OCCURRENCE OF SEVERAL GLYCOPROTEINS IN GLYOXYSOMAL MEMBRANES OF CASTOR BEANS

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1. Introduction

It is generally assumed that glycoproteins are constituents of the cell surface or belong to those species of proteins determined to be secreted. The fact that they also are found in lysosomes [1] or in lysosomal equivalents like in yeast vacuoles [2] is taken as indication that these organelles might be regarded as part of the extracellular compartment.

However, the occurrence of glycoproteins in true cytoplasmic organelles has been reported repeatedly. Thus glyoxysomal malate synthase from castor beans [3] and from cucumber [4] and isocitrate lyase from cucumber [5] have been claimed to be glycoproteins.

We were not able to confirm the reports on the glycoprotein nature of malate synthase and isocitrate lyase. During this investigation it turned out, however, that at least 12 *N*-glycosylated polypeptides are present in glyoxysomal membranes of castor beans.

2. Materials and methods

Seeds of castor bean (*Ricinus communis*) were soaked overnight and germinated in vermiculite for 2 days at 30°C.

Cellular proteins were radioactively labeled by incubating slices of endosperm tissue with D-[1-^{14}C]-glycosamine in presence or absence of tunicamycin. Slices ~1 mm thick were placed on moist filter paper. D-[1-^{14}C]Glucosamine (5 μCi, 57 Ci/mol; Amersham Buchler) or *N*-acetyl-[1-^{3}H]glucosamine (3 Ci/mmol) or [^{3}H]glucosamine (18.8 Ci/mmol) or [^{35}S]methionine (1.25 Ci/mol), all from Amersham Buchler, were applied to the surface of each slice. Tunicamycin (15 μg) in 20 μl 0.3 mM NaOH or only NaOH (control) was applied 15 min before labeling with [^{14}C]-glucosamine. The tissue was incubated for times indicated at 30°C in darkness.

Homogenisation of endosperm tissue and the separation of organelles by sucrose density gradient centrifugation were performed essentially as in [6].

Gradients were centrifuged for 16 h at 20 000 rev./min in a SW 27 rotor in a Beckman L2-65B ultracentrifuge. After centrifugation 0.5 ml fractions were collected and the sucrose content of the individual samples was determined refractometrically. Sucrose gradient fractions which contained the protein bands of mean buoyant densities of 1.12 g/ml (ER), 1.18 g/ml (mitochondria) and 1.24 g/ml (glyoxysomes) were pooled separately.

The membrane proteins of the glyoxysomes were separated from matrix proteins by osmotic shock: 3 vol. 50 mM Tricine buffer (pH 7.5) were added to each sample. After shaking for 30 min the membranes were recovered by centrifugation for 2 h at 42 000 rev./min and resuspended in 50 mM Tricine buffer (pH 7.5). All pooled samples were dialysed against water until the refractometrically determined sucrose content was zero.

After freeze drying the samples were prepared for 10% SDS–polyacrylamide gel electrophoresis as in [7]. After electrophoresis the gels were fixed and the proteins stained with Coomassie blue R-250. The incorporation of [^{14}C]glucosamine was detected by fluorography [8].

To identify [^{14}C]glucosamine, the radioactivity incorporated into glyoxysomal proteins was released after freeze drying by treating the samples with 1 ml 2 N HCl or 1 ml H_2O (control) at 100°C for 3 h. After evaporation [^{14}C]glucosamine was identified by paper chromatography on Whatman no. 1 in 1-butanol–

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acetic acid–ethyl acetate–water (4:2.5:3:4, by vol.).

For pronase digestion labeled membrane fractions were treated as in [9].

3. Results

During germination the endosperm tissue of castor beans is mainly active in producing glyoxysomes for fat to sucrose conversion [10]. A number of enzymes of the dolichol pathway of protein glycosylation drastically increase in activity during germination [11,12]. It was of interest, therefore, whether (and possibly how) the increased rate of glycoprotein synthesis might be related to the formation of glyoxysomal glycoproteins.

When it was tried to confirm the reports on the glycoprotein nature of malate synthase [3,4] negative results were obtained with a number of methods. Thus malate synthase purified according to [13] yielded considerably <0.5 mol glucosamine/mol polypeptide after hydrolysis (separated and determined by amino acid analyzer). Less than 4% of malate synthase was found to bind to con A–Sepharose and also PAS staining on the gel (trying to avoid any of the many possible artefacts) was negative. Incubation of endosperm tissue slices with tunicamycin (an inhibitor of the initial reaction of the dolichol pathway [14–16]) for 12–24 h did not result in an inhibition in the increase of malate synthase activity. During this incubation the activity of the enzyme increased 3–5-fold in the tissue slices. For isocitrate lyase, too, no indications were obtained that the enzyme might be a glycoprotein; this has been stated before [4].

However, the tissue did synthesize glycoproteins during this time of germination, because *N*-[^{3}H]acetylglucosamine was incorporated into trichloroacetic acid-insoluble material (fig.1a) and this incorporation was inhibited 60–70% by tunicamycin (fig.1b). When the trichloroacetic acid-insoluble material was treated with pronase, 64% of the radioactivity was converted into trichloroacetic acid-soluble material. To find out, whether radioactive *N*-acetylglucosamine was incorporated into glyoxysomal proteins, the tissue was homogenized under isotonic conditions and the organelles separated in a sucrose gradient [6]. The glyoxysomal fraction was collected and by measuring the ER marker enzyme NADH: cytochrome *c* reductase it was shown that ER contamination was certainly <5%. The glyoxysomes were dialyzed for 24 h to remove

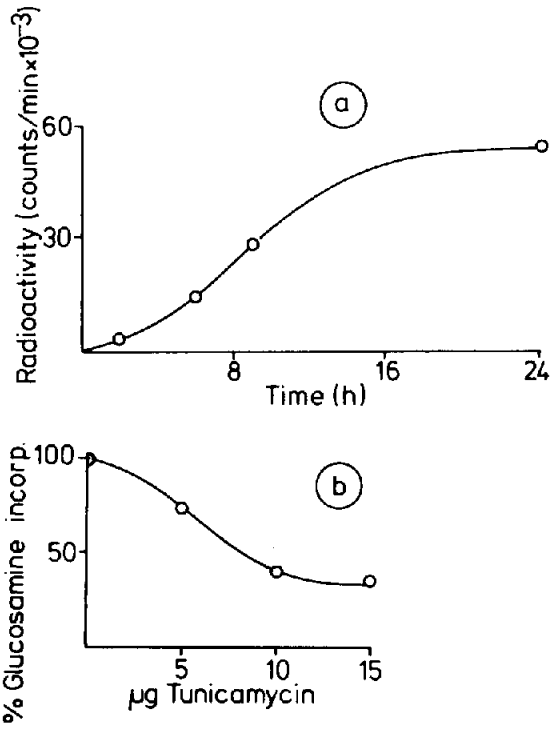

Fig.1. (a) Time course of in vivo incorporation of *N*-acetyl-[1-^{3}H]glucosamine into trichloroacetic acid-insoluble material. After grinding the trichloroacetic acid precipitate of the crude extract of one tissue slice was collected by filtration on Whatman GF/A glass–fiber paper and washed with 10% trichloroacetic acid, chloroform/methanol (2:1) and chloroform/methanol/water (10:10:3). The radioactivity on the filters was determined by scintillation counting. (b) Effect of tunicamycin on the in vivo incorporation of [^{3}H]glucosamine (12 h) into trichloroacetic acid-insoluble material.

the sucrose, solubilized in SDS and the proteins were then separated on a SDS slab gel. The banding pattern a,b of fig.2 represent the Coomassie blue stain of such an experiment, whereas c,d represent the radioautogram of the identical gels. As can be seen [^{14}C]glucosamine is incorporated into a large number of polypeptides of various sizes and this incorporation is completely blocked with tunicamycin. The glycosylation inhibitor did not affect the total amount of protein on the gel (cf. a,b) and it also did not affect the incorporation of [^{35}S]methionine in a parallel experiment (fig.2, gel e,f). To make sure that the radioactivity in the glyoxysomal proteins is indeed glucosamine, an aliquot of the glyoxysomal fraction was hydrolyzed and the radioactivity released was analyzed by paper chromatography. As can be seen in fig.3 all the radio-

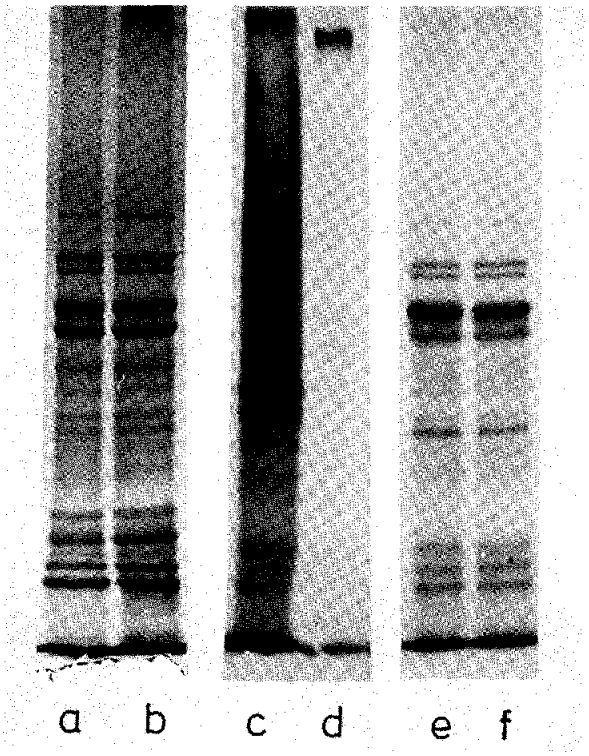

Fig.2. SDS–polyacrylamide gel electrophoresis of the glyoxysomal fraction collected from 10 endosperm slices which were labeled with D-[1-^{14}C]glucosamine in absence (b,c) or presence of tunicamycin (a,d); Coomassie blue stain (a,b) and fluorography (c,d) of identical gel. Fluorography of a parallel glyoxysomal sample labeled with [^{35}S]methionine in absence (e) or presence of tunicamycin (f).

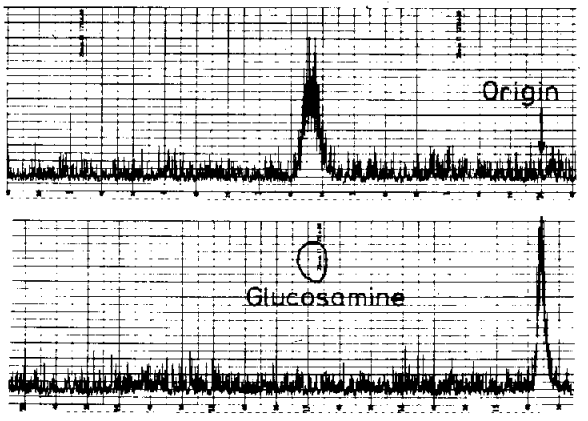

Fig.3. Paper-chromatogram of radioactivity released from glyoxysomal fraction after treating it with 2 N HCl (top) or water (control; bottom) at 100°C for 3 h. The glyoxysomal fraction was collected from 10 endosperm slices labeled with D-[1-^{14}C]glucosamine.

activity was released by acid hydrolysis and ran on paper like the glucosamine standard.

Finally it was of interest to find out whether the glycoproteins obviously present in glyoxysomes are part of the glyoxysomal membrane or the glyoxysomal matrix. The experiment labeling proteins with [^{14}C]glucosamine in vivo was repeated, therefore, and after separation of the glyoxysomes one half of it was treated as before, whereas the other half was osmotically shocked to separate matrix from membrane proteins. Fig.4 shows that the pattern of radioactive bands in the total glyoxysomal fraction (c) corresponds exactly to the pattern of the membrane proteins (b), whereas no radioactivity was found with the matrix proteins (a). The Coomassie bands of the same gels indicated that ~50% of the total protein was released by the osmotic shock as matrix proteins (not shown).

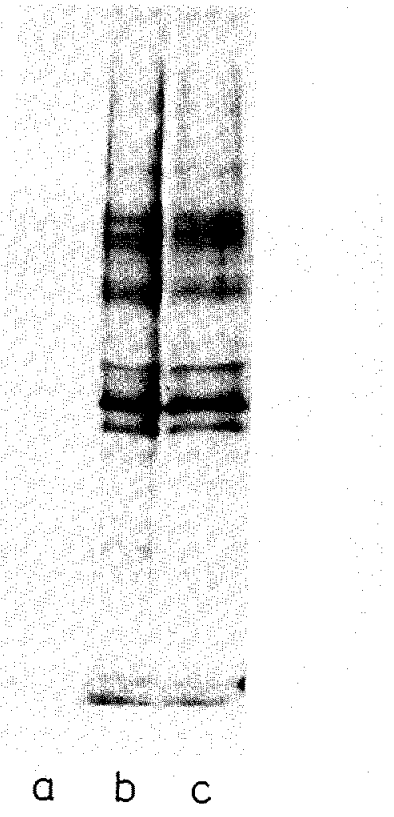

Fig.4. Fluorogram of SDS–polyacrylamide gels of D-[1-^{14}C]-glucosamine labeled glyoxysomes before (c) and after osmotic shock (b = membrane; a = matrix proteins).

4. Discussion

It has been shown that glyoxysomal membranes contain >12 polypeptides which are *N*-glycosylated. The 5 major bands correspond to glycoproteins of M_r 90 000, 71 000, 56 000, 47 000 and 43 000. They do not coincide with any of the major Coomassie bands, nor with the newly synthesized [^{35}S]methionine labelled proteins (fig.2). This either means that the glycoproteins are minor components of the glyoxysomal membrane; they might, for example, constitute small intermediate pools which loose their carbohydrate portion during a final processing.

The existence of glycoproteins in glyoxysomes from castor beans has been postulated before for malate synthase and for a number of matrix proteins [3]. It has not been possible to confirm either of these results. The same laboratory has shown however [17] that from purified glyoxysomal membranes glycopeptides can be released by hydrolysis, which contain arabinose, mannose, galactose and *N*-acetylglucosamine as the main sugars. Although the absolute amount of sugar reported (34% carbohydrate, 66% protein) seems to be too high, these results in principle are in agreement with the observation reported here.

In [18] malate synthase was convincingly demonstrated to be synthesized in the cytoplasm. The previous assumption [19,20] that malate synthase originates at the ER became doubtful when it was shown [21] that the membrane-associated form of the enzyme only accidentally co-migrates with microsomes under certain experimental conditions; this result has been confirmed in this laboratory (U. B., unpublished). The observation [18] of the cytoplasmic origin of malate synthase fits very well the data that the enzyme is not a glycoprotein. Also for isocitrate lyase it has been shown for *Neurospora* that this enzyme is only synthesized on free ribosomes [22].

Since *N*-glycosylation of proteins proceeds at the ER [11,23,24] and since a large number of glycoproteins are present in the membranes of glyoxysomes the postulated interrelationship between ER and glyoxysomes [20] may nevertheless exist to some extent.

Glycosylated proteins in intracellular organelles have so far only been definitely shown to exist in lysosomes and lysosomal vacuoles [1,2]. Their additional presence in the ER and the Golgi body, on the other hand, might simply be a consequence of the location of the glycosylating and carbohydrate processing reactions at the membranes of these organelles. If the results reported here are taken together with the reports on the occurrence of glycoproteins in membranes of lysosomes [25] and lysosome-like vacuoles [26] and with the various indications concerning the existence of glycoproteins in membranes of mitochondria [17,27] and in protein bodies [28], one is inclined to assume that all membrane-bound cell organelles contain glycoproteins.

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