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Activity of Thylakoid-bound Ribosomes in Pea Chloroplasts[1]

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ABSTRACT

Pea (*Pisum sativum*) chloroplast thylakoid membranes were prepared by washing in hypotonic buffers. These membranes contained bound ribosomes which were active in protein synthesis when supplemented with soluble components from a strain of *Escherichia coli* low in ribonuclease. After dissolving the membranes by Triton and purification of the ribosomes, sucrose density gradient profiles indicated the presence of polysomal material as well as monomeric ribosomes. Most of the products of protein synthesis remained associated with the thylakoid membranes even after ribosomes were removed completely by high salt concentrations in the absence of Mg^{2+}. Of the newly formed products, 50% could be digested by pronase, while the remainder were protected by their association with the thylakoid membranes. The products are likely to be a mixture of intrinsic and extrinsic membrane proteins, with only the former completely protected by the membranes from attack by proteases.

Both higher plant and algal chloroplasts contain prokaryotic type (70S) ribosomes, responsible for synthesis of a number of the plastid polypeptides.

Electron microscope observations (12, 13, 17) and biochemical tests (11–13, 23–25, 28, 38) indicate that some of these ribosomes are intimately associated with the green thylakoid membranes, while others are free in the stroma. In chloroplasts of mature pea leaves approximately 20% of the 70S ribosomes were estimated to be bound to thylakoid membranes (38). A similar proportion of thylakoid-bound ribosomes was reported by Chua *et al.* (12, 13) in *Chlamydomonas*, but Margulies and Michaels (23, 24) found 50% of the chloroplast ribosomes bound if chloramphenicol was present during cell harvesting. Activity in protein synthesis (11, 16, 28, 38) and gradient profiles showing polysomal structure (13, 26) all indicate the presence of mRNA as well as ribosomes on the membranes.

An attractive possibility, but not an exclusive one, is that the ribosomes bound to thylakoid membranes might be making proteins which are inserted *in situ* into the membrane. The association of newly incorporated amino acids with thylakoid membranes has been noted several times, and both in *Chlamydomonas* (28) and pea (16) chloroplasts the products of this incorporation have been shown to include some discrete polypeptides as seen on SDS gels.

Much of the experimental design to demonstrate formation of membrane proteins is derived from work with RER, in which bound ribosomes form nascent polypeptides that penetrate through the membrane into the internal cisterna, prior to transport to the cell surface for secretion. These proteins are said to be "vectorially discharged" into the vesicle, and one criterion is the association with the vesicle of nascent chains discharged by puromycin (31, 34). Analogous association of puromycin-discharged nascent chains with *Chlamydomonas* thylakoid membranes was noted by Margulies and Michaels (24). A more compelling proof for vectorial discharge is provided by testing the nascent peptides for susceptibility to proteases. When the peptides have entered ER vesicles they are no longer hydrolyzed (5, 6). This criterion has not yet been applied to the products of thylakoid-bound ribosomes.

The nature of the ribosome-membrane association has been investigated both in ER (1, 7, and summarized in 26 and 32), and in *Chlamydomonas* chloroplasts (23). It was found, in both systems, that some proportion of membrane-bound ribosomes were released by the action of high salt (500 mM KCl) in the presence of Mg^{2+}, and more by the addition of puromycin to the high salt. It was concluded that ribosomes are bound to membranes by two types of interaction: electrostatic bonds, and a link between the nascent peptide chain and the membrane.

While a number of studies have been devoted to protein synthesis by isolated higher plant chloroplasts, either intact or freshly broken (3, 4, 9–11, 16, 18, 22, 27, 29, 30, 39), less attention has been paid to the activity of membrane-bound ribosomes by themselves (11, 16, 25, 38). In the present work we have studied protein synthesis by isolated, washed pea chloroplast thylakoid membranes when supplemented with an extract from *Escherichia coli* as a source of soluble factors. The system has been characterized with respect to Mg^{2+} optimum, sensitivity to inhibitors, and extent of reinitiation. Information has been obtained concerning the fate of the products formed.

MATERIALS AND METHODS

Preparation of Chloroplast Membranes. All operations were carried out at 4 C. Thirteen- to 18-day-old Progress No. 9 pea (*Pisum sativum*) leaves were ground briefly in 9 volumes of 200 mM Tris (pH 8.5), 200 mM sucrose, 30 mM Mg acetate, 60 mM KCl, 5 mM β-mercaptoethanol in a Waring Blendor. The brei was filtered through eight layers of cheesecloth and two layers of Miracloth and spun at 3,600*g* for 5 min over an 8-ml cushion consisting of 0.5 M sucrose, 200 mM Tris (pH 8.5), 30 mM Mg acetate, 60 mM KCl, and 5 mM β-mercaptoethanol. The pellet obtained was resuspended gently in a small volume of grinding buffer and the centrifugation step repeated. The resultant pellet was suspended in 10 ml of 40 mM Tris (pH 8.5), 30 mM Mg acetate, and 60 mM β-mercaptoethanol ("breaking buffer") (about 3 ml/10 g of original leaf wt), and centrifuged for 10 min at 12,000*g*. The pellet, which contains the thylakoid membranes, was washed two more times in breaking buffer, then an additional two times using 40 mM Tris (pH 8.6), 10 mM Mg acetate, 20 mM KCl, and 5 mM β-mercaptoethanol. Membranes prepared in this manner were used for *in vitro* protein synthesis, ribosome isolation, and RNA assay.

Detachment of Ribosomes from Thylakoids and Their Isolation. When membrane-bound ribosomes were to be detached and

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isolated, 0.1% DEPC[5] was added to the buffer used in homogenizing the leaves. Chloroplasts were sedimented as usual (above), then resuspended in fresh buffer with DEPC and recentrifuged. The "breaking buffer" did not include DEPC. While adding DEPC to the original homogenizing medium gave an increased yield of detached ribosomes, adding it to the medium in which chloroplasts were broken open resulted in a complete failure to detach the ribosomes.

Membranes, washed once in homogenizing buffer, three times in breaking buffer, and twice in the final hypotonic buffer, were dissolved by adding a solution of 200 mM Tris (pH 8.5), 30 mM Mg acetate, 60 mM KCl, 5% Triton, and 5 mM β-mercaptoethanol in a proportion of 10 ml/mg membrane Chl. The solubilized membranes were clarified by centrifugation at 12,000*g* for 20 min and the clear green supernatant was layered over a discontinuous sucrose gradient comprising 2 ml each of 1.5 M and 2 M sucrose in 40 mM Tris (pH 8.5), 10 mM Mg acetate, 20 mM KCl, and 5 mM β-mercaptoethanol in polycarbonate bottles. The samples were spun at 50,000 rpm for 3.5 hr in the 50.2 Ti rotor in a Beckman L5-50 ultracentrifuge. After the run, the supernatants were aspirated off, the sides of the tubes washed well, the pellets resuspended in a very small volume (50–100 μl) of resuspension buffer, and clarified by centrifugation at 9,750*g* for 10 min. The absorbancies at 260 and 280 nm were determined and the samples were stored as beads in liquid N_2.

RNA Determination. This was carried out as described previously (2), using samples of 100 to 200 μg of membrane Chl in the assay. Briefly, RNA was detached from the membrane by incubating in 1 M KCl, 40 mM Tricine at 30 C, then co-precipitated with $MgPO_4$ in ethanol. The precipitate was redissolved in 10 mM NaCl-Na acetate at pH 5.6, and incubated with pronase for 30 min at 37 C. The assay for RNA was the RNase-sensitive enhancement of fluorescence of ethidium bromide (15), measured in an Aminco photofluorimeter.

Preparation of *E. coli* Extract. Twenty-five g of frozen cell paste of *E. coli* strain K-12, ATCC 10798 were melted and washed in 20 mM HEPES (pH 7.6), 10 mM Mg acetate, 100 mM KCl, 2 mM $CaCl_2$, and 5 mM β-mercaptoethanol (grinding buffer). The cells were collected by centrifugation (9,500*g* for 10 min), frozen in liquid N_2, and ground with twice their weight of alumina, adding a small amount of alumina at a time in order to keep the homogenate as a thick paste. Grinding buffer was then added until a slurry formed and it was centrifuged at 15,000*g* for 15 min. The supernatant obtained was incubated with 3 μg/ml of DNase for 10 min at 4 C, and then centrifuged at 30,000*g* for 30 min. One ml of "energy cocktail" (see below) was added to each 10 ml of the supernatant ("S-30") from this centrifugation, the mixture was incubated for 60 min at 30 C, placed on a Sephadex G-25 column, and eluted with 20 mM HEPES (pH 7.6), 120 mM KCl, 5 mM Mg acetate, and 5 mM β-mercaptoethanol. This procedure leads to chain termination and runoff of bacterial ribosomes from their mRNA. Five fractions of 5 ml each following the void volume were collected and tested for activity in supporting protein synthesis by washed chloroplast membranes. Control experiments showed that the S-30 fraction by itself had negligible activity in protein synthesis; less than 5% of that observed after adding chloroplast membranes in the usual proportion. The extract was stored as frozen beads in liquid N_2.

***In Vitro* Protein Synthesis.** *In vitro* protein synthesis reaction mixtures (total volume = 240 μl) consisted of 2 mM HEPES (pH 7.6), 120 mM KCl, 10 mM Mg acetate, 0.1 mM each of 19 amino acids, and 5 μCi of [^{3}H]leucine (61 Ci/mmol), 80 μM GTP, 40 μl of S-30, 4.3 mM creatine phosphate, 0.4 mM ATP, and 210 μg/ml of creatine phosphokinase (these three comprising the energy cocktail), and either 0.4 A_{260} units of ribosomes or washed chloroplast membranes containing 100 μg of Chl. All additions except the S-30 were made at 0 C, and the reaction was started by addition of S-30 and transfer to a 30 C water bath. At the end of the reaction 10-μl aliquots were removed to Whatman 3MM filter paper discs and the hot trichloroacetic acid-insoluble radioactive material recovered, processed, and counted as described by Mans and Novelli (21). To measure radioactive product bound to the membranes, 1 ml of ribosomal resuspension buffer made 2% with respect to cold leucine was added to each reaction mixture, the membranes were collected by centrifugation at 9,750*g* for 10 min and resuspended in a small volume of resuspension buffer (200–300 μl). One hundred-μl aliquots of the resuspended membranes were absorbed by Whatman filter paper discs and processed as described above. Values for background counts were obtained from a reaction mixture made 0.1 M with respect to EDTA before addition of S-30. Counting efficiency was found to be 8% for this system.

Pronase, Puromycin, and Detergent Treatment of Reaction Mixtures. Membranes were collected from *in vitro* protein synthesis reaction mixtures as described above. The pellets were resuspended in 300 μl of 40 mM Tris (pH 8.5), and 20 mM KCl. To the suspensions were added sodium deoxycholate to 1%, Triton X-100 to 0.5%, and/or pronase at 100 μg/mg of Chl, or puromycin at 1 mM. Fifty-μl aliquots were removed from the mixtures at different times, and the hot trichloroacetic acid-insoluble material recovered and processed in the standard fashion.

Sucrose Density Gradients. One and five-tenths A_{260} units of ribosomal material were made 2% with respect to glutaraldehyde and layered on 10 to 34% sucrose density gradients which had been prepared by the method of Stone (36). Glutaraldehyde was used to preserve the ribosome structures during density gradient centrifugation (37). The volume of each gradient was 4.8 ml, and they were centrifuged at 50,000 rpm in the SW 50 rotor in a Beckman L5-50 ultracentrifuge. The tubes were punctured using an ISCO needle-holding assembly and displaced from the bottom upward with 60% glycerol using a Gilson "minipuls 2" pump. A_{254} was monitored using a 4-mm Internation Equipment Corp. flow cell in a Gilford recording spectrophotometer.

Source of Reagents. [^{3}H]Leucine was from Schwarz/Mann, Orangeburg, N.Y. Lot No. BR-2412, 61 Ci/mmol; pronase (protease type VI) and creatine phosphate from Sigma Chemical Co.; creatine phosphokinase from Calbiochem; and *E. coli* cells from Miles Laboratories. The sucrose used in all gradients and buffers was density gradient grade RNase-free sucrose from Schwarz/Mann.

RESULTS

Characteristics of Protein Synthesis by Washed Chloroplast Membranes. The optimum Mg^{2+} concentration for amino acid incorporation by washed membranes in this system was 10 mM (data not shown). This is very similar to optima for protein synthesis by whole chloroplasts reported earlier (9, 10). Incorporation rates on the basis of both Chl and membrane RNA are noted in Table I. These range up to about 0.15 pmol of leucine/μg of RNA · hr. Ribosomes detached from the membrane appear to be about 50% as active.

Washed chloroplast membranes incorporate amino acids into protein rapidly for the first 15 min, then more slowly for periods of up to 1 hr (Fig. 1). We have observed that the rate of incorporation continues to be linear for periods of up to 1 hr or more if the concentration of soluble extract is increased by a factor of 2 (Fig. 2). The lack of inhibition by aurintricarboxylic acid at 20 mM indicates that little or no reinitiation occurred, and extensive inhibition by 50 μg/ml of chloramphenciol and complete inhibition by 10 μg/ml of RNase (Fig. 1) are characteristics expected of protein synthesis by 70S ribosomes. The full susceptibility to RNase also indicates the absence of intact chloroplasts (22) or contaminating bacteria.

[5] Abbreviations: S-30: soluble factors for protein synthesis derived from *Escherichia coli* cells; DEPC: diethylpyrocarbonate.

Table I. Protein Synthesis by Washed Pea Chloroplast Membranes and by Ribosomes Detached from Them.

Procedures for preparing membranes and ribosomes and for amino acid incorporation are listed in Materials and Methods. All chloroplast membrane reaction mixtures contained 100 μg chlorophyll. The amount of RNA in the membranes and in detached ribosomes was determined by ethidium bromide fluorescence. The reaction rates in pmoles was calculated from the known specific activity of ^{3}H-leucine used, and known counting efficiency, assuming no contamination by unlabeled leucine in the washed membranes, ribosomes, or E. coli soluble fraction.

Expt.	Ribosome Source	^{3}H-leu Incorporation	RNA per Reaction μg	CPM/ μg RNA·hr	pmoles Leu/ μg RNA·hr
I	Thylakoid Membranes	29,288[a]	20	1,460	0.136
II	Thylakoid Membranes	90,575[a]	51	1,776	0.164
III	Detached Ribosomes	15,697[b]	20	784	0.073
IV	Detached Ribosomes	5,912[b]	12	493	0.046

[a] cpm/100 μg chlorophyll/hr

[b] cpm/reaction mix/hr

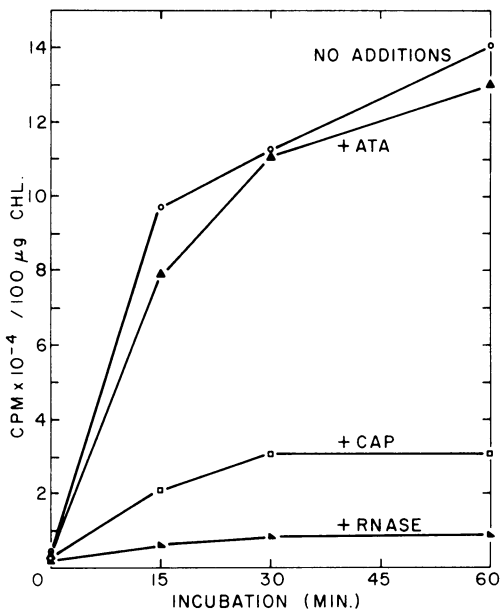

FIG. 1. Effects of chloramphenicol (CAP), aurintricarboxylic acid (ATA), and RNase on *in vitro* protein synthesis by washed thylakoid membranes.

Relation of Products to Thylakoid Membranes. A question of considerable interest is whether proteins synthesized by the thylakoid-bound ribosomes will remain with the membranes, as extrinsic or intrinsic membrane proteins. Simple washing of membranes after incorporation had occurred released no more than 30% of the trichloroacetic acid-insoluble counts (Fig. 2), whether the reaction was terminated at 20, 40, or 80 min. This could have been due to retention of nascent chain-containing ribosomes by the thylakoids, or to retention of newly formed proteins only.

A more critical criterion for the incorporation of newly formed proteins into the inner parts of membrane vesicles in that of protection from attack by proteolytic enzymes (33). When chloroplast membranes taken from a 1-hr incubation with radioactive amino acids were exposed to 100 μg of pronase/mg of Chl for 20 min at 4 C, 44% of the incorporated material was digested (Table II, line 2). Digestion of the products had come to an end point with this length of incubation, since time course studies showed almost no more digestion between 20 min and 4 hr (data not shown).

The incomplete nature of the digestion could have been due either to protection of the nascent peptides by the ribosomes, or to their incorporation into the deeper parts of the membranes. In order to test the extent of protection by ribosomes, these were dissociated and removed by incubating labeled membranes in high salt buffer (750 mM KCl) lacking Mg^{2+} prior to exposure to pronase (2, 23, 33). Removal of ribosomes in this fashion did not decrease the amount of bound product at all (Table II, line 3), nor was there any increase in susceptibility of labeled products to attack by pronase (line 4 compared to line 2). These results eliminate the possibility that retained products are present due to retention of the ribosomes.

If protection from pronase is due to incorporation of some of the peptide products into the membranes, this protection might disappear when the membranes are dissolved by detergents. Although 5% Triton X-100 dissolved thylakoid membranes to submicroscopic particles, no increase in pronase susceptibility resulted (data not shown), perhaps due to replacement of the membranes by detergent micelles as a protective coating. However, the combination of 1% deoxycholate and 0.5% Triton seemed to permit more complete attack by pronase (Table III). The combination of the pronase and detergents solubilized 70 to 75% of the newly formed polypeptides, compared to only 50% digested by pronase alone. In order to determine whether artificial discharge of nascent peptide chains by puromycin affects their susceptibility to proteolytic attack, labeled membranes were incubated first with puromycin and then with pronase. The degree of proteolysis of bound product was unchanged by puromycin treatment (compare line 4 with line 3 in Table IV).

Removal of Polysomes from Thylakoid Membranes. A number of procedures were investigated in attempts to remove the ribosomes and/or polysomes from these thylakoid membranes and display them on sucrose density gradients. The use of 1% deoxycholate as is common for ER-bound ribosomes was not possible, since especially at 4 C it precipitates with the 10 mM Mg^{2+} needed to preserve 70S ribosome structure (9). The best procedure identified so far is to pretreat the membranes with 100 μg of pronase/mg of Chl then solubilize the membranes with 5% Triton. The ribosomes are recovered by centrifuging the mixture layered over 1.5 and 2 M sucrose for 3.5 hr in the Beckman 50.2 Ti rotor at 50,000 rpm. Without pronase longer centrifuging times were needed to obtain an equivalent yield of ribosomes, and in addition the sucrose density profiles showed anomalous patterns (data not shown).

The profile of *A* at 254 nm obtained on a linear 10 to 34%

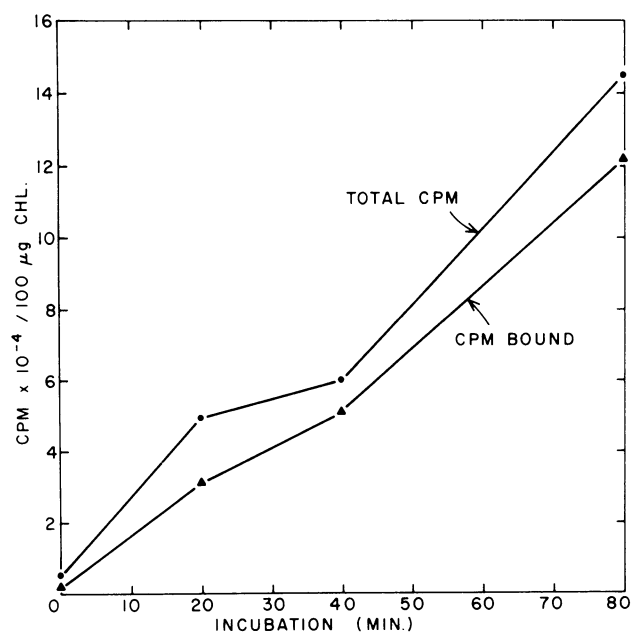

FIG. 2. Association of newly formed protein with thylakoid membranes during subsequent washings. For details see text.

Table II. Susceptibility of the Bound Products of In vitro Protein Synthesis to Attack by Pronase.

Following amino acid incorporation, labeled thylakoid membranes were centrifuged, then resuspended in the digestion mixture with or without pronase as indicated. Those membranes washed in 750 mM KCl - 40 mM Tris pH 8.5 to dissociate ribosomes were resuspended in the mixture briefly, then centrifuged a second time before resuspension in the digestion mixture. All membranes were incubated in the digestion mixture for 20 min before processing and counting. Details in Materials and Methods. Control membranes were processed for counting directly from the amino acid incorporation mixture without intervening wash steps. These had 19,600 cpm/100 μg chlorophyll. All values are the average of 4 determinations.

750 mM KCl Wash	Pronase	Acid-Insoluble Radioactivity	% of Control
-	-	18,200[a]	93
-	+	10,900	56
+	-	18,400	94
+	+	11,277	58

[a] cpm/100 μg chlorophyll

Table III. Detergent Potentiation of the Digestion of New Proteins by Pronase.

Labeled thylakoid membranes were centrifuged and washed in 750 mM KCl-40 mM Tris pH 8.5, and resuspended in the digestion mixture as described in Table II. Detergents used were 0.5% Triton and 1% sodium deoxycholate in 40 mM Tris pH 8.5, 20 mM KCl.

Digestion Mixture		Expt. I		Expt. II	
Detergents	Pronase	Residual Counts	% of Control	Residual Counts	% of Control
-	-	9,800[a]	100	54,500	83
-	+	4,700	47	33,700	53
+	-	9,000	91	46,800	73
+	+	2,700	28	16,600	26

[a] cpm/100 μg chlorophyll in hot acid-insoluble material

Table IV. Effect of Puromycin on the Release of Bound Products of In vitro Protein Synthesis and on their Susceptibility to Attack by Pronase.

Labeled thylakoid membranes were obtained as described in Table II. They were then incubated with or without puromycin (1 mM) as indicated for 15 min at 4 C (Incubation I), centrifuged again, and resuspended in the digestion mixture (Incubation II). In cases where samples were treated with both puromycin and pronase in this step, the former was added, samples were incubated at 4 C for 15 min, and pronase was then added. Digestion with and without pronase was 20 min in each case.

Incubation I	Incubation II		Residual	% of
Puromycin	Puromycin	Pronase	cpm	Control
-	-	-	28,700	99
+	-	-	31,200	107
-	-	+	15,400	53
-	+	+	16,400	63

sucrose density gradient of the ribosomes released by pronase and Triton is shown in Figure 3A (30-min centrifugation time). Peaks corresponding to 1, 2, 3, and 4-mers can be identified by their position relative to an 80S ribosomal standard which was run on a parallel gradient (Fig. 3B). Incubation of the ribosomes with 10 μg/ml of pancreatic ribonuclease at 30 C resulted in the breakdown of the polysome population to monosomes (Fig. 3C). Some ribosomal subunits are also apparent. (Centrifugation time for the RNase-treated ribosomes was 1 hr to allow ribosomal subunits to be displayed on the gradient.) The ribosomal profile shown in Figure 3A is predominantly polysomal.

When the yield of RNA in the ribosomal pellet was compared with the value obtained on the same membrane preparation for bound RNA, it was found that recovery was very low (170 μg of RNA/mg of Chl for total bound RNA *versus* 30 μg of RNA/mg of Chl recovered in the ribosomal pellet). When much longer (18–22 hr) centrifugation times were used, the amount of RNA recovered in ribosomal pellets approximated the values frequently

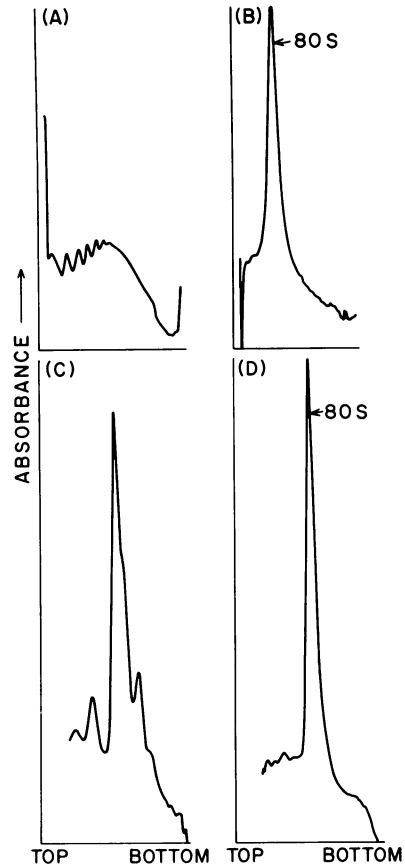

FIG. 3. Ribosome profiles obtained from sucrose density gradients. A: chloroplast membrane ribosomes (A_{260} = 1.5/gradient) were made 2% with respect to glutaraldehyde, layered onto 10 to 34% sucrose density gradients, and centrifuged for 35 min at 50,000 rpm in the SW 50 rotor. B: ribosomes (80S) isolated from wheat germ by the method of Marcus (1974) treated as in A. C: chloroplast membrane ribosomes as in A, but incubated with 10 μg/ml of RNase at 30 C before the addition of glutaraldehyde. D: 80S ribosomes from wheat germ, treated as in C. Centrifugation was for 1 hr in this case.

obtained for chloroplast membrane-bound RNA. Sucrose density gradient profiles of ribosomes obtained from these longer centrifugations show a preponderance of monomers (data not shown).

DISCUSSION

Washed chloroplast membranes from pea leaves were quite active in protein synthesis when supplemented with an extract from the RNase-deficient strain of *E. coli*. This supernatant is a convenient source of tRNA and enzymes specific for the prokaryotic type of ribosomes, is relatively low in RNase activity, and permitted rates of protein synthesis as rapid or greater than those found using either a whole pea chloroplast supernatant (16) or the more laboriously prepared soluble factors from entire pea leaves (38). The system exhibited the expected features of prokaryotic protein synthesis: a high Mg^{2+} optimum, and sensitivity to chloramphenicol. The sensitivity to RNase and digestion of products by pronase (especially after using detergents) indicated that bacterial contamination was at most a minor problem with the present system. The lack of inhibition by aurintricarboxylic acid indicated little or no reinitiation occurred, and even if competent runoff bacterial ribosomes were present they did not participate in the reactions observed.

Ribosomes detached from the membranes with Triton and then purified were only 50 to 75% as active, on an RNA basis, as the ribosomes still on the membranes. In fact the loss of activity may have been even larger. The isolated ribosomes were sedimented

through dense sucrose layers for 9.5 hr, which is not enough time to collect the entire population, and selects for the more active polysomes. Presumably monomeric ribosomes and subunits present in the original membrane-bound population contributed to the RNA content but not to protein synthesis.

Most of the products formed by washed thylakoid membranes remained membrane-associated after the reaction was terminated. This was true at various times during the reaction (Fig. 1) and even after the ribosomes were decomposed and released from the membranes by treatment with 750 mM KCl in the absence of Mg^{2+} (Table II). Therefore new proteins must be strongly associated with thylakoid membranes throughout the elongation process. Similar association of bound ribosome products with the membranes was noted earlier for *Chlamydomonas* (25, 28) and pea (16) chloroplasts.

While it is tempting to use the term "vectorial discharge" for the process leading to this association, the original meaning had to do with penetration by the growing peptide chain through the entire width of the ER membrane and entry into the lumen of the vesicle. Thus, newly formed products of the RER were largely inaccessible to attack by proteases (5, 6, 19, 33, 34) in the absence of detergents. In the present case the broad spectrum protease mixture, pronase, digested about 50% of the new proteins even when detergents were not used. These susceptible proteins cannot be in the intrathylakoid space, therefore, but are probably either extrinsic membrane proteins (35), or intrinsic proteins oriented so that some part is at the external face of the thylakoid membranes. A less likely possibility is that this class includes proteins adsorbed nonspecifically to the surface of the thylakoids.

Treating the membranes with a low level of Triton plus 1% deoxycholate resulted in proteolysis of an additional 25% of the newly formed proteins. The remaining resistant peptides at this point were probably very hydrophobic, intrinsic membrane proteins, not readily exposed to the aqeuous phase even by detergents which disrupted much of the membrane structure.

By contrast, vigorous pronase digestion of thylakoids from intact pea chloroplasts labeled *in vitro* (14) was reported to destroy virtually all of the incorporated label. In the experiments of Eaglesham and Ellis the concentration of pronase was 10-fold higher than that used here, and the temperature was 37 C instead of 4 C; these experimental differences may account for the differing results. Pronase was used to probe for exposed proteins only in the present work; in the earlier work it was used to indicate the protein nature of the products and some membrane disruption may have occurred to permit more complete exposure of intrinsic proteins.

It is interesting to note that Chen and Wildman (11) reported a residual 20% of the product of *in vitro* protein synthesis by isolated chloroplasts which was bound to the thylakoid membranes and not released by the action of 1% deoxycholate. This value is close to the residual 25% of the membrane products which we find resist protease attack in spite of the presence of deoxycholate. Chen and Wildman also concluded that some of the products of protein synthesis by isolated chloroplasts included a group of intrinsic membrane proteins.

As with ribosomes bound to thylakoid membranes of *Chlamydomonas* (in ref. 25 compare Fig. 2A with Fig. 5), pronase treatment is needed to extract polysomes from pea chloroplasts which show a clear profile on sucrose density gradients. Margulies and Michaels (24) suggested that in *Chlamydomonas,* proteolytic enzymes might digest hydrophobic portions of nascent chains which would otherwise bind together adjoining polyribosomes. In the case of pea thylakoids it seems more likely that pronase acts at the site of binding of the polyribosomal complex to the membrane, since pronase permitted a marked increase in the yield of detached ribosomes. It is relevant that mild treatment of rough microsomes with proteases released all bound ribosomes (8).

Our data do not provide a firm estimate of what per cent of bound ribosomes are present in polysomes. Some profiles (Fig. 3) show a considerable proportion present as polysomes. However, these ribosomes were the ones that had previously sedimented in 3.5 hr through two layers of dense sucrose, a procedure that is highly selective for polysomes (20). Longer centrifugation times in the preliminary purification brings down material in which polysomes are very scarce, perhaps due to traces of contaminating RNase but also perhaps reflecting to a greater extent the full nature of the original population (20). It is also relevant that at least 50% of the ribosomes are detached from these thylakoid membranes by high salt (+ Mg^{2+}), indicating that they were attached by electrostatic bonds only without participation of nascent chains (data not shown). Most of the newly formed proteins were not removed by even more severe high salt conditions, with Mg^{2+} missing (Fig. 2). Unlike ER-bound ribosomes whose major product may be a soluble protein destined for export from the cell (19, 34), the products of the thylakoid-bound polysomes are likely to include a variety of membrane proteins, some of them extrinsic or superficial and therefore susceptible to proteolysis, and others intrinsic and likely to be hydrophobic in nature.

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