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Development of a universal glycosyltransferase assay amenable to high-throughput formats

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Abstract

The development of a general 1-Zn(II) NDP sensor assay for rapid evaluation of GT activity is described. The 1-Zn(II) NDP sensor assay offers submicromolar sensitivity, compatibility with both purified enzymes and crude cell extracts, and exquisite selectivity for nucleoside diphosphates over the corresponding NDP-sugars. Thus, the 1-Zn(II) NDP sensor assay is anticipated to offer broad applicability in the context of GT engineering and characterization.

Keywords

glycosyltransferase; enzyme; evolution; engineering; carbohydrate; sugar nucleotide

Complex carbohydrates are found in a wide range of biomolecules in cells, including polysaccharides, proteoglycans, glycolipids, glycoproteins, and antibodies. They play important roles in a number of biological processes such as cell growth, cell-cell interactions [1], immune response [2], inflammation [3], and viral and parasitic infections [4]. The attachment of carbohydrates to the biomolecules is catalyzed by glycosyltransferases (GTs) which transfer a monosaccharide unit from a nucleotide or lipid sugar donor to acceptor substrates in a regio- and stereospecific manner. Given the importance of carbohydrates in biology and medicine, the development of methods for glycan synthesis and modification remains a major focus of research [5-8].

While both chemical and enzymatic methods have been developed for glycan synthesis, enzymatic processes are often advantageous due to both their efficiency as well as their stringent regio- and stereochemical control [9, 10]. However, the lack of availability of suitable glycosyltransferases (GTs), and/or the requisite sugar nucleotide donors [11], for targeted glycosyl-bond formation often restricts the alternative application of enzymes. Thus, technologies to enable the generation of tailor-made GTs, either via rational design and/or directed evolution [10, 12], are anticipated to greatly augment the utility of GTs in this regard. Although there are recent examples in which GTs were successfully evolved to modulate their substrate specificity [13-15], in all cases the corresponding assays were

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developed for a specific acceptor. While other GT assays, including radiochemical assays, immunological assays, pH-based assays, or phosphatase-coupled assays exist [16, 17, 18], each has limits in the context of high throughput screening. In this study, we describe the development of a truly general fluorescence-based GT assay, based upon a xanthene-based Zn(II) complex nucleoside diphosphate chemosensor [19]. Given this **1**-Zn(II) NDP sensor assay is highly sensitive, is compatible with both purified enzymes or crude extracts, and relies upon a sensor for the general leaving group of most Leloir-type GT-catalyzed reactions, the assay is anticipated to have broader applicability.

Materials and methods

Unless otherwise specified, all chemicals and enzymes were reagent grade or better obtained from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA) and were used without further purification. Recombinant *Streptomyces antibioticus* wild-type oleandomycin glycosyltransferase (OleD) and corresponding mutants (OleD-ASP, OleD-AIP and OleD-TDP16) were produced and purified as described previously [14, 20, 21]. Absorbance readings were performed on a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA, USA) and fluorescence was measured by a BMG Labtech FLUOstar Optima plate reader (microtiter plate scale, Durham, NC, USA). Mass spectrometric data were obtained on either a Waters (Milford, MA) LCT time-of-flight spectrometer for electrospray ionization (ESI) or a Varian ProMALDI (Palo Alto, CA) Fourier transform ion cyclotron resonance mass spectrometer (FTICR) equipped with a 7.0 T actively-shielded superconducting magnet and a Nd:YAG laser.

Preparation of ligand **1**

Ligand **1** was prepared as previously described without modifications starting from orcinol and ethyl orsellinate [19, 22, 23].

TDP binding assay

Complex **1**-2Zn(II) stock solution was prepared by dissolving ligand **1** (2.5 mM) and ZnCl₂ (6.3 mM, 2.5 equivalent) in 10 mM HCl. The **1**-Zn(II) NDP sensor assay solution was prepared by adding the complex stock solution (10 μ L) to the assay buffer (10 mL) containing 50% methanol in 25 mM HEPES (pH 7.4), 10 mM NaCl, 1 mM MgCl₂. TDP or TDP-Glc at different concentrations (0.01, 0.02, 0.04, 0.08, 0.16, 0.31, 0.62, 1.25, 2.50, 5.00, 10.00 μ M, final concentrations) was added to the assay solution (200 μ L, final volume) in a 96 well plate and the fluorescence was measured at 520 nm with excitation at 485 nm. The dissociation constant was obtained by calculating the free TDP concentration at which $\Delta F/\Delta F_{\text{max}}$ equals 0.5 (ΔF , fluorescence intensity change; ΔF_{max} , maximum fluorescence intensity change).

Enzyme assays

Representative GT (wtOleD or OleD variants, 1 μ M final concentration) was added to the reaction buffer containing 10 mM Tris (pH 8.0), 1 mM TDP-Glc, 1 mM 4-MU and 1 mM MgCl₂, and the mixture incubated at room temperature. For each GT activity determination, an aliquot of the GT reaction mixture (5 μ L) was added to the **1**-Zn(II) NDP sensor assay solution (195 μ L) and the fluorescence was measured at 520 nm as described for the TDP binding assay.

The corresponding 4-MU fluorescence assay (where 4-MU glycosylation directly correlates to a reduction in 4-MU fluorescence) was conducted as previously described [14, 24]. Briefly, for this study the GT reaction mixture (10 μ L) was added to 10 mM Tris (pH 8.0, 990 μ L) and the fluorescence was measured at 460 nm with excitation at 355 nm.

Crude cell extract assays

Cells from OleD-expressing bacterial cell cultures (25 mL) were harvested by centrifugation (4000 rpm) and frozen at -80°C . The frozen cell pellets were thawed on ice, resuspended in the lysis buffer (2 mL) containing 50 mM Tris (pH 8.0), lysozyme (1 mg/mL, 50 kU/mL) and benzonase (125 U/mL, Novagen, San Diego, CA, USA, Cat# 70746-3), and incubated on ice for 1 hour. Removal of the cell debris by centrifugation (12,000 rpm) afforded crude cell extracts. OleD assays with crude cell extracts were carried out as described for the assays with purified enzymes by adding crude cell extracts (1 μL for 100 μL reaction, 1%) instead of purified enzymes to the reaction buffer. A Z factor for the assay containing TDP16 at 100 min was calculated by using the equation, $Z = 1 - (3\sigma_s + 3\sigma_c)/|\mu_s - \mu_c|$ where σ_s and σ_c are denoted for the standard deviations of the sample signal and control signal, and μ_s and μ_c for the means of the sample signal and control signal [25].

OleD Kinetics

Kinetics were performed with constant concentrations of OleD-TDP16 (1 μM) and TDP-Glc (1 mM) in 50 mM Tris-HCl (pH 8.0) containing 1 mM MgCl_2 while varying 4-MU concentrations (0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mM). TDP production was assessed using the 1-Zn(II) NDP sensor assay at 20, 60, 180 seconds by fluorescence change at 520 nm. Initial reaction velocities, obtained as the slope of best fit to the initial linear portion of the reaction time course, were subsequently fit to the Michaelis-Menten equation.

Results and discussion

Given nearly all LeLoir GT-catalyzed reactions produce NDP as a product, a sensitive NDP sensor would be advantageous for the development of a general GT assay strategy. Among the fluorescence-based NDP sensors that have been developed for biochemical applications [19, 26, 27], the xanthene-based Zn(II) complex [Fig. 1, 1-Zn(II)] offers both high sensitivity and selectivity for NDP over NDP-sugar (the requisite GT substrate). The complex contains two sites of 2,2'-dipicolylamine-Zn(II) and xanthene as a fluorescent sensing unit for nucleoside polyphosphates. This chemosensor selectively senses nucleoside di- or triphosphates with a large fluorescence enhancement ($F/F_0 > 15$) and strong binding affinity (about 1 μM of apparent dissociation constant, K'_d), whereas no detectable fluorescence change is induced by monophosphate species, NDP-sugars or various other anions. Therefore we expected the complex 1-Zn(II) could serve as enabling feature for the development of a general NDP sensor-based GT assay.

The xanthene-based ligand **1** was prepared as previously described starting from the commercially available compounds, orcinol and ethyl orsellinate [19, 22, 23]. To test the feasibility of 1-Zn(II) NDP sensor assay in the context of a GT assay, the well-studied macrolide-inactivating GT from *S. antibioticus* (OleD) was selected as a model system [14, 20, 21, 28]. As a first step, the binding affinity of 1-Zn(II) to TDP-Glc and TDP, the OleD substrate and product, respectively, was assessed in a 96-well plate format (Fig. 2). As anticipated, the large fluorescence increase at 520 nm directly correlated with an increase of [TDP] while increasing [TDP-Glc] had no effect. Thus, this standard analysis confirmed the complex to provide submicromolar sensitivity and cleanly distinguish between NDP and NDP-sugar [19], providing the selectivity (TDP $K'_d = 0.44 \mu\text{M}$) and sensitivity required for a general GT assay.

Next, the 1-Zn(II) NDP sensor assay was applied to an in vitro GT assay containing purified enzymes. OleD-WT and three GT variants which display different proficiencies (ASP, AIP, TDP16 [14, 20, 21]) were employed as a representative GT series with TDP-Glc and 4-methylumbelliferone (4-MU) serving as the glycosyl donor and acceptor, respectively.

Notably, the established order of 4-MU/TDP-Glc turnover across this series was TDP16 > ASP > AIP with no conversion expected using WT [14, 20, 21]. Consistent with this, an enzyme-dependent and time-dependent increase of fluorescence was observed which directly correlates to the variant efficiency of NDP production (and corresponding glucosyltransfer) among the series of reactions evaluated (Fig. 3). As expected, controls lacking enzyme, NDP-sugar or acceptor also lacked Δ fluorescence. As further confirmation, the validated quenching of 4-MU fluorescence upon 4-MU 7-*O*-glucosylation measured in parallel at 460 nm with excitation at 355 nm (Fig. 3) [14], revealed an identical trend of catalyst proficiency to that determined by the **1-Zn(II)** NDP sensor assay. For the most active variant TDP16, steady state kinetic parameters were also determined using the **1-Zn(II)** NDP sensor assay in a 96-well plate format (Fig. S1). Saturation was observed by varying 4-MU at a fixed concentration of TDP-Glc (1 mM) to provide an apparent K_M of 0.24 ± 0.011 mM and k_{cat} of 12.3 ± 0.45 min⁻¹ ($k_{cat}/K_M = 51$ mM⁻¹ min⁻¹) and these parameters are comparable to those previously determined via a discontinuous HPLC assay for TDP16 [21].

Finally, to assess the high throughput applicability of **1-Zn(II)** NDP sensor assay, we examined crude extract compatibility. Specifically, the plate-based **1-Zn(II)** NDP sensor assay was applied to crude cell extracts from OleD-expressing cells (*E. coli* BL21). Each cell extract for the four OleD variants was added to the reaction mixture containing TDP-Glc and 4-MU, and the mixture was transferred to the assay solution in a 96-well plate and the fluorescence was measured at 520 nm with excitation at 485 nm (Fig. 4). Based upon this analysis, the observed crude extract reactivity trends were identical prior assessments using homogenous catalysts. Importantly, this study clearly demonstrates the **1-Zn(II)** NDP sensor assay to be fully compatible with crude extract analyses as controls lacking expressed GT or less active GT displayed little to no detectable background signal (a Z factor of 0.82 was determined for the assay containing TDP16 at 100 min [25]). In addition, this study clearly demonstrates the ability of the **1-Zn(II)** NDP sensor assay, even in a plate-based crude extract format, to distinguish among a range GT mutants which display differing proficiencies.

In conclusion, a general **1-Zn(II)** NDP sensor assay has been developed for rapid evaluation of GT activity. The assay as described is sensitive, amenable to both purified enzymes and crude cell extracts, and, given the **1-Zn(II)** NDP sensor selectivity for all five nucleoside diphosphates ($K'_d < 1$ μ M for ADP, TDP, UDP, GDP or CDP [19]) over the corresponding NDP-sugars ($K'_d > 20$ μ M), is anticipated to offer broad applicability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

GT	glycosyltransferase
OleD	oleandomycin glucosyltransferase
WT	wild type

TDP	thymidine diphosphate
TDP-Glc	thymidine 5'-diphospho- α -D-glucose
4-MU	4-methylumbelliferone
UDP	uridine diphosphate
ADP	adenosine diphosphate
CDP	cytidine diphosphate
GDP	guanosine diphosphate
NDP	nucleoside diphosphate

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Fig. 1.
Schematic illustration of the NDP sensing mechanism of **1-2Zn(II)**.

Fig. 2. Fluorescence intensity change of **1**-2Zn(II) with different concentrations of TDP (◆) and TDP-Glc (■). Each data point represents an average based upon assays conducted in triplicate. Measurement conditions: 50% methanol in 25 mM HEPES (pH 7.4), 2.5 μM ligand **1**, 6.3 μM ZnCl₂, 10 mM NaCl, 1 mM MgCl₂, excitation at 485 nm, emission at 520 nm.

Fig. 3.

GT assay results for OleD-WT and variants by **1-2Zn(II)** (top) and **4-MU** (bottom). Fluorescence was measured at 520 nm with excitation at 485 nm for **1-2Zn(II)** and at 460 nm with excitation at 355 nm for **4-MU**. Each data point represents an average based upon assays conducted in triplicate. GT reaction conditions: 10 mM Tris (pH 8.0), 1 mM TDP-Glc, 1 mM MgCl₂, 1 mM 4-MU, 1 μ M OleD, room temperature; (+), in the presence of 4-MU; (-), in the absence of 4-MU; the control contains no enzyme. Assay conditions: for **1-2Zn(II)**, 5 μ L of the GT reaction mixture was added to 195 μ L of the assay solution containing 50% methanol in 25 mM HEPES (pH 7.4), 2.5 μ M ligand **1**, 6.3 μ M ZnCl₂, 10 mM NaCl, 1 mM MgCl₂; for **4-MU**, 10 μ L of the GT reaction mixture was added to 990 μ L of 10 mM Tris (pH 8.0).

Fig. 4.

GT assay results with crude cell extracts from OleD WT and variants expressing cells by **1-2Zn(II)**. Fluorescence was measured at 520 nm with excitation at 485 nm. Each data point represents an average based upon assays conducted in triplicate. GT reaction conditions: 10 mM Tris (pH 8.0), 1 mM TDP-Glc, 1 mM MgCl_2 , 1 mM 4-MU, 1 % crude cell extracts (1 μL for 100 μL GT reaction), room temperature; (+), in the presence of 4-MU; (-), in the absence of 4-MU; the blank contains the cell extract from a blank vector expression. Assay conditions: 5 μL of the GT reaction mixture was added to 195 μL of the assay solution containing 50% methanol in 25 mM HEPES (pH 7.4), 2.5 μM ligand **1**, 6.3 μM ZnCl_2 , 10 mM NaCl, 1 mM MgCl_2 .