Synthesis and Secretion of Hydroxyproline-containing Macromolecules in Carrots

III. METABOLIC REQUIREMENTS FOR SECRETION

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MARCIA R. DOERSCHUG AND MAARTEN J. CHRISPEELS
Department of Biology, John Muir College, University of California, San Diego, La Jolla, California 92037

ABSTRACT

Using pulse-chase experiments with radioactive proline, it is possible to study the rapid transfer from the cytoplasm to the cell wall of the hydroxyproline-rich protein found in the cell walls of higher plants. The secretion of this protein is not obligatorily coupled to protein synthesis. Secretion is completely inhibited by uncouplers of oxidative phosphorylation and strongly inhibited by the inhibitors of electron transport, cyanide and azide. It is concluded that the transfer of proteins from the cytoplasm to the cell wall is an energy-requiring step.

The plant cell wall contains a glycoprotein or glycoproteins rich in hydroxyproline (for a review see Ref. 13). The protein moiety of this glycoprotein is synthesized in the cytoplasm and subsequently transferred to the cell wall (3, 7, 18, 19). Little is known about the mechanism of this secretory process. It appears to be mediated by membranous organelles in the cytoplasm (3, and Dashek, personal communication). Using aged disks of carrot phloem parenchyma, we present evidence that the transport of the hydroxyproline-rich glycoproteins from the cytoplasm to the cell wall is an energy-requiring process, which is not obligatorily coupled to protein synthesis.

MATERIALS AND METHODS

Fresh carrots (Daucus carota cv.) were purchased in local supermarkets and stored at 5 C. Disks measuring 1 mm in thickness and 8 mm in diameter were incubated ("aged") for 24 hr at 30 C on a rotary shaker (1 g in a 50-ml Erlenmeyer flask containing 10 ml of 2 mM acetate buffer, pH 4.8, and 50 μg/ml of chloramphenicol [15]). All labeling experiments were done with [14C]proline (specific radioactivity 1.8 mc/mg and uniformly labeled) purchased from New England Nuclear Corp. Following the incorporation of radioactive proline, the tissue was rinsed three times with cold 1 mM proline and then exhaustively homogenized in 10 ml of cold water with a cold mortar and pestle.

Determination of Radioactivity in Protein-bound Proline and Hydroxyproline. The entire homogenate was centrifuged at 1000g for 3 min to sediment the cell wall. Aliquots of the supernatant (the cytoplasm) were precipitated with an equal volume of 15% trichloroacetic acid, and the proteins were collected on membrane filters (B, from Schleicher and Schuell). The cell walls were purified by three consecutive washes in cold water and finally resuspended in 9 ml of water. Aliquots of the cell wall suspension were mixed with 4 volumes of ethanol and collected on membrane filters (EHWP 2500 from Millipore). The filters and collected cellular material were sealed in a vial with 5 ml of 6 N hydrochloric acid and autoclaved for 90 min (120 C and 22 lbs pressure). After removal of the hydrochloric acid by evaporation under vacuum, the residue was dissolved in water and spotted on Whatman 3MM paper. Proline and hydroxyproline were separated by descending chromatography for 15 hr in isopropyl-formic acid–water, 15:2:2 (8). The resulting chromatogram was cut into 1-inch strips, and radioactivity in proline and hydroxyproline was determined by liquid scintillation counting.

Determination of ATP. One gram of tissue was heated for 6 min at 80 C with 3 ml of 66% ethanol in water. The tissue and the ethanol extract were quickly cooled and then shaken for 1 hr at 5 C. Aliquots of the ethanol extracts were then diluted 25-100-fold, and the ATP was determined by the firefly luciferin-luciferase assay (9), with commercially purchased ATP as a standard. The intensity of the emitted light was monitored with a liquid scintillation counter. In preliminary experiments we established that ATP is not broken down during the isolation process and that it is not necessary to homogenize the disks to extract the ATP.

Metabolic inhibitors were obtained from the following sources: cycloheximide ("actidione") was a gift from The Upjohn Company, Kalamazoo, Michigan; m-chloro(carbonyl cyanide) phenylhydrazone and p-trifluoromethoxy(carbonyl cyanide) phenylhydrazone were gifts from E. I. DuPont de Nemours and Company; sodium azide and potassium cyanide were purchased from Matheson, Coleman and Bell, and J. T. Baker Chemical Co., respectively. Potassium cyanide was dissolved as follows: 65 mg in 10 ml of 0.2 N acetate buffer, pH 3.8. This gives a final pH of 4.8.

RESULTS AND DISCUSSION

A pulse-chase experiment with radioactive proline which demonstrates the disappearance of protein-bound [14C]hydroxyproline from the cytoplasm as well as its concomitant appearance in the cell wall is shown in Figure 1. After the radioactivity is chased with free proline, protein-bound [14C]-hypro in the cytoplasm continues to increase for a few minutes, and then it

1 Abbreviations: hypro: hydroxyproline; CKCCP: m-chloro(carbonyl cyanide) phenylhydrazone; FCCP: p-trifluoromethoxy(carbonyl cyanide) phenylhydrazone.

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declines rapidly. This decline shows a half-time of approximately 15 min. More protein-bound 14C-hypro arrives in the cell wall than disappears from the cytoplasm. Peptidylhydroxyproline is formed through the hydroxylation of peptidylproline (5), and this process lags behind the incorporation of proline by several minutes (6). Conversely, hydroxylation will proceed for several minutes after incorporation of 14C-proline is arrested with free proline.

To examine whether cycloheximide, a potent inhibitor of cytoplasmic protein synthesis in higher organisms, affects the secretory process itself, we labeled carrot disks for 20 min and then followed the transfer of protein-bound 14C-hypro from the cytoplasm to the cell wall in the absence and presence of the inhibitor. Table I shows that cycloheximide does not affect the secretory process and that protein secretion is independent of concomitant protein synthesis. A control experiment demonstrated that under our conditions cycloheximide at 10 μg/ml does indeed inhibit protein synthesis. Addition of cycloheximide to carrot disks which are incorporating 14C-proline arrests all further incorporation of the amino acid almost immediately (less than 2 min) and nearly completely (more than 95%) (see Fig. 8 in Ref. 3).

The observation that protein secretion proceeds normally in the absence of concomitant protein synthesis is consistent with the results of Chrispeels and Varner (6) showing that the release of ribonuclease by aleurone cells proceeds normally when protein synthesis is blocked. The same is true for the secretion of proteins by the pancreatic exocrine cell (11). Similarly, Morré et al. (16) observed that the formation of slime droplets (carbohydrate secretion) by root caps is not inhibited by puromycin.

To determine whether secretion is a process dependent on metabolic energy, we measured its temperature coefficient (Q10). Carrot disks were labeled with 14C-proline for 171/2 min, and the radioactivity was chased with free proline for 2½ min. These operations were performed at 30 C. Secretion was followed at 4, 14, and 22 C by transferring the flasks to suitable water bath shakers. The rate of arrival of protein-bound 14C-hypro in the cell wall (Fig. 2) indicates that the over-all process of secretion has a temperature coefficient of 2.0, which is well within the range of temperature coefficients for known metabolic processes. This by itself is insufficient evidence to implicate metabolic energy in secretion. Indeed, in at least one case, diffusion of polar molecules through lipid layers has a temperature coefficient of 2 in the physiological temperature range (17).

The uncoupling of protein synthesis from protein secretion makes it possible to use inhibitors of energy metabolism to investigate whether or not protein secretion is an energy-requiring process. Indeed, such inhibitors are known to affect protein synthesis, but any effect which they may have on secretion itself is not mediated through their effect on synthesis. The transfer of the hypro-rich protein from the cytoplasm to the wall is completed in 20 to 30 min. This limited our choice of inhibitors of energy metabolism to those which penetrate fast and reach their maximal effectiveness within a few minutes. This was determined by examining their effect on the ATP pool of the tissue, or on the rate of 14C-proline incorporation into proteins. Some compounds (e.g., antimycin A and N2) had to be rejected because too much time elapsed between their addition and the time of maximal effectiveness. The inhibitors used in this study all caused a rapid drop in the ATP level of the tissue and inhibited protein synthesis within minutes of their addition.

We determined the effect of two uncouplers of oxidative phosphorylation, n-chloro(carboxyl cyanide) phenylhydrazone and p-trifluoromethoxy(carboxyl cyanide) phenylhydrazone (10), and of two inhibitors of electron transport, sodium azide and

### Table I. Effect of Cycloheximide on Secretion

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<thead>
<tr>
<th>Time after Chase Is Applied</th>
<th>Secretion</th>
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<tbody>
<tr>
<td></td>
<td>Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>min</td>
<td>cpm/hr</td>
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<tr>
<td>2½</td>
<td>73,000</td>
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<td>22½</td>
<td>46,600</td>
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Fig. 1. Secretion of protein-bound 14C-hypro. Carrot disks (1 g) were aged for 22 hr, then labeled for 20 min with 1 μc of 14C-proline in 10 ml. The radioactivity was chased with 1 ml of 0.1 m proline, and samples were taken after 0, 2½, 5, 15, and 60 min. Protein-bound 14C-hypro was determined on aliquots of the cytoplasm and cell wall precipitated with trichloroacetic acid and alcohol, respectively.

Fig. 2. Temperature coefficient of secretion. Carrot tissue (1 g) was aged for 22 hr and then labeled for 17½ min with 1 μc of 14C-proline per flask and 10 ml of 2 x 10^6 m carrier proline. The radioactivity was chased with 1 ml of 0.1 m proline for 2½ min, and the flasks were then taken out of the 30 C water bath and moved to 4, 14, and 22 C water baths. Samples were collected at the times indicated and homogenized in water. Protein-bound 14C-hypro in the cell wall was determined after purification of the walls, as described under "Materials and Methods." Rates of arrival in the cell calculated as cpm of 14C-hypro per hr of secretion are as follows: 4 C, 7,500 cpm/hr; 14 C, 14,000 cpm/hr; 22 C, 24,500 cpm/hr.
potassium cyanide, on the process of secretion. Carrots were labeled for 17½ min, and the radioactivity was briefly (2½ min) chased with proline. At that time we added the inhibitors to the incubation media and then followed the disappearance of protein-bound hydroxyproline from the cytoplasm and its appearance in the cell wall. The uncouplers, CICCP and FCCP, used at a concentration of $10^{-4}$ M completely prevent secretion (Table II). The inhibitors, cyanide at 2 mm and azide at 0.2 mm, allowed secretion at a slow rate. More complete inhibition was obtained with sodium azide at a final concentration of 0.5 mm.

We also studied the effect of several inhibitors of energy metabolism on the ATP pool of the cell. One gram of carrot disks incubated for a day at 30°C was found to contain about 30 m&mu;moles of ATP. Upon addition of either cyanide or CICCP to the medium there is a rapid drop in the ATP content of the tissue (Fig. 3). After about 30 min the ATP level falls to about one-fifth to one-sixth of the control level. This is interpreted to mean that ATP continues to be utilized at least for some time after the inhibitors are added, but that its synthesis is rapidly inhibited upon the addition of either cyanide or CICCP. It is not clear whether this residual level is maintained through continuous synthesis and utilization of ATP or whether it represents ATP which is unavailable to those processes which normally use ATP.

In several mammalian systems protein secretion is also an energy-requiring process (1, 2, 12). In protein secretion by the exocrine cell of the pancreas the energy-requiring step is on the proximal side of the Golgi apparatus (between the Golgi apparatus and the outside of the cell). Although we do not yet know the secretory pathway of proteins in plant cells, it is pertinent to speculate on the nature of the energy-requiring step. We do know that protein secretion in our system is mediated by cytoplasmic organelles (3) and that the protein in question is actually a glycoprotein. Most of the hydroxyproline residues are glycosylated with arabinose (5, 14). Glycosylases involved in glycoprotein synthesis have recently been found in association with several membranous organelles (Golgi apparatus, smooth endoplasmic reticulum, cell membrane) isolated from mammalian cells. Similar studies with plant cells have not yet been published. Glycosylation is an energy-requiring step since it involves the transfer of a sugar residue from a sugar nucleotide to a protein. Therefore, at least part of the energy requirement for protein secretion can be accounted for by the need for ATP to synthesize the carbohydrate moiety of the glycoproteins which are on their way to the cell wall. The cytoplasmic location of the energy-requiring step may well be identical with the localization of the glycosylating enzymes.

**Table II. Effect of Metabolic Inhibitors on Secretion**

All conditions as in Figure 1. Proline chase was added 17½ min after the beginning of labeling and the inhibitors 20 min after the beginning of labeling. Final concentrations of inhibitors: CICCP and FCCP, 10 &mu;M; CN<sup>-</sup>, 2 &mu;M; azide, 0.2 &mu;M.

<table>
<thead>
<tr>
<th>Time after Chase Is Applied</th>
<th>Secretion</th>
<th>CICCP</th>
<th>FCCP</th>
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<tr>
<td>min</td>
<td>cpm of protein-bound %C hydroxyproline/g tissue in cell wall</td>
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<tr>
<td>2½</td>
<td>10,000</td>
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<tr>
<td>22½</td>
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**CONCLUSION**

The question which we have tried to answer here is whether the transport of the hydroxy-rich protein or proteins from the cytoplasm to the cell wall is a physical process, limited by diffusion, or whether it is a metabolic process dependent on cellular energy. The results show that protein secretion is not obligatorily linked to protein synthesis and that it is an energy-requiring process which is inhibited by metabolic inhibitors. These inhibitors cause the ATP level of the cells to fall very rapidly.

**LITERATURE CITED**


