Mechanism-based Labeling Defines the Free Energy Change for Formation of the Covalent Glycosyl-enzyme Intermediate in a Xyloglucan endo-Transglycosylase*

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Xyloglucan endo-transglycosylases (XETs) are key enzymes involved in the restructuring of plant cell walls during morphogenesis. As members of glycoside hydrolase family 16 (GH16), XETs are predicted to employ the canonical retaining mechanism of glycosyl transfer involving a covalent glycosyl-enzyme intermediate. Here, we report the accumulation and direct observation of such intermediates of PttXET, which yield relatively stable, kinetically competent, covalent glycosyl-enzyme adducts. The free energy change (ΔG0) for the formation of the covalent GalGXXXG-enzyme as 6.3–8.5 kJ/mol (1.5–2.0 kcal/mol). The intermediates thus far are those from GH16 and clan GH-B enzymes (11). The canonical retaining mechanism is a double-displacement mechanism in which a covalent glycosyl-enzyme intermediate is formed by attack of an enzyme nucleophile (typically a carboxylic amino acid side chain) on the anomic carbon of the saccharide acceptor, yielding transglycosylation (9, 10) employed by GH16 and clan GH-B enzymes (11). The apparent incongruity in classification has its basis in the anomeric configuration-retaining mechanism of glycolyl transfer (9, 10) employed by GH16 and clan GH-B enzymes (11). The canonical retaining mechanism is a double-displacement mechanism in which a covalent glycosyl-enzyme intermediate is formed by attack of an enzyme nucleophile (typically a carboxylic amino acid side chain) on the anomic carbon of the substrate. The formation and breakdown of this intermediate is facilitated by general acid/base catalysis mediated by a second carboxylic amino acid side chain. The glycolyl-enzyme may partition either to water, yielding substrate hydrolysis, or to a saccharide acceptor, yielding transglycosylation (9, 10). In xyloglucan-active enzymes from GH16, this partitioning gives rise to xyloglucan-specific endo-β1,4-glucanase activity (endo-xyloglucanase, EC 3.2.1.151) and xyloglucan:xyloglucan transferase activity (XET, EC 2.4.1.207), respectively (recently reviewed in Ref. 8). Consequently, the abbreviations XTH (for xyloglucan endo-transglycosylase/hydrolase) is often used to refer to the subfamily of genes that encode these enzymes (2).

Kinetic analyses have shown previously that XETs from GH16 operate via a Ping Pong Bi Bi mechanism (12, 13), whereas evidence for the covalent nature of the glycosyl-enzyme has been provided by trapping catalytically competent xyloglucan-enzyme complexes on solid supports (14). Unfortunately, such complexes are both too heterogeneous and too large to be observed directly by mass spectrometry (MS) or crystallography, which would provide conclusive proof of a covalent adduct in the XET mechanism and allow the elucidation of atomic features of the glycosyl-enzyme. The problem is further complicated by the observation that strict XETs such as...
PttXET16–34 (formerly PttXET16A, 13, 15–17) and BobXET16A (18) do not use activated aryl β-glycosides or β-glycosyl fluorides of xylogluco-oligosaccharides as glycosyl donors (15, 16). This effectively precludes the steady-state accumulation of a glycosyl-XET complex using Withers’s fluorosugar methodology or by manipulation of the relative rates of formation and breakdown of the intermediate using activated donor substrates in combination with mutation of the catalytic acid/base residue (19–23).

On the other hand, XETs use short xylogluco-oligosaccharides containing at least one internal unbranched Glc (G) residue, e.g. XXXGXXXG (see Fig. 1) as glycosyl donors (Refs. 8, 13, and 15 and references therein). However, these substrates are themselves glycosyl acceptors (8, 13), and as shown in this study, attempts to observe a steady-state accumulation of a glycosyl-enzyme using XXXGXXXG were unsuccessful. To overcome these challenges, we synthesized two xylogluco-oligosaccharide donor substrates, GalGXXXG and GalGXXXGXXXG (see Fig. 1), bearing axial C-4 hydroxyl groups on their nonreducing termini. We show that such “blocked” substrates, which are incapable of acting as glycosyl acceptors, act as glycosyl donors for hybrid aspen PttXET16–34, leading to the time- and concentration-dependent accumulation of a catalytically competent, covalent xylogluco-oligosaccharide-XET adduct. Furthermore, quantitative MS and high-performance capillary electrophoresis as described previously (13, 15).

Labeling of PttXET16–34 with the Donor Substrates GalGXXXG (1) and GalGXXXGXXXG (2)—Labeling experiments were performed in a total reaction volume of 24 μl by adding appropriate concentrations of ligand to a solution of PttXET16–34 (17 μM final concentration) in 20 mM sodium acetate buffer (pH 5.5), followed by incubation at 25 °C. The donor substrate concentrations were 1.25 mM for GalGXXXG and 200 or 255 μM for GalGXXXGXXXG. Samples for electrospray ionization (ESI)-MS analysis were withdrawn at selected time points and diluted to final protein concentrations of 1–5 μM in 5% aqueous acetonitrile containing 0.1% formic acid. The PttXET16–34 nucleophile mutant E85G was used in a similar experiment, in which 1 μM GalGXXXGXXXG was added to 30 μM enzyme solution.

Substrate concentrations in the range 0–1880 μM were used in a titration experiment of PttXET16–34 with GalGXXXGXXXG. Samples for ESI-MS analysis were taken after a reaction time of 2 min.

To follow the hydrolysis rate of the xylogluco-oligosaccharide PttXET16–34 intermediates, the excess donor substrates were removed by ultrafiltration of the enzyme reaction mixture using 10-kDa Biomax centrifugal filters (Millipore), followed by re-dilution with 20 mM NaOAc (pH 5.5; 3 × 100 μl). After buffer exchange, samples were withdrawn at appropriate time intervals for ESI-MS analysis. Reactivation of dialyzed GalG and GalGXXXG-PttXET16–34 with XXXG was performed by adding 1.25 mM and 20 μM oligosaccharide, respectively, to the labeled enzyme.

### EXPERIMENTAL PROCEDURES

**General**—Ultrapure water with a resistivity (ρ) = 18.2 mega-ohms/cm was produced using a Milli-Q system (Millipore) and used in all experiments. All other chemicals and solvents were of reagent grade or better and were obtained from Sigma, Aldrich, or Fluka unless noted otherwise.

**Proteins**—Xyloglucan endo-transglycosylase-(16–34) from the hybrid aspen *Populus tremula × tremuloides* (PttXET16–34, formerly PttXET16A, GenPept AA87142) was expressed in *Pichia pastoris* as described previously (17). The enzyme concentration was determined from *A*280 measurements (68490 M⁻¹ cm⁻¹). The PttXET16–34 catalytic nucleophile variant E85G was produced using standard site-directed mutagenesis techniques (24). Horse heart myoglobin was obtained from Sigma as a powder and dissolved in ultrapure water prior to dilution to 6 μg/ml (0.36 μM) in 5% aqueous acetonitrile containing 0.1% formic acid.

**Synthesis and Kinetic Analysis of Xylogluco-oligosaccharide Donor Substrates** (see Fig. 1) GalGXXXG (1, β-d-Galp(1→4)-β-d-GlcP(1→4)-[α-d-Xylp(1→6)]-β-d-GlcP(1→4)-[α-d-Xylp(1→6)]-β-d-GlcP(1→4)-β-d-GlcP(1→4)-β-d-GlcP(1→4)-β-d-GlcP(1→4)-[α-d-Xylp(1→6)]-β-d-GlcP(1→4)-β-d-GlcP(1→4)-β-d-GlcP(1→4)-[α-d-Xylp(1→6)]-β-d-GlcP(1→4)] and GalGXXXGXXXG (2, β-d-Galp(1→4)-β-d-GlcP(1→4)-[α-d-Xylp(1→6)]-β-d-GlcP(1→4)-[α-d-Xylp(1→6)]-β-d-GlcP(1→4)-[α-d-Xylp(1→6)]-β-d-GlcP(1→4)-[α-d-Xylp(1→6)]-β-d-GlcP(1→4)-[α-d-Xylp(1→6)]-β-d-GlcP(1→4)-β-d-GlcP(1→4)-β-d-GlcP(1→4)-β-d-GlcP(1→4)]

![Image](https://via.placeholder.com/150)

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**Trapping of Covalent Glycosyl-XET Intermediates**

To follow the hydrolysis rate of the xylogluco-oligosaccharide PttXET16–34 intermediates, the excess donor substrates were removed by ultrafiltration of the enzyme reaction mixture using 10-kDa Biomax centrifugal filters (Millipore), followed by re-dilution with 20 mM NaOAc (pH 5.5; 3 × 100 μl). After buffer exchange, samples were withdrawn at appropriate time intervals for ESI-MS analysis. Reactivation of dialyzed GalG and GalGXXXG-PttXET16–34 with XXXG was performed by adding 1.25 mM and 20 μM oligosaccharide, respectively, to the labeled enzyme.
age model, and 0.4-Da peak width at half-height). The selected m/z range included all major multiple-charged ([M + nH]+) peaks of PttXET16–34 and glycosyl donor adducts. Calculation of the relative proportions of free and glycosyl-enzymes was based on the peak heights of the two major glycoforms of PttXET16–34 (calculated masses of 33,854 Da (GlcNAc2Man9) and 34,016 Da (GlcNAc2Man9)17) relative to their glycosyl-enzyme counterparts. All peaks were symmetrical; use of peak areas from centroid spectra gave indistinguishable results when tested. This analysis assumes identical chromatographic behavior (protein species were always observed to co-elute) and ionization efficiencies (charge-state distributions appeared to be unaltered) of the free and glycosyl-enzymes.

Oligosaccharide Analysis by HPAEC-PAD—Analysis of oligosaccharide products formed by the reaction of PttXET16–34 with GalGXXXGXXGXXG was performed using the HPAEC-PAD method (gradient B) as described previously (27). Samples analyzed by liquid chromatography-MS were flash-frozen in liquid N2, thawed by addition of NaOH (5 M), and analyzed by liquid chromatography-MS were flash-frozen in liquid N2, thawed by addition of NaOH (5 M), and placed in a cooled autosampler compartment (8 °C) prior to HPAEC-PAD. Chromatographic peaks were identified by direct comparison with known standards, except in the case of GalGXXXGXXGXXG, which were identified by extrapolation of the retention times of XXXG, GalGXXXG, (XXXG)2, GalG(XXXG)2, (XXXG)3, and (XXXG)4. Molar detector response factors of XXXG, GalGXXXG, (XXXG)2, and GalG(XXXG)2 were determined from linear plots of oligosaccharide concentration versus PAD signal. For quantitation of GalG(XXXG)3, the response factor for GalG(XXXG)2 was used, which may underestimate the concentration of this product by 10%.

**RESULTS**

Labeling of PttXET16–34 with the Donor Substrate GalGXXXGXXG (1)—It has been shown previously that xyloglucanases and XETs preferentially cleave the glycosidic bond of unbranched glucose residues (G units) in xyloglucan oligo- and polysaccharide donor substrates (8, 13). The novel oligosaccharides GalGXXXGXXG (1) and GalGXXXGXXGXXG (2) were synthesized as minimal glycosyl donor substrates for PttXET16–34 that would allow formation of a covalent glycosyl-enzyme intermediate in the first step of the canonical retaining mechanism (10) but would be incapable of acting as glycosyl acceptors because of the presence of the axial 4-OH on the terminal galactosyl residue. For both substrates, it was anticipated that productive binding of the shared GalGXXXG motif in the negative subsites of PttXET16–34 (16) would lead to formation of a common GalGXXXG-XET intermediate with concomitant production of GG or XXXG from substrates 1 and 2, respectively (Fig. 1).

Unexpectedly, the kinetics with GalGXXXGXXG and XXXG-ANTS as donor and acceptor substrates, respectively, showed transfer of the GalG moiety to the acceptor, whereas no GalGXXXG transfer was observed (Table 1). Consistent with this observation, protein MS analysis of the reaction of GalGXXXGXXG with PttXET16–34, in the absence of a competent acceptor substrate, indicated that the enzyme cleaved the substrate after the third X unit from the reducing end of the oligosaccharide to yield a GalG-PttXET16–34 glycosyl-enzyme. Fig. 2A shows the reconstructed ESI-MS of recombinant PttXET16–34 (17), observed as three enzyme isoforms bearing GlcNAcMan9–10 N-glycans (calculated masses of 33,854, 34,016, and 34,178 Da). Time-dependent accumulation of a GalG-PttXET16–34 intermediate with 1:1 stoichiometry (calculated mass increase of 324.3 Da and observed mass increase of 324 Da) can be clearly observed after 4 h (Fig. 2B) and 26 h (Fig. 2C) reactions with 1.26 mM GalGXXXGXXG. Following rapid (~20 min) buffer exchange of the 4-h sample by ultrafiltration to remove unreacted substrate (Fig. 2D), the covalent intermediate was observed to be hydrolytically stable over a period of 30 min (Fig. 2E) to 20 h (essentially identical to Fig. 2E; data not shown). The high mass resolution of the ESI-time-of-flight-MS, in addition to the data on the dialyzed sample, allowed the covalent nature of the intermediate to be assigned unambiguously; noncovalent binding of lactose (Galβ(1→4)Glc) would yield well resolved peaks 18 Da higher than those observed.

**TABLE 1**

| Substrate | Transglycosylation product | Leaving group | Initial rate, v0[E−]| Rate relative to XXXGXXGXXG |
|-----------|---------------------------|---------------|-------------------|---------------------------|
| XXXGXXGXXG | XXXGXXGXXG-ANTS | XXXG | 78.4 | 100 | Ref. 15 |
| XXXGXXGXXG | XXXGXXGXXG-ANTS | GG | 0.43 | 0.5 | Ref. 15 |
| GalGXXXGXXG | GalGXXXGXXG-ANTS | XXXG | 0.98 | 1.2 | Ref. 25, this study |
| GalGXXXGXXGXXG | GalGXXXGXXGXXG-ANTS | XXXGXXGXXG | ND | | Ref. 25, this study |
| GalGXXXGXXGXXG | GalGXXXGXXGXXG-ANTS | XXXGXXGXXG | 234.3 | 299 | Ref. 25, this study |

* ND, not detectable.

*GalGXXXGXXG-ANTS was not detected up to 3 h under standard conditions but was observed after prolonged incubation (24 h) along with XXXGXXGXXG-ANTS arising from GalGXXXGXXG-ANTS acting as a donor substrate (cleavage site highlighted).
The intermediate was, however, kinetically competent, as demonstrated by incubation of the dialyzed 4-h sample with the xylogluco-heptasaccharide XXXG. After 30 min in the presence of XXXG, nearly all of the enzyme was reverted back to the free form (Fig. 2F). After dialysis, a sample incubated with GalGXXXGGG for 26 h was similarly reactivated after 100 min by incubation with 1.25 mM XXXG (data not shown).

The endolytic cleavage of GalGXXXGGG at the glycosidic bond preceding the lactosyl unit (GalG), rather than that following the cellobiosyl unit (GG), reflected results from kinetic analyses showing that interactions of the donor substrates are dominated by a high affinity of the XXXG moiety for the positive, rather than the negative, subsites in PttXET16–34 (25). As shown in Table 1, glycosyl transfer of GalG from GalGXXXGGG (Fig. 1) onto the fluorescent acceptor substrate XXXG-ANTS (13) occurred at a similar initial rate as transfer of the same group from the homologous donor GalGXXXG. Most notably, transfer of GalGXXXG from GalGXXXGGG onto XXXG-ANTS is not observed by capillary electrophoresis under standard initial rate conditions (up to 3 h of incubation with 3 μM PttXET16–34) (13, 25). 17 μM PttXET16–34 was used in the labeling experiments over 4–26 h. These substrates are nevertheless poor donors for PttXET16–34; initial rates of glycosyl transfer are 1% of that observed for the reference donor substrate XXXGXXXG under the same conditions (Table 1). XXXGXXXG clearly exploits multiple binding interactions in both the positive and negative subsites in PttXET16–34 (15, 25).

**Labeling of PttXET16–34 with GalGXXXGXXXG**—Consistent with the data above, GalGXXXGXXXG was observed to be a facile donor substrate for PttXET16–34, with initial rate data indicating that the predominant mode of cleavage occurs at the glycosidic bond following the reducing end.
XXXG moiety (Fig. 1 and Table 1). Analogous to substrate 1, the presence of galactose at the nonreducing end rendered GalGXXXGXXXG incompetent as an acceptor substrate, in this case allowing rapid accumulation of covalent GalGXXXG-PttXET16–34 enzyme intermediates with heterogeneity arising from variable N-glycosylation of the enzyme (Fig. 3A; cf. Fig. 2A). The mass increase of 1369.5 Da observed for all glycoforms is consistent with a 1:1 stoichiometry between the enzyme and the GalGXXXG label (calculated increase of 1369.2 Da). No labeling was observed when the PttXET16–34 catalytic nucleophile mutant E85G was used. Likewise, labeling was not observed in time course experiments when XXXGXXXG was used as a donor substrate at equimolar and higher concentrations (1702 to 0.5 mM) with the wild-type enzyme (data not shown).

Interestingly, prolonged incubation of substrate 2 (255 μM) with PttXET16–34 resulted in accumulation of species of higher mass than the covalent GalGXXXG-enzyme glycoform intermediates. Mass analysis indicated that these species did not arise from multiple labeling of PttXET16–34 with GalGXXXG moieties but rather from the accumulation of GalGXXXGXXXG-PttXET16–34 over a period of 8 h (calculated mass increase for PttXET16–34 of 2414.1 Da and observed mass increase of 2414.1 Da) (Fig. 3). Analysis of oligosaccharide products by HPAEC-PAD after treatment of similar reactions with NaOH indicated the presence of the expected products XXXG and GalGXXXG, excess substrate 2, and GalGXXXGXXXGXXXG (see “Titration of PttXET16–34 with GalGXXXGXXXG” below). The appearance of this longer oligosaccharide may be explained by kinetically slow cleavage of GalGXXXGXXXG at the glycosidic bond of the lactosyl unit to release XXXGXXXG, which subsequently acts as a glycosyl acceptor for the GalGXXXG-PttXET16–34 intermediate, yielding GalG(XXXG)₃ and free enzyme. A minimal reaction scheme that accounts for all of the observed protein and oligosaccharide species is shown in Fig. 4A. Analysis of the protein MS data as a function of time and [GalGXXXGXXXG] in the range 17–1250 μM indicated that a steady-state was rapidly established for all GalGXXXGXXXG concentrations in <2 min (Fig. 3 and data not shown). Supporting this interpretation, the high-performance capillary electrophoresis analysis of the reaction between GalGXXXGXXXG and XXXG-ANTS showed that GalG transfer occurred only after 90% accumulation of the GalGXXXGXXXG-ANTS initial transglycosylation product (25).

Notably, the GalG-PttXET16–34 intermediate was never observed to accumulate in this system (Figs. 3 and 7). Initial rate kinetic data (Table 1), together with protein MS data (Fig. 2), indicated that formation of this intermediate was slow relative to the rate of formation of GalGXXXG-PttXET16–34. GalG-PttXET16–34 must nevertheless be formed in the reaction sequence leading to the production of GalG(XXXG)₃. The lack of accumulation of GalG-PttXET16–34 implies that this intermediate is rapidly broken down by transglycosylation onto XXXGXXXG (the reverse of its formation) or XXXG produced by the formation of GalGXXXG-PttXET16–34 (Fig. 4A).

Whereas the rate of hydrolysis of GalG-PttXET16–34 was undetectable, as described above, the rate of hydrolysis of GalGXXXG-PttXET16–34, although still extremely slow, was measurable. Fig. 5 shows the protein mass spectra of PttXET16–34 as a function of time after removal of unreacted...
GalGXXXGXXXG by ultrafiltration. Quantitation of the fractional peak heights\(^7\) for the two major glycoforms of the free and glycosyl-enzymes indicated that the half-life for GalGXXXG-PttXET\(_{16–34}\) was 176 ± 12 min (\(k = 0.0057 \pm 0.0004 \text{ min}^{-1}\)) (Fig. 6). In contrast, equilibrium was re-established in <2 min in a reaction containing 200 \(\mu\text{M}\) GalGXXXGXXXG and 17 \(\mu\text{M}\) PttXET\(_{16–34}\) (60% present as the glycosyl-enzyme) after the addition of 20 \(\mu\text{M}\) XXXG (data not shown).

These data are consistent with earlier observations that PttXET\(_{16–34}\) is an overwhelmingly predominant XET (EC 2.4.1.207) with negligible xyloglucan activity (EC 3.2.1.151). Previously, PttXET\(_{16–34}\) was shown to hydrolyze xyloglucan (1 g/liter) at a rate of <0.0076 min\(^{-1}\) (an upper limit based on the assay parameters; no hydrolysis was detected)\(^{17}\). In comparison, characterized endo-xyloglucanases, including Tropaeolum majus NXG1 from GH16 (8), have \(k_\text{cat}\) or \(v_0/\text{[E]}_0\) values in the range 10\(^0\)–10\(^4\) min\(^{-1}\) (see Refs. 8 and 27–29 and references therein).

Titration of PttXET\(_{16–34}\) with GalGXXXGXXXG—The observation that the relative proportions of the free and glycosyl-enzymes could be rapidly shifted according to Le Chatelier’s principle provided one indication that the dominant equilibrium in the reaction mixture was that shown in Fig. 4B. Consequently, titration of PttXET\(_{16–34}\) with substrate 2, together with product analysis by MS and HPAEC, allowed estimation of the \(K_\text{eq}\) and \(\Delta G^0\) of formation of the covalent glycosyl-XET intermediate. Fig. 7 shows the mass spectra of PttXET\(_{16–34}\) following incubation with initial concentrations of GalGXXXGXXXG in the range of 0–1880 \(\mu\text{M}\). In these cases, the reaction time was limited to 2 min, and only the free enzyme and the covalent GalGXXXG-PttXET\(_{16–34}\) adduct were present (Fig. 7). Neither GalG-PttXET\(_{16–34}\) nor the higher order adduct GalGXXXGXXXG-PttXET\(_{16–34}\) accumulated, even at high substrate concentration (cf. Fig. 3). The fractional peak heights for the two major glycoforms were used to quantify the dependence of the proportion of the glycosyl-enzyme on the concentration of GalGXXXGXXXG.

Fitting Equation 1,

\[
\text{Fraction GalGXXXG-XT} = \frac{(\text{fraction GalGXXXG-XT})_{\text{max}}}{(1 + K_{\text{app}}/[\text{GalGXXXGXXXG}]_0)}
\]

(Eq. 1)

to the data shown in Fig. 8 yielded \(K_\text{app} = 104 \pm 14 \mu\text{M}\) and (fraction GalGXXXG-XT)_{\text{max}} = 0.878 ± 0.037, where \(K_{\text{app}}\) is the concentration of GalGXXXGXXXG at 50% labeling. The observed (fraction GalGXXXG-XT)_{\text{max}} value of <1.0 likely reflects, to a large extent, a decline in the concentration of active enzyme relative to total protein; MS data collected on the same

\(7\) In this and subsequent analyses, the use of peak heights was for practical convenience; the highly symmetrical MS peaks yielded identical results when peak areas were used.
Trapping of Covalent Glycosyl-XET Intermediates

PttXET16–34 sample 2 months previous to those shown in Figs. 7 and 8 exhibited a (fraction GalGXXXG-XET) value of 0.93 ± 0.02 at [GalGXXXGXXXG]₀ = 1275 μM. Together, these data imply that in the steady state, the equilibrium assumption that considers only those species shown in Equation 1 is likely to be valid, with only a minor amount (<5%) of free PttXET16–34 potentially formed via alternative pathways (Fig. 4).

HPAEC-PAD Analysis of Oligosaccharide Products Formed from the Action of PttXET16–34 on GalGXXXGXXXG—At short time scales (<10 min) while [GalGXXXGXXXG] was still high and other oligosaccharides were present in low concentration, the pathway involving GalG-PttXET16–34 leading to the formation of GalG(XXXG)₃ (Fig. 4) and accumulation of GalGXXXGXXXG-PttXET16–34 was not reflected in the protein MS data (Fig. 7; cf. Fig. 5). However, even at short reaction times (2 min), product analysis with HPAEC-PAD indicated that GalG(XXXG)₃ was formed in limited amounts, dependent upon the initial concentration of GalGXXXGXXXG (Table 2). GalG(XXXG)₃ presents three potential cleavage sites for PttXET16–34 attack (indicated by dashes): GalG-XXXG-XXXG—XXXG. Cleavage at the lactosyl unit is strongly disfavored (Table 1). Only cleavage of the glycosidic linkage represented by the em dash would lead to a unique glycosyl-enzyme; however, the low concentration of this bond (one-half the concentration of GalG(XXXG)₃) relative to GalGXXXGXXXG effectively precludes this event.

The ratio of GalGXXXG (formed from the basic hydrolysis of GalGXXXG-PttXET16–34) to XXXG was consistently 20% lower than the 1:1 ratio expected from the equilibrium shown in Fig. 4 (Table 2). This excess of GalGXXXG can be explained by rapid reaction of XXXG with GalG-PttXET16–34 to regenerate the free enzyme. GalGXXXG is itself a poor donor substrate, which reacts with PttXET16–34 only slowly at high concentration (Fig. 3) and thus accumulates in the reaction.

Calculation of the Free Energy Change for the Formation of the Glycosyl-PttXET16–34 Intermediate—The ability to measure the ratio of free PttXET16–34 and GalGXXXG-PttXET16–34, together with quantitation of the pseudo-equilibrium concentrations of XXXG and GalGXXXGXXXG, presents the unique opportunity for the calculation of the equilibrium constant for formation of a covalent glycosyl-enzyme intermediate in PttXET16–34 (Equation 2).

\[
K_{eq} = \frac{[\text{GalGXXXG-PttXET16-34}]}{[\text{XET}] \times [\text{GalGXXXGXXXG}_{\text{obs}}]} \quad \text{(Eq. 2)}
\]

The free energy change for formation of the glycosyl-enzyme is related to the equilibrium constant by \( \Delta G^0 = -RT\ln K_{eq} \) where \( R \) is the ideal gas constant (8.314 J m⁻¹ K⁻¹) and \( T \) is the absolute temperature of the reaction (298 K). Table 2 shows the calculated \( K_{eq} \) and \( \Delta G^0 \) values for three concentrations of GalGXXXGXXXG that lie in the linear portion of the plot of (fraction GalGXXXGXXXG-PttXET16–34) versus [GalGXXXGXXXG] (Fig. 8). These values are in reasonable agreement and indicate that the \( \Delta G^0 \) value for formation of the glycosyl-enzyme is ~−8.5 kJ/mol (2.0 kcal/mol).

Alternatively, the \( K_{eq} \) value may be derived from the data shown in Fig. 8, given that \( K_{obs} \) is the concentration of GalGXXXGXXXG at which [PttXET16–34] and [GalGXXXG-PttXET16–34] are equal. Assuming that (fraction GalGXXXG-XET)max represents the fraction of active enzyme in the PttXET16–34 sample and that [XXXG] is equivalent to one-half the concentration of active enzyme at [GalGXXXGXXXG] =

![Figure 8. Titration of PttXET16–34 with GalGXXXGXXXG.](image)

**TABLE 2**

| Nominal added [GalGXXXGXXXG]₀ | Observed [XXXG]µM | Observed [GalGXXXG]µM | Observed [GalGXXXGXXXG]µM | Observed [GalG(XXXG)₃]µM | Observed [total sugar]µM | [GalGXXXG-XET][XET]µM | Keq | \( \Delta G^0 \) kJ/mol |
|-------------------------------|--------------------|------------------------|-----------------------------|---------------------------|--------------------------|------------------------|------|---------------------------|
| 50 μM                         | 3.0 ± 0.1          | 3.5 ± 0.1              | 43.1 ± 0.1                  | 0.6 ± 0.1                 | 50.2 ± 0.1               | 0.39 ± 0.04             | 0.027 | 8.86                      |
| 100 μM                        | 4.0 ± 0.1          | 5.0 ± 0.1              | 87.4 ± 0.6                  | 1.7 ± 0.1                 | 98.1 ± 0.1              | 0.56 ± 0.03             | 0.025 | 9.05                      |
| 200 μM                        | 4.9 ± 0.1          | 7.1 ± 0.1              | 167.2 ± 2                   | 4.1 ± 0.1                 | 183 ± 2                 | 1.55 ± 0.19             | 0.045 | 7.58                      |

* All oligosaccharide concentrations were determined by HPAEC-PAD.

* Values were determined from protein MS data using heights of peaks at 33,850 and 34,012 Da (PttXET16–34) and 35,220 and 35,382 Da (GalGXXXG-PttXET16–34), respectively. Nominal [PttXET16–34] = 17 μM based on A₀₂₈₀ measurements.

* \( K_{eq} \) = ([GalGXXXG-XET][XET])/[XXXG]_obs/(GalGXXXGXXXG)_{obs}.

* \( \Delta G^0 = -RT\ln K_{eq} \).
mediate. Catalytic acid/base mutants of some have been devised to facilitate the accumulation of this inter-

book mechanism” of this paradigmatic retaining glycosidase (33).

glycosyl-enzyme. The trapping of a covalent intermediate on hen mutants to further slow the hydrolytic breakdown of the (fluoro-

required the additional use of catalytic acid/base glycosidase

strates that allow the formation of the glycosyl-enzyme at rea-

conceivable summary). These compounds are, in effect, slow sub-

residue in a number of wild-type glycosidases (see Ref. 22 for a

in the labeling and identification of the catalytic nucleophile

good anomeric leaving groups have been particularly effective

enzymes, followed by direct observation using MS and x-ray

elegant methods using designed substrate analogs have been

substrates in combination with rapid enzyme inactivation by a

ysis has been achieved for glycoside hydro-

lases and phosphorylases using radiolabeled variants of natural

tics. Classically, this has been

The compounds XXXGXXXG, GalGXXXGGG, and Gal-

GXXGXXXG (8), the axial C-4 hydroxyl group of the lactosyl (Galβ(1→4)Glc) unit on the nonreducing terminus prevented GalGXXXGGG and GalGXXGXXXG from acting as glycosyl acceptors. Consequently, accumulation of covalent GalG-XET and GalGXXG-XET glycosyl-enzyme intermediates was clearly observed by MS, respectively.

Notably, the saccharides undergoing nucleophilic substitution were electronically unperturbed (containing a natural leaving group and no substitutions on the sugar ring), and the enzyme was the wild-type variant in these systems. The results can thus be directly extrapolated to the natural reaction: the action of PttXET16–34 on xyloglucan polysaccharides involves formation of a hydrolytically stable, covalent glycosyl-enzyme intermediate. Furthermore, this intermediate is likely to be involved in catalysis by all XETs and endo-xyloglucanases from GH16. Taken together with the vast body of data from kinetic studies (9, 10) and trapping experiments (20–23), studies on unperturbed systems such as PttXET16–34 and glycogen-debranching enzyme (36) present some of the strongest evidence that wild-type glycosyl–enzymes are covalent in both α- and β-retaining enzymes.

Free Energy of Formation of a Covalent XET Glycosyl-enzyme—Brief incubation of PttXET16–34 with GalGXXGXXXG established a rapid equilibrium condition for the formation of the covalent GalGXXG-enzyme intermediate (Fig. 4B). Measurement of the ratio of free and glycosyl-enzymes by mass spectrometry, together with the analysis of all major sugar species by HPAEC-PAD, allowed us to estimate the $\Delta G^0$ value for this process as 6.3–8.5 kJ/mol (1.5–2.0 kcal/mol). Interestingly, the limited data that exist for similar systems of enzymatic glycosyl transfer indicate that $\Delta G^0$ values for the formation of glycosyl-enzymes can be negative. For example, Sinnott and co-workers (38) have reported experimentally determined $\Delta G^0$ values ranging from −19 to −24 kJ/mol (−4.5 to −5.7 kcal/mol) for a series of evolvants of the Escherichia coli β-galactosidase ebg. For the transfructosylation reaction catalyzed by Bacillus subtilis levansucrase, early elegant work by Chambert and Gonzy-Tréboul (39) gave a

\[
K_{\text{eq}} = \frac{[E]}{2K_{\text{app}} - [E]} \quad (\text{Eq. 3})
\]

where \([E]_t = [\text{PttXET16–34}]_{\text{app}} \times (\text{fraction GalGXXG-XET})_{\text{max}}\). Substitution of 17 μM for [PttXET16–34]_{\text{app}}, 0.878 for (fraction GalGXXG-XET)_{\text{max}}, and 104 μM for \(K_{\text{app}}\) yields a $\Delta G^0$ value of 6.3 kJ/mol (1.5 kcal/mol).

The largest potential sources of error in the determination of \(\Delta G^0\) are the apparent concentrations of the oligosaccharide products (up to 20%, from the equilibrium assumption), the relative proportions of labeled and free enzyme (∼10%), and the active enzyme concentration (10%). The individual calculation methods weight these errors differently but nonetheless give comparable results. Ultimately, it is the relative initial con-

centrations of the enzyme and its reversible label that dominate the equilibrium measurement and \(\Delta G^0\) determination.

**DISCUSSION**

**Covalent Nature of the Glycosyl-enzyme in Glycosidases and Transglycosylases—**In addition to data from kinetic isotope effect studies (9, 10), the strongest evidence for the covalent nature of the glycosyl-enzyme intermediate in retaining glyco-
side hydrolases and transglycosylases has come from trapping and direct observation of the intermediate by a number of techniques. Classically, this has been achieved for glycoside hydro-
lases and phosphorylases using radiolabeled variants of natural substrates in combination with rapid enzyme inactivation by a pH shift or denaturation (30–32). More recently, a number of elegant methods using designed substrate analogs have been devised by Withers and co-workers (reviewed in Refs. 20–23) for the mechanism-based trapping of both α- and β-glycosyl-
enzymes, followed by direct observation using MS and x-ray crystallography.

In particular, 2-deoxy-2-fluoro- and 5-fluorosugars bearing good anomeric leaving groups have been particularly effective in the labeling and identification of the catalytic nucleophile residue in a number of wild-type glycosidases (see Ref. 22 for a concise summary). These compounds are, in effect, slow sub-

strates that allow the formation of the glycosyl-enzyme at reason-

able rates but that dramatically reduce the rate of enzyme deglycosylation, thus favoring accumulation of the intermediate. However, in some cases, Withers’ fluorosugar methodology has required the additional use of catalytic acid/base glycosidase mutants to further slow the hydrolytic breakdown of the (fluoro-
glycosyl-enzyme. The trapping of a covalent intermediate on hen egg white lysozyme represents one of the most important uses of this combined strategy (19), leading to the revision of the “text-

book mechanism” of this paradigmatic retaining glycosidase (33).

Whereas fluorination of the sugar ring perturbs the elec-

tronic structure of the glycosyl-enzyme itself, other methods have been devised to facilitate the accumulation of this inter-

mediate. Catalytic acid/base mutants of some α-retaining (34) and β-retaining (35) glycosidases are able to react with sub-

strates bearing good leaving groups to form glycosyl-enzymes with comparatively low rates of turn over, thus allowing direct observation by crystallography. Another elegant approach involves the use of blocked donor substrates that are incapable of acting as glycosyl acceptors to trap glycosyl-enzyme intermediates of transglycosidases. 4-MeO-deoxy-α-maltotriosyl fluoride has been successfully employed to trap covalent maltotriosyl-enzyme inter-

mediates on both a glycogen-debranching enzyme (36) and a cyclodextrin glycosyltransferase (37). Notably, a significant hydrolysis rate necessitated the additional use of a catalytic acid/ base mutant in case of the cyclodextrin glycosyltransferase, whereas the covalent intermediate could be trapped directly on the wild-type glycogen-debranching enzyme. As such, this latter example arguably represents the most clear-cut evidence so far of the covalent nature of a (β-)glycosyl-enzyme intermediate, as both the enzyme and intermediate are natural.

This study provides an additional example of the successful use of blocked donor substrates to label a wild-type enzyme. The compounds XXXGXXXG, GalGXXXGGG, and Gal-

GXXGXXXG (Fig. 1) were all competent donor substrates for PttXET16–34, the dominant modes of cleavage of which were dictated by favored binding of the XXXG moiety in the positive enzyme subsites. Whereas XXXGXXXG functions as its own ac-

ceptor substrate leading to the production of XXXG and XXXGXXXGXXG (8), the axial C-4 hydroxyl group of the lactosyl (Galβ(1→4)Glc) unit on the nonreducing terminus prevented GalGXXXGGG and GalGXXGXXXG from acting as glycosyl acceptors. Consequently, accumulation of covalent GalG-XET and GalGXXG-XET glycosyl-enzyme intermediates was clearly observed by MS, respectively.

**Trapping of Covalent Glycosyl-XET Intermediates**

Brief incubation of PttXET16–34 with GalGXXGXXXG established a rapid equilibrium condition for the formation of the covalent GalGXXG-enzyme intermediate (Fig. 4B). Measurement of the ratio of free and glycosyl-enzymes by mass spectrometry, together with the analysis of all major sugar species by HPAEC-PAD, allowed us to estimate the $\Delta G^0$ value for this process as 6.3–8.5 kJ/mol (1.5–2.0 kcal/mol). Interestingly, the limited data that exist for similar systems of enzymatic glycosyl transfer indicate that $\Delta G^0$ values for the formation of glycosyl-enzymes can be negative. For example, Sinnott and co-workers (38) have reported experimentally determined $\Delta G^0$ values ranging from −19 to −24 kJ/mol (−4.5 to −5.7 kcal/mol) for a series of evolvants of the Escherichia coli β-galactosidase ebg. For the transfructosylation reaction catalyzed by Bacillus subtilis levansucrase, early elegant work by Chambert and Gonzy-Tréboul (39) gave a

the expression for the equilibrium constant can be sim-

plified to Equation 3,

\[ K_{\text{eq}} = \frac{[E]}{2K_{\text{app}} - [E]} \quad (\text{Eq. 3}) \]
much smaller value of $-1.9 \pm 0.8 \text{ kJ/mol} (-0.460 \pm 0.200 \text{ kcal/mol})$, indicating "that the high energy of the glycosidic linkage of sucrose is preserved in the fructosyl-enzyme intermediate." (The coherent nature of this intermediate is discussed in Ref. 30.)

As XETs catalyze the rearrangement of xyloglucan chains by cleavage and subsequent re-formation of a $\beta(1\rightarrow4)$ glycosidic bond, the substrates and products are enthalpically equivalent. XET-catalyzed transglycosylation reactions are therefore entropically driven, as manifested in the broadening of molecular mass distributions observed when XETs act on xyloglucan (2). Thus, like levansucrase, XETs also preserve the energy of the glycosidic bond in the covalent glycosyl-enzyme, although in XETs, this species is slightly less stable than the donor substrate (cf. PttXET16–34, 1.5–2.0 kcal/mol; B. subtilis levansucrase, $-0.460 \pm 0.200 \text{ kcal/mol}$).

PttXET16–34 has been shown previously to have no detectable hydrolytic activity when acting on xyloglucan polysaccharide (17). This study indicates that covalent GalGXXXG-PttXET16–34 is hydrolytically unstable (Fig. 6) in the complete absence of sugar acceptor. Indeed, given the free energy of hydrolysis of a $\beta$-glycosidic bond, it would be remarkable if the glycosyl-enzyme were indefinitely stable in dilute aqueous solution. Given the long half-life of the intermediate (176 ± 12 min), it is highly unlikely that PttXET16–34 acts as a xyloglucanendo-hydrolase (EC 3.2.1.151) under physiologic conditions. This could occur only if the concentrations of free acceptor substrates (xyloglucan oligo- or polysaccharides) were essentially nil, in which case, donor substrate hydrolysis would be extremely slow (for comparison, the archetypal GH16 endoxylanoglucanase T. maritima XNJG1 exhibits hydrolytic rates ($v_0$ [[$E_0$]) of $-5 \text{ min}^{-1}$ for xyloglucan oligo- and polysaccharides at or near enzyme saturation) (8).6

Unfortunately, attempts to obtain crystallographic data on PttXET16–34 glycosyl-enzymes have thus far been unsuccessful. This appears to be due to the fact that the preferred crystal packing of this enzyme places a second molecule of the asymmetric unit in the negative enzyme subsites (16). However, such atomic level information will be essential to fully elucidate the determinants of transglycosylation versus hydrolysis in XETs and xyloglucanases of GH16, a topic of continuing interest in our laboratories (8).

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