Glycosylation of Exogenous Protein by Endoplasmic-Reticulum Membranes from Castor-Bean (*Ricinus communis*) Endosperm

By Robert B. MELLOR, Lynne M. ROBERTS and J. Michael LORD
Postgraduate School of Biological Sciences, University of Bradford, Bradford,
Yorkshire BD7 1DP, U.K.

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Endoplasmic-reticulum membranes isolated from the endosperm tissue of 3-day-old castor-bean (*Ricinus communis*) seedlings catalysed the enzymic transfer of the sugar moiety from an oligosaccharide-lipid to a chemically unfolded form of ribonuclease A.

Studies in this laboratory have established that several constituent proteins of the endoplasmic reticulum and glyoxysomal membranes, isolated from germinating castor-bean (*Ricinus communis*) endosperm tissue, are glycosylated (Mellor & Lord, 1978, 1979a). These proteins are readily labelled when intact endosperm tissue is incubated with GDP-[¹⁴C]mannose (Mellor & Lord, 1979b). The analysis of isolated cellular fractions established that the endoplasmic reticulum is the exclusive cellular location of enzymes catalysing the synthesis of dolichol monophosphate mannose and a mannose-containing oligosaccharide-lipid (Mellor & Lord, 1979b). In the light of current concepts, we assume that the oligosaccharide moiety of the oligosaccharide-lipid is transferred to protein by enzymes that are also located in the endoplasmic reticulum (Hsu *et al.*, 1974; Lucas *et al.*, 1975). This glycosylation step may occur co-translationally while the nascent polypeptide chain receiving the oligosaccharide is still attached to the ribosome (Kiely *et al.*, 1976; Lingappa *et al.*, 1978).

Convincing evidence that the oligosaccharide-lipid functions as an intermediate in glycoprotein biosynthesis depends, however, on demonstrating a direct transfer of the sugar moiety from the lipid to a polypeptide acceptor. Pless & Lennarz (1977) showed that carbohydrate-depleted and chemically denatured glycoproteins can function as oligosaccharide acceptors in a cell-free system containing hen oviduct membranes. In the present study we have utilized this glycosylation system to demonstrate that the endoplasmic-reticulum membranes isolated from castor-bean endosperm cells catalyse the core glycosylation of sulphitolyzed ribonuclease A.

Materials and Methods

Endosperm halves (20) excised from 3-day-old castor-bean seedlings were homogenized, and cellular organelles were fractionated by sucrose-density-

gradient centrifugation as described previously (Bowden & Lord, 1976). Collected gradient fractions containing the endoplasmic-reticulum membranes, located by using cholinephosphotransferase as a marker (Lord *et al.*, 1973) were pooled. The vesicles were osmotically disrupted to release soluble matrix protein by adding 2 vol. of 50 mM-Tricine {N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}, pH 7.5, containing, where appropriate, 0.5 M-KCl. The membranes were collected by centrifugation at 100 000g_{av.} and 2°C for 30 min. The pellet, resuspended in 50 mM-Tris/HCl, pH 7.2, was used as the source of enzyme.

Bovine pancreatic ribonuclease A (Sigma, type XII-A) was sulphitolyzed as described by Pechère *et al.* (1958). Reagents were removed by a series of dialyses (Tarentino *et al.*, 1974) and the sulphitolyzed protein was freeze-dried.

[¹⁴C]Mannose-labelled oligosaccharide-lipid was prepared by incubating a particulate enzyme preparation with 1 µCi of GDP-[¹⁴C]mannose (The Radiochemical Centre; specific radioactivity 173 Ci/mol) in 5 ml of 50 mM-Tris/HCl, pH 7.5, 10 mM-MgCl₂ and 2 mM-β-mercaptoethanol. The particulate enzyme was obtained by centrifuging the homogenate from 40 3-day-old endosperm halves at 20 000g_{av.} and 2°C for 15 min. After 60 min at 30°C, 20 ml of chloroform/methanol (1:1, v/v) was added, and after mixing the phases were separated by centrifugation. The organic phase, which contained dolichol monophosphate mannose, was discarded and the aqueous phase and interface material were re-extracted with 10 ml of chloroform. After phase separation, the aqueous phase and interface material were centrifuged to pellet insoluble particles. The pellet was washed twice with 50% (v/v) methanol and the washings were discarded. The washed pellet was resuspended in 10 ml of chloroform/methanol/water (10:10:3, by vol.) and the mixture was left for 15 min at room temperature to extract oligosaccharide-lipid. After removing the residue by centrifugation, the extract was evaporated

to dryness under vacuum at 35°C. The [^{14}C]mannose-labelled oligosaccharide-lipid residue was redissolved in a small volume of 0.4% sodium deoxycholate. In this way, 1.5×10^5 – 2×10^5 c.p.m. of [^{14}C]mannose-labelled oligosaccharide-lipid were prepared.

[^{14}C]Mannose-labelled oligosaccharide-protein was synthesized in a reaction mixture (final vol. 1.0 ml) containing endoplasmic-reticulum membranes (up to 250 μg of protein), 0.2% sodium deoxycholate, 50 mM-Tris/HCl, pH 7.2, 10 mM-MgCl₂, 10 mM-MnCl₂, [^{14}C]mannose-labelled oligosaccharide-lipid (3×10^3 – 7×10^3 c.p.m.) and sulphitolyzed ribonuclease A (up to 150 μg). The mixture was incubated at 30°C and at intervals portions (0.25 ml) were removed and added to 1.0 ml of 10% (w/v) trichloroacetic acid. The precipitate was filtered on to a Whatman GF/A filter disc and then washed successively with 10% trichloroacetic acid, chloroform/methanol (1:1, v/v) and chloroform/methanol/water (10:10:3, by vol.). Washed filter discs were immersed in 10 ml of scintil-

lant (Bray, 1960) and counted for radioactivity at an efficiency of 80–85%.

Results and Discussion

Washed endoplasmic-reticulum membranes isolated from 3-day-old castor-bean endosperm tissue catalysed the incorporation of [^{14}C]mannose-labelled oligosaccharide into trichloroacetic acid-insoluble product in a reaction mixture supplemented with [^{14}C]mannose-labelled oligosaccharide-lipid and sulphitolyzed ribonuclease A (Fig. 1). Significant incorporation of radioactivity into protein product did not occur in the absence of sulphitolyzed ribonuclease A or when this chemically denatured form of the exogenous protein was replaced by native ribonuclease A. Washed glyoxysomal membranes did not catalyse the glycosylation of exogenous protein, and the activity of the endoplasmic-reticulum membranes was markedly described by washing in buffers

Fig. 1. Synthesis of [^{14}C]mannose-labelled oligosaccharide-protein

Trichloroacetic acid-insoluble product formation was followed by using the reaction mixture described in the text with: O, 200 μg of washed endoplasmic-reticulum membrane protein; ●, 200 μg of KCl-washed endoplasmic-reticulum membrane protein; Δ, 200 μg of washed glyoxysomal membrane protein; ▲, sulphitolyzed ribonuclease A omitted; □, sulphitolyzed ribonuclease A replaced with native ribonuclease A.

Fig. 2. [^{14}C]Mannose-labelled oligosaccharide-protein synthesis by collected fractions after sucrose-density-gradient centrifugation

An extract of 20 endosperm halves from 3-day-old seedlings was applied to a sucrose gradient and centrifuged for 3 h at 53000g_{av} and 2°C. Sulphitolyzed-ribonuclease A-dependent synthesis of glycosylated product was determined for each collected 1.0 ml fraction as described in the text. The sucrose concentration of each fraction was measured refractometrically (----). ER, M and G designate fractions containing peak activities of marker enzymes for endoplasmic reticulum, mitochondria and glyoxysomes respectively.

containing 0.5 M-KCl (Fig. 1). These data are in good agreement with the findings of Pless & Lennarz (1977), who used a similar assay system containing hen oviduct membranes. Accordingly we accept their interpretation that sulphitolysis facilitates glycosylation of bovine ribonuclease A by unfolding the native protein conformation to expose the carbohydrate acceptor site at asparagine residue 34 (Plummer & Hirs, 1964).

The enzymic transfer of [^{14}C]mannose-labelled oligosaccharide from the oligosaccharide-lipid to sulphitolyzed ribonuclease A was dependent on the concentrations of membrane protein (up to 250 μg), sulphitolyzed ribonuclease A (up to 150 μg) and [^{14}C]mannose-labelled oligosaccharide-lipid (up to 7000 c.p.m.) (results not shown).

The intracellular location of enzymic activity catalysing the glycosylation of exogenous protein was confirmed by assaying collected fractions after sucrose-density-gradient centrifugation of a crude homogenate. Each collected fraction was halved, one half being assayed in the presence of sulphitolyzed ribonuclease A and the other in its absence. The results of this experiment are shown in Fig. 2, the activity of each fraction supplemented with sulphitolyzed ribonuclease A having been corrected for endogenous activity in the absence of acceptor. A major peak of particulate activity was obtained (fraction 9) corresponding to a visible protein band at a mean buoyant density of 1.12 g/ml. This fraction has previously been identified as ribosome-denuded endoplasmic-reticulum membranes (Lord *et al.*, 1973). The greatest proportion of the total enzymic activity, however, remained in the soluble fraction at the top of the gradient (fractions 1–6, Fig. 2). The significance of this soluble activity is unclear. We have previously shown that the endoplasmic reticulum is the exclusive cellular location of enzymes catalysing the synthesis of dolichol monophosphate mannose and mannose-containing oligosaccharide-lipid (Mellor & Lord, 1979b). It is possible that this soluble glycosylating activity represents enzyme lost from the endoplasmic reticulum during tissue homogenization. Washing the membranes with 0.5 M-KCl markedly decreased their glycosylation activity (Fig. 1), and this treatment is known to release peripheral proteins associated with the luminal surface of the microsomal vesicles (Huang & Beevers, 1973; Bowden & Lord, 1978).

The present results demonstrate that, in addition to

its capacity to synthesize oligosaccharide-lipid, the endoplasmic reticulum also catalyses the transfer of the oligosaccharide moiety from the lipid carrier to exogenously supplied acceptor protein. We assume this activity '*in vitro*' in the presence of denatured exogenous protein reflects the role of the endoplasmic reticulum in the core glycosylation of nascent proteins in castor-bean endosperm cells. The importance of the endoplasmic reticulum as a site of glycosylation in this plant tissue was indicated when this cellular fraction was shown to acquire radioactive protein before it appeared in other organelles when intact endosperm tissue was incubated with GDP-[^{14}C]mannose (Mellor & Lord, 1979b).

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