GLYCOLIPID FORMATION IN *VOLVOX CARTERI* f. *NAGARIENSIS*

Effects of tunicamycin and showdomycin

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1. Introduction

Inhibitors of glycosylation have become useful tools in elucidating the biological function of glycoproteins and the processes proper of their formation. Recent studies in vitro in a variety of mammalian tissues and plants have shown that antibiotics such as amphomycin, bacitracin, diumycin, tsushimycin or tunicamycin represent more or less potent inhibitors of certain steps in the lipid-mediated glycosylation pathway [1–3]. However, the observed inhibition patterns are quite complex and the results obtained with a number of membrane systems are, at least in part, contradictory. Without further investigation, therefore, the usefulness of these inhibitors remains dubious and their value for understanding of the reactions concerned rather limited.

Exploratory studies in our laboratory have demonstrated that particulate membrane fractions of the green alga *Volvox carteri* f. *nagariensis* are capable of synthesizing Dol-P- and Dol-PP-sugar derivatives, suggesting a glycolipid pathway of protein glycosylation which may be common to plant and animal kingdom [5]. In order to further characterize those enzymes which catalyze the formation of Dol-P-glucose and Dol-PP-*N*-acetylglucosamine, we have investigated the influence of two antibiotics, viz. tunicamycin and showdomycin, on their activity. Evidence is presented showing that under conditions in vitro, tunicamycin strongly inhibits the UDP-*N*-acetylglucosamine: dolichyl phosphate-*N*-acetylglucosamine-1-phosphate transferase (EC 2.4.1.), the first enzyme of the dolichol cycle, whereas Dol-P-glucose synthesis is affected only at considerably higher concentrations of the antibiotic. Showdomycin (2-(β-D-ribofuranosyl)maleimide), on the other hand, blocks both transferases with identical kinetics. This inhibition is probably due to an irreversible reaction of the antibiotic with essential thiol groups of the enzymes under investigation.

Abbreviations: Dol-P, Dol-PP, dolichyl mono (di) phosphate, DTT, dithiothreitol; NDP-sugar, nucleoside diphosphate sugar

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2. Materials and methods

2.1. *Materials*

UDP-[^{14}C]glucose (spec. act. 240 Ci/mol), UDP-*N*-acetyl-[^{14}C]glucosamine (spec. act. 346 Ci/mol) and GDP-[^{14}C]mannose (spec. act. 108 Ci/mol) were obtained from The Radiochemical Centre (Amersham). Unlabeled sugar nucleotides, uridine monophosphate, iodoacetamide and dolichylphosphate were from Sigma GmbH (Taufkirchen). Showdomycin and *N*-ethylmaleimide were purchased from Serva (Heidelberg). Tunicamycin was the generous gift of Dr R. L. Hamill, Lilly Research Laboratories, (Indianapolis, IN). All other chemicals were of the highest purity available.

2.2. *Growth of* Volvox carteri *and membrane preparation*

Cultures (500 ml) of the strains HK 10 (female) and 69-1b (male) of *Volvox carteri* f. *nagariensis* were grown axenically in Provasoli *Volvox*-medium according to [4] and harvested after 4–6 days. Particulate membrane fractions were prepared from asexual colonies essentially as in [5]. Studies in vivo with tunicamycin were performed on the male strain.

2.3. *Glycosyl transferase assay*

Standard assay mixtures contained the following

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components in a final volume of 100 μl: 5 μg Dol-P, NDP-[^{14}C]sugar (10^4–10^5 cpm), 0.6% Triton X-100, 10 mM metal ions as indicated, 2 mM DTT, 20 mM Tris–HCl (pH 7.4) and an appropriate amount of the membrane preparation (100–200 μg). The incubations were performed at room temperature (22–25°C) for the times indicated and terminated by addition of 1 ml chloroform/methanol (2:1, v/v). The glycolipids were isolated following the multiple extraction procedure in [6].

2.4. *Inhibition studies*

The particulate membrane fraction was preincubated at 0°C with tunicamycin, showdomycin, or UMP for 15 min and the remaining transferase activity tested by the above standard procedure at room temperature. Controls were run with buffer alone.

2.5. *General procedures*

Protein was determined by the Lowry-method with bovine serum albumin as a standard [7]. Glycolipids and free sugars were separated on silica gel thin layer sheets (Merck, Darmstadt) using the following solvent systems: chloroform/methanol/ammonia (1 M) (65:25:4 and 60:35:6, by vol.) or *n*-butanol/acetic acid/water (4:1:1, by vol.). Radioactivity was measured in a Delta 300 scintillation counter (Searle Analytic Inc.) using as scintillation mixture 4 g 2,5-diphenyloxazole, 0.2 g 1,4-bis-2(5-phenyloxazolyl)-benzene, 60 g naphtalene and 20 ml ethylene glycol in 100 ml methanol, filled up with dioxane to 1000 ml. Radioautograms were taken on Sakura QH X-ray film.

3. Results and discussion

3.1. *Influence of metal ions and detergents on the synthesis of Dol-P-glucose, Dol-P-mannose, and Dol-PP-*N*-acetylglucosamine*

The formation of Dol-P-glucose, Dol-P-mannose and Dol-PP-*N*-acetylglucosamine by a particulate membrane fraction from *Volvox carteri* as well as their chemical characterization was described in [5]. To increase the yield of these glycolipids for the subsequent inhibition experiments we have studied systematically the effect of divalent metal ions and detergents on their synthesis. The results are summarized in table 1. The transfer of [^{14}C]sugars from their respective nucleoside diphosphates to chloroform–methanol-soluble glycolipid derivatives is stimulated by addition of certain divalent cations in the following order of effectiveness: $Mn^{2+} > Mg^{2+} \gg Ca^{2+}$ for the synthesis of Dol-P-glucose; $Mg^{2+} \gg Mn^{2+}, Ca^{2+}$ for the transfer of mannose and *N*-acetylglucosamine-1-phosphate to Dol-P. No glycolipid formation was found with Fe^{2+} and Fe^{3+}, or in the presence of ethylenediamine tetraacetate (EDTA, 2 mM). Only small amounts of glycolipids are synthesized when detergents are omitted (fig.1). Enzyme activity is maximal with 0.6% Triton X-100. Other non-ionic detergents (Lubrol WX, Brij 56) and ionic detergents of the sulfobetaine type are less effective or do not stimulate glycolipid formation at all (not shown). The critical micelle concentration of Triton X-100 is 0.24 mM [8]; i.e., ~60-times lower than the detergent concentration necessary for optimal transferase activity. This suggests

Table 1
Effect of metal ions on the formation of Dol-P-glucose, Dol-P-mannose, and Dol-PP-*N*-acetylglucosamine in *Volvox carteri* membrane preparations

Metal ion added (10 mM final conc.)	Dol-P-glucose (pmol)	Dol-P-mannose (pmol)	Dol-PP-*N*-acetyl-glucosamine (pmol)
None	<0.01	<0.01	<0.01
EDTA (2 mM)	<0.01	<0.01	<0.01
Mg^{2+}	2.00	0.22	2.17
Mn^{2+}	2.46	0.08	1.64
Ca^{2+}	0.19	<0.01	<0.01
Fe^{2+}	<0.01	<0.01	<0.01
Fe^{3+}	<0.01	<0.01	<0.01
Co^{2+}	<0.01	0.07	0.04
Ni^{2+}	<0.01	0.02	<0.01

Enzyme activity was measured under standard assay conditions in the presence of metal ions as indicated; nucleotide sugar was 0.7 μM; incubation was for 30 min

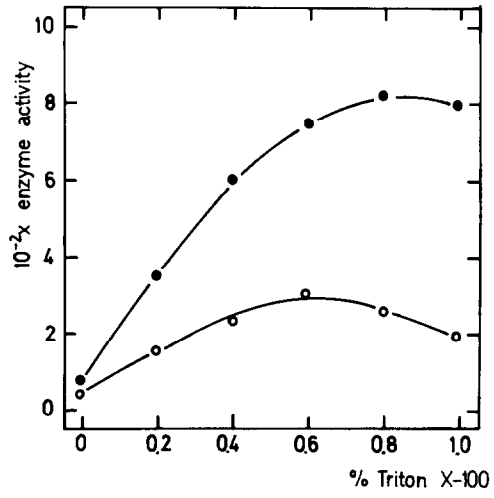

Fig.1. Effect of increasing Triton X-100 concentration on the formation of Dol-P-glucose and Dol-PP-*N*-acetylglucosamine by *Volvox carteri* membrane preparations. Glycosyl transferase activity was assayed as in section 2; nucleotide sugar was 0.7 μM; incubation was for 30 min; (•——•) Dol-P-glucose; (○——○) Dol-PP-*N*-acetylglucosamine.

that the observed enhancement of glycolipid synthesis in the presence of 0.6% Triton X-100 is only in part due to the solubilization of exogeneously added Dol-P, but mainly caused by the disruption of the microsomal membrane vesicles, thus facilitating accessibility of the respective glycosyltransferases.

3.2. *Effect of tunicamycin on the formation of Dol-PP-*N*-acetylglucosamine, Dol-PP-di-*N*-acetylchitobiose and Dol-P-glucose*

Tunicamycin was first reported by Tkacz and Lampen [9] to inhibit specifically the synthesis of Dol-PP-*N*-acetylglucosamine. Subsequent work with a number of animal and plant tissues confirmed this observation [1,10,11]. Although its mechanism of action is not understood in detail, tunicamycin is assumed to act as a multi-substrate analogue, the lipid moiety mimicking the dolichol phosphate group, and the uracil–carbohydrate–glucosamine structure resembling UDP-*N*-acetylglucosamine. When a microsomal fraction of *Volvox carteri* is incubated with UDP-*N*-acetylglucosamine and various amounts of tunicamycin, a concentration-dependent inhibition of Dol-PP-*N*-acetylglucosamine formation is observed (fig.2). Inhibition of the enzyme of 50% was obtained at 0.020–0.025 μg tunicamycin/ml, this value, however, varies slightly with the amount of membranous protein present in the incubations. This concentration dependence is consistent with the assumption that the antibiotic is a tight-binding and reversible (probably competitive) inhibitor, since an irreversible inhibition would be expected to give linear loss of activity with tunicamycin concentration and an intersection with the abscissa at an inhibitor concentration corresponding stoicheiometrically to the concentration of the enzyme.

An enzyme activity has been observed in the *Volvox* system catalyzing the transfer of a second *N*-acetylglucosamine residue to Dol-PP-*N*-acetylglucosamine, thus giving rise to a chitobiosyl lipid [5]. Using Dol-PP-*N*-[^{14}C]acetylglucosamine as the acceptor and unlabeled UDP-*N*-acetylglucosamine as the glycosyl donor, we found no inhibition of Dol-PP-di-*N*-acetylchitobiose synthesis at tunicamycin concentrations that blocked the formation of the lipid bound monosaccharide completely. Fig.2 shows, on the other hand, that the formation of Dol-P-glucose is inhibited by tunicamycin. Considerably higher (70–80-times) concentrations of tunicamycin are, however, required to achieve a comparable inhibition.

In vivo studies have demonstrated that administration of tunicamycin to the culture medium resulted

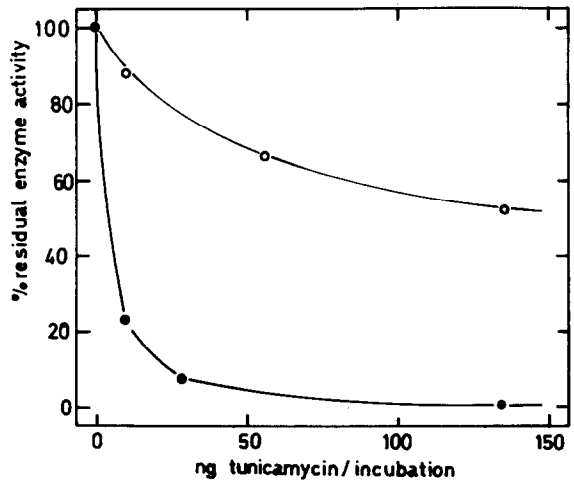

Fig.2. Inhibition of Dol-PP-*N*-acetylglucosamine and Dol-P-glucose synthesis by tunicamycin. The particulate membrane fraction of *Volvox carteri* spheroids was prepared in the presence of 2.5 mM DTT and preincubated for 15 min with varying amounts of the antibiotic. Remaining glycosyl transferase activity was measured as in section 2. Donor: (•——•) UDP-*N*-acetyl-glucosamine; (○——○) UDP-glucose; tunicamycin M_r = ~830.

in a changed morphological pattern of *Volvox* colonies: Increasing concentrations of the antibiotic from 0.05–0.5 μg/ml in the medium was accompanied by a striking alteration in the reproductive cycle of the alga such as inversion and hatching of daughter colonies, whereas the motility of parental colonies was far less affected. In the same concentration range (<0.1 μg/ml, R. Gilles, personal communication) of tunicamycin, the capability of *Volvox* gonidia for sexual differentiation triggered by the inducer glycoprotein [4] was lost. These observations as well as the high specificity of tunicamycin to interfere with the lipid-mediated glycosylation pathway [12] suggests that N-linked glycoproteins are involved in these developmental and differentiation processes.

3.3. *Inhibition of Dol-P-glucose and Dol-PP-*N*-acetylglucosamine synthesis by showdomycin*

Studies [13] had demonstrated the nucleoside antibiotic showdomycin to be quite effective in inhibiting the glycosyl-phosphoryl-dolichol synthesis in aorta tissue. Since high amounts of Dol-P-glucose are synthesized by a microsomal preparation from *Volvox carteri*, we were interested to see whether this antibiotic would exert a similar inhibitory effect on the Dol-P-glucose formation in the alga. Indeed, the corresponding transferase was inhibited in a rapid and concentration-dependent manner. This inhibition was more pronounced when membranes were used that had been prepared in the absence of DTT (fig.3). In addition, the synthesis of Dol-PP-*N*-acetylglucosamine was blocked with an identical reaction rate when the same incubation conditions were employed.

The inhibitory effect of showdomycin on both enzymes was lost, however, when the antibiotic was preincubated with an excess of DTT (table 2). Based on the structural relationship of showdomycin to uridine and maleimide, it seemed likely that the antibiotic itself may be 'inactivated' by covalent binding of the maleimide part to the sulfhydryl groups of DTT, suggesting that the inhibition of the above glycosyl transferases may be caused by a similar chemical reaction of possibly essential SH-groups at the active site of the enzyme.

To clarify this hypothesis, we have tested the influence of various SH-alkylating reagents on their ability to inhibit the Dol-P-glucose formation. As seen from table 2, summarizing the results of these studies, we found that the glycosyl transferase is inactivated by reaction with *N*-ethylmaleimide and that the inactiva-

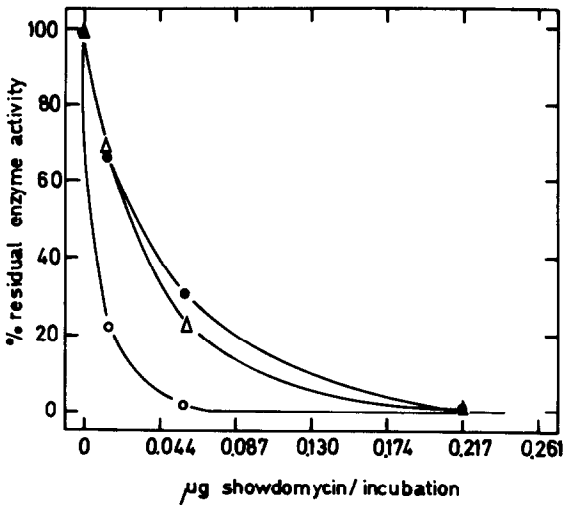

Fig.3. Effect of showdomycin on the formation of Dol-PP-*N*-acetylglucosamine and Dol-P-glucose by *Volvox carteri* membrane preparations. The particulate membrane fraction was preincubated with showdomycin for 15 min and the remaining enzyme activity measured by the standard procedure in section 2: (△——△) Dol-PP-*N*-acetylglucosamine, and (●——●) Dol-P-glucose, synthesized by membranes prepared in the presence of 1.25 mM DTT; (○——○) Dol-P-glucose, synthesized by membranes prepared without DTT; showdomycin M_r = 229.

Table 2
Effect of various alkylating agents on glycolipid formation in *Volvox carteri* membrane preparations

Reagent added (2.0 mM final conc.)	Dol-P-glucose formed (cpm)
None	910
Showdomycin	<20
Showdomycin, preincubated with DTT (4 mM)	1030
Showdomycin (2 mM), UMP (0.6 mM) and membrane fraction; DTT (6.0 mM) added after 15 min	200
N-Ethylmaleimide	<20
Iodoacetamide	800
UMP (0.6 mM)	230
UMP (2 mM)	<20

Membranes were prepared without DTT in the homogenation buffer and preincubated for 15 min at room temperature with the reagents indicated. The remaining enzyme activity was assayed as in section 2. For measuring the protective effect of UMP, membranes were preincubated in the presence of showdomycin (2.0 mM) and UMP (0.6 mM). After 15 min, the mixture was made 6 mM in DTT, and the residual transferase activity determined after further 15 min reaction time

tion rate was comparable to that obtained with showdomycin. The enzyme could be protected against inhibition by showdomycin or *N*-ethylmaleimide by the addition of UMP, indicating a reaction of the inhibitors at the active site. Inactivation of the transferase by iodoacetamide required longer preincubation times as is expected from its lower reactivity as compared to *N*-ethylmaleimide [14].

We conclude that the inhibition of Dol-P-glucose and Dol-PP-*N*-acetylglucosamine synthesis by showdomycin is caused by an irreversible reaction of the maleimide structure of the antibiotic with essential nucleophilic groups (probably thiol groups) near or at the active site of the glycosyl transferases. The similar inhibition rates using showdomycin or *N*-ethylmaleimide indicate that inhibition by the former is probably not due to its alleged substrate similarity but rather to its reactivity as a maleimide derivative. This interpretation finds support in the observation that glycolipid formation in liver is not inhibited by either *N*-ethylmeleimide or showdomycon, and does not require DTT for maximum activity. Thus, in contrast to the mammalian liver system, the algae glycolipid synthesizing enzymes seem to possess essential cysteine residues in or nearby the active centre.

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