

Triton-Stimulated Nucleoside Diphosphatase: Characterization¹

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Summary

Our previous report indicated a Triton-stimulated NDPase was specifically associated with Golgi membranes isolated from corn roots. Characterization of the enzyme indicates that UDP is the slightly preferred substrate with Mn^{2+} the preferred divalent cation. Monovalent cations do not further activate the Triton-stimulated UDPase activity. The enzyme has a pH optimum at 6.5 and a temperature optimum between 38-40°C. Kinetic analyses indicate that UDP- Mn^{2+} is the substrate for the enzyme.

Properties of the Triton-stimulated NDPase are compared to other membrane associated NDPases isolated from plants, animals and fungi. Characteristics and subcellular location of NDPase activity are discussed in relation to the possible biochemical role of the enzyme.

Keywords: Golgi; Membranes; Nucleoside 5'-diphosphatase; Triton X-100.

1. Introduction

Little information is available on the properties of the latent NDPase associated with Golgi membranes of higher plants. Because measurement of latent activity is a cumbersome process, no thorough characterization has been reported and consequently, the biological role of the enzyme is unclear. A partial characterization of a membrane associated NDPase was performed with a crude particulate fraction isolated from onion root tips [9]. Characterization was performed on fresh prepara-

tions and hence properties of the "latent" NDPase [18] were not determined. A recent characterization of a Golgi associated latent NDPase isolated from a brown alga has been reported [2], however, the properties are considerably different from those presented in this paper.

Our previous work with corn roots showed that a Triton-stimulated NDPase activity has an identical distribution and location to that of the Golgi associated cold storage-activated (latent) NDPase in linear sucrose density gradients [19]. Characterization of the Triton-stimulated NDPase is necessary to determine the optimum conditions for detecting activity, especially if this enzyme activity proves to be a useful marker for Golgi membranes in plant cell homogenates. Furthermore, characterization should provide greater insight as to the biochemical function of the enzyme as well as general knowledge about the function of the Golgi apparatus.

2. Materials and Methods

2.1. Plant Material

Corn seeds (*Zea mays* L. WF9Ht × Mol7Ht) were germinated in the dark for 4 days at 26°C [11]. Primary roots (6-8 cm in length) were excised with stainless steel scissors and immediately rinsed twice with cold deionized distilled water.

2.2. Centrifugation Procedures

Cell disruption was performed as described earlier [19]. Our previous results showed 36% of the total Triton-stimulated UDPase activity was pelleted between 1,000 to 13,000 × g and 56% sedimented between 13,000 to 80,000 × g [19]. Cell homogenates were centrifuged at 1,000 × g × 5 minutes and the supernatant was then centrifuged at

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80,000 × g × 35 minutes with a Type 42.1 rotor in a Beckman L5-65B ultracentrifuge. The 1,000 to 80,000 × g pellet was suspended in 0.25M sucrose + 1 mM dithiothreitol in 15 mM Tris-Mes pH 7.2 and overlaid on a step gradient composed of 8 ml of 26% sucrose and 28 ml of 32% sucrose (W/W). The step gradient was designed from the results of linear sucrose density centrifugation [19]. Step gradients were centrifuged at 80,000 × g for 2 hours in an SW 28 rotor and membranes were collected from the 26%/32% sucrose interface.

2.3. Enzyme Assays

K⁺-stimulated ATPase activity was determined as described by LEONARD and VANDERWOUDE [12]. NDPase activity was measured in the presence of 3 mM UDP + 3 mM MnSO₄ in 30 mM Tris-MES pH 6.5, unless otherwise indicated in the text. Triton-stimulated (or activated) NDPase was determined by subtracting the activity in the absence of detergent from that measured in the presence of 0.03% detergent. Assays were incubated at 37 °C for 15 to 20 minutes with 18 to 27 μg of membrane protein unless otherwise indicated. Triton X-100 interference of the Fiske and SubbaRow assay was eliminated by using sodium dodecyl sulfate in the color reagents [5, 19, 21]. All phosphorylated substrates were prepared as described [7]. NADH cytochrome c reductase activity was assayed according to HODGES and LEONARD [7] in the presence of antimycin A [13]. Protein was estimated according to LOWRY *et al.* [14].

3. Results

3.1. Discontinuous Sucrose Gradient Centrifugation

Our previous study [19] showed that the nearest contaminants of Golgi membranes purified in sucrose gradients were likely to be PM and ER. The degree of contamination by the tonoplast could not be determined since no reliable marker for vacuole membrane has been reported. Specific activities of the PM, Golgi, and ER markers in the 1,000 to 80,000 × g fraction prior to sucrose gradient separation were compared to specific activities of the markers in the membrane fraction collected at the 26%/32% interface after centrifugation (Table 1).

Triton-stimulated UDPase activity (Golgi) was enriched 4 fold while the K⁺-stimulated Mg²⁺-ATPase (a marker for PM in corn roots; ref. 12), and antimycin A insensitive NADH cyt c reductase activity (ER) both showed a decrease in specific activity (Table 1). These data confirm our choice of a step gradient design used to purify and further characterize the Triton-stimulated UDPase.

3.2. Characterization of the Golgi Associated NDPase

Triton-stimulated UDPase activity was linear for 30 minutes (Fig. 1) and was linear with respect to

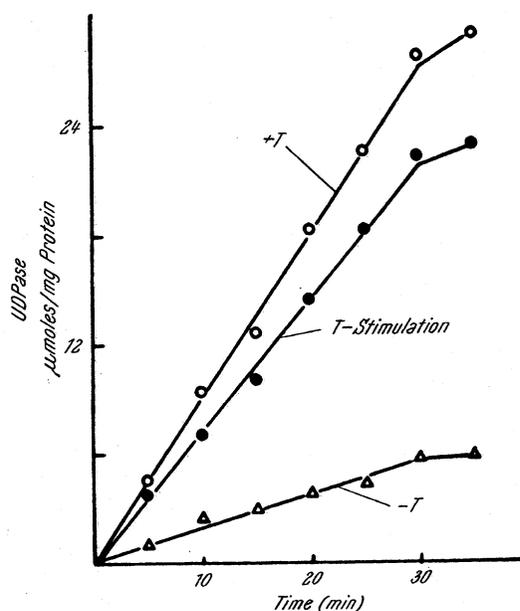


Fig. 1. Time course for Triton-stimulated UDPase activity associated with Golgi membranes isolated from corn root homogenates. Membranes were purified by differential centrifugation followed by discontinuous sucrose gradient separation. See Materials and Methods for details

Table 1. Enrichment of the Triton-stimulated UDPase activity associated with Golgi membranes isolated from corn root homogenates. A 1,000-80,000 × g pellet (crude) was overlaid on a two step gradient composed of 26% and 32% sucrose (W/W) and centrifuged for two hours at 80,000 × g. The interface (purified membranes) was collected and assayed as described in Materials and Methods

Fraction	UDPase Activity μmoles Pi/mg protein · hr			2 ⁺ Mg-ATPase Activity	NADH Cytochrome c Reductase Activity μmoles/mg protein · min
	- Triton	+ Triton	Triton- Stimulation	K ⁺ Stimulation	+ Antimycin A
Crude	8.84	20.44	11.60	5.14	0.299
Purified	11.08	56.64	45.56	1.97	0.281

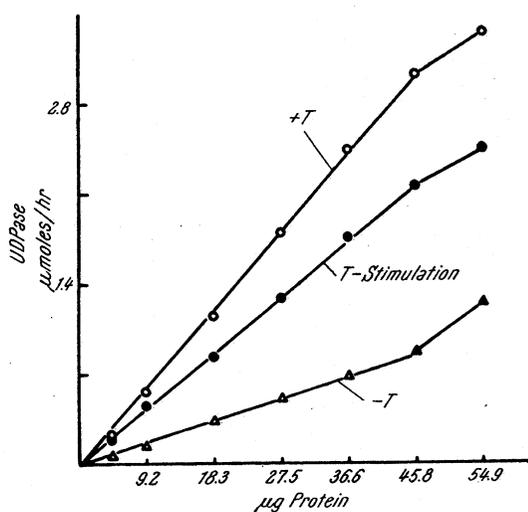


Fig. 2. Protein dependence curve for Triton-stimulated UDPase activity associated with Golgi membranes isolated from corn root homogenates. See Materials and Methods for membrane isolation and purification details

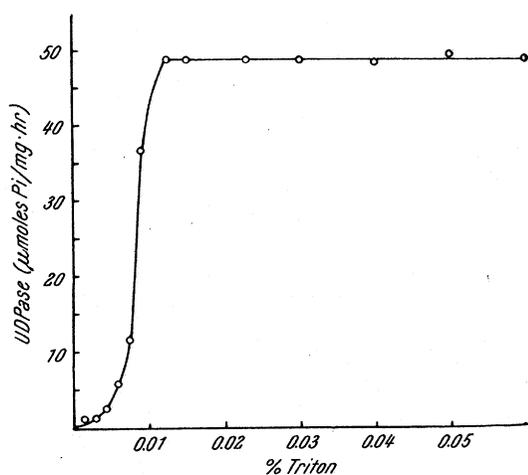


Fig. 3. Triton X-100 dependence for UDPase activity associated with Golgi membranes isolated from corn root homogenates. Assays were performed with 20 μg of membrane protein and similar results were obtained with 31 μg of membrane protein

membrane protein concentration up to 45 μg (Fig. 2). Maximum detergent activation occurred at 0.015% Triton X-100 (Fig. 3) and 0.03% detergent was used in all further assays. The presence of SDS in the Fiske and SubbaRow reagent prevented precipitation by Triton X-100 [19].

Other detergents or surfactants (all at 0.03% final concentration) such as digitonin, saponin, octyl glucopyranoside and SDS did not interfere with the Fiske and SubbaRow procedure. Digitonin and saponin activated the latent UDPase (Table 2) while

Table 2. Detergent-activation of a Golgi associated UDPase isolated from corn roots and purified by discontinuous sucrose gradient centrifugation [22]. Detergent-stimulation was determined by subtracting the activity measured in the absence of detergent from that measured in its presence. All compounds were tested at 0.03% final concentration. See Materials and Methods for assay conditions and for further information about the interference of Triton X-100 and deoxycholate with the Fiske and SubbaRow procedure

Detergent or Surfactant	UDPase Activity μmoles Pi/mg protein · hr	
	0	Detergent-stimulation
0	13.68	
Sodium dodecyl sulfate	4.45	0
Octylglucopyranoside	14.09	0.41
Deoxycholate	20.82	7.14
Saponin	57.04	43.36
Digitonin	58.95	45.27
Triton X-100	60.29	46.61

octylglucopyranoside and SDS did not produce substantial activation under our assay conditions. Deoxycholate treatment activated the latent UDPase but like Triton X-100, the modified Fiske and SubbaRow procedure [19] was necessary to prevent turbidity. A detergent concentration-dependent activation of UDPase was only performed with Triton X-100 (Fig. 3) and optimal conditions for other detergents was not determined.

Triton-stimulated UDPase activity had a temperature optimum between 38-40 °C (Fig. 4) although consider-

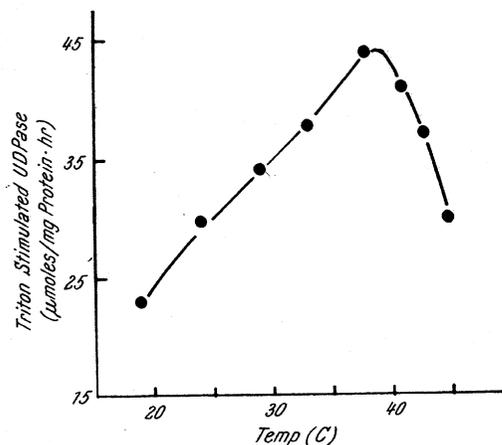


Fig. 4. Temperature optimum for Triton-stimulated UDPase activity associated with Golgi membranes isolated from corn root homogenates

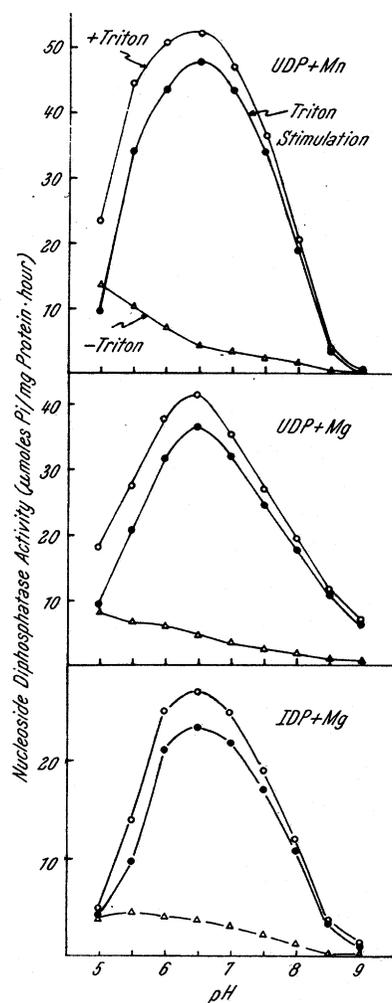


Fig. 5. Optimum pH for Triton-stimulated nucleoside diphosphatase activity associated with Golgi membranes isolated from corn root homogenates. Three different combinations of nucleoside diphosphate plus divalent cation were tested

able activity was detected at 18°C. The pH optimum was determined by using UDP + MnSO₄, UDP + MgSO₄ and IDP + MgSO₄ (Fig. 5). IDP + MgSO₄ was used

because previous workers, using corn root homogenates, assayed for latent IDPase activity at pH 7.5 in the presence of 3 mM IDP + 3 mM MgSO₄ and 50 mM KCl [12]. In all three combinations, the Triton-stimulated activity exhibited an optimum at pH 6.5 (Fig. 5).

The Triton-stimulated NDPase substrate preference was UDP > IDP > GDP >>> CDP > ADP > ATP (Table 3). Five out of five times, UDP was the slightly preferred substrate, however, in 2 out of 5 experiments, the order of preference between IDP and GDP was reversed. The divalent cation specificity in the presence of UDP was Mn²⁺ > Ca²⁺ > Mg²⁺ > Fe²⁺ (Table 4) regardless of the anion used (sulfate or chloride). The divalent cation specificity in the presence of IDP was Mn²⁺ > Mg²⁺ > Ca²⁺ > Fe²⁺ (Table 5) and the significance of the reversal of preference between Ca²⁺ and Mg²⁺ was unclear to us.

Monovalent cations (KCl, NaCl, CsCl, and LiCl at 50 mM) did not further activate the Mn²⁺-UDPase (data not shown) and consequently, KCl was omitted from the assay during characterization studies. Cesium chloride appeared to inhibit the Triton-stimulated UDPase, however, we have found that CsCl interfered with the modified Fiske and SubbaRow procedure [5, 21]. Concentrations of CsCl greater than 15 mM reduced color development of the phosphorus assay (data not shown). No other monovalent cations tested interfered with the modified Fiske and SubbaRow procedure.

3.3. Enzyme Kinetics

When UDP is held constant and MnSO₄ varied, the Triton-activated UDPase exhibits saturation kinetics (Fig. 6). To determine if the hyperbolic saturation represents a simple Michaelis-Menten enzyme, a linear transformation of the Michaelis-Menten equation was

Table 3. Substrate specificity of the Triton-stimulated nucleoside diphosphatase activity associated with Golgi membranes isolated from corn root homogenates. Membranes were purified as in Table 1. Assays were performed at 37°C at pH 6.5 with 3 mM MnSO₄ + 3 mM phosphorylated substrate

Substrate	µmoles Pi/Mg protein · hr		
	- Triton	+ Triton	Triton-stimulation
UDP	15.04	61.54	46.50
IDP	12.33	55.46	43.13
GDP	13.67	52.86	39.19
CDP	5.96	9.96	4.00
ADP	7.25	10.83	3.58
ATP	10.42	11.04	0.62

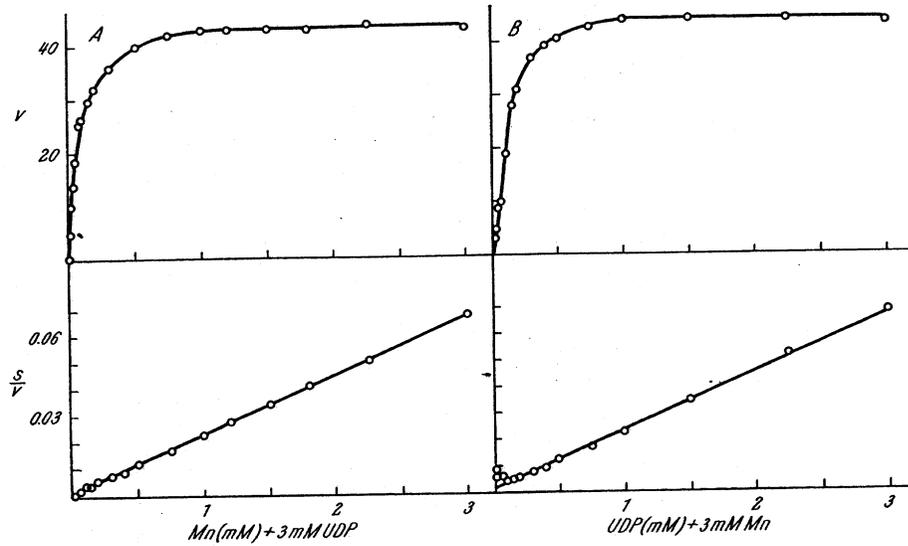


Fig. 6. Enzyme kinetics of the Triton-stimulated UDPase activity associated with Golgi membranes isolated from corn root homogenates. (A, Top) V vs S plot where $V = \mu\text{moles Pi/mg protein} \cdot \text{hr}$ and $S = \text{variable concentration of MnSO}_4 \text{ (mM)}$ with 3 mM UDP held constant. Bottom: Linear transformation of the Michaelis-Menten equation (Hane's plot). $K_M = 0.065 \text{ mM UDP}$ and $V_{max} = 45 \mu\text{moles/mg protein} \cdot \text{hr}$. (B) Enzyme kinetics of the Triton-stimulated UDPase activity associated with Golgi membranes isolated from corn root homogenates. Top: $V = \mu\text{moles Pi/mg protein} \cdot \text{hr}$, and $S = \text{variable concentration of UDP (mM)}$ with 3 mM MnSO_4 held constant. Bottom: Linear transformation of the Michaelis-Menten equation (Hane's plot). $K_M = 0.07 \text{ mM MnSO}_4$ and $V_{max} = 46 \mu\text{moles/mg protein} \cdot \text{hr}$

Table 4. Divalent cation specificity for the Triton-stimulated nucleoside diphosphatase associated with Golgi membranes isolated from corn root homogenates. 3 mM UDP was used with 3 mM divalent cation (sulfate or chloride anion) and assays were performed at pH 6.5. See Materials and Methods for details of the assay

Divalent Cation	UDPase Activity ($\mu\text{moles Pi/mg Protein} \cdot \text{hr}$)					
	Sulfate anion			Chloride anion		
	- Triton	+ Triton	Triton-Stimulation	- Triton	+ Triton	Triton-Stimulation
0	8.53	15.78	7.25	8.53	15.78	7.25
Mn	14.25	59.03	44.78	13.00	59.66	46.66
Ca	14.44	53.91	39.47	13.25	54.81	41.56
Mg	13.63	38.75	25.12	12.81	38.56	25.75
Fe	6.19	23.50	17.31	6.09	21.78	15.69

Table 5. Divalent cation specificity for the Triton-stimulated nucleoside diphosphatase associated with Golgi membranes isolated from corn root homogenates. 3 mM IDP was used with 3 mM divalent cation (see Table 3)

Divalent cation	IDPase Activity ($\mu\text{moles Pi/Mg protein} \cdot \text{hr}$)		
	- Triton	+ Triton	Triton-Stimulation
0	4.56	12.50	7.94
MnSO_4	12.68	54.32	41.64
MgSO_4	10.08	41.72	31.64
CaSO_4	9.22	33.96	24.74
FeSO_4	3.28	22.58	19.30

plotted. A K_M of 0.065 mM and V_{max} of 45 μ moles/mg protein \cdot hr was determined from a Hane's plot (S/V vs S) which showed a straight line (Fig. 6 A). When $MnSO_4$ was held constant and the concentration of UDP varied (Fig. 6 B), and identical saturation of the Triton-stimulated UDPase was observed (compare to Fig. 6 A). The Hane's plot (Fig. 6 B), indicated second substrate inhibition under the assay conditions of high $MnSO_4$ (3 mM) with very low amounts of UDP (0.02-0.1 mM). The K_M was determined to be 0.07 mM and V_{max} was 46 μ moles/mg protein \cdot hr (Fig. 6 B). Since the K_M and V_{max} for UDP and $MnSO_4$ are virtually the same, it is likely that UDP- Mn^{2+} is the actual substrate for the Triton-stimulated UDPase.

4. Discussion

4.1. Properties of Nucleoside Diphosphatases

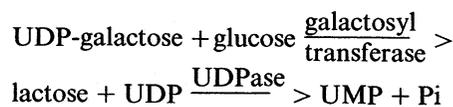
Partial characterization of membrane associated nucleoside diphosphatases isolated from plants [2, 9, 18, 23], animals [10] and fungi [4, 6] have been reported. Current available evidence indicates that the NDPase has a slightly acid pH optimum of 6.5, prefers Mn^{2+} over other divalent cations, does not require monovalent cations, and prefers UDP, GDP, and IDP over other phosphorylated substrates. Triton treatment usually activates nucleoside diphosphatase activity [2, 9, 10, 18, 19, 22] with one exception [23]. The NDPase appears to be a simple Michaelis-Menten enzyme with a low K_M for NDP (Figs. 6 A and 6 B, Refs. 4, 6, 10). The Triton-stimulated UDPase from corn roots has the lowest K_M reported for higher plants (Figs. 6 A and 6 B). Our results indicate that Mn-UDP is the actual substrate and should be used in equimolar concentrations (Figs. 6 A and 6 B) during enzyme assays. This result is considerably different from the high levels of divalent cation (20 mM) used in relation to NDP for detecting NDPase activity in Golgi bodies isolated from brown alga [2].

4.2. Role of the Nucleoside Diphosphatase

Currently, there are two interpretations for the latent NDPase activity associated with subcellular membranes. According to RAY *et al.* [22] the NDPase activity represents a partially inactivated glucan synthetase, *i.e.*, the NDPase and glycosyl transferase (glucan synthetase) are one and the same enzyme. This conclusion was inferred from the observation that the activation of Golgi associated IDPase activity isolated from etiolated pea seedlings follows a time course similar to the decline of Golgi associated glucan synthetase. Furthermore,

treatment with digitonin or Triton X-100 activated the IDPase and inhibited the glucan synthetase. MORRÉ *et al.* [18] isolated Golgi membranes from onion stems and found a progressive loss of glucan synthetase activity with time while the increase in IDPase activity was more abrupt. This result suggests the presence of two distinct enzymes.

The second interpretation, which postulates a role for the NDPase, presumes that NDPase and glycosyl transferase are two different enzymes. The latent UDPase activity associated with Golgi membranes from mammalian cells has been implicated in a coupled reaction involving a glycosyl transferase [10]:



Free UDP inhibits the transferase and the UDPase quickly removes the inhibitory product. Support for the role of NDPase activity is provided by the observations that free NDP (usually UDP or GDP) inhibits glycosyl transferase reactions in plants (glucan synthetase, ref. 22; sterol glucosyl transferase, ref. 23) and fungi (chitin synthetase, ref. 4). NDPase activity relieves or prevents product inhibition of the glycosyl transferase reaction [6, 10, 23]. Consistent with the postulated role for the NDPase are the cytochemical reports which have correlated the localization of polysaccharide in the Golgi apparatus with the presence of NDPase activity [3, 16]. The NDPase would presumably remove free NDP and allow polysaccharide synthesis to occur.

Sugar derivatives of UDP and GDP are most commonly used in glycoprotein glycosyl transferase reactions associated with Golgi [20] and ER [15, 17, 20]. NDPase, which prefers UDP and GDP, has not been implicated in glycoprotein synthesis. Since subcellular fractionation studies have not confirmed the localization of NDPase in plant ER [19], it may be that the enzyme is not involved in "core" glycosylation reactions and only involved with Golgi associated glycosyl transferase reactions which add to the "core" [1].

The low K_M of the Triton-stimulated UDPase reported here (Figs. 6 A and 6 B) support the potential role of the enzyme for maintaining levels of UDP in the Golgi apparatus. Very low levels of UDP have been shown to inhibit a UDP-glucose sterol transferase in soybean seeds and only a NDPase with a very low K_M would rapidly remove free NDP. However, it is unclear whether each type of Golgi associated glycosyl

transferase has its own NDPase [6] or whether there is only one NDPase present which simply acts as a general safety valve by maintaining low levels of free nucleoside diphosphates. It is also unclear whether the specific association of NDPase activity and glucan synthetase reported by RAY *et al.* [22] is an exception rather than the rule.

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