Early dolichyl sugar synthesis during differentiation of non-growing brine-shrimp (Artemia salina) embryos

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Incubation of cell-free extracts of Artemia salina with sugar nucleotides resulted in the formation of dolichyl derivatives. These enzymic activities were detected early in the development of the encysted Artemia embryos. Dolichyl sugars seemed to act as intermediates in the glycosylation of proteins, which may be involved in the initiation of cellular differentiation.

Dolichol-linked sugars are intermediates in the glycosylation of some eukaryotic and viral proteins (Schachter, 1978; Parodi & Leloir, 1979). We previously reported biosynthesis both in vivo and in vitro of some insect dolichyl derivatives (Quesada-Allue et al., 1976a,b; Quesada-Allue & Belocopitow, 1978), which also seemed to be involved in the glycosylation of insect proteins (Quesada-Allue, 1978, 1980; Butters & Hughes, 1980), thus confirming that this mechanism is a general feature of eukaryotic organisms.

Because of its unusual biology, the brine shrimp (Artemia salina) has become a useful system for studies on developmental biochemistry (Finamore & Clegg, 1969; Hentschel & Tata, 1976). In conditions of extreme salinity its development stops and the embryos (mainly gastrulae) are encysted, entering a dormant, ametabolic stage. After the cysts are desiccated and then rehydrated, development continues. In the initial postgastrula development of encysted Artemia embryos, cellular differentiation and morphogenesis take place without the occurrence of cell division, DNA synthesis or growth (Hentschel & Tata, 1976).

Since I recently obtained evidence for the biosynthesis of dolichol-linked sugars by crustacean enzymes (Quesada-Allue, 1978), the Artemia system offers a unique opportunity to study the activation of the dolichol pathway and its relations with the process of differentiation. When A. salina cryptobiotic embryos are activated, a number of rapid molecular changes take place. The present paper describes the early biosynthesis of dolichyl phosphate mannose and dolichyl phosphate glucose. The present evidence is the first to support a relationship between dolichol pathways and differentiation in the complete absence of growth and cell division.

Experimental

The analytical procedures used in the present paper have been described previously (Quesada-Allue & Belocopitow, 1978; Quesada-Allue, 1980). The same results were obtained with different batches of Artemia cysts.

After treatment with 5% NaClO, to remove the chorion layer and contaminants (Finamore & Clegg, 1969), encysted embryos of Artemia were immersed in cold 4% NaCl containing streptomycin and penicillin. Viable cysts were activated by incubation at 20°C for the desired period. The activated cysts were extensively washed and divided into two identical aliquots. Those in one of them were carefully disaggregated in a large-clearance glass/glass Kontes homogenizer to obtain entire cells. The washed cells remained viable for at least 8 h (Trypan Blue test) and showed permeability to labelled amino acids. The other portion of cysts was transferred to a pre-cooled mortar, suspended in liquid N₂ and homogenized in 80 mM-Tris/HCl buffer, pH 7.5, containing 4 mM-NaCl, 15 mM-KCl, 5 mM-MgCl₂, 2 mM-EDTA, 2 mM-2-mercaptoethanol, 100 mM-sucrose, 1 mM-butylated hydroxytoluene and 1% glycerol. The broken material was further fractionated as described by Quesada-Allue & Belocopitow (1978) by successive centrifugations to obtain a microsome-enriched fraction. The latter fraction showed an appreciable synthesis of polypropyl sugar-like substances, whereas other fractions have only trace activities.

The incubation mixtures contained, in a final volume of 60 μl: 6 mg of microsomal protein, 80 mM-Tris/maleate buffer, pH 8.0, 30 mM-2-mercaptoethanol, 13 mM-MgCl₂, 6 mM-KCl, 1.5 mM-EDTA and GDP-[³¹⁴C]mannose (2 × 10⁵ c.p.m.) or...
UDP-[\textsuperscript{14}C]glucose (3 \times 10^4 c.p.m.). The mixtures were incubated for 30 min at 25°C, stopped, processed and counted for radioactivity as described by Quesada-Allue et al. (1976b). The lower phases of partitions of the incubation mixtures (Folch et al., 1957) were used as a source of lipid-linked monosaccharides (Quesada-Allue et al., 1976b).

Orotate decarboxylase activity was measured by the method of Jänne & Williams-Ashman (1971).

**Results and discussion**

When GDP-[\textsuperscript{14}C]mannose or UDP-[\textsuperscript{14}C]glucose were used as sugar donors in cell-free experiments, the main acid-labile lipidic substances biosynthesized behaved respectively as dolichyl phosphate [\textsuperscript{14}C]mannose and dolichyl phosphate [\textsuperscript{14}C]glucose. The identity was assigned by the following criteria.

(a) Anion-exchange chromatography on DEAE-cellulose columns (Quesada-Allue & Belocopitow, 1978) showed that both mannosylated and glucosylated *Artemia* substances were retained by the resin and were co-eluted by salts together with \(^3\text{H}\)-labelled liver dolichyl phosphate glucose (Fig. 1a).

(b) The biosynthesis was stimulated by exogenous addition to incubation mixtures of insect or liver dolichyl phosphate (Fig. 1b).

(c) The putative dolichyl phosphate [\textsuperscript{14}C]mannose and dolichyl phosphate [\textsuperscript{14}C]glucose chromatographed like authentic samples in silica-gel G thin-layer plates (Figs. 1b and 1c) (Quesada-Allue et al., 1976a,b).

(d) The substances were broken down by mild acid hydrolysis and the liberated products chromatographed in several systems with mannose and glucose (Fig. 1c).

The labelled mannosylated and glucosylated protein-like material obtained after complete delipidation of the incubation mixture (Quesada-Allue, 1980) was insoluble in hot 10% trichloroacetic acid (Fig. 2a). When treated with *Streptomyces griseus* protease for 3 days at 37°C, 80% of the radioactivity became soluble in trichloroacetic acid and migrated in paper electrophoresis with both acid and basic buffers. As judged by gel filtration in

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6. glucose; 7, mannose; 8, solvent front. (c) The same as (b) but *Artemia* lipid-linked [\textsuperscript{14}C]mannose (○) was chromatographed. An identical sample of the latter was dried and treated with 200 \(\mu\)l of 0.01 M-HCl for 30 min at 98°C. After HCl evaporation, labelled material was dissolved both with chloroform/methanol (3:2, v/v) and water, mixed and chromatographed in a parallel lane (□). A standard of liver dolichyl phosphate [\textsuperscript{14}H]glucose was also present (✓).
biosynthesis becomes detectable in the cell-free system 2h after immersion of cysts (Fig. 2a), preceding the glycosylation of microsomal protein-like material. Ornithine decarboxylase, an enzyme whose activity increases markedly when certain undifferentiated cells are activated (Jänne & Williams-Ashman, 1971), was not detected until 6.5h after immersion. In the undisrupted-cells experiment (Fig. 2b), the incorporation of [2-3H]-leucine into hot 10% trichloroacetic acid-insoluble material is detectable 1h after immersion of the cysts. Cycloheximide diminished the incorporation of radioactivity, probably indicating that polypeptide elongation had been inhibited. When [3H]-mannose was used as tracer it was taken up by the cells but, under the conditions of the assay, glycosylation of proteins could not be detected.

The data on leucine incorporation into polypeptide-like material using the intact cell system is in agreement with those obtained in cell-free systems by others (Clegg, 1966; Hentschel & Tata, 1976). Thus our evidence strongly suggests that dolichyl phosphate derivative synthesis was activated early and could be detected simultaneously with the re-assumption of polypeptide elongation. In addition, these pre-programmed (Muthukrishnan et al., 1975) polypeptides might be some of the ones that became glycosylated in cell-free experiments. Since the embryos of Artemia salina undergo some morphogenetic events in the complete absence of growth and cell division (Finamore & Clegg, 1969; Hentschel & Tata, 1976), it appears that dolichyl-linked sugars are biosynthesized early, probably to act as intermediates in the glycosylation of proteins (Clegg, 1966; Osuna et al., 1977; Nagainis & Warner, 1979), which, in turn, are somehow related to the initiation of cellular differentiation (James & Tata, 1980).

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References

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Sephadex G-15 the putative glycopeptides diminished in size during the treatment with proteinase.

The Artemia metabolic machinery remains quiescent up to a complete hydration, 40–60 min after immersion of encysted gastrulae (Finamore & Clegg, 1969). Dolichyl phosphate monosaccharide
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