The effect of cycloheximide on the glycosylation of lactating-rabbit mammary glycoproteins

David A. WHITE and Brian K. SPEAKE

Department of Biochemistry, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K.

(Received 12 May 1980/Accepted 13 June 1980)

1. Cycloheximide inhibited immediately the incorporation of L-[4,5-³H]leucine and D-[2-³H]mannose into mammary proteins, suggesting that the mannosylation of mammary glycoproteins requires the continued supply of newly synthesized polypeptides. 2. The incorporation of radioactivity from *N*-acetyl-D-[1-¹⁴C]glucosamine into protein was not inhibited until approx. 30 min after cycloheximide addition. Much (greater than 90%) of this radioactivity was present as *N*-acetylgalactosamine. 3. *N*-Glycosylation appears to be inhibited immediately by cycloheximide due to a lack of newly synthesized acceptor polypeptides, whereas *O*-glycosylation continues for 30 min, the time taken for acceptor peptides to move from their site of synthesis to the Golgi region and for completion of glycosylation. 4. There was a transient increase in the incorporation of mannose into lipid-linked oligosaccharide in the presence of cycloheximide, followed by a decrease in the radioactivity in this fraction. 5. The major lipid-linked oligosaccharide extracted from explants incubated for 2 h in the presence of cycloheximide (6–7 monosaccharide units) was smaller than that extracted from control explants (10–12 monosaccharide units).

N-Glycosylation of mannose-containing glycoproteins in the mammary gland involves the transfer of oligosaccharide from the lipid-linked oligosaccharide intermediate to protein (Speake & White, 1978) and inhibition of formation of the intermediate by tunicamycin results in inhibition of protein *N*-glycosylation (Speake & White, 1979). There is considerable evidence in other systems indicating that transfer of the oligosaccharide is a co-translational process (e.g. Kiely *et al.*, 1976; Rothman & Lodish, 1977; Berman & Kuehl, 1977). A comparison of protein *N*-glycosylation in cell-free and explant preparations of lactating rabbit mammary gland (White, 1978; Speake & White, 1978) suggests that a high rate of protein glycosylation is dependent on the continued synthesis of those polypeptides that are acceptors of oligosaccharide from the lipid-linked oligosaccharide. The present paper describes the effects of inhibition of protein synthesis by cycloheximide on protein glycosylation in mammary explants.

Materials and methods

Animals

Primiparous lactating rabbits of the New Zealand White strain (albinos) were obtained from the Joint

Animals Breeding Unit, University of Nottingham School of Agriculture, Sutton Bonington, Leics., U.K. The animals were at least 6 months old at the time of mating. Explants from mammary glands were obtained from rabbits in mid-lactation (10–15 days *post partum*).

Materials

Glucose-free Medium 199 was obtained from Wellcome Research Laboratories, Beckenham BR3 3BS, Kent, U.K. Sheep prolactin (NIH-P-S11) (26.4 i.u./mg) was a gift from the Pituitary Hormone Distribution Program, National Institute of Arthritis and Metabolic Diseases, Bethesda, MD, U.S.A. Ox insulin was a gift from Mr. V. J. Birkinshaw, Boots Pure Drug Co., Nottingham, U.K. An acid hydrolysate of amylose was kindly provided by Dr. J. F. Kennedy, Department of Biochemistry, University of Birmingham, Birmingham, U.K. D-[2-³H]Mannose, *N*-acetyl-D-[1-¹⁴C]glucosamine and L-[4,5-³H]leucine were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Cortisol 21-acetate and cycloheximide were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. All other chemicals were obtained from commercial sources.

Preparation of mammary explants

Explants of lobuloalveolar mammary tissue were prepared as described previously (Speake *et al.*, 1975).

Measurement of incorporation of D-[2-³H]mannose, N-acetyl-D-[1-¹⁴C]glucosamine and L-[4,5-³H]leucine into endogenous acceptors of mammary explants

Duplicate groups of 20 explants were incubated for various periods of time in 1 ml of glucose-free Medium 199 containing glycerol (1 mg/ml), insulin (5 g/ml), prolactin (1 µg/ml) and cortisol (1 µg/ml). The incubation medium also contained 10 µCi of D-[2-³H]mannose (sp. radioactivity 2 Ci/mmol), 2.0 µCi of N-acetyl-D-[1-¹⁴C]glucosamine (sp. radioactivity 52 Ci/mol) or 5.0 µCi of L-[4,5-³H]leucine (sp. radioactivity 53 Ci/mmol). Only a single radioactive substrate was added to each incubation. Cycloheximide was added at time zero of the incubation.

At the end of the incubation period, each group of explants was washed with Medium 199 and homogenized in chloroform/methanol (2:1, v/v; 3 ml) and dolichol-linked monosaccharide, dolichol-linked oligosaccharide and protein fractions were isolated as previously described (Speake & White, 1978).

Paper-chromatographic procedure

The products of mild acid hydrolysis of the lipid-linked oligosaccharide fraction were analysed by descending paper chromatography on Whatman 3MM paper in the solvent system isobutyric acid/water/aq. NH₃ (sp.gr. 0.880) (57:39:4, by vol.). Saccharides were detected by using alkaline AgNO₃ (Trevelyan *et al.*, 1959). Radioactive zones were detected by liquid-scintillation counting of 1 cm strips of the chromatogram (Speake & White, 1978).

Mild acid hydrolysis

Mild acid hydrolysis of dolichol-linked oligosaccharides was carried out as described previously (Speake & White, 1978).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results

All values represent means of duplicate determinations and are typical of at least three experiments.

The effect of cycloheximide concentration on the incorporation of [2-³H]mannose and [4,5-³H]leucine into protein

Cycloheximide had pronounced inhibitory effects

Fig. 1. The effect of cycloheximide concentration on the incorporation of [2-³H]mannose and [4,5-³H]leucine into protein by mammary explants

Duplicate groups of 20 explants were incubated for 30 min at 37°C under O₂/CO₂ (19:1) in 1 ml of Medium 199 (glucose-free) containing 10 µCi of D-[2-³H]mannose (■) or 5.0 µCi of L-[4,5-³H]leucine (●) in the presence of increasing concentrations of cycloheximide. At the end of the incubation period each group of explants was homogenized in chloroform/methanol (2:1, v/v; 3 ml). A lipid-free protein fraction was prepared as described by Speake & White (1978) and the radioactivity in this fraction was determined.

on the incorporation of both substrates into mammary proteins (Fig. 1). Leucine incorporation was inhibited by 50% and mannose by 30% at cycloheximide concentrations of 2 µg/ml. Greater than 80% inhibition of leucine incorporation into protein was seen at concentrations of cycloheximide above 10 µg/ml. The inhibition of mannose incorporation into protein did not appear as striking as with leucine. However, analysis of the protein pellet by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate revealed that greater than 90% of the radioactivity in this fraction was associated with oligosaccharide lipid running at the dye front and not with protein. After initial precipitation with trichloroacetic acid it was not possible to extract all of the lipid-linked oligosaccharide with chloroform/methanol/water (10:10:3, by vol.) and this step was later omitted from the extraction procedure. Thus cycloheximide inhibited both mannose and leucine incorporation into protein. Subsequent experiments were performed by using cycloheximide at 50 µg/ml.

The effect of cycloheximide on the incorporation with time of [4,5-³H]leucine, [2-³H]mannose and [1-¹⁴C]glucosamine into protein

Inclusion of cycloheximide in the incubation medium caused an immediate and sustained

inhibition of incorporation of leucine (Fig. 2) and mannose (Fig. 3a) into mammary proteins in the first 60 min. A slight increase in the incorporation of mannose occurred after this time. Incorporation of *N*-acetylglucosamine into protein in the presence of cycloheximide was linear and similar to the control for the first 30 min of incubation, but was inhibited after this time such that after a further 30 min the inhibition was 50% of the control (Fig. 3b).

Fig. 2. The effect of cycloheximide (50 μ g/ml) on the incorporation of [4,5- 3 H]leucine into protein by mammary explants

Explants were incubated with [4,5- 3 H]leucine in the absence (●) or presence (■) of cycloheximide and lipid-free protein pellets were prepared as described in the legend to Fig. 1.

The effect of cycloheximide on the incorporation of [2- 3 H]mannose into lipid-linked oligosaccharides

The incorporation of [2- 3 H]mannose into lipid-linked oligosaccharides in the presence of cycloheximide was inhibited by 50% after 2 h incubation (Fig. 4). There was a transient increase in the incorporation at 15 min, but further incubation resulted in a decrease in the radioactivity in this fraction to a level 50% of the control at 60 min.

Fig. 4. The effect of cycloheximide (50 μ g/ml) on the incorporation of [2- 3 H]mannose into lipid-linked oligosaccharide by mammary explants

Explants were incubated with [2- 3 H]mannose in the absence (●) and presence (■) of cycloheximide as described in the legend to Fig. 1. The lipid-linked oligosaccharide fraction was prepared as described by Speake & White (1978) and the radioactivity in this fraction was determined.

Fig. 3. The effect of cycloheximide (50 μ g/ml) on the incorporation of [2- 3 H]mannose and *N*-acetyl[1- 14 C]glucosamine into protein by mammary explants

Explants were incubated with (a) [2- 3 H]mannose or (b) *N*-acetyl[1- 14 C]glucosamine in the absence (●) or presence (■) of cycloheximide and lipid-free protein pellets were prepared as described in the legend to Fig. 1.

Fig. 5. Paper chromatography of the oligosaccharides released by acid hydrolysis from lipid-linked oligosaccharides synthesized by explants incubated with [^3H]mannose in the absence (a) and presence (b) of cycloheximide

The oligosaccharides were chromatographed on Whatman 3 MM paper in isobutyric acid/water/aq. NH_3 (sp.gr. 0.880) (57:39:4, by vol.) with glucose oligomers of known chain lengths as standards. The chromatogram was cut into segments 1 cm in length and the radioactivity was determined by liquid-scintillation counting. The bars and corresponding numbers represent the position of migration of standard glucose oligomers, i.e. G_1 represents the position of $(\text{Glc})_1$, G_2 represents $(\text{Glc})_2$ etc.

Analysis of the size of the oligosaccharides linked to lipid after 3 h in the presence and absence of cycloheximide is shown in Fig. 5. The oligosaccharide released by acid hydrolysis of the control oligosaccharide lipid was estimated to be of 10–12 monosaccharide units, whereas that of the inhibited incubation appeared to consist of only 6 monosaccharide units.

Discussion

As mentioned in the introduction there is evidence

from immunoprecipitation studies of nascent ribosome-bound polypeptides that *N*-glycosylation is a co-translational process (e.g. ovalbumin synthesis in hen oviduct; Kiely *et al.*, 1976). Inhibition of protein synthesis in bovine thyroid slices by addition of puromycin blocked the transfer of oligosaccharide from the donor oligosaccharide lipid to protein and resulted in an increase in the radioactivity associated with the lipid-linked intermediate (Spiro *et al.*, 1976). A close relationship between the synthesis of polypeptide acceptor and formation of the core oligosaccharide attached to lipid in canine kidney cells was also suggested by Schmitt & Elbein (1979). Inhibition of protein synthesis by addition of cycloheximide was accompanied by a substantial inhibition in the incorporation of radiolabelled mannose into lipid-linked oligosaccharides and it was suggested that accumulation of larger sized lipid-linked oligosaccharides leads to feedback inhibition of one of the earlier enzymes in the pathway.

In the present study inhibition of protein synthesis resulted in an inhibition of protein mannosylation suggesting that mannose-containing oligosaccharides are incorporated into newly synthesized polypeptides. In contrast with this the incorporation of radiolabel from *N*-acetyl[1- ^{14}C]glucosamine into protein was not greatly inhibited until approx. 30 min after addition of cycloheximide. As described in a previous paper (Speake & White, 1979) much of this radioactivity was present as galactosamine residues of casein, the major glycoprotein synthesized by lactating mammary gland. Thus although *N*-glycosylation of proteins is inhibited immediately by cycloheximide addition, *O*-glycosylation as measured by incorporation of galactosamine continues for a further 30 min. This length of time probably represents the time taken for newly completed casein polypeptides present at time zero to move from the rough endoplasmic reticulum and for their glycosylation in the Golgi region. Radioactivity in protein at this time could be precipitated with anti-(rabbit casein) antiserum. The transient increase in the incorporation of mannose into lipid-linked oligosaccharide in the presence of cycloheximide suggests that, in the absence of newly synthesized acceptor polypeptide, oligosaccharide accumulates initially on the lipid carrier. Subsequently a decrease in the radioactivity of this fraction was found and was probably due to degradation of the oligosaccharide by glycosidases. Thus the size of the lipid-linked oligosaccharide extracted from tissue incubated in the presence of cycloheximide for 2 h was considerably smaller than that extracted from control tissue incubations.

In view of the results obtained with tunicamycin (Speake & White, 1979) and in the present study with cycloheximide it is possible to describe a simple

model for protein glycosylation in mammary tissue. Casein is glycosylated as a post-translational process, probably in the Golgi, by sequential transfer of monosaccharide units from nucleotide sugars to protein. No evidence of lipid-linked galactosamine has been obtained in previous studies. A number of other polypeptides are initially glycosylated as a co-translational process or soon after completion of synthesis by transfer of an oligosaccharide from dolichyl pyrophosphate.

The authors thank the Medical Research Council for a fellowship to B. K. S. and for financial support of this work.

References

- Bergman, R. W. & Kuehl, W. M. (1977) *Biochemistry* **16**, 4490–4496
- Kiely, M. L., McKnight, G. S. & Schimke, R. T. (1976) *J. Biol. Chem.* **251**, 5490–5495
- Lowry, O. H., Rosebrough, N. J., Farr, A. R. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Rothman, J. E. & Lodish, H. F. (1977) *Nature (London)* **269**, 775–780
- Schmitt, J. W. & Elbein, A. D. (1979) *J. Biol. Chem.* **254**, 12291–12294
- Speake, B. K. & White, D. A. (1978) *Biochem. J.* **170**, 273–283
- Speake, B. K. & White, D. A. (1979) *Biochem. J.* **180**, 481–489
- Speake, B. K., Dils, R. & Mayer, R. J. (1975) *Biochem. J.* **148**, 309–320
- Spiro, M. J., Spiro, R. G. & Bhoyroo, V. D. (1976) *J. Biol. Chem.* **251**, 6400–6408
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1959) *Nature (London)* **166**, 444–445
- White, D. A. (1978) *Biochem. J.* **170**, 479–486