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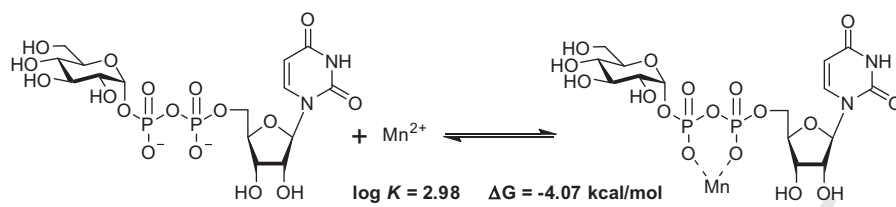
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Graphical abstract

Relaxometric determination of binding between Mn(II)-UDP and Mn(II)-UDP-glucose in aqueous solution

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Relaxometric determination of binding between Mn(II)–UDP and Mn(II)–UDP-glucose in aqueous solution

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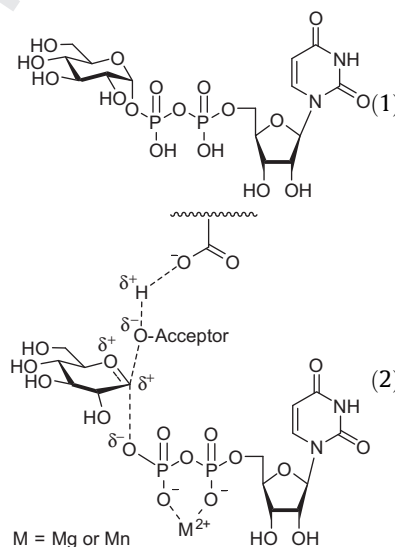
ABSTRACT

The applicability of relaxometry for the determination of formation constants of Mn(II)–UDP (logK = 3.78) and Mn(II)–UDP-glucose (logK = 2.98) complexes is demonstrated. The obtained value indicates a well-defined interaction between Mn(II) and UDP-glucose in aqueous solution (pH = 5.50) with $\Delta G = -4.07$ kcal/mol.

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1. Introduction

Glycosyltransferases catalyze the biosynthesis of glycosidic linkages to produce oligo- and polysaccharides as well as a wide variety of other natural products by conjugating sugars to lipids, proteins, nucleic acids, antibiotics, or several types of other small molecules.¹ The so-called Leloir-type enzymes use sugar diphosphonucleotides (NDP-sugars, e.g., UDP-glucose **1**) as their glycosyl donor substrates. In these derivatives the sugar moiety, together with the acceptor, is responsible for the specificity of the reaction while the pyrophosphate acts as a leaving group and also as a chelator for the cofactor metal ion (usually Mg(II) or Mn(II)) in most of the GT-A fold structures.² The metal ion facilitates departure of the nucleoside diphosphate by stabilizing the developing negative charge as visualized by a simplified representation of a computed model of the transition state **(2)** for a reaction catalyzed by an inverting glycosyltransferase.³



Some data on the interaction of Mn(II) and UDP-sugars were reported in the seventies. Thus, from evaluation of ESR titration of Mn(II) with UDP-galactose a $K_{\text{diss}} = 14.5 \pm 1.1$ mM value (pH 8.0, 0.08 M *N*-methylmorpholine (NMM) containing 0.08 M KCl at 26 ± 2 °C) was obtained.⁴ In that paper⁴ a $K_{\text{diss}} \sim 19$ mM obtained for Mn(II)–UDP-glucose from proton relaxation enhancement experiments was cited from Ref.⁵ In another paper reference was

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made to unpublished observations stating that the apparent dissociation constant for the $\text{Mn(II)-UDP-galactose}$ complex was 7.5 mM, however, details and circumstances of the determination were not indicated.⁶ Other ESR studies at 18 °C at pH = 7.4 allowed the determination of an association (stability) constant $K = 58.3 \text{ M}^{-1}$ for $\text{Mn(II)-UDP-glucose}$.⁷ Since that time, to the best of our knowledge, there has been only one report on the interaction of Mn(II) ions and diphosphate containing molecules studied by isothermal titration calorimetry reporting a stability constant $K = 169 \text{ M}^{-1}$ for $\text{Mn(II)-UDP-glucose}$ (in 100 mM HEPES buffer, pH 7.5 at 37 °C, ionic strength unknown).⁸ The data referring to the $\text{Mn(II)-UDP-glucose}$ system were converted into comparable $\log K$ (stability constant) values which are collected in Table 1 (entries 7–9).

Given the variance shown by the above data we set out to determine stability constants for the complex of Mn(II) with UDP-glucose by methods other than those applied so far. Such data can be useful in mechanistic evaluations of glycosyltransferase catalyzed reactions. For comparison, complex formation between Mn(II) and UDP as a model system has been also investigated.

2. Experimental

2.1. Reagents

UDP and UDP-glucose were purchased from Sigma-Aldrich and Carbosynth, respectively, and were used without further purification. The concentrations of their stock solutions were determined via pH-potentiometry with the help of Gran functions.⁹

The Mn(II) stock solution was prepared by dissolving $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (Reanal) in tri-distilled water, which contained a known amount of HCl to minimize hydrolysis and oxidation of the Mn(II). The Mn(II) concentration of the stock solution was confirmed by gravimetric analysis via precipitation as $\text{MnNH}_4\text{PO}_4 \cdot \text{H}_2\text{O}$, while pH-potentiometry was used to determine the acid concentration.

2.2. Potentiometric studies

The pH-potentiometric titrations were made with a Radiometer pHM 93 instrument equipped with a Metrohm combined electrode (type 6.0234.100). The titrant was added from a Metrohm 715 Dosimat automatic burette. The measurements were carried out at 25.0 °C and at an ionic strength of 0.2 M (KCl). Solutions of HCl and carbonate-free KOH (ca. 0.2 M, used as the titrant) were prepared from Merck products and their concentrations were determined by pH titrations. The electrode system was calibrated according to Irving et al.¹⁰ to convert pH readings into hydrogen

ion concentrations. The pH-potentiometric titrations were performed at $2.0 \leq \text{pH} \leq 11.0$ (or until precipitation occurred). The ligand concentration was $1 \times 10^{-3} \text{ M}$ and the metal-to-ligand ratio ranged from 1:1 to 1:5. The initial volume of the samples was 10.0 mL. The experimental results were utilized to establish the stoichiometry of the species and to calculate the stability constants. Species stoichiometry and stability constants were determined with the computer program PSEQUAD.¹¹ Volumes of the titrant were fitted and the accepted fittings were always below $1 \times 10^{-2} \text{ mL}$.

2.3. Relaxometry

Relaxometric measurements¹² were made on a Bruker Minispec MQ-20 instrument operating at 20 MHz. The spin-lattice relaxation time, T_1 , was measured with this technique by the inversion-recovery method.

The relaxivity of the Mn(II) aqua was determined in a separate experiment using the published methodology.¹² The volume of the samples was 1 mL, and the concentration of the metal ion varied in the range 0.2×10^{-3} – $20 \times 10^{-3} \text{ M}$.

For the investigated systems, the volume of the samples was 0.5 mL, and the ionic strength was 0.2 M KCl at 25 °C. To set the pH NEP (*N*-ethyl-piperazine with a $\log K_2 = 5.58$ (0.02) at $I = 1.0 \text{ M}$ KCl and 25 °C, pH = 5.50) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH = 7.57) buffer solutions were used. The measurements on Mn(II)-UDP samples were performed at pH = 5.50 only, while at both pH values on the $\text{Mn(II)-UDP-glucose}$ system. All studies were carried out under an inert atmosphere (Ar).

For the Mn(II)-UDP and $\text{Mn(II)-UDP-glucose}$ systems the metal ion concentration in the samples was set to $2 \times 10^{-3} \text{ M}$ while the metal to ligand ratio varied in the range of 1:(0.25–3) and 1:(0.25–6), respectively. For the Mn(II)-UDP system, the pH-dependence was also studied at $4.9 \leq \text{pH} \leq 6.6$ at a metal-to-ligand ratio of 1:2. The $\text{Mn(II)-UDP-glucose}$ system was also investigated by titrating the samples with Mn(II), giving metal-to-ligand ratios varying from the initial 1:5 to 5:1.

Relaxivity values were calculated using the observed $(1/T_1)$ values and the equilibrium concentration of the complex calculated from:¹²

$$[\text{MnH}_x\text{L}] = \frac{1/T_1^{\text{Mn}}[\text{Mn}]_t - 1/T_1'}{1/T_1^{\text{Mn}} - 1/T_1^{\text{MnH}_x\text{L}}}$$

where $1/T_1' = 1/T_1 - 1/T_w$ and $[\text{Mn}]_t = [\text{Mn}] + [\text{MnH}_x\text{L}]$.

$1/T_w$ = diamagnetic contribution to the relaxation rate ($1/T_1$ in the absence of Mn(II)).

Table 1
Comparison of stability constants for Mn(II)-UDP and Mn(II)-UDP-glucose (UDP-Glc) complexes

Entry	Equilibrium process	Constant ^a (logK)	Method	Conditions ^b	Ref.
1	$\text{Mn(II)} + \text{UDP}^{2-} = [\text{Mn(UDP)}]$	4.14(5)	pH-metry	25 °C, 0.2 M KCl	This work
2		4.07	pH-metry	25 °C, 0.1 M NaNO ₃	13
3		3.45	Calorimetry	37 °C, 0.1 M HEPES, pH 7.5	8
4		3.51	ESR titration	18 °C, pH 7.4	7
5		3.94	Unknown ^c	Unknown ^c	17
6		3.78 (2)	Relaxometry	25 °C/0.2 M KCl, pH 5.50	This work
7	$\text{Mn(II)} + \text{UDP-Glc}^{2-} = [\text{Mn(UDP-Glc)}]$	2.23	Calorimetry	37 °C, 0.1 M HEPES (pH 7.5)	8
8		1.72	Proton relaxation enhancement	Unknown ^d	5
9		1.77	ESR titration	18 °C, pH 7.4	7
10		2.98 (7)	Relaxometry	25 °C, 0.2 M KCl, 0.05 M NEP, pH 5.50	This work
11		3.57 (13)	Relaxometry	25 °C, 0.2 M KCl, 0.04 M HEPES, pH 7.57	This work

^a Standard deviations in the last significant digit are given in parentheses.

^b Abbreviations: HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, NEP: *N*-ethyl-piperazine.

^c The referred book was unavailable to us, therefore, the conditions of the measurement remained unknown. The given value was cited in Ref. 6.

^d The referred book was unavailable to us, therefore, the conditions of the measurement remained unknown. The given value was cited in Ref. 4.

$1/T_1^{\text{Mn}}$ and $1/T_1^{\text{MnHxL}}$ are the relaxivities of the Mn(II) and the complex formed.
 $x = 1$ Mn{H(UDP)}
 $x = 0$ Mn(UDP-glucose).
 L = UDP or UDP-glucose.

The computer program PSEQUAD¹¹ was used to obtain the stability constants from the calculated equilibrium concentrations of the complexes.

3. Results and discussion

The main goal of this work was to determine the strength of interaction between Mn(II) and UDP-glucose **1**. The pH-metric titration of UDP-glucose provided clear evidence for dissociation of a single proton in the measurable pH-range, which (based on chemical evidences and literature support¹³) belongs unambiguously to the deprotonation of the neutral N(3)H (see Chart 1) of the nucleobase residue. According to this result, the diphosphate moiety of **1** releases a proton in the very acidic region ($\text{pH} \ll 2$) and the UDP-glucose²⁻ form predominates from the beginning of the measurable pH-range. As a consequence, any pH-effect cannot belong to the metal-ion complexation of the diphosphate residue and pH-potentiometry cannot be applied to study complex formation between Mn(II) and UDP-glucose **1**. Owing to the very low intensity of the spin-forbidden d-d bands of the high-spin d⁵ Mn(II) complexes UV-visible spectrophotometry could not be applied to this system either.¹⁴ However, formation of the Mn(II)-UDP-glucose complexes can be followed by measuring the spin-lattice relaxation rate ($1/T_1$) of the water protons, since the complex formation between the Mn(II) ion and the anionic ligand reduces the number of metal-coordinated water molecules, thus providing a useful technique for quantitative analysis of the binding equilibrium. To check the applicability of this method to the Mn(II)-UDP-glucose system, a model system, Mn(II)-UDP, was studied first. The dissociation constants of the species existing

at pH ~ 2 of UDP-glucose²⁻ **1** and UDP²⁻ **3** (Chart 1) were determined by pH-potentiometry and the values (which are in very good agreement with the literature¹³) are shown in Chart 1.

3.1. Mn(II)-UDP model system

Stability constants for Mn(II)-UDP found in the literature are listed in Table 1 (entries 2–5). In this system the complex formation is accompanied by a measurable pH-effect, thereby, prior to the relaxometric measurements, the stability constant for the Mn(II)-UDP complex could be determined via pH-potentiometry. Representative titration curves are shown in Figure 1.

The calculated stability constant for the Mn(II) + UDP²⁻ = [Mn(UDP)] process (Table 1, entry 1) is in good agreement with the literature value,¹³ also determined by pH-potentiometry (entry 2; exclusive coordination of UDP via the diphosphate moiety was proven in that paper¹³). The somewhat bigger difference between our value and that determined by isothermal titration calorimetry⁸ (entry 3) or ESR titration⁷ (entry 4) is possibly due to the significantly different conditions of temperature and ionic strength.

The relaxivity of $8.14 \pm 0.03 \text{ mM}^{-1} \text{ s}^{-1}$, determined from an individual measurement, for Mn(II)aqua is in good agreement with the literature.¹⁴ The relaxivity of $9.91 \pm 0.58 \text{ mM}^{-1} \text{ s}^{-1}$ obtained for [Mn(UDP)] was significantly different, thus, the relaxivity (water-proton relaxation rate) is in principle applicable to the determination of the stability constant of the complex (Table 1, entry 6). A comparison of the stability constants obtained by relaxometry and by pH-potentiometry (entries 1 and 6, respectively) shows an acceptable agreement, particularly, if the somewhat different conditions (see Section 2) are also taken into account.

3.2. Mn(II)-UDP-glucose system

After proving the applicability of the relaxometric method for stability constant determination in the model system, measurements were performed on the Mn(II)-UDP-glucose system. The experimental data could be convincingly fitted by assuming the existence of the complex [Mn(UDP-glucose)] ($R_{[\text{Mn(UDP-glucose)}]} = 8.82 \pm 0.40 \text{ mM}^{-1} \text{ s}^{-1}$ at pH = 5.50; $R_{[\text{Mn(UDP-glucose)}]} = 8.91 \pm 0.39 \text{ mM}^{-1} \text{ s}^{-1}$ at pH = 7.57). It is important to note that the result obtained at pH = 7.57 carries a higher uncertainty as compared to that at pH = 5.50, because at higher pH deprotonation of N(3)H takes place to a small extent (ca. 2–3 %). Thus, intermolecular prototropic exchange processes between the protonated and deprotonated species might alter the relaxation rate of the water proton and the relaxivity, as well. Therefore, we think that the result obtained at pH 5.50 is more reliable. The logarithmic stability constants obtained for the Mn(II)-UDP-glucose complex are shown in Table 1, entries 10 and 11. The difference between these values, apparently due to the change of pH, probably reflects the above processes.

The stability constant calculated for the [Mn(UDP-glucose)] (entry 10) is lower with ca. one log unit than that of [Mn(UDP)] (entry 6). A similar difference between these two constants was also found by calorimetry⁸ (compare entries 3 and 7) and by ESR titration⁷ (entries 4 and 9). This difference is probably due to a decrease in the basicity of the diphosphate moiety upon substitution of the terminal OH-group by a carbohydrate residue. The direct correlation between the basicity of a coordinated diphosphate residue and the stability of the corresponding metal complex is detailed elsewhere.¹³ The stability of the Mn(II)-UDP-glucose complex is moderate, corresponding to a Gibbs energy change of $\Delta G = -4.07 \text{ kcal/mol}$. To demonstrate this, the extent of complex formation has been calculated as a function of the analytical concentration of Mn(II). The solid line in Figure 2 refers to the 1:1 concentration ratio of Mn(II) to UDP-glucose and shows that

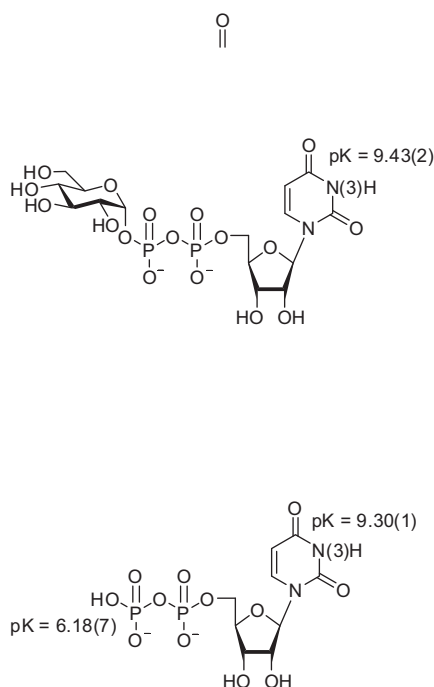


Chart 1. UDP-glucose²⁻ **1** and UDP²⁻ **3** (the predominant forms of the compounds existing at the beginning of the measurable pH-range, ca. pH 2). The pKs belonging to the neutral N(3)H residues and the terminal OH group of **3** are shown with standard deviations in parentheses.

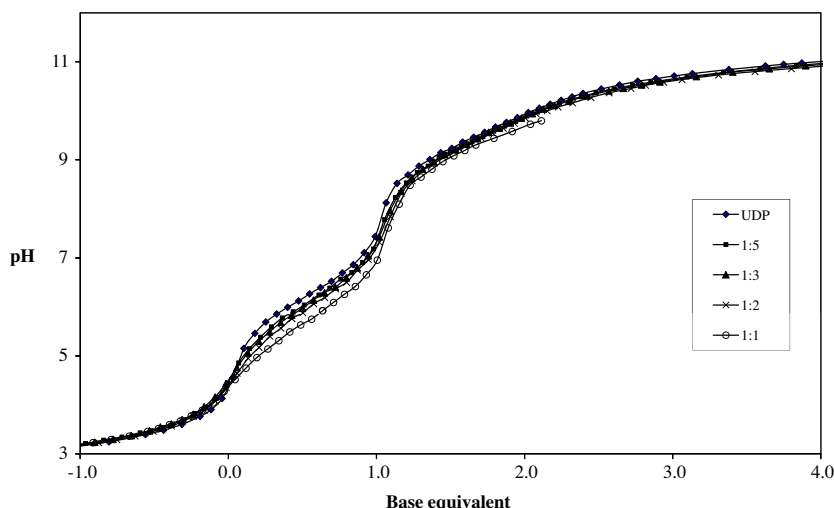


Figure 1. Potentiometric (pH) titration curves for UDP (◆) and Mn(II)-UDP at metal-to-ligand ratios of: 1:5 (■), 1:3 (▲), 1:2 (×) and 1:1 (○) with $c_{\text{ligand}} = 1 \times 10^{-3} \text{ mol dm}^{-3}$. Negative base equivalents refer to acid solutions.

the fraction of the complex (ratio of the complex to the total concentration of the metal ion) is significant for analytical concentrations above ca. 10^{-4} M . Formation of the complex becomes negligible below 10^{-4} M (pc is above 4) and is practically zero at and below 10^{-5} M . However, as is clearly shown by the dashed line, if the Mn(II) concentration is decreased only at a constant concentration (1.0 mM) of UDP-glucose, a well defined ratio (about 50%) of the total Mn(II) remains complexed even at $\text{pc}_{\text{Mn(II)}} = 6$ ($1.0 \mu\text{M}$).

For physiological (intracellular) concentration of Mn(II) various values can be found in the literature from 10^{-8} M to $2-3 \times 10^{-5} \text{ M}$, while a better concord exists for that of UDP-glucose ($2-4 \times 10^{-4} \text{ M}$). It follows from the above consideration that under physiological conditions formation of the Mn(II)-UDP-glucose complex is very minor or even negligible.

The higher stability of the Mn(II)-UDP complex (actually one of the products of a reaction catalyzed by a glycosyltransferase) compared to that of the Mn(II)-UDP-glucose complex (one of the

substrates of the reaction) must have implications for understanding the catalytic mechanism. The observed difference in the stabilities certainly reflects the enhanced leaving ability of UDP upon Mn(II) complexation. However, in the environment of the enzyme's active site a more complicated interplay of several other factors (e.g., binding of the metal ion to additional complexing residues like the frequent DXD motif, geometry of the complex, interactions of the active site residues with other parts of the substrate/product, conformational changes of the protein during the catalytic process) have to be considered. In this respect a very recent study suggests higher stabilization of a glycosyltransferase upon simultaneous addition of UDP-glucose and Mn(II) as compared to that of UDP and Mn(II). Such issues need, no doubt, further experimental studies and theoretical analyses.

In conclusion, as a result of this work (i) the applicability of relaxometry for the determination of the stability constant of the Mn(II)-UDP-glucose complex has been demonstrated; (ii) as a consequence of the moderate stability of that complex, if manga-

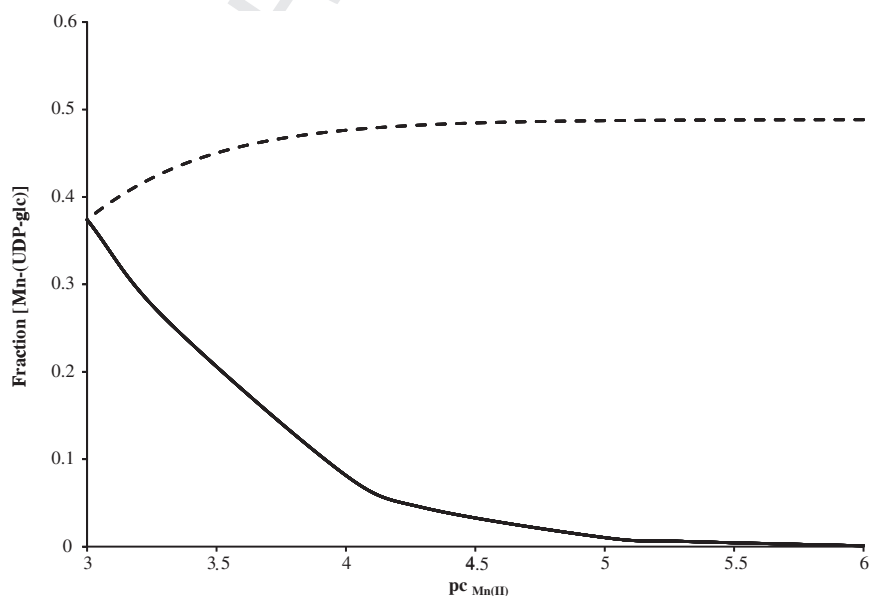


Figure 2. Fraction of complexed Mn(II) as a function of $[\text{Mn(II)}]$, at 1:1 Mn(II) to UDP-glucose ratio (solid line) and at constant 1.0 mM UDP-glucose concentration, where the ratio of Mn(II) to UDP-glucose is varied from 1:1 to 1:1000 (dashed line).

nese(II) and UDP-glucose are present at equimolar concentration in the sample, the complex is formed in measurable fraction only above an analytical concentration of 10^{-5} M; (iii) at high ligand excess a large fraction of Mn(II) is complexed even at micromolar concentrations of the metal ion.

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