as an acceptor.

It is likely that the active species are not actually water molecules, but residues in disordered regions of the polypeptide.

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1. 1. Nomenclature and abbreviations for GT-A fold.
2. 2. Methods.
3. 3. Results.
4. 4. Discussion.
5. 5. Conclusions.
6. 6. References.

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respective Gln and Arg residues in this position. The closest polar groups to donor sugar C1, O5 and phosphate O3 vary considerably, can carry either positive or negative charges, and often their mutation does not inhibit catalysis (e.g. [25]). Furthermore, glycosyl transfer still proceeds when O5 is substituted with sulfur [56], and as such an intermediate thiocarbenium ion is unlikely to be stabilized to the same extent by donation from sulfur as in a regular oxocarbenium intermediate.

It is noteworthy that the active site architecture for proximal dipoles is conserved and distinct for retaining and inverting enzymes (Fig. 3, Table 2). For the inverting enzymes the C1 bond to the leaving group O3 lay at acute angles from adjacent polar residues (Fig. 3A: 67° to His308 Ne of GlcAT-1; Fig. 3B: 80° to Lys279Nε and 75° to Trp314 Nε of GalT1). In retaining enzymes the corresponding angles are obtuse (Fig. 3C: 162° to His78 Ne of LgtC; Fig 3D: 155° to Lys 346 Nε and 155° to Glu303 Oε of GTA). Significantly, this positions the polar groups and enzyme macrodipoles that stabilize the retaining and inverting transition states to lie approximately orthogonal to each other (Fig. 3). The orientations of the protein macrodipoles are conserved among retaining enzymes where they lie roughly perpendicular to the nucleophile approach as expected for stabilization of developing partial cationic charge without influencing leaving group departure or nucleophilic attack. The macrodipoles of inverting enzymes are similarly conserved, but are oriented parallel to the line of nucleophile approach, oriented to assist such an attack.

Further, the proximity of nucleophile (Nu) and electrophile (C1) in the retaining enzymes places tight constraints on the extent of dissociation possible before nucleophilic approach becomes the dominant interaction. The position of acceptor nucleophiles modeled from deoxy-acceptor crystal structures are observed at distances much less than 3 Å (2.5 Å for GTA and 2.2 Å for LgtC) from donor C1, whereas they would be expected to reside greater...
than 3 Å away to allow UDP dissociation prior to nucleophilic attack [57]. The computed transition states for glucopyranosyl fluoride solvolyces place the acceptor oxygen 3.02 Å from C1 in the S_{N}1 transition state and at 2.25 Å in the associative “front side” transition state [57]; the corresponding distance computed for the “S_{N}2-like” transition state of a galactosyl transferase is 2.3 Å [58]. Nu and C1 can be much greater than 3 Å in a precatalytic conformation as is observed for both modeled systems of inverting enzymes (4.4 Å for GlcAT-I and 4.2 Å for GaT1).

Comparing the biologically active GTA structures to inverting enzymes such as galactosyltransferases [44] GaT1 reveals that the enzymes bind distinct metal-nucleotide-sugar conformers (Figs. 2 & 3), where the metal coordinating angle <b-M-c is less than <b-M-f for inverting enzymes and the opposite for retaining enzymes (Table 2). Inverting enzymes position C1 for inline nucleophilic attack from the acceptor at an angle nearly 180° to the leaving group, while retaining enzymes position these groups at roughly 90° with respect to the C1-leaving group axis (Fig. 2 165° inverting GaT1; 74° in retaining GTA). This is accomplished by inverting and retaining enzymes orienting their metal-nucleotide-sugar binding Rossmann folds approximately perpendicular to one another (Figure 4). Although geometrically distinct, in-line (inverting) and orthogonal transition states are not dissimilar; retaining enzymes apparently orient their acceptors to an apical position of a trigonal bipyrimal transition state with the leaving group occupying one of the equatorial positions. This orientation is formally accessible as a pseudo-rotation of the trigonal bisaccharide geometry of the S_{2}2 transition state, which is facilitated by structurally conserved obtuse oriented enzymatic dipoles for retaining enzymes, and is complemented by conserved acute dipoles for inverting enzymes (Fig. 3). Concurrent opening of the H-C1-Nu angle would result in associative retention of the donor’s anemic stereochemistry (Fig. 1C). A similar reaction pathway has been suggested based on structural studies involving glycomimetic inhibitors [36,58] and quantum chemical calculations [59], however the proposed mechanisms were still referred to as “S_{N}1-like” implying the mechanism proceeds with a rate-limiting associative transition state and an intermediate of some finite lifetime.

Analysis of other Published Data

NMR analysis of donor hydrolysis facilitated by retaining glycosyltransferase enzymes in the absence of acceptor indicates that the cleaved monosaccharides are attacked by water from a retained position [59,60]. This is inconsistent with a dissociative mechanism, as the steric constraints imparted by the enzyme’s fully liganded closed position would not be at play, with solvent molecules occupying both equatorial and axial positions.

Glycosyltransferases are bi-substrate enzymes and some mechanistic features can be inferred from the overall kinetic schemes observed. Double displacement should follow ping-pong kinetics as it develops a covalent intermediate, which can be identified on a Lineweaver-Burke plot as parallel lines at varied donor substrate concentrations; as is seen for trans-sialidase, for which such a mechanism has good precedent [61]. This is not observed for retaining glycosyltransferases such as MshA, asingle values for the acceptor K_M have been reported even when detailed bisubstrate Michaelis–Menten kinetic data has been collected [62]. This kinetic evidence does not support a 2-step mechanism with a covalent intermediate for this Leloir retaining glycosyl-transferase.

The double displacement mechanism has strong precedent for enzymes that do not use metallic co-factors (reviewed in [34]) such as glycoside hydrolases, in which covalent glycosyl-enzyme intermediates have been trapped in crystal structures by using fluororilated substrates (e.g. [63,64]). Such a species should be easier to trap for a glycosyltransferase as the strong donor leaving groups would leave a covalent sugar-enzyme intermediate in an energy well with the second attack being the rate limiting step [39], and there have been intensive attempts to trap such an intermediate. The only reports of enzyme-glycosyl intermediates have come from two independent ESI-MS studies, which identified apparent covalent intermediates using postulate nucleophile mutants [25,26]. One case showed the covalent species substituted remotely from the acceptor and produced at a rate much slower than enzymatic turnover, an observation of limited relevance to the catalytic mechanism. The other case showed the enzyme-glycosyl intermediate bound to the mutated cysteine; however, such an intermediate has not been observed by means other than MS. It has been suggested that these species could be the results of charged carboxation monosaccharides introduced in the gas phase by the electrospray conditions that undergo reaction with enzyme nucleophiles to produce such glycosylated species (reviewed in [65]). Kinetic isotope effect data [36] are also strong evidence against a stable covalent intermediate.

While double-displacement should follow ping-pong bi-substrate kinetics, S_{N}1 and associative mechanisms should follow either random associative or Theorell-Chance mechanisms. The latter is followed by a retaining galactosyl transferase [66]. The distinction between these is whether or not the ternary species builds to an extent that is kinetically significant. A developed S_{N}1 intermediate must avoid water attack, so a well-structured ternary complex formed in a random associative scheme is a reasonable possibility. There is no need for a long-lived ternary complex in an orthogonal mechanism. Thus the observation of Theorell-Chance kinetics is consistent with, but does not compel an orthogonal mechanism for the group transfer transition state.

Mechanistic Proposal

The foregoing establishes that there is little direct evidence that Leloir retaining glycosyltransferases utilize a double-displacement or a fully developed S_{N}1 mechanism (Fig. 1A or E). The focus therefore shifts to the dissociative pathway S_{N}2 and the orthogonal pathway (Fig. 1D or C). The distinction between an S_{N}and an orthogonal mechanism is found in the reaction profile and the timing of bond formation and bond breakage: if nucleophilic attack precedes or is concurrent with leaving group dissociation [59] with no enzymatic cage required to stabilize an oxocarbenium intermediate then there can be little dissociative character to the mechanism. The physical organic literature describes an associative mechanism as A_{N}D_{S} indicating a single transition state with association of the nucleophile fully concurrent with departure of the leaving group. An alternative in which dissociation is slightly ahead of association would be D_{A}A_{N} but in this case as well there is a single transition state without an intermediate. The S_{N}2 pathway must involve an intermediate in a two-step process. It is described as D_{S}A_{N}D_{S} or D_{N}A_{S}A_{N} [66] with the notations denoting differing depths of the energetic well occupied by the intermediate of the two-step process.

The available structural and kinetic data presented above are most consistent with an orthogonal mechanism of the D_{N}A_{S} type. The assumed geometric and energetic consequences of the various relevant transition states and intermediates are sketched in Figure 5 to visually highlight the distinctions. The geometrical changes of the alternative mechanisms are illustrated in the More O’Ferrall-Jencks diagram (Fig. 5 left). The geometric consequences of the orthogonal mechanism are that the bond-making and bond-breaking phases are more closely coordinated than in
the S\textsubscript{N}1 trajectory. The energetic consequences are given in Fig. 3 right with the curves offset for clarity. The orthogonal mechanism involves a single barrier without intermediate, while the S\textsubscript{N}1 and the more dissipative S\textsubscript{N}2 reaction profiles involve and intermediate with two transition states. The geometric location of the transition states is indicated in Fig. 3 left with asterisks. The proposed orthogonal transition state likely lies close in energy to the transition state leading to a S\textsubscript{Ni} intermediate. The key issue is that these pathways differ solely in the number of barriers and intermediates invoked. The D\textsubscript{Ni}A process we favour in fact is identical in energetic profile with the one determined computationally [38]. These authors described their trajectory as “Syl-like”. We disagree with this description as the trajectory does not involve an intermediate, so cannot be S\textsubscript{Ni} by definition [66]; our use of “orthogonal” makes this distinction clearer.

Conclusions

The foregoing structural and kinetic analyses are most consistent with an orthogonal pathway for glycosyltransferases that retain anameric stereochemistry. From the structural perspective, retaining and inverting enzymes are observed to bind and to act upon distinct conformers of the metal nucleotide sugar complex. The donor substrate trajectory architecture observed for retaining enzymes is conserved so as to present the transferring monosaccharide anemic electrophile from an orthogonal orientation. The distances observed between the approaching nucleophile and C1 are too close to support full development of dissociation in the structures of both LgtC and GTA.

A double displacement mechanism requires an appropriately positioned and structurally conserved nucleophile in the active site. The active sites of many retaining enzymes do not contain well-positioned candidate nucleophiles, and those that have been proposed are often not sequentially or spatially conserved. In many cases, alamine mutagenesis of the proposed nucleophiles does not always abolish enzyme activity [26,36].

Structural and kinetic evidence lies in favor of a single step orthogonal displacement. The substitution is positioned to initiate nucleophilic attack and proceed through a trigonal bipyramidal transition state with incoming acceptor

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