VECTORIAL DISCHARGE OF NASCENT POLYPEPTIDES ATTACHED TO CHLOROPLAST THYLAKOID MEMBRANES

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SUMMARY. Chloroplast thylakoids with attached ribosomes were isolated from *Chlamydomonas reinhardti*. They were allowed to incorporate labeled amino acids into polypeptides. Labeled membranes were recovered from the reaction mixture, and a portion was treated with puromycin. The amount of labeled polypeptides released to the medium, and to the membranes by puromycin was determined by comparing radioactivity in soluble protein before, and after untreated, and puromycin-treated membranes were solubilized with the detergent Nonidet P-40. About 20% of the radioactive protein associated with the membranes was in nascent chains which were terminated by puromycin. Essentially all of terminated nascent chains remained with the membranes, and thus, were vectorially released. The results support the hypothesis that polypeptides which are synthesized by thylakoid-bound ribosomes are being incorporated into the membranes as they are synthesized.

INTRODUCTION. Ultrastructural studies have shown that some chloroplast ribosomes are associated with the chloroplast thylakoids in arrangements suggestive of polyribosomes (1-4). Thylakoids have been isolated from *Chlamydomonas reinhardti* with ribosomes attached to them (1,3). Over 50% of the thylakoid-bound ribosomes of the isolated membranes are polyribosomes (Margulies, M.M. and Michaels, A., unpublished). The thylakoid-ribosome complex incorporates labeled amino acids into nascent and completed polypeptides *in vitro* (5). Nascent polypeptide chains are important to the attachment of chloroplast ribosomes to thylakoids (1,3). These results led us to postulate that the thylakoid proteins which were synthesized by chloroplast membrane-bound ribosomes were incorporated into the membranes as they were synthesized (1).

Proteins synthesized in one subcellular compartment can be transferred or localized in another by vectorial release of polypeptides from membrane-bound ribosomes (6-8). This directional release also provides a mechanism by which proteins might be incorporated into the structure of membranes (9). Vectorial

release on termination of nascent chains with puromycin has been demonstrated with nascent polypeptides attached to the ribosomes of the endoplasmic reticulum (8), and for nascent polypeptides attached to ribosomes on the cytoplasm side of the mitochondrial envelope (6). Our experiments were designed to determine whether vectorial (i.e. directional) release of nascent polypeptides attached to thylakoid-bound ribosomes might play a role in the biosynthesis of thylakoid membranes.

MATERIALS AND METHODS. Chloroplast membranes were isolated from chloramphenicol-treated cells of *C. reinhardti*, as previously described (5). Chloramphenicol was added to cultures to preserve the ribosome-chloroplast membrane association during harvesting and breaking of cells (3). Amino acid incorporation was carried out with 80 μCi of tritiated amino acids per ml of reaction mixture, but otherwise as described previously (5). The tritiated amino acid mixture was obtained from New England Nuclear, Boston, Mass. (Cat. No. 250), and had an average specific radioactivity of 9.95 nCi/pmole. Samples were counted on filter paper discs (10) at an efficiency of 6.1%, giving a calculated specific radioactivity of 1,340 cpm/pmole amino acid. Amino acid incorporation was allowed to proceed for 2 h, and the reaction mixture was cooled to 0°C. The membranes were recovered, and washed once with buffer (25 mM KCl, 25 mM $MgCl_2$, 25 mM Tris-HCl, 1 mM dithiothreitol, pH 7.5) by centrifugation. The washed membranes were stored at -80°C. Eighty-five percent or more of the radioactive protein in the reaction mixture was recovered in the washed membranes. Membrane samples, containing 0.1 mg of chlorophyll, and about 10,000 cpm in 0.5 ml of buffer, were incubated for 1 hr at 0°C with, or without 1 mM puromycin, and with, or without 0.05 ml of Nonidet P-40 (5)/mg membrane chlorophyll. In the experiment presented, Nonidet was added at the start of the 1 h incubation at 0°C. However, Nonidet can be added at the end of the 1 h incubation at 0°C (i.e. after treatment with puromycin) with the same result (see Discussion). The treated membranes were then analyzed by centrifugation on sucrose density gradients. These were prepared from 0.31 and 1.4 M sucrose solutions in buffer, as already described (11), but with these exceptions: 11.5 ml of gradient was prepared instead of 12.0 ml; 0.5 ml of a cushion of 2 M sucrose in buffer was layered under the gradient; 0.5 ml of buffer was layered on top of the gradient; the 0.5 ml sample was layered between this overlay, and the gradient. The total volume in the centrifuge tubes was 13.0 ml. The gradients were centrifuged for 1 h at 40,000 rpm, at 0 to 2°C in a Spinco SW 40 rotor. They were scanned at 254 nm, divided into 0.306 ml samples, and two-0.1 ml samples were taken from each fraction for determination of radioactive protein. Material that pelleted to the bottom of the tube was resuspended, and radioactivity determined. Gradient fractions 1-4 contained soluble protein, 5-11 ribosomes (subunits and monomeric ribosomes), 12-35 polyribosomes, and 36-43 membranes. 80 S cytoplasm monoribosomes in 15,000 x g homogenate supernatants sedimented to fraction 11 when analyzed on replicate gradients.

RESULTS. Essentially no radioactivity was found in the soluble protein region of the gradients when labeled membranes were analyzed without detergent treatment, whether or not the sample had been treated with puromycin (Table I, columns A and B). Ninety-five percent of radioactivity was recovered with the membranes. When membranes were treated with detergent, 20% of radioactivity

Table I

Vectorial Release of Nascent Polypeptides

Gradient Fraction	Treatment of membranes				Δ puromycin	
	Without detergent		With detergent		Without detergent	With detergent
	Without puromycin	With puromycin	Without puromycin	With puromycin		
	(A)	(B)	(C)	(D)	(B - A)	(D - C)
	percent cpm					
Soluble protein	1.0	2.1	20.6	42.5	+ 1.1	+ 21.9
Ribosomes	2.3	1.6	15.0	19.3	- 0.7	+ 4.3
Polyribosomes	2.2	0.5	42.7	25.4	- 1.7	- 17.3
Membranes	94.5	95.8	21.6	12.9	+ 1.3	- 8.7
	cpm recovered from gradient (9,190 put on)					
Total	7,320	7,170	7,270	7,820		

was found with soluble protein (Table I, column C), indicating that that portion of radioactivity was in completed proteins (5). However, if membranes were treated with both puromycin, and detergent, 42% of radioactivity was in soluble proteins (Table I, column D). The difference of 21% between membranes treated with detergent, with, or without puromycin (D minus C) indicates that that portion of radioactive protein was discharged as a result of the action of puromycin. Ninety-five percent of the radioactive protein discharged by puromycin was discharged to the membrane vesicles ((D-C) - (B-A)/(D-C)). The increase in radioactive protein in the soluble fraction produced by puromycin was accompanied by a decrease from 43 to 25% of radioactivity in the polyribosome fraction (Table I, columns C and D).

DISCUSSION. Over 85% of label in peptides at the end of 2 h amino acid incorporation by chloroplast membranes from *C. reinhardti* remains with membranes (5). Some of the label bound to the membranes appears to be in completed membrane proteins, because it is solubilized by detergents, and gives discrete high molecular weight, radioactive bands on acrylamide gel electrophoresis (5). However, a portion appears to be in nascent protein, because it is associated with polyribosome-like particles which are released by detergent-treatment of membranes (5, and Table I in this work). These results suggest that the radioactivity associated with these polyribosome-like particles is the precursor of the completed radioactive membrane proteins, the latter having become attached, and incorporated into the membrane while still nascent protein (i.e. they become incorporated into the membrane without going through a stage where they are not attached to the membrane) (3,5). Our observation of directional release by puromycin of label associated with thylakoid polyribosomes argues for this interpretation. We have obtained similar patterns of radioactive protein distribution to those presented in Table I: 1) when isolated membranes were treated for 1 h with puromycin, and then treated with detergent, or 2) when puromycin was added during amino acid incorporation instead of after recovery of membranes.

Vectorial discharge of nascent polypeptides has been demonstrated with microsomes (8), and also with mitochondria (6). Microsomes, mitochondria, and the chloroplast thylakoid fragments used in our preparations (3) can all be considered as spaces enclosed by membranes. Some of the nascent polypeptides of microsomes are released to the membrane-enclosed space upon nascent protein-termination with puromycin (8). Neither in our experiments, or in the experiments with mitochondria (6) has it been demonstrated whether the peptides which are vectorially released are in the membranes, or in the spaces enclosed by the membranes. However, unlike the endoplasmic reticulum (from which microsomes are derived), the chloroplast thylakoids have no documented secretory function. Only in the phycobilin-containing chloroplasts of cryptophyte algae

has the space within the thylakoids been reported to contain protein (12). Thus, in cryptophytes, ribosomes attached to thylakoids could function in secretion. Our experiments only show that a portion of nascent polypeptides produced by ribosomes on thylakoid membranes is vectorially discharged to the thylakoids. Whether the discharged protein remains with the membrane itself or is discharged into the lumen is not known. However, the lack of documented secretory function for thylakoids suggests that vectorial release occurs to the membranes, and not to the spaces enclosed by them.

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