Acta Biochimica Polonica

Vol. 41 No. 3/1994

QUARTERLY

A recent approach to the study of dolichyl monophosphate topology in the rough endoplasmic reticulum

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Key words: Dol-P, amphomycin, N-glycosylation, membrane topology

Amphomycin though withdrawn as an antibiotic against the gram-positive bacterial infection, can certainly serve as an excellent tool for determination of the topology of Dol-P in the endoplasmic reticulum membranes, which has been otherwise impossible.

Glycosylation of the asparagine residue(s) present in the consensus sequence Asn-X-Ser/Thr of the eukaryotic cell glycoproteins is initiated in the luminal face of the rough endoplasmic reticulum (ER) [1] by a cotranslational transfer of $Glc_3Man_9GlcNAc_2$ from its isoprenoid-lipid carrier, i.e., dolichol. Over the years substantial progress has been made in our understanding how the precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ is assembled on dolichol. Apart from the involvement of multienzyme complex responsible for sequential transfer of *N*-acetylglucosamine, mannose and finally glucose, the availability of respective nucleotide diphosphate sugar donors as well as their dolichyl monophosphate sugar derivatives are essential for this process. It has been shown that the endoplasmic reticulum membranes have the capacity to translocate UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-glucose from the cytosol to the lumen but lack the ability to do the same with GDP-mannose [2, 3]. This prompted the speculation that formation of Dol-PP-GlcNAc/Dol-PP-$GlcNAc_2$ and Dol-P-Glc occurs either in the lumen or on the cytoplasmic face from which they are translocated to the lumen. However, in the absence of a GDP-mannose transporter, Dol-PP-$GlcNAc_2$-Man_5 and Dol-P-Man must be formed on the cytoplasmic face of the ER membranes before being elongated to form Dol-PP-GlcN-Ac_2Man_9. This argues strongly for a bi-modal distribution of Dol-P in the ER membranes and is in contradiction to the earlier observation that all Dol-P requiring enzymes share a common Dol-P pool [4]. To address the topology of Dol-P in the ER membranes a lipopeptide, amphomycin, and its antibody were suggested as excellent probes. A brief account of the amphomycin/anti-amphomycin system has therefore been presented to justify this claim.

Chemistry of amphomycin

Amphomycin is a naturally occurring peptide and is produced by the microorganism *Streptomyces canas*. It is a straight chain undecapeptide (Asp-MeAsp-Asp-Gly-Asp-DABe-Val-Pro--{DAB^t-PIP}) with either 3-isododecenoic or 3-anteisotridecenoic acid attached to the NH_2-terminal aspartic acid residue by an amide linkage [5]. Physicochemical characteristics indicate that amphomycin is an acidic surface active peptide with an isoelectric point (pI) of 3.5–3.6. It is soluble in water and lower alcohols, and forms both sodium and calcium salts. It was initially introduced as an antibiotic against gram-positive bacteria but its use was discontinued because of serious side effects, i.e., induction of hemolysis [6].

Amphomycin's ability to inhibit glycan chain assembly

In the late 1970's, it was documented that amphomycin inhibits the synthesis of peptidoglycan in gram-positive bacteria by blocking the transfer of phospho-*N*-acetyl-muramyl-pentapeptide from its UDP derivative to undecaprenyl monophosphate [7, 8]. Because of a close structural similarity between the undecaprenyl monophosphate and the dolichyl monophosphate, glycosylation reactions involving Dol-P in pig aorta, plants, bovine brain and rat parotid acinar cells, and subsequently formation of N-linked glycoproteins [9–11], were also inhibited by amphomycin. This inhibition, however, was reversed by the addition of exogenous Dol-P [10]. Synthesis of the monosaccharide-lipids Dol-PP-GlcNAc, Dol-P-Man and Dol-P-Glc, were inhibited by amphomycin when studied in cell free systems containing fixed concentration of Dol-P and respective sugar nucleotides [10]. Detailed kinetic studies were, however, performed only with the Dol-P-Man synthase activity. Dol-P-Man synthase catalyzes the biochemical reaction GDP-mannose + Dol-P ⇔ Dol-P-Man + GDP, and the enzymatic activity was found in higher eukaryotes, eukaryotic protozoa (yeast) and in plants. Incubation of ER membranes from calf brain with varying concentrations of GDP-mannose in the presence or absence of amphomycin showed normal kinetics and no significant changes in apparent K_m for GDP-mannose (1.08 μM in the presence of, and 1.37 μM in the absence of amphomycin). However, the V_{max} was reduced to 0.17 pmol/mg of protein × min^{-1} in the presence of amphomycin as compared to 1.86 pmol/mg of protein × min^{-1} in its absence. This strongly suggested that amphomycin neither affected the binding of GDP-mannose to the enzyme nor did it interact directly with GDP-mannose or with the enzyme. On the other hand, it has been suggested that the interaction between amphomycin and Dol-P might be possible. To test this hypothesis Dol-P-Man synthase activity was measured as a function of Dol-P concentration. It was found that the shape of the substrate-saturation curve was changed from a rectangular hyperbola to a sigmoid curve. The Hill coefficients (n) for this reaction were found to be 2.02 and 1.22 in the presence and absence of the inhibitor, and the corresponding K_m values for Dol-P were 333 and 47.3 μM, respectively. The latter data clearly indicate that the interaction between the amphomycin and Dol-P reduced the affinity of the enzyme for Dol-P. Additional evidence that amphomycin interacted with Dol-P came from a double-reciprocal plot of $1/v-v_o$ against 1/M often used for kinetic analysis of the allosteric enzymes where $1/v-v_o = (1+B/K_1)(K_{ia}K_b/AB) + K_b/B + (K_a/A+1) \times K_2/V(1+B/K_1)\ 1/M + 1/V$; and where v_o = initial velocity in the absence and v = velocity in the presence of a modifier, and K_1 and K_2 are the dissociation constants of substrate (B) and modifier (m), respectively from the allosteric sites. The equation predicted that the plot of $1/v-v_o$ against 1/M will be a straight line in one combination and a parabola if combined twice. In fact, the results indicate that the double-reciprocal plot was linear in the absence of amphomycin, whereas it was a parabola in its presence; this explains the strong interaction between Dol-P and amphomycin [12]. Additional experiments were performed in which ER membranes containing prelabelled Dol-^{32}P and Dol-P-[^{3}H]Man were mixed and then each lipid was extracted with $CHCl_3$-CH_3OH (2:1, v/v) in the presence or absence of amphomycin. Amphomycin interfered with the extraction of Dol-^{32}P but not of Dol-P-[^{3}H]Man or of the major membrane phospholipids [10] suggesting amphomycin's affinity for nonglycosylated Dol-P.

Evidence for the interaction between Dol-P and amphomycin

To study this interaction the unsubstituted primary amino group of DAB^e (D-erythro-α,β-diaminobutyric acid) of amphomycin was radiolabelled with tritium by reductive methylation [13]. [^{3}H]Amphomycin (10000 c.p.m.) was then incubated with Dol-P (10 μg) suspended in 20 mM Tris/HCl, pH 7.0, containing 0.2% Triton X-100 at room temperature and in the presence of 10 mM $CaCl_2$, and analyzed over a Bio-Gel A 1.5 m column (0.64 cm × 25 cm). It was observed that the bulk of the radioactivity emerged after the blue dextran peak (void volume, V_o) but much earlier than the amphomycin peak (V_1). This suggested an interaction between these two compounds [12] and perhaps the formation of a complex. Comigration of [^{3}H]amphomycin and [^{32}P]Dol-P in a dual-

label experiment containing [^{32}P]Dol-P and [^{3}H]amphomycin further confirmed the complex formation [12].

Structural features of the specificity of the interaction

Inhibition by amphomycin of the translocation step during peptidoglycan synthesis in gram-positive bacteria and that of monosaccharide-lipid synthesis in higher organisms [7, 8, 11] raised the question of the existence of a common structural element in amphomycin and the carrier lipids. It has been demonstrated that amphomycin formed complexes with undecaprenyl monophosphate, retinyl monophosphate, as well as with the perhydromonoene retinyl monophosphate [12, 14]. No complex, however, was formed either with [^{32}]dolichyl pyrophosphate or with [^{14}C]undecaprenyl pyrophosphate, or with free dolichol and polyprenol. To assess the common structural link, amphomycin was treated with mild acid (0.25 N acetic acid at 100°C for 2 h), a condition that was shown earlier to liberate the fatty acetylated aspartic acid residue at the NH_2 terminus; [5] and then interacted with dolichylmonophosphate in an identical manner. The net result was no complex formation. This led to the conclusions that amphomycin (i) does not discriminate between the α-saturated (dolichol) and α-unsaturated (undecaprenol) polyprenyl monophosphates; (ii) does not recognize the pyrophosphate derivatives or the free alcohols; (iii) must preserve the fatty acylated aspartic acid residue at NH_2 terminus for its native configuration. Otherwise, the complex formation was rapid: incubation of the reaction mixture in ice or at room temperature prior to the analysis did not improve the binding any further, and both hydrophobic and polar interactions were involved in stabilizing the complex.

Influence of membrane phospholipids and divalent cations on the Dol-P : amphomycin interaction

It has been established beyond any doubt that amphomycin interacts with Dol-P in eukaryotic cells inhibiting synthesis of Dol-PP-GlcNAc, Dol-P-Man, and Dol-P-Glc in the ER membranes. Since the ER membranes also contain other phospholipids, the question was whether amphomycin would react with major membrane phospholipids in the same manner as it does with Dol-P? To answer this question a variety of phospholipids were brought into reaction with [^{3}H]amphomycin and then monitored over the Bio-Gel A 1.5 column. The elution profile of [^{3}H]amphomycin in the presence of phosphatidic acid, phosphatidylserine, phosphatidylinositol, and phosphatidylcholine indicated that these phospholipids seemed to have interact with amphomycin to varying degrees and emerged at positions different from that of the dolichyl monophosphate-amphomycin complex. More stricking results were obtained when an equimolar mixture of phosphatidylserine and Dol-P reacted with [^{3}H]amphomycin. The radioactivity migrated to the area where dolichyl monophosphate-amphomycin normally emerged [15]. This conclusively proved that amphomycin is highly specific for Dol-P.

The amphomycin complexing with Dol-P was also dependent upon the nature of the divalent cation present, and was examined by incubating [^{3}H]amphomycin (10000 c.p.m.) with Dol-P (50 µg) in the presence of either 10 mM $CaCl_2$ or $MnCl_2$ or $MgCl_2$ or $CdCl_2$, and monitoring the mixture on the same Bio-Gel column. The complex formation corresponded to 80% in the presence of Ca^{2+}, 4% in the presence of Cd^{2+} and was none in the presence of Mn^{2+} or Mg^{2+}. Moreover, addition of a 10-fold molar excess of EDTA over [Ca^{2+}] completely abolished the complex formation. This, in fact, supported the earlier observations that only 2–3 µg of amphomycin was required for a 50% inhibition of Dol-P-Man/Dol-P-Glc synthesis in the presence of Ca^{2+} but much higher concentration of amphomycin were needed when Ca^{2+} was replaced by either Mn^{2+}, Mg^{2+}, Ni^{2+} or Cd^{2+} [10]. The inability of amphomycin to form a complex *in vitro* in the presence of either Mn^{2+} or Mg^{2+} on the one hand, and the inhibition of Dol-P-Man/Dol-P-Glc synthesis at higher concentrations in the presence of the same two metal ions on the other hand, presented the problem. This could be resolved by taking into account the fact that amphomycin used in all these experiments was a Ca^{2+} salt and for all experimental purposes it was solubilized with acetic acid followed by neutralization with sodium hydroxide. Therefore, the released Ca^{2+} remained in the solution and, on increasing the aliquot size, not only amphomycin concentra-

tion was raised but also that of Ca^{2+}. Pseudo inhibition of Dol-P-Man/Dol-P-Glc synthesis observed in the presence of either Mn^{2+} or Mg^{2+} and a large amount of amphomycin was in fact due to the presence of Ca^{2+} ions.

From these studies it may very well be concluded that, during the complex formation, the phosphate head group of dolichyl/undecaprenyl monophosphate and the fatty acylated aspartic acid residue at the NH_2-terminus of amphomycin were held together by a calcium bridge (Fig. 1).

Anti-amphomycin antibody and its characterization

The current knowledge of the Dol-P:amphomycin interaction stimulated us to develop a probe by which the topology of Dol-P in the ER membrane could be correctly established. This would have fundamental importance for understanding the various steps leading to formation of Dol-PP-$GlcNAc_2$-Man_9Glc_3 at the luminal side of the ER membrane. The immediate approach to the problem was to develop a monoclonal antibody against amphomycin, assuming that amphomycin will elicit appropriate immune responses when injected into mice.

By screening of hybridomas, some clones secreting an antibody specific for amphomycin were identified. Isotyping of the antibodies proved that they belonged to the immunoglobulin subclasses IgG + IgM. High titer antibody was then produced by injecting the hybrid cells into Balb/c mice and collecting the ascitic fluid. The specificity of the immunoglobulin present in the ascitic fluid was tested against amphomycin by enzyme-linked immunoassay (ELISA) as well as by dot-blot analysis on nitrocellulose strips [15].

Previous experience suggested that the fatty acylated aspartic acid residue at the NH_2-terminus of amphomycin was essential for Dol-P binding. Therefore, to understand its role in the antibody production and antigen recognition, both native and mild acid-hydrolyzed amphomycin were subjected to the ELISA test. The antibody was equipotent for both species, suggesting that the antibody binding site of amphomycin is distinct from the Dol-P binding site and is critical for the studies described below. This fact was also confirmed by a partial

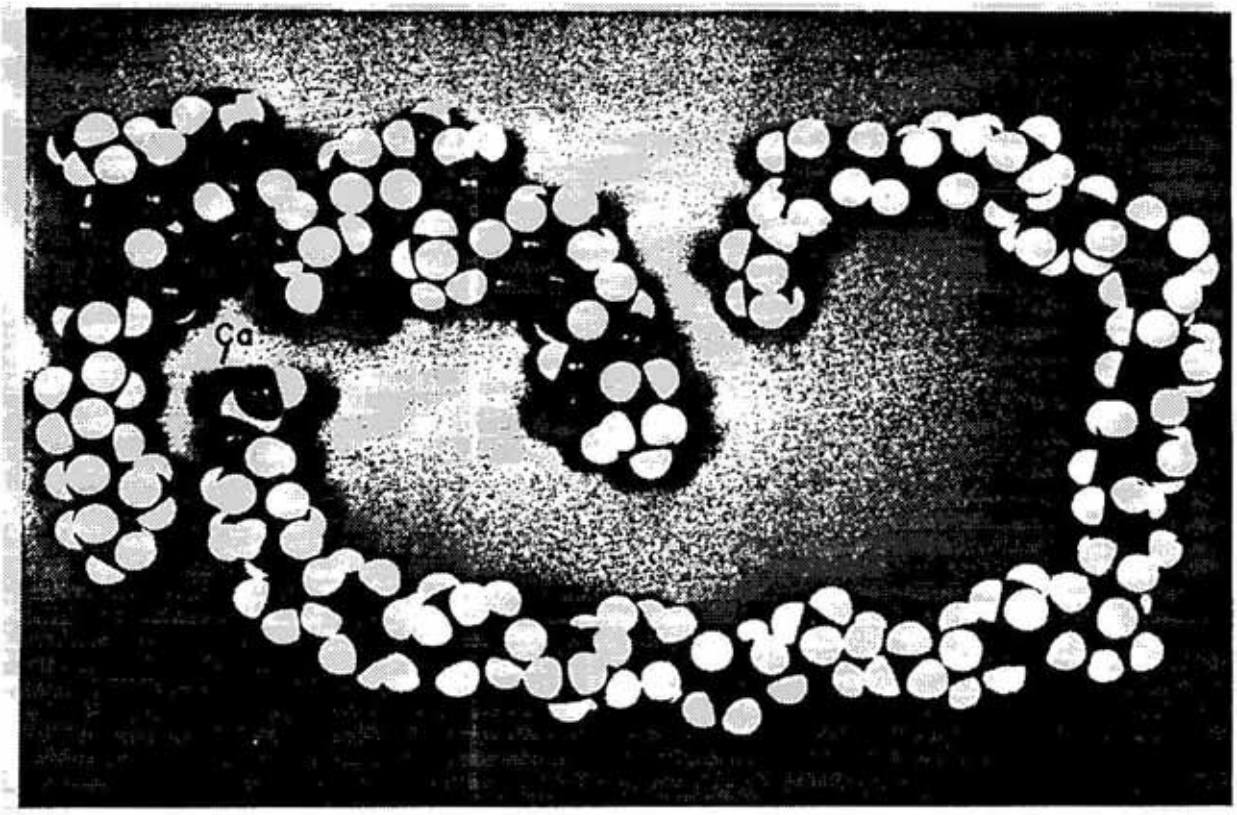

Fig. 1. Site of interaction between Dol-P and amphomycin. A space filling model of Dol-P (lower) and amphomycin (upper).

recovery of the Dol-P-Man synthase activity in the reaction mixture which was exposed to amphomycin pretreated with the antibody.

Immunochemical localization of amphomycin in the cell

Various control experiments provided convincing evidence that the mouse monoclonal antibody was indeed active against amphomycin. The next approach was to locate amphomycin by immunocytochemical methods following its insertion into the cell. The rationale for this study was its strong affinity for Dol-P upon entry into the cell, were amphomycin should self-associate with, and bind to, Dol-P. The condition used to introduce amphomycin into the cell was identical to that used for antibody (i.e., treatment of cells for 30 s with ice-cold methanol) and which preserved the intracellular structures. Under these circumstances amphomycin should bind only to those Dol-P molecules that face the cytoplasmic side of the ER membranes. If the information thus far generated on amphomycin holds true, then this process could be monitored by indirect immunofluorescence microscopy. When a monolayer culture of capillary endothelial cells were fixed and exposed to amphomycin, followed by anti-amphomycin antibody, followed by FITC-conjugated goat anti-mouse IgG, fluorescence was detected in the perinuclear region of the cell and in an area reminiscent of endoplasmic reticulum. All other areas, including plasma membrane and Golgi apparatus remained insensitive (Fig. 2).

The author greatfully acknowledges L. Kent Stitzer for reading the manuscript.

Fig. 2. Immunocytochemical localization of Do-P in capillary endothelial cells. Magnification, × 980.

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