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Glycoprotein Biosynthesis in *Chlamydomonas*[1]

I. *IN VITRO* INCORPORATION OF GALACTOSE FROM UDP-[^{14}C]GALACTOSE INTO MEMBRANE-BOUND PROTEIN

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ABSTRACT

A crude membrane fraction from *Chlamydomonas reinhardii* was found to catalyze D-galactose transfer from UDP-galactose to endogenous proteins. Highest incorporation rates were achieved by incubation at 25°C and pH 7.5 in the presence of 10 millimolar Fe^{2+}. Hydrolytic studies on the labeled polymer revealed that radioactivity was attached to protein via an alkali-stable and acid-labile linkage. Identification of galactose as the only labeled sugar in the acid hydrolysate and results of a tentative estimation of the molecular weight of the charged alkaline degradation product indicate that monomeric galactose units are transferred to form an *O*-glycosidic bond with peptidyl hydroxyproline. No indications were found for a similar linkage to serine which, in contrast to the hydroxyproline-*O*-glycoside linkage, is acid-stable but is cleaved by β-elimination. Chromatography of the sodium dodecyl sulfate-solubilized polymer on Sepharose-6B demonstrated that galactosyl residues are mainly associated with proteins which are of considerably higher molecular weight than are the majority of sodium dodecyl sulfate-denatured membrane proteins in this fraction.

Glycoproteins can be classified according to the nature of their carbohydrate-peptide linkages. Although the β-*N*-acetylglucosaminyl-asparagine linkage seems to be ubiquitous in all living organisms, there are considerable differences between plants and animals in the nature of their *O*-glycosidic bonds (8, 17). A major group among the *O*-glycosidic plant glycoproteins studied so far are hydroxyproline-rich cell wall glycoproteins. As the result of Lamport's (9) suggestion that so-called 'extensin' may play an important role in the regulation of cell elongation in higher plants, much attention has been paid to elucidation of its chemical structure, metabolism (5, 10, 11) and secretion (1–3). A major difficulty in all these studies is the fact that the extensin molecule cannot be isolated from the native cell wall without breaking covalent bonds.

The cell wall of the unicellular green alga *Chlamydomonas reinhardii* consists of a multilayered structure composed of a number of crystalline glycoproteins (16). In many respects, these are similar to extensin: (a) they are rich in hydroxyproline and their overall amino acid composition seems to be almost identical; (b) they are rich in arabinose and galactose (12) and carry the typical L-arabinosyl-hydroxyproline linkage and (c) between one and four consecutive arabinose residues are often found to be linked to peptidyl hydroxyproline (14).

In addition to arabinose, a variety of other sugars are linked to hydroxyproline in glycoproteins of noncellulosic algae. This finding, together with the occurrence of Hyp-*O*-Gal linkages in glycoproteins from *Chlamydomonas reinhardii* and some other species of noncellulosic green algae (13), seems to represent a fundamental difference between algae and higher plants. Similar structures, however, have been found to occur in extracellular proteoglycans and in cellular fractions of suspension-cultured *Acer pseudoplatanus* cells (15). In these cells and in their culture medium, an arabinogalactan containing some xylose and fucose is bound to hydroxyproline via galactose and, probably in some cases, via glucose. Therefore, it is conceivable that somewhat larger, heteromer Hyp-glycosides and the Hyp-*O*-Gal linkage are common features of algal and higher plant glycoproteins in general.

The crystalline lattice of *Chlamydomonas* cell walls is easily disrupted under mild chaotropic conditions to give a solution containing several different high mol wt glycoproteins. On dialysis, the glycoproteins reassemble to yield a crystalline product indistinguishable from the original cell wall in terms of its chemical structure and morphology (4). Thus, *Chlamydomonas* provides a particularly good system for studying native, nondegraded cell wall glycoproteins, which are probably representative of *O*-glycosidic plant glycoproteins in general. Recent work on this subject has concentrated on analyses of the chemical structure of *Chlamydomonas* glycoproteins. I am studying glycoprotein biosynthesis in *Chlamydomonas reinhardii*, using an *in vitro* system, in which crude homogenates transfer glycosyl residues from various sugar nucleotides to endogenous acceptor proteins. In the present work, the *in vitro* incorporation of galactose from UDP-galactose into membrane proteins is reported.

MATERIALS AND METHODS

Culture of Chlamydomonas. Vegetative cells of *C. reinhardii* wild type strain 90, from the Sammlung von Algenkulturen (Göttingen, Federal Republic of Germany), were grown as described recently (7). The cultures were constantly illuminated by fluorescent light (7,000 lux) and aerated with filtered 3% CO_2 in air. Cells were harvested by centrifugation at the end of the log phase of growth, washed twice in deionized H_2O, and used immediately for preparation of particulate enzyme.

Chemicals. UDP-[^{14}C]galactose (250–360 mCi/mmol) was obtained from the New England Nuclear Co. Al_2O_3 was from Martinswerk GmbH, Bergheim/Erft, Federal Republic of Germany. All other chemicals were from the Sigma Chemical Co., Taufkirchen, or from E. Merck, Darmstadt, Federal Republic of Germany.

Preparation of Particulate Enzyme. Wet-packed cells harvested from 6 L of culture were ground with Al_2O_3 without further additions in a chilled mortar. All subsequent steps were performed at 0 to 4°C. The stiff paste was suspended in 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 5 mM β-mercaptoethanol, 5 mM $MgCl_2$ and centrifuged at 1,000*g* for 10 min. The supernatant was centrifuged

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at 48,000*g* for 30 min, and the pellet was resuspended in 5 to 10 ml of 50 mM Tris-maleate (pH 7.5), 10 mM β-mercaptoethanol, 40% (v/v) glycerol, and, unless otherwise stated, 3 mM $MgCl_2$. This crude particulate enzyme preparation normally contained 8 to 12 mg protein per ml. Transferase activity was found to be stable for several months when the preparation was stored at −20°C.

Enzyme Assay. Galactosyl transferase was measured in a total volume of 50 μl. Unless stated otherwise, 5 μl 0.1 M $FeSO_4 \cdot 7\ H_2O$ and 5 μl UDP-[^{14}C]galactose (220,000 dpm) were added to 40 μl of crude particulate enzyme preparation, and the reaction mixture was incubated at 25°C for 30 min. The reaction was stopped by transferring the incubation mixture onto a paper disc (Whatman 3 MM, approximately 1.5-cm diameter) and placing the soaked paper in a chilled 10% (w/v) TCA solution. The paper was extensively washed in 5% (w/v) TCA, methanol, and chloroform: methanol (2:1, v/v), then dried in a stream of air, and counted in a toluene-based scintillation cocktail. In all experiments, controls were carried out in which UDP-[^{14}C]galactose was added after TCA precipitation. After the washing procedure, these samples were found to be practically free of label.

Alkaline Degradation. Labeled glycoproteins on paper discs were treated with 0.2 N NaOH for 5 h at 50°C (β-elimination) or with 0.2 M $Ba(OH)_2$ for 6 h at 100°C. Under both conditions, 90 to 95% of the original radioactivity was released during the incubation. NaOH hydrolysates were neutralized by treatment with Dowex 50W-X8(H^+), and $Ba(OH)_2$ hydrolysates were neutralized by adding an equivalent amount of sulfuric acid to precipitate Ba^{2+} ions.

Acid Hydrolysis. Samples were hydrolyzed with 2 N TFA[2] in sealed ampules for 90 min at 120°C. TFA was removed by evaporation and washing three times with water; then the residues were dissolved in a small volume of water and subjected to HPLC or PC.

HPLC. HPLC was performed on a Shodex Ionpak S 801 column obtained from Macherey a. Nagel GmbH, Düren, Federal Republic of Germany, equipped with a syringe-loading sample injector (Rheodyne, Model 7120). The column was run at a pressure at 125 p.s.i. at 40°C, with water as the eluant.

PC. Descending PC using Whatman No. 1 paper (46 × 57 cm) was performed in the following solvent systems; (a) ethylacetate: acetic acid:formic acid:H_2O (18:3:1:4); and (b) 1-butanol:pyridine: H_2O:acetic acid (60:40:30:3).

RESULTS

Synthesis of Labeled Polymer. Incubation of *Chlamydomonas reinhardii* particulate preparations with UDP-[^{14}C]galactose in the presence of divalent cations resulted in the incorporation of label into a TCA-insoluble, but SDS-soluble, fraction. Rates of incorporation were linear for a few min and then declined rapidly (Fig. 1). The transfer reaction displayed maximum activity at pH 7.5, and slightly higher incorporation rates were found in Tris-maleate buffer than were found in Tris-HCl. Standard incubations were carried out at 25°C; the use of higher temperatures did not result in significantly higher incorporation rates (data not shown in detail). The effect of various divalent cations on the rate of sugar transfer is illustrated in Figure 2. The greatest stimulation was achieved with 10 mM Fe^{2+} ions in the incubation mixtures. When the assay mixture was supplied with Mg^{2+} ions, in addition to Fe^{2+} or Mn^{2+} ions (final concentration, 10 mM each), the stimulatory effect of the cations was found not to be additive. The membranes used in this experiment were prepared as described in "Materials and Methods," with the exception that Mg^{2+} was omitted. Galactosyl transferase activity of membranes prepared in this way was reduced by 20 to 30%.

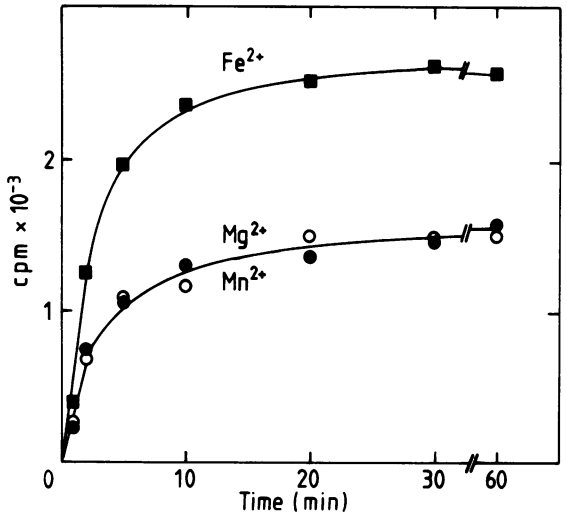

FIG. 1. Time-dependence of the transfer of galactose from UDP-[^{14}C]galactose to endogenous proteins in the presence of Fe^{2+}, Mg^{2+}, or Mn^{2+} (10 mM). Standard conditions were used, except that the membranes were prepared in the absence of Mg^{2+}.

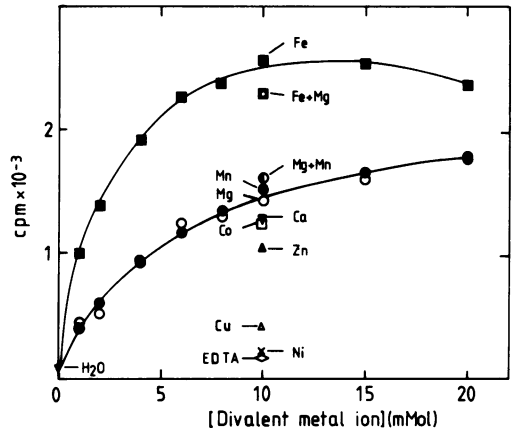

FIG. 2. Divalent metal ion requirement for the transfer of galactose from UDP-Gal to endogenous proteins. In the samples containing two different ions, their concentrations were 10 mM each. Standard conditions were used, except that the membranes were prepared in the absence of Mg^{2+}.

Properties of the Labeled Polymer. Labeled polymer was quantitatively eluted from the paper discs by extraction with 25 mM Tris-HCl buffer containing 0.25% SDS. The extract was concentrated and passed through a Sephadex G-50 column, and the fractions were monitored for radioactivity and *A* at 280 nm. The results (Fig. 3A) indicate that radioactivity is present exclusively in high mol wt compounds. Radioactive material on paper discs was subjected to β-elimination and $Ba(OH)_2$ and acid hydrolysis, and the hydrolysates were analyzed by chromatography on the Sephadex G-50 column (Fig. 3, B–D). The breakdown products were further analyzed by ion exchange chromatography on Sephadex A-50, by a HPLC system which separates charged molecules from small oligosaccharides and monosaccharides, and by PC. The results clearly indicated that labeled breakdown products in the alkaline hydrolysates were exclusively present in a charged form (data not shown in detail). The fact that radioactive galactose was not detectable after β-elimination rules out the possibility that *in vitro* synthesis of Ser-*O*-Gal or Thr-*O*-Gal linkages occurred in the galactose transferase assay. The occurrence of charged degradation products after β-elimination, with similar properties to

[2] Abbreviations: TFA, trifluoroacetic acid; PC, paper chromatography.

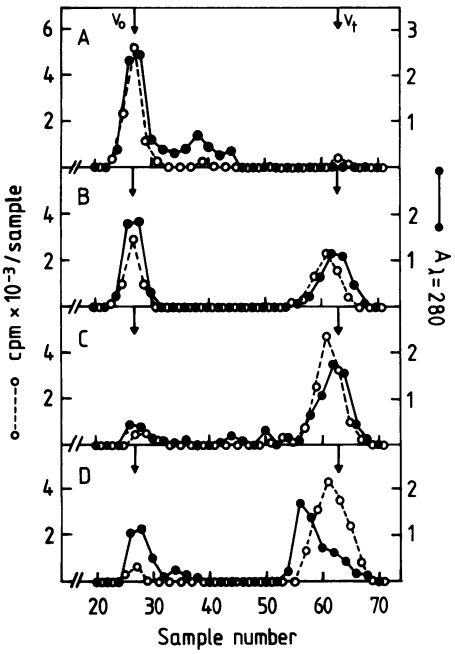

FIG. 3. Sephadex G-50 gel chromatography of the labeled polymer fraction before (A) and after hydrolytic treatment. B, 0.2 *N* NaOH, 5 h, 50°C; C, 0.2 M $Ba(OH)_2$, 6 h, 100°C; D, 2 *N* TFA, 90 min, 120°C. The column (1 × 75 cm) was equilibrated and eluted with 10 mM Tris-HCl buffer (pH 8.0).

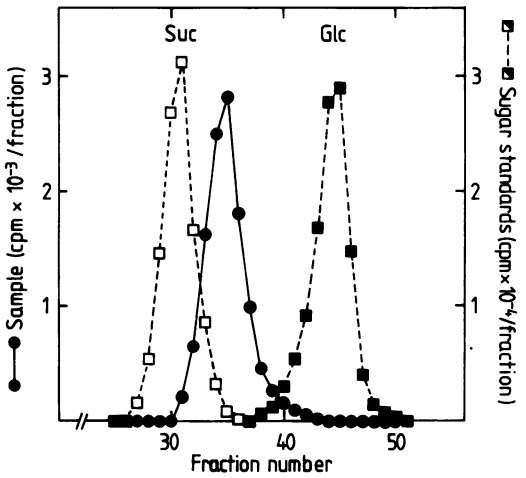

FIG. 4. Sephadex G-10 gel chromatography of the product from alkaline hydrolysis of the labeled polymer with 0.2 M $Ba(OH)_2$. In a preliminary run, the column (1 × 75 cm, 10 mM Tris-HCl buffer [pH 8.0]) was calibrated by the separation of a mixture of [^{14}C]sucrose and [^{14}C]glucose standards. Radioactivity was monitored in alternate fractions in both runs, and the data are presented in the same figure.

those obtained after $Ba(OH)_2$ treatment, reflects incomplete alkaline degradation under the former less vigorous hydrolytic conditions. The radioactive breakdown product obtained after acid hydrolysis (Fig. 3D) was identified as monomeric galactose by HPLC and PC in both solvent systems (data not shown).

Gel filtration on a Sephadex G-10 column was used to estimate the mol wt of the radioactive material released from the polymer by alkaline hydrolysis. In a preliminary run, the column was calibrated by separating a mixture of [^{14}C]sucrose and [^{14}C]glucose standards. The neutralized $Ba(OH)_2$ hydrolysate was then passed through the column, and the eluate was monitored for radioactivity. The radioactivity was eluted from the column in a single peak

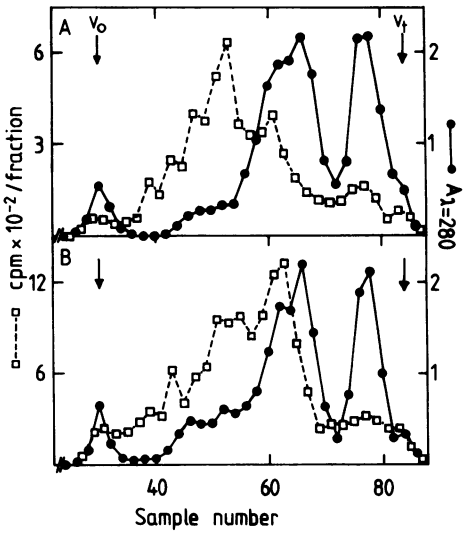

FIG. 5. Sepharose-6B gel chromatography of the SDS-solubilized, labeled polymer fraction. Crude membranes prepared in the absence of divalent cations were incubated with UDP-[^{14}C]galactose in the presence of 10 mM Mg^{2+} (A) and 10 mM Fe^{2+} (B), respectively. The gel had been equilibrated and was run with 25 mM Tris-HCl buffer (pH 8.0) containing 0.25% (w/v) SDS.

at an elution volume slightly greater than that for sucrose (Fig. 4). Based on its elution volume, the mol wt of the radioactive material was estimated to be approximately 300 (daltons), which is similar to that of Hyp-Gal.

Taken together, the results suggest that, in this *in vitro* system, galactose is transferred from UDP-Gal to peptide-bound Hyp. To obtain some preliminary information about the number of components in the polymer product and their mol wt, labeled glycoprotein, which was synthesized in the presence of optimum Fe^{2+} (10 mM) or Mg^{2+} (20 mM), was eluted from the paper discs, concentrated, and run on a Sepharose-6B column. The eluate was analyzed for radioactivity and *A* at 280 nm. The data (Fig. 5) do not reveal how many labeled protein species are present, but it can be seen that a large proportion is of higher mol wt than is the bulk of the SDS-denatured membrane proteins. Qualitatively, the pattern of glycosylated proteins seems to be similar for both samples. But, in addition to the higher rate of incorporation in the presence of Fe^{2+} compared with Mg^{2+}, a significantly larger amount of lower mol wt glycoprotein(s) is labeled.

DISCUSSION

The particulate fraction of a cell homogenate from *C. reinhardii* catalyzed the transfer of galactose from UDP-galactose to acceptor macromolecules, which appeared to be a small number of high mol wt polypeptides. Several lines of evidence indicate that monomeric galactosyl residues are covalently linked to peptidyl hydroxyproline. On alkaline degradation of the polymeric product in 0.2 M $Ba(OH)_2$, radioactivity was found exclusively in a charged molecule of low mol wt. Hyp-*O*-glycosides are known to be stable under these conditions (20). β-Elimination resulted in partial release from the polymer of a labeled low mol wt compound which had the same properties as did the labeled material in the $Ba(OH)_2$ hydrolysate. In contrast with the stability of Hyp-*O*-glycosides during β-elimination, the corresponding serine- and threonine-*O*-glycoside linkages are hydrolyzed completely. As no neutral sugar residues were detected in the β-elimination mixture, it is evident that no Ser-*O*-glycoside or Thr-*O*-glycoside linkages were synthesized by this *in vitro* system under the conditions used. Further evidence for the presence of Hyp-*O*-Gal linkages was obtained by estimating the mol wt of the alkaline degradation

product by means of Sephadex G-10 gel filtration (Fig. 4). A single radioactive peak which ran slightly more slowly than sucrose, was found used as a marker for disaccharides, and corresponded to a mol wt of approximately 300 (daltons). This estimated value compares well with the actual mol wt of 293 daltons for Hyp-Gal. Although these results do not prove conclusively that the labeled hydrolysis product is Hyp-Gal, it is clear that a single sugar residue is linked to some charged molecule of similar mol wt to hydroxyproline. After acid hydrolysis of the polymer or the alkaline degradation product (data not shown), radioactivity was found exclusively in a monosaccharide, which was identified as galactose by HPLC and PC. It is well documented that the Hyp-*O*-Ara linkage is acid-labile (11), and this property has recently been confirmed for Hyp-*O*-Gal (19).

Enzymic activity of glycosyltransferases often shows a strict requirement for the presence of divalent cations, and the galactosylation system described here is no exception. Surprisingly, however, Fe^{2+} ions resulted in higher incorporation rates than did Mg^{2+} or Mn^{2+} ions, which are well documented in the literature as the most effective ions in other systems (6). To my knowledge, the only other report (18) in which Fe^{2+} apparently increased glycosyltransferase activity concerns a mannosyltransferase system from *Fusarium solani*. However, the authors point out that the observed activity in the presence of Fe^{2+} did not reflect incorporation of labeled mannose into glycoprotein, since gel filtration of the product in the presence of SDS demonstrated that radioactivity was associated with low mol wt compounds.

A high percentage of the labeled glycoproteins is of considerably higher mol wt than is the bulk of SDS-denatured membrane proteins. Comparison of the elution profiles for samples prepared in the presence of Fe^{2+} and Mg^{2+} (Fig. 5) suggests that, in both cases, the same proteins became labeled, although incorporation was higher with Fe^{2+}, particularly for labeled proteins of lower mol wt. However, examination of the alkaline and acid hydrolysates of the polymer prepared in the presence of Mg^{2+} gave results similar (data not shown) to those obtained for samples prepared in the presence of Fe^{2+}. This indicates that the same linkage was formed under both sets of conditions.

Electrophoretic analysis of the cell wall of *C. reinhardii* has revealed the presence of at least eight SDS-soluble glycoproteins, of which the major ones were in the mol wt range of 150,000 to 300,000 (daltons) (16). That predominantly large protein molecules were labeled in the *in vitro* assay suggests that these may represent precursors of cell wall glycoproteins. Support for this hypothesis would be obtained if it could be demonstrated conclusively that Hyp-*O*-Gal linkages in *C. reinhardii* are found only in cell wall glycoproteins, as suggested by Miller *et al.* (13). The presence of hydroxyproline-galactoside linkages in the cell wall of *Codium* (another noncellulosic green alga) has also been reported briefly (14). Because Miller *et al.* used crude cell wall preparations of *C. reinhardii* in their experiments, it remains possible that Hyp-Gal-containing glycoproteins either are not constituents of or are not restricted to the cell wall.

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