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COMMUNICATIONS

Published on Web 11/29/2006

Chemical Rescue of α3-Galactosyltransferase. Implications in the Mechanism of Retaining Glycosyltransferases

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Glycosyltransferases (GTs) comprise a large class of enzymes involved in the biosynthesis of polysaccharides and the carbohydrate moieties of glycoproteins, glycolipids, and glycosaminoglycans. Glycoside bond formation by sugar nucleotide-dependent glycosyltransferases (Leloir-type GTs) proceeds with either inversion or retention of the anomeric configuration.[1,2] Whereas the mechanism of inverting GTs seems clearly established, an S_N2 reaction in a single displacement step, the mechanism of retaining GTs is still controversial. A double displacement mechanism was proposed by analogy to retaining glycoside hydrolases.[3] Such a mechanism transferred to retaining GTs would involve the participation of an enzyme nucleophile and a general acid/base catalyst, typically Asp or Glu residues (Figure 1a). In the first step, the nucleophile attacks the anomeric carbon of the donor leading to the glycosyl-enzyme intermediate (an acid catalyst or a metal ion may be required to assist the exocyclic C1−O bond cleavage of the donor). In the second step, the acceptor, activated by a general base, attacks the intermediate to give the product with overall retention of configuration.

When the first 3D structure of a retaining GT was solved, that of LgtC from *Neiseria meningitidis*,[4] no carboxylic amino acid (Asp or Glu) was localized close to the anomeric carbon of the donor substrate, and a glutamine residue was tentatively suggested to be the enzyme nucleophile. To prove it and trap a covalent glycosyl−enzyme intermediate, different strategies adopted from the work with retaining glycoside hydrolases were employed.[5] Failure to provide clear evidence for a double displacement mechanism prompted the authors to propose a new mechanism, rather unusual and with limited chemical precedence,[6] in which the reaction proceeds via a front side single displacement, also known as S_Ni mechanism.[4] In this "concerted one-step" mechanism the nucleophilic hydroxyl group of the acceptor attacks the anomeric carbon at the same side from which the UDP leaving group departs, reaching a highly dissociative oxocarbenium ion-like transition state (asynchronous C1•••O-acceptor bond formation and C1•••O−UDP bond cleavage) (Figure 1b). This mechanistic view has also been extended to other retaining GTs, for example, trehalose-6-phosphate synthase OtsA,[7] trehalose phosphorylase,[8] and glycogen phosphorylase,[9] a non-Leloir GT for which an S_Ni mechanism had been proposed several years ago.[1]

A different scenario may apply to other GTs for which the 3D structures of enzyme−substrate complexes indicate the presence of a carboxylic amino acid residue in a suitable position to act as an enzyme nucleophile. This is the case of family 6 GTs which include mammalian α3-galactosyltransferases (α3GalT)[10] and blood group GTs (GTA and GTB).[11] For bovine α3GalT, Glu317 was tentatively proposed as the enzyme nucleophile,[12] but also a role in acceptor binding became apparent from the structure of the enzyme in complex with UDP and lactose acceptor.[13] Mutation by glutamine (E317Q) yielded an enzyme with increased K_M for the acceptor and 2400-fold lower k_{cat}, a moderate reduction in activity when compared to the effects of substitutions for the catalytic

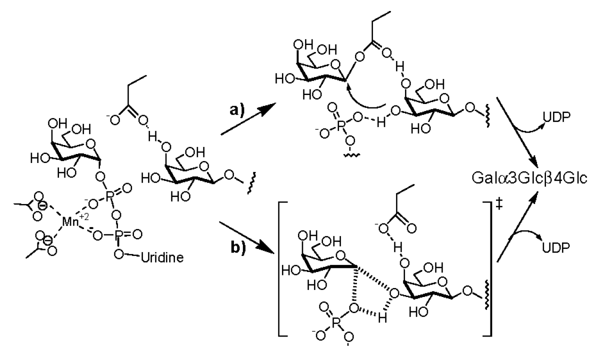

Figure 1. Proposed alternative mechanisms for retaining α3-galactosyltransferase: (a) double displacement mechanism; (b) front side single displacement (S_Ni) mechanism.

nucleophile in retaining glycosidases.[14] Because of that, it was suggested that Glu317 is not the nucleophile and that α3GalT does not utilize a double displacement mechanism. Instead, a single displacement S_Ni mechanism was sought following the proposal for LgtC. However, no systematic studies to trap an intermediate have been conducted on any family 6 GT.

A puzzling result came into play when the glutamine residue of LgtC was mutated to glutamate in an attempt to increase its nucleophilicity and recheck the involvement of that residue in covalent catalysis.[15] Surprisingly, a covalent intermediate was trapped but with the sugar substrate bound to an adjacent aspartate residue, which appeared far away from the reaction center in the X-ray structure of the enzyme-donor complex.

In summary, there is no definitive evidence for one or another mechanism for retaining GTs and the debate remains open. With this background, we here apply a "chemical rescue methodology" to bovine α3GalT on inactive mutants at key residues of the active site with the aim of providing new mechanistic information for the debate. Well established as a mechanistic probe for retaining glycoside hydrolases, chemical rescue stands for enzyme reactivation of inactive mutants by exogenous small molecules; addition of an exogenous nucleophile such as azide to mutants in which the catalytic nucleophile or the general acid/base has been replaced by alanine reactivates the enzyme leading to the corresponding α- or *β*-glycosyl azide adduct, the stereochemistry of which correlates with the function of the mutated residue.[16]

Bovine α3GalT was recombinantly expressed in *E.coli* as the soluble catalytic domain after removing the N-t transmembrane domain.[17] Wild-type and mutant proteins at Glu317 (tentative nucleophile) and Asp316 were purified to homogeneity as reported.[18] Enzyme activities and kinetic parameters for transferase (with UDPGal donor and lactose acceptor), hydrolase (with UDPGal in the absence of acceptor), and rescue of inactive mutants (with added sodium azide) were determined by monitoring both UDP release by the pyruvate kinase/lactate dehydrogenase (PK/LDH) coupled assay, and product formation by the radiometric assay using labeled UDPGal (UDP-[1-^{3}H]Gal).

10.1021/ja0659931 CCC: $33.50 © 2006 American Chemical Society

Table 1. Kinetic Parameters for WT and Mutant α3GalTs

	reaction	k_{cat} (s^{-1})	K_M (donor)
WT	transferase[a]	1.3	12.5 μM
	hydrolase	2.2×10^{-2}	12 μM
D316A	transferase[a]	1.3×10^{-3}	70 μM
E317A	transferase[a]	$<10^{-4}$ [c]	n.d.[d]
	hydrolase	2.0×10^{-4}	0.9 mM
	rescue[b]	2.5×10^{-2}	2.5 mM

[a] At 10 mM lactose acceptor. [b] Rescued activity (GalβN_3 formation) at 200 mM sodium azide. [c] Specific activity at 50 μM UDPGal and 10 mM lactose: v/[E]= 1.8×10^{-5} s^{-1}. Conditions: 13 mM HEPES buffer, 50 mM KCl, 13 mM $MnCl_2$, 0.13 mg·mL^{-1} BSA, pH 7.0, 30 °C (radiometric assay). [d] n.d. = not determined.

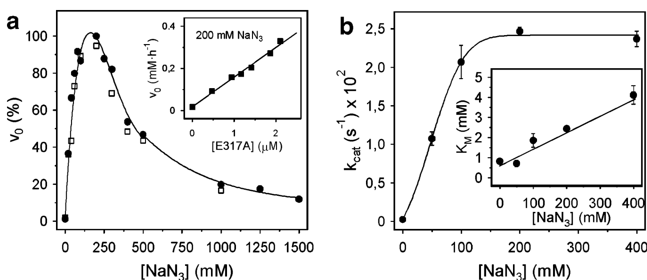

Figure 2. Rescue of E317A by sodium azide: (a) activity by the PK/LDH coupled assay (rate of UDP release vs azide concentration) at 2 mM UDPGal and (●) 10 mM lactose acceptor or (□) no acceptor. The inset shows dependence on enzyme concentration at 2 mM UDPGal, 200 mM NaN_3. Panel b shows the kinetic parameters as a function of sodium azide (radiometric assay).

For the wild-type enzyme, increasing concentrations of sodium azide had an inhibitory effect (first-order kinetics) on both transferase ($IC_{50} \approx 300$ mM) and hydrolase ($IC_{50} \approx 170$ mM) activities at 50 μM UDPGal with (10 mM) or without lactose acceptor, respectively. Removal of the carboxyl side chain in the E317A mutant rendered an inactive enzyme (Table 1) with k_{cat} values of about 10^4-fold lower for transferase and 10^2-fold lower for hydrolase activities than for that of the wild-type enzyme. When sodium azide was added to the E317A mutant, reactivation as determined by UDP release (PK/LDH coupled assay) was observed (Figure 2a), the same behavior obtained with or without lactose as acceptor. Therefore, the reaction observed does not correspond to transglycosylation (trisaccharide product formation) but to the formation of a non-phosphorylated galactose derivative other than the hydrolysis product (free galactose) as shown by TLC. With 2 mM UDPGal as substrate, maximal reactivation was obtained at 200 mM azide, followed by a decrease in activity at higher azide concentrations. Kinetics of enzyme reactivation were further analyzed by monitoring product formation by the radiometric assay in the absence of lactose acceptor. Initial rates versus UDPGal concentration (50 μM to 10 mM) followed normal saturation (Michaelis−Menten) curves at different azide concentrations (0−400 mM). Kinetic parameters as a function of sodium azide concentration are plotted in Figure 2b, and selected data (at 200 mM sodium azide) given in Table 1. Values of k_{cat} follow a saturation curve reaching a maximal value of 2.5×10^{-2} s^{-1}, more than a 100-fold increase compared to the residual transglycosidase activity of the E317A mutant, and approximately the same value as k_{cat} for hydrolase activity of the wild-type enzyme. The values of K_M show a linear dependence with azide concentration, and therefore k_{cat}/K_M first increases up to 50 mM sodium azide but then decreases at higher concentrations, explaining the apparent inhibition in Figure 2a. The product from the rescue reaction after a prolonged reaction time in the presence of alkaline phosphatase (to remove UDP) was purified by acetylation with Ac_2O/pyridine followed by silica gel chromatography. The ^{1}H NMR spectrum identified the product as β-D-galactosyl azide, with a characteristic doublet at δ 4.60 ppm (J 8.7 Hz) assigned to the anomeric proton (synthetic reference was obtained as in ref 19).

To check whether the rescue by azide is specific for the 317 position, with azide binding in the cavity created in the E317A mutant, two control experiments were performed. First, Asp316, the neighboring residue to the target Glu317, was mutated to alanine. The D316A mutant had a 1000-fold lower k_{cat} than the wild type enzyme (Table 1). The pH profile on k_{cat}/K_M(donor) for the transferase reaction was similar for D316A and WT enzymes, with the same kinetic pK_a in the acidic limb of 5.5, indicating that Asp316 does not interact with the essential Glu317. Under the same conditions used for the E317A mutant, addition of sodium azide (up to 1 M) did not rescue the activity of the D316A mutant. Second, other mutations at position 317 were analyzed for possible rescue by azide. Mutants E317Q and E317I have the carboxyl group removed but do not leave a cavity as in the case of the alanine mutant. Both mutant enzymes were inactive, and the addition of azide (10, 50, or 200 mM) did not rescue their activity.

In this Communication we have reported the first successful "chemical rescue" on a retaining glycosyltransferase. Azide acts as nucleophile to give β-D-galactosylazide when the side chain of Glu317 has been removed and a cavity is left. This result fits well with the double displacement mechanism where Glu317 acts as the catalytic nucleophile, since its role can be replaced by the exogenous nucleophile upon mutation to Ala, the same behavior obtained with retaining glycosidases. However, the fact of rescue does not fully discard the alternative S_Ni mechanism. Whether the ability of being rescued is a signature of GTs operating by a double displacement mechanism as opposed to others acting by a different mechanism (i.e., S_Ni) requires to extend this methodology to other GTs.

Acknowledgment. Work supported in part by Grant BFU2004-06377-C02-02 from MEC. We thank Javier Martínez-Baigorri for the characterization of the E317Q and I mutants.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0659931