TRANSPORT OF PROTEINS INTO MITOCHONDRIA AND CHLOROPLASTS

NAM-HAI CHUA and GREGORY W. SCHMIDT

From The Rockefeller University, New York 10021

I. INTRODUCTION

It has been well established that mitochondria and chloroplasts are not autonomous organelles. These organelles are capable of nucleic acid and protein synthesis, but many soluble and membrane proteins that become localized in them are initially synthesized on cytoplasmic ribosomes (cf. references 47 and 122). A fundamental question of considerable current interest is: How is the transport of such proteins through the delimiting membranes of the organelle envelope accomplished? A related concern is whether there are discriminatory mechanisms to ensure the specific incorporation of a product of cytoplasmic protein synthesis into its destined organellar location.

Although the problem of transport of proteins into mitochondria and chloroplast has been investigated for more than a decade, most of the early studies employed indirect approaches and yielded results that were equivocal. Only with the recent advances in techniques for mRNA purification (8, 88), in vitro protein synthesis (92, 110), and isolation of purified mitochondria (89, 121) and chloroplasts (98, 100) with intact outer membranes has it been possible to analyze in vitro transport of proteins into these organelles in a systematic and direct manner. Interest in this area has also been stimulated and encouraged by advances made in the understanding of how secretory proteins are transferred across microsomal membranes (cf. reference 22) and how certain plant and microbial toxins are transported into

cells (cf. references 104 and 108). This review will focus on recent papers dealing with the subcellular locations of the sites of synthesis of mitochondrial and chloroplast proteins and with the transport of these proteins into the respective organelles.

II. STRUCTURES OF MITOCHONDRIA AND CHLOROPLASTS

Chloroplasts and mitochondria are alike in that both are enclosed by two layers of delimiting membranes (Fig. 1). However, there is one striking difference between the two organelles: The inner mitochondrial membrane invaginates to form cristae; in chloroplasts, although the inner envelope membrane frequently invaginates during ontogeny, such images are rare in the fully developed organelle (cf. reference 55). Electron microscope (55) and biochemical (cf. reference 63) analyses have firmly established that there is no continuity between the inner envelope membrane and the thylakoid membranes. Thus, while there are only two compartments (intermembrane and matrix space) in mitochondria, there are in fact three compartments (intermembrane, stroma, and thylakoid space) in chloroplasts (Fig. 1).

Although the basic structural plan of mitochondria described above remains roughly the same among organisms from widely different taxonomic groups, the structures of chloroplasts vary considerably especially among the lower photosynthetic eukaryotic organisms. The structures presented schematically in Fig. 1 apply only to chloroplasts of higher plants and green algae (cf. reference 32). In Cryptomonads, Chrysomonads, and Haptophytes, the chloroplasts apparently are enclosed by the so-called chloroplast endoplasmic reticulum
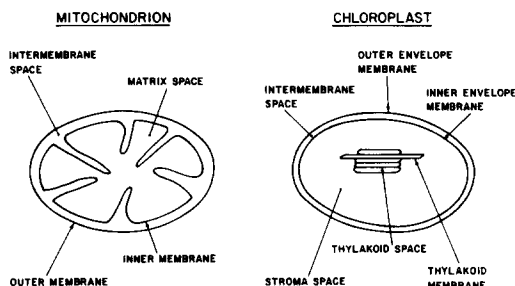
FIGURE 1   Schematic diagrams of a mitochondrion and a chloroplast.

(ER),[1] which is a cisterna of rough ER continuous with the nuclear envelope (cf. references 13, 32, and 44). In Euglenoids and Dinoflagellates, the chloroplast envelope is made up of three membranes, and it has been suggested that the additional membrane layer is derived from the chloroplast ER (cf. references 13, 32, and 44).

III. PERMEABILITY PROPERTIES OF ORGANELLES

The permeability properties of chloroplasts and mitochondria have been studied mainly with respect to low molecular weight substances. The permeability barrier to metabolites is located in the inner membrane of mitochondria (cf. reference 40) or the inner envelope membrane of chloroplasts (cf. reference 63). The outer membranes of both organelles, on the other hand, are freely permeable to charged and uncharged small molecules.

The permeability of the organelle outer membrane toward larger molecules has also been examined. Thus, the outer membranes of both animal (133) and plant mitochondria (91) are impermeable to cytochrome c (mol wt, 12,000) and, presumably, to proteins of higher molecular weights. In spinach chloroplasts, Heldt and Sauer (64) reported that the outer envelope membrane is impermeable to dextran. However, because the dextran fraction used was not specified, the molec-

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[1] *Abbreviations used in this paper:* C, cytoplasmic; CAT, carboxyatractyloside; CF 1, chloroplast coupling factor; CP II, chlorophyll-protein complex II; ER, endoplasmic reticulum; GDH, glutamate dehydrogenase; L, large subunit of RuPBCase; M, matrix; MDH, malate dehydrogenase; Met, methionine; pS, precursor to S; RSER, rapidly sedimenting endoplasmic reticulum; RuPBCase, ribulose-1,5-bisphosphate carboxylase; S, small subunit of RuPBCase.

ular weight cut-off of this membrane is still unknown.

IV. SUBORGANELLAR LOCALIZATIONS OF PROTEINS

Because mitochondria have two compartments and chloroplasts have three, organelle proteins that are synthesized in the cytosol have to tranverse one, two, or even three membrane barriers to reach their final destinations. Therefore, in considering the mechanisms of protein transport into an organelle, it is necessary to define the compartment in which the protein is localized. Furthermore, in the case of membrane proteins, the detailed mechanisms by which they reach their final destinations may depend on their topography in the plane of the membrane and on whether they are integral or peripheral components. For these reasons, we will briefly summarize the distribution of proteins in the different compartment of mitochondria and chloroplasts and the localizations of major membrane proteins. More detailed reviews of the suborganellar localization of proteins and membrane protein topography have appeared recently (2–4, 35, 58, 60, 131). Finally, we describe in greater detail the properties of mitochondrial and chloroplast outer membranes because of their presumptive roles in protein transport.

1. Mitochondria

A. OUTER MEMBRANE: The purified outer membrane, which accounts for ~4% of the total mitochondrial proteins, contains ~50% (wt/wt) lipids and 50% (wt/wt) proteins (cf. references 35 and 40). A number of enzymatic activities are associated with the outer membranes (cf. references 35 and 40). One of these enzymes, NADH-cytochrome b_5, is immunologically identical to that found in the ER (84). However, other enzymes, such as monoamine oxidase and kynurenine hydroxylase, are localized exclusively in the outer membranes (cf. references 35 and 40).

So far, outer membranes have been isolated from rat liver (94, 124), beef heart (62), *Neurospora crassa* (103), potato (91), and mung bean (91). The polypeptide patterns of these preparations are distinct from those of the inner membrane derived from the same source. One striking feature is the presence of a 30,000-dalton component in all outer membrane preparations. Unfortunately, the function and topological disposition

of this and other outer membrane polypeptides is unknown. Pulse-labeling experiments in the presence of specific inhibitors indicate that probably all of the outer membrane proteins are synthesized on cytoplasmic ribosomes (cf. reference 122).

B. INTERMEMBRANE SPACE: A list of enzymatic activities that have been localized to the space between the outer and the inner mitochondrial membranes can be found in references 2 and 40. The sites of synthesis of these enzymes have not been defined but, presumably, these enzymes are made on cytoplasmic ribosomes.

C. INNER MEMBRANES: The inner mitochondrial membrane accounts for about one-fifth of the total protein in the organelle (124). It is composed of 25% (wt/wt) lipids and 75% (wt/wt) proteins (60). Approx. 30-40% of the membrane proteins are peripheral because they can be extracted by procedures that do not disrupt the lipid bilayer (cf. reference 58).

In addition to respiratory electron transport and oxidative phosphorylation, many other enzymatic activities are localized to the inner mitochondrial membranes (2). Only 30-40% of the inner membrane proteins are involved in the respiratory chain and ATP-synthesizing system (35). Integral components of the inner membrane include the four multienzyme complexes of the respiratory chain: NADH-coenzyme Q reductase (complex I), succinate-coenzyme Q reductase (complex II), QH_2-cytochrome c reductase (complex III), and cytochrome c oxidase (complex IV) (cf. references 35 and 58). Cytochrome c oxidase is perhaps the best characterized complex with regard to orientation of individual subunits in the plane of the membrane. In beef heart mitochondria, the enzyme complex is a transmembrane protein consisting of six polypeptide subunits (41). Eytan et al. (41) have shown that subunits II, V, and VI are exposed to the cytoplasmic (C) side, subunit III is situated on the matrix (M) side, and the remaining two subunits, I and IV, are presumably located in the middle of the membrane because they are inaccessible to p-diazonium benzene sulfonate which does not penetrate the inner membrane.

The most abundant integral enzyme of the inner membrane, comprising 6% of the protein of beef heart mitochondria, is the carboxyatractyloside (CAT)-binding protein (mol wt, 29,000) which catalyzes ATP-ADP exchange across the membrane (83). Because of its ability to translocate adenine nucleotides, it must be a transmembrane protein exposed at both the C and M sides.

Among the peripheral membrane proteins, cytochrome c and F_1-ATPase have been investigated extensively with respect to their structures and locations in the inner membrane. Cytochrome c is a basic protein of mol wt ~12,000 and is located on the C side (35, 60). The F_1-ATPase, on the other hand, consists of five nonidentical subunits which are attached as a complex to the M side of the inner membrane (cf. reference 35).

A great majority of the inner mitochondrial membrane polypeptides are synthesized outside the organelle (cf. reference 122). This includes integral membrane proteins of all possible orientations, e.g., the CAT-binding protein and subunits IV-VII of yeast cytochrome c oxidase, and also peripheral membrane proteins which are located on either the C side (e.g. cytochrome c) or the M side (e.g. F_1-ATPase subunits).

D. MATRIX SPACE: Matrix proteins make up ~60-70% of the total mitochondrial protein (124). A complete list of the enzymes located in this mitochondrial compartment has been reported by Altman and Katz (2). In rat liver mitochondria, the urea cycle enzyme, carbamyl-phosphate synthetase I (mol wt, 165,000), accounts for ~15-20% of all mitochondrial protein (30). Most of the matrix enzymes (122) and ribosomal protein (54, 85) are synthesized in the cytosol.

2. Chloroplasts

A. OUTER AND INNER ENVELOPE MEMBRANE: The chloroplast envelope is composed of two distinct membranes separated by an intermembrane space. Centrifugation of osmotically shocked chloroplasts on discontinuous sucrose gradients yields an envelope fraction substantially free of contamination by other chloroplast components (38, 112, 113), but it does not allow the separation of the inner and outer envelope membranes. Thus, all the biochemical experiments reported so far have been performed with the mixture.

Early permeability studies with intact chloroplasts identified the inner envelope membrane as the osmotic barrier (64). Transport of ions and metabolites through this membrane is mediated by several distinguishable translocators, but none has yet been isolated and purified (cf. reference 63). However, by analogy with other transport systems, such as the CAT-binding protein (83), these translocators are presumably integral proteins of the inner envelope membrane. The enve-

lopes are devoid of cytochrome b_5 and NADPH-cytochrome c oxidoreductase activities (38) but contain acyl-CoA synthetase, bound acylase, phosphatidic acid phosphatase, and galactosyl transferase (38, 70). These enzymes probably function at the inner membrane. No enzymatic activity has yet been attributed to the outer envelope membrane, and it is not known whether any enzymes reside in the intermembrane space.

SDS-polyacrylamide gel electrophoresis of the spinach envelope fraction resolves >30 polypeptide bands, 7 of which are predominant (95). In contrast to the polypeptides of the thylakoid membranes and stromal fraction, most of the envelope polypeptides are distributed in the high molecular weight region (30,000-100,000). The function of none of these polypeptides has been identified. Because only three envelope polypeptides are synthesized by intact chloroplasts (69, 99), the remainder are probably made in the cytosol.

B. THYLAKOID MEMBRANES: The thylakoid membrane is the site of photosynthetic electron transport and phosphorylation. The membrane is composed of 50% (wt/wt) lipids and 50% (wt/wt) proteins. The protein complement is a heterogeneous mixture of polypeptides which can be resolved by SDS-gradient gel electrophoresis into >30 bands (3, 4, 24, 27). The molecular organization of the thylakoid membrane and the structure and function of some of the membrane polypeptides have been summarized by Trebst (131) and Anderson (3, 4).

As with the outer chloroplast membrane components, the biogenesis of the thylakoid membrane requires both cytoplasmic and chloroplast protein syntheses (cf. references 19, 39, 47, and 123). In *Chlamydomonas reinhardtii*, in vivo pulse-labeling studies have shown that 24 out of a total of 33 major thylakoid membrane polypeptides resolved by SDS-gradient gel electrophoresis are made outside the organelle (25). These polypeptides include both integral and peripheral components of the membrane (Matlin and Chua, unpublished material) and, together, they constitute 70% of the total membrane protein mass (25). Only a few of the cytoplasmically synthesized membrane polypeptides have been assigned functions. Prominent among these is the apoprotein of chlorophyll-protein complex II (CP II) which functions in harvesting light energy (cf. references 3 and 4). The protein moiety of CP II

accounts for ~25-30% of the total thylakoid membrane proteins, and it consists of at least two integral polypeptides (90) of similar molecular weights (cf. reference 4). Several peripheral membrane proteins including ferredoxin, ferredoxin-NADP reductase (cf. reference 19), plastocyanin (53, 61), and probably two subunits of the chloroplast coupling factor (CF 1) (96, 39) are products of cytoplasmic protein synthesis. There is solid evidence that ferredoxin-NADP reductase (cf. references 17 and 131), CF 1 (cf. reference 131), and plastocyanin (17) are all located on the outer surface (stroma side) of the thylakoids. Whether there are any cytoplasmically synthesized peripheral proteins which finally become localized on the inner surface (thylakoid space) of the thylakoids is not known.

The localization of CP II has been investigated in at least two different plant systems. The *Acetabularia* complex contains two polypeptide subunits of 21,500 and 23,000 daltons, but only the larger one is accessible to enzymatic iodination and pronase digestion (6). In contrast, both polypeptide components of the broad bean CP II are reduced by 2,000 daltons after trypsinization of membrane vesicles (129). Because the apoprotein of CP II is integral to the membrane, both polypeptide subunits must be embedded in the lipid bilayer and, from their limited susceptibility to protease, exposed to the outer surface of the thylakoids. Recent techniques for preparing inverted thylakoid membrane vesicles (5) should facilitate the determination of the orientations of both polypeptides.

C. STROMAL SPACE: Approx. 50% of the total chloroplast proteins are soluble and compartmentalized in the stromal space. These proteins include enzymes of the photosynthetic CO_2 fixation pathway as well as those involved in the synthesis of amino acids, fatty acids, and photosynthetic pigments (cf. references 48 and 86). Ribulose-1,5-bisphosphate carboxylase (RuBPCase) is one of the few stromal proteins which have been isolated and purified to homogeneity. This enzyme, which accounts for ~70% of the total stromal protein, is made up of eight copies each of large (mol wt, 55,000) and small (mol wt, 12,000) subunits (77). The large subunit is synthesized inside the chloroplast whereas the small subunit is made on cytoplasmic ribosomes (cf. references 39 and 47). In addition to the small subunit of RuPBCase, most of the other stromal

proteins, as well as chloroplast ribosomal proteins, are also synthesized on cytoplasmic ribosomes (cf. references 19, 26, 47, and 123).

V. HYPOTHETICAL MODES OF PROTEIN TRANSPORT ACROSS ORGANELLE ENVELOPE

1. Transfer of Proteins Across Membranes in Other Biological Systems

In formulating hypothetical models for the transport of proteins into mitochondria and chloroplasts, it is instructive to examine mechanisms of protein transport across membranes in other biological systems. The passage of nucleic acid-protein complexes across biological membranes will not be considered here because there is no evidence that cytosolic proteins are imported into organelles in this form. On the basis of the available data, there are two general cases in which proteins have been clearly shown to traverse delimiting membranes. The first is the segregation of secretory proteins into the cisternae of the rough ER (cf. reference 107), and the second is the entry of certain proteinaceous plant and microbial toxins into cells (cf. reference 108). The possible mechanisms of protein transport in these two cases are briefly reviewed here.

A. SEGREGATION OF SECRETORY PROTEINS INTO ER CISTERNAE ("THE SIGNAL HYPOTHESIS"): The synthesis and segregation of secretory proteins is a classic example of selective protein transport into a membrane-bounded compartment. The mechanism of transport of secretory proteins across the ER membrane has been outlined by the Signal Hypothesis, the details of which can be found elsewhere (14–16, 22, 97). Briefly, the hypothesis proposes that all secretory proteins are initially synthesized as precursors which contain, at their N-termini, a short chain extension (16–30 amino acid residues) designated as the signal peptide. The signal peptide is envisioned to have a very special function. Upon its emergence from ribosomes it binds specifically to receptors on the ER membrane leading to the attachment of the translating polysomes to the latter. The resulting ribosome-membrane junction allows the exclusive synthesis of secretory proteins on rough ER and the co-translational transport of these proteins into the ER cisternae. The signal peptide is metabolically short-lived and is removed by an ER-bound protease even while translation of the message is in progress.

It is important to emphasize that, according to the Signal Hypothesis, secretory proteins are transported into the ER cisternae only during but not after translation (14, 15). Thus, translation of mRNA for a number of secretory proteins in a cell-free system results in the synthesis of precursor molecules ~2,000 daltons larger than the corresponding mature secretory proteins (14, 15, 22). In contrast, the mature secretory proteins are recovered when stripped microsomes are included in the in vitro system during translation. In the latter experiments, the secretory proteins are resistant to proteolysis, indicating that they have been sequestered into the microsomal lumen. Presecretory proteins synthesized in vitro, on the other hand, are not segregated upon subsequent incubation with the same preparation of microsomal vesicles. These results demonstrate conclusively that the transport of secretory proteins into microsomes is obligatorily coupled to translation and cannot occur after completed synthesis of the precursor. There is morphological evidence that certain plant storage proteins are synthesized by ribosomes bound to the membranes of protein bodies (20). The segregation of these proteins is probably mechanistically like that for secretory proteins.

Because of the postulated function of the signal peptide, knowledge of its amino acid sequence may provide clues as to how it fulfills its role. The partial and complete signal sequences of a large number of presecretory proteins are available and they are all characterized by a preponderance of hydrophobic amino acid residues (cf. reference 22). These results suggest that hydrophobicity may play an important role in the interaction between the signal peptide and the ER membrane.

B. ENTRY OF PLANT AND MICROBIAL TOXINS INTO CELLS: Certain proteinaceous toxins of plant and microbial origin are known to traverse plasma membranes of sensitive eukaryotic (31, 104, 108) and prokaryotic cells (104, 105, 130) before exerting their lethal effects. One of the most studied toxins is the diphtheria toxin which is produced by a lysogenized strain of Corynebacterium diphtheriae carrying the coryne-phage β (31, 108, 109). The toxin inhibits protein synthesis in animal cells by catalyzing the transfer of ADP-ribose from NAD to the elongation factor, EF-2. It is known that the toxin structural gene is encoded by the phage genome (cf. references 31 and 109). The toxin is synthesized as a

single polypeptide chain of mol wt 63,000 containing two disulfide bridges and can be cleaved by mild proteolytic treatment to yield a "nicked" product containing two chains, A and B, derived from the N- and C-terminal portions, respectively, of the parent molecule. After limited proteolysis, the A and B chains are still held together by a single disulfide bridge. Although the isolated A chain possesses the toxic ADP-ribosylating activity, it is not toxic when administered to animals or incubated with HeLa cells. The B chain is required for binding of the entire toxin molecule to specific receptors on plasma membranes. A nonsense phage mutation has been characterized which causes the synthesis of a nontoxic, abbreviated protein (mol wt, 45,000) lacking a 17,000-dalton segment at the C-terminus. Because the mutant protein (CRM 45) is unable to bind to toxin-specific receptors, it is likely that the C-terminal region of the B chain is important for this function.

In an attempt to elucidate the mechanism of transfer of diphtheria toxin across plasma membrane, Boquet et al. (18) compared the detergent-binding properties of A and B chains and of native toxins, either intact or "nicked." It was found that the B chain contains a binding site for Triton X-100 but that this hydrophobic domain is not exposed in the native toxin unless the latter is first denatured with 0.1% SDS. In contrast, the undenatured CRM 45 mutant protein and its truncated B fragment are able to insert into detergent micelles, indicating that the hydrophobic domain in the mutant protein is exposed. Thus, the B chain of diphtheria toxin appears to consist of two domains: an N-terminal region which is hydrophobic and a C-terminal hydrophilic region which is involved in receptor binding. Boquet et al. (18) have proposed the following hypothetical sequence of events surrounding the attachment of diphtheria toxin to plasma membrane and the subsequent entry of the A chain into the cytoplasm. The intact toxin is first bound to specific receptors on the plasma membrane via the C-terminal region of the B chain. This binding induces a conformational change such that the hydrophobic domain of the B chain becomes inserted into the plasma membrane. Alternatively, it is conceivable that, before insertion, a limited proteolytic step is needed to cleave off the hydrophilic C-terminal segment, thereby generating a molecule similar to the CRM 45 mutant protein. In either case, Boquet et al. (18) postu-

lated that the hydrophobic domain of the B chain may form a channel in the plane of the membrane either by itself or in association with putative receptor proteins in the plasma membrane. The A portion of the toxin is drawn into the channel as the latter is being formed. The toxin is cleaved into A and B chains when the junction between the two chains reaches the cytoplasmic face of the plasma membrane. Reduction of the disulfide bridges by intracellular glutathione allows the A chain to enter the cytoplasm while the B chain is left in the plasma membrane.

Other toxins known to penetrate the plasma membranes of animal cells possess structural features very similar to those of diphtheria toxin in that these proteins also contain domains required for membrane receptor interaction and transport. Thus, two plant toxins, abrin and ricin, which inhibit protein synthesis in animal cells are also made of two nonidentical subunits, A and B, held together by a single disulfide bond (104, 108). The B chain binds to a glycoprotein receptor in the membrane thereby facilitating the transfer of the A chain into the cytoplasm where it inactivates 60S ribosomal subunits (106). Other examples are cholera toxin and Pseudomonas toxin, both of which traverse animal cell membranes in much the same way as diphtheria toxin. For instance, the A and B subunits of cholera toxin are not linked by disulfide bonds but, instead, are held together by noncovalent forces (46). The B subunits bind specifically to a membrane receptor which has been identified as ganglioside G_{M1} (120). In the case of Pseudomonas aeruginosa, the toxin has a mol wt of 66,000 and, under appropriate conditions, it also catalyzes ADP-ribosylation of the elongation factor (31, 68). This toxin could be activated in vitro simply by denaturation of the protein and simultaneous reduction of its disulfide bonds; no proteolytic cleavage seems to be required (87). However, whether limited proteolysis is an obligatory step preceding entry of this toxin into sensitive cells has not yet been established.

The passage of proteinaceous toxins across the plasma membrane is not a phenomenon restricted to eukaryotes. Many proteinaceous bacteriocins are effective only when they reach the cytoplasm of sensitive bacterial strains (cf. references 104 and 105). One of the best characterized bacteriocins is colicin E3 which is a 62,000-dalton polypeptide containing a specific RNase activity (105). The toxin cleaves the 16S RNA of the 30S

ribosomal subunit, thereby inactivating protein synthesis. Colicin E3 is secreted as a complex with a 9,500-dalton peptide by strains of *Escherichia coli* which carry the colicinogenic factor. The small peptide inhibits the enzymatic activity of colicin E3 and thereby confers immunity within the colicin-producing strains. Sensitive bacteria are as susceptible to purified colicin E3 as to the colicin E3-immunity protein complex; therefore, it follows that the immunity protein is not required for uptake of colicin E3 into the cells in its active form. Unlike diphtheria toxin, the colicin E3 protein is transported into the cytoplasm without any proteolytic cleavage.

It should be emphasized that the transfer of proteinaceous toxins across plasma membrane differs significantly from the transfer of secretory proteins across ER membrane in one important respect. In the former, the entry of toxins into sensitive cells does not depend on concomitant translation of the toxin molecules. Hence, the transport process is a post-translational event involving proteins whose synthesis has already been completed.

There is strong evidence that the entry into cells of diphtheria toxin as well as other plant and microbial toxins mentioned above (cf. references 31, 104, 105, and 108) does not occur by pinocytosis of the plasma membrane. In contrast, proteins such as transferrin, asialoglycoproteins, serum lipoproteins, and lysosomal hydrolases are internalized by cells through pinocytosis (cf. reference 104). Because there is no morphological evidence that proteins are imported into mitochondria and chloroplasts by pinocytosis of the organellar envelopes, the transport mechanisms of these proteins will not be elaborated here. Detailed discussions concerning the uptake of these proteins by cells can be found in reference 104.

2. Transport of Proteins into Organelles

The subcellular localization of cytoplasmic ribosomes that are engaged in the synthesis of organellar proteins determines the mechanism by which these proteins are transported into the organelle. The cytoplasmic ribosomes could be bound to the ER membranes, bound to the outer membranes of organelles, or free in the cytosol. These three possible modes of synthesis of organelle proteins are further elaborated in the following discussion

A. SYNTHESIS ON ROUGH ER: By analogy with secretory proteins, synthesis of organellar proteins by ribosomes bound to the ER would also represent obligatory translocation of the polypeptide chain across a membrane during translation (cf. reference 128). This mechanism requires the ER to be a vector for integration of proteins into organelles. Occasional observations of apparent continuity between the ER membranes and the outer membrane of mitochondria (43) or outer envelope membrane of chloroplasts (33) might implicate the ER as a passive corridor which restricts diffusion of newly synthesized proteins destined for organelles. Alternatively, the ER may be regarded as an active vector if the organellar proteins are packaged in vesicles which fuse specifically with outer membranes and then release their contents into the organelles.

B. SYNTHESIS ON RIBOSOMES BOUND TO ORGANELLE OUTER MEMBRANES: Synthesis of proteins on ribosomes bound to organelle outer membrane would ensure direct insertion of newly synthesized proteins into the organelle without intervening transport steps (21, 78–81).

C. SYNTHESIS ON FREE RIBOSOMES: Proteins destined for transport into organelles could be synthesized in the cytosol on free ribosomes (23, 36, 118). After synthesis, the proteins would then recognize specific receptors on organellar outer membranes. Interaction with the latter would somehow facilitate their transport into the organelle.

Modes A and B are homologous mechanisms of protein transport across the membrane because both require the synthesis of organellar proteins on membrane-bound ribosomes. The two modes differ only in how the proteins are handled after membrane traversal. Similar to secretory proteins (cf. reference 22), the synthesis and transport of organellar proteins in modes A and B are tightly coupled; the transfer of proteins across membranes is strictly dependent on concomitant translation. As predicted by the Signal Hypothesis (14, 16), organellar proteins would be synthesized as precursors containing, at their N-termini, a transient signal sequence specific for either the ER membrane or the outer membrane of the organelle. It is obvious that to ensure specificity with respect to the target organelle, the signal sequences of these proteins would have to be different from those of secretory proteins.

If organellar proteins are synthesized on free instead on membrane-bound ribosomes, as in model C, the synthesis and transport of these proteins are not coupled; rather, the two processes

are separated not only in their subcellular locations but also in time. In this case, the transfer of proteins into mitochondria and chloroplasts is a post-translational event, much like the transfer of toxins across plasma membranes (cf. section V 1 B). To ensure specific transport into the target organelle, the proteins may be synthesized as larger precursors containing an additional sequence. The extra peptide, which may be located at either the N- or the C-terminus, is distinct from the signal peptide in that it does not trigger the binding of translating polysomes to the organellar envelope (36). Rather, it is functionally equivalent to the toxin B subunit (cf. references 104 and 108) in that it binds to specific receptors on the outer membrane of the organelle and somehow facilitates the post-translational transport of the precursor into the organelle. In the case of an N-terminal extension, the binding properties of this extra peptide may not be expressed until the entire precursor molecule has been synthesized and has attained a required conformation. Otherwise, the N-terminal chain extension on nascent polypeptides would interact directly with outer membrane receptors during translation. Alternatively, post-translational modifications (e.g., methylation, phosphorylation, limited proteolysis) of the extra sequence may be required to generate a conformation permitting binding. Experimental evidence pertaining to these models is discussed in the next section.

VI. CYTOPLASMIC SITES OF SYNTHESIS OF ORGANELLAR PROTEINS AND THEIR TRANSPORT INTO ORGANELLES

1. Synthesis on Rough ER

The realization that mitochondria are not autonomous organelles led to attempts to identify the sites of synthesis of mitochondrial proteins in other cell fractions. One approach, introduced by Beattie et al. (10), was to pulse-label tissues, isolate subcellular fractions, purify mitochondrial proteins from each fraction, and determine the time-course of appearance of radioactive mitochondrial proteins. Rat liver cytochrome c is one of the mitochondrial proteins whose sites of synthesis have been investigated extensively in this manner. Earlier work, which has been reviewed by Schatz and Mason (122), indicated that when either radioactive amino acids (50, 73, 74) or δ-aminolevulinic acid (34, 111) were used as precur-

sors for in vivo labeling, cytochrome c with high specific radioactivity seemed to be concentrated in association with the ER.

In contrast, the recent studies of Robbi et al. (116, 117) show that rat liver cytochrome c is not made on rough ER. These authors conducted an exceedingly careful in vivo pulse-labeling study on cytochrome c synthesis in rat liver (117). They first quantitated cross-contamination of the various subcellular fractions by marker enzyme analysis and estimated the extent of cytochrome c redistribution during the fractionation procedure by adding labeled cytochrome c to the liver homogenate. From such studies, Robbi et al. (117) concluded that much of the "microsomal" cytochrome c results from the in vitro release of the protein from mitochondria and its subsequent avid association with ribosomes and/or microsomes.

Robbi et al. (116) next examined cytochrome c synthesis during pulse-labeling in vivo and quantitated the distribution of newly synthesized cytochrome c in various cell fractions by a sensitive radioimmunoassay. At early time points, the specific radioactivity of cytochrome c is higher in the microsomes compared to mitochondria; however, the bulk of the newly made cytochrome c is localized in the latter. Thus, Robbi et al. (116) concluded that cytochrome c is not made on rough ER and that the apparent high specific activity of "cytochrome c" which they and previous workers have recovered in microsomes is, in fact, a highly labeled contaminant which co-purified with cytochrome c. Robbi et al. (116) proposed that cytochrome c is synthesized either by 80S ribosomes bound to the mitochondria (21, 78-81) or at an extra-mitochondrial site (free ribosomes?) which has not yet been identified. In either case, the newly synthesized cytochrome c must be transferred rapidly into the mitochondria to account for the kinetic observations.

Another approach toward identification of the site of synthesis of organellar proteins is to prepare, from fractionated tissues, polyribosomes which are then employed to complete synthesis of their nascent polypeptide chains in vitro. This approach is valuable to the extent that pure polysome preparations can be obtained and the resulting translation products can be characterized biochemically. It has been reported that complete formation of cytochrome c can be achieved with in vitro protein-synthesizing systems comprised of rat liver microsomes and supernate factors (51, 52, 72), and that microsomes can transfer proteins

directly to mitochondria in mixtures of the two preparations in vitro (71). Gonzalez-Cadavid and Cordova (51) recently found that cytochrome c is a translation product of both free and membrane-bound polyribosomes in vitro. Yet, because there are more than twice as many membrane-bound as free polysomes in liver cells, they reasoned that cytochrome c is mainly synthesized by ribosomes bound to the ER. This conclusion is at variance with that of Robbi et al. (116, 117); however, a more quantitative analysis of the protein-synthesizing capacity of polysomes from each cell fraction may help to resolve the discrepancy.

Both in vivo and in vitro methods have been used to provide evidence that soluble enzymes of the mitochondrial matrix are products of protein synthesis by ribosomes bound to the ER of rat liver (12, 49, 75). Bingham and Campbell (12) used rat liver microsomes to direct protein synthesis in vitro. After 30 min, the incubation mixture was treated with nonionic detergent, and malate dehydrogenase (MDH) was purified from the resulting supernate by ion exchange chromatography in the presence of carrier MDH. Radioactivity was incorporated into protein corresponding to peaks of MDH activity, but because antibody was not used to verify to purity of newly synthesized MDH in these studies, the possibility that the product labeled in vitro is a persistent contaminant of MDH cannot be ruled out. Whether free polyribosomes synthesize MDH was not determined in this study.

Godinot and Lardy (49) examined the in vivo synthesis of rat liver glutamate dehydrogenase (GDH) during pulse-labeling. A well-characterized antibody was employed to precipitate the enzyme from microsomal and mitochondrial cell fractions. The immunoprecipitate was then subjected to SDS-polyacrylamide gel electrophoresis which was found to be essential for removal of significant amounts of radioactivity not associated with GDH. Moreover, Godinot and Lardy added radioactive GDH to rat liver cell homogenates and followed its fate during subsequent cell fractionation. The exogenous protein was used to indicate how GDH is redistributed when inadvertently released from broken mitochondria. Finally, the cross-contamination of microsomes and mitochondria was estimated from the activities of marker microsomal and mitochondrial enzymes in each cell subfraction. Thus, they could demonstrate with confidence that GDH in association with microsomes possesses 3–4 times greater spe-

cific radioactivity than the mitochondrial enzyme as a result of an in vivo pulse-label. Kawajiri et al. (75) have also found that the GDH associated with microsomes possesses high specific radioactivity relative to that localized in mitochondria at the early time-points after an in vivo pulse-label. Both groups of workers have concluded from these studies that GDH is synthesized by ER-bound ribosomes.

It is important to bear in mind that in rat liver there is a large unlabeled pool of GDH in mitochondria relative to that associated with microsomes (75). Therefore, comparing the changes of specific radioactivity of GDH in mitochondria and microsomes with time following a pulse-label may not accurately reflect its biosynthetic origin. As Robbi et al. (116, 117) point out, it is equally important to determine the rate of appearance of absolute amounts of newly synthesized protein among the cell fractions during in vivo pulse-labeling experiments. The conclusion of Godinot and Lardy (49) and Kawajiri et al. (75) that microsomal GDH is the primary precursor for mitochondrial GDH would be strengthened considerably if they had demonstrated that the bulk of the newly synthesized enzyme is associated with microsomes at the early time points.

Kawajiri et al. (75) also employed a more direct method to ascertain the cytosolic site of synthesis of GDH in rat liver. They showed that the nascent polypeptide chains released from rough microsomes by EDTA and nonionic detergent could be immunoprecipitated by antibodies against GDH. Free polysomes, on the other hand, contained no GDH antigenic determinants. These results, which provide compelling evidence that GDH is a product of protein synthesis by ER-bound ribosomes, were supported by the observation that ^{125}I-labeled Fab fragments prepared from anti-GDH IgG bind preferentially to detergent-solubilized polysomes from microsomal pellets. However, Kawajiri et al. (75) found that the GDH-synthesizing polysomes discharge their nascent polypeptide chains to the cytoplasmic surface of the microsomes. This extra-mitochondrial pool of GDH was shown to be localized on the cytoplasmic side of the ER by three criteria: (a) susceptibility of microsomal GDH to exogenous proteases, (b) its accessibility to antibody, and (c) its release from microsomes by washing with moderately high ionic strength buffers. When Kawajiri et al. (75) subfractionated microsomes, they found that most of the GDH sediments in low

density regions of sucrose gradients. The GDH-enriched fraction also contained substantial amounts of MDH and marker enzymes for microsomal membranes. Because the GDH- and MDH-rich membranes are considerably less dense than the bulk of smooth microsomes, Kawajiri et al. (75) suggested that, after synthesis on rough ER, both GDH and MDH are concentrated in association with specialized smooth ER vesicles which they called "microparticles." They proposed that these microparticles somehow function as vehicles for transport of proteins into mitochondria. Purified microsomal GDH and MDH were found to possess an affinity for isolated "microparticles," but such adsorption of the enzymes purified from mitochondria could not be detected (76). This was explained to be a consequence of post-translational modifications which are exemplified by pI differences of the cytoplasmic and mitochondrial forms of GDH. It was suggested that such modifications were involved in the transport of GDH from one cell fraction to another.

There are at least two conceptual difficulties which render the mechanism of transport of GDH and MDH into mitochondria as proposed by Kawajiri et al. (75) hard to visualize. First, how might "microparticles" facilitate transport of proteins into mitochondria? Such vesicles could fuse with mitochondrial outer membranes, and this should result in the release of their contents into the intermembrane space; however, proteins adsorbed onto the cytoplasmic face of the vesicle would remain outside the mitochondria. Second, it has been previously shown that probably all ER-bound polysomes in rat liver discharge their nascent polypeptide chains vectorially into the ER lumen (cf. reference 119). Thus, the "membrane-bound" polysomes which apparently synthesize GDH are of an unusual sort. The ribosome-membrane junction in rough ER is stabilized by the nascent chains and the bound ribosomes are not released by high salt buffers (cf. reference 119). Dodd (37) reported that GDH is capable of binding to the negatively charged polar head groups of phospholipids. Such an interaction should be destabilized at high ionic strength, and, in fact, Kawajiri et al. (76) found that microsomes could be freed of GDH and MDH with 0.2 M salt. In view of these findings (37, 76), it would be important to determine whether polysomes with nascent GDH might also be released from microsomes under these conditions. If this were the case, it might be supposed that the GDH-synthesizing polysomes interact nonspecifically with microsomal membranes. Yet, it would be difficult to explain how an artifactual adsorption of free polysomes could be so specific and complete that it resulted in the lack of any nascent polypeptides in the free polysome fraction reactive with anti-GDH IgG. For these reasons, the nature of the interaction of ER membranes and GDH-synthesizing polysomes should be studied further.

Although no compelling evidence to support the concept of synthesis of organellar proteins on rough ER has yet appeared, electron micrographs occasionally reveal an apparent, but not proven, continuity of the ER with the outer envelope membrane of chloroplasts (33) and outer mitochondrial membrane (43, 101). A candidate for association between the ER and mitochondria is the fraction of rat liver microsomes which has been observed to co-sediment with mitochondria during low-speed centrifugation (126). The "rapidly sedimenting endoplasmic reticulum" (RSER) is separated from the mitochondria only after rigorous homogenization in the presence of 0.5 M KCl and 20 mM EDTA. Shore and Tata (126) have examined the in vivo rate at which proteins synthesized by cytoplasmic ribosomes are incorporated into two classes of rat liver mitochondria: mitochondria associated with RSER and mitochondria which sediment at relatively higher centrifugal forces. These studies indicated that the RSER-associated mitochondria incorporate newly synthesized proteins at half the efficiency of the "free" mitochondria. Accordingly, Shore and Tata (126) concluded that the association of RSER and mitochondria does not facilitate protein transport.

Shore and Tata (127) have also attempted to discern whether the RSER is specialized in regard to synthesis, as opposed to transport, of mitochondrial proteins. Polyadenylated RNA was purified from various rat liver cell fractions and used to direct protein synthesis in cell-free translation systems. Antibodies against mitoplasts (mitochondria devoid of outer membranes and soluble proteins in the intermembrane space) were employed to precipitate the products synthesized in vitro. Shore and Tata (127) concluded that rough microsomes, and not RSER or free ribosomes, are primarily responsible for cytoplasmic synthesis of mitochondrial proteins. Unfortunately, the immunoprecipitate obtained with the polyspecific antibodies was contaminated by translation products which were not mitochondrial proteins. Ef-

forts to surmount this problem failed. In view of the technical difficulties that Shore and Tate (127) encountered, their conclusions should be substantiated by further study.

It remains possible that mitochondrial proteins are synthesized by ribosomes bound to the ER membranes. As a consequence, such newly synthesized mitochondrial proteins may be packaged in vesicles derived from either the smooth ER or the Golgi apparatus. Selective transport is then achieved through specific fusion of these vesicles with the outer mitochondrial membrane (128). However, as mentioned previously, there is neither morphological nor biochemical evidence for this mechanism.

So far, there is no biochemical evidence to implicate the synthesis of chloroplast proteins on the rough ER. On the basis of morphological observations, Gibbs (45) has suggested that, in *Ochromonas*, chloroplast proteins may be synthesized on bound ribosomes of chloroplast ER. This membrane occurs in association with chloroplasts of only certain algae and therefore its possible role in the transport of protein into chloroplasts does not extend to those of higher plants and green algae.

2. Synthesis on Ribosomes Bound to Organelle Outer Membranes

Vectorial discharge of proteins synthesized by ribosomes bound to the outer membranes of organelles would provide a reasonably straightforward mechanism for transport of proteins into mitochondria and chloroplasts. Substantial evidence for the existence of such bound cytoplasmic ribosomes exists only for yeast mitochondria. Arrays of ribosomes proximate to the mitochondrial outer membranes are apparent in electron micrographs of *Saccharomyces* (78, 79) and *Rhodotorula* (82) spheroplasts. In *Rhodotorula*, however, spatial considerations, as noted by Keyhani (82), probably preclude coupled synthesis and transport of proteins into mitochondria of this organism even though the ribosomes appear to be highly ordered with respect to the outer membrane. Cytoplasmic ribosomes are more intimately associated with mitochondrial outer membrane in *Saccharomyces* (78) and, in fact, can be seen even when the mitochondria are purified (79).

A series of elegant experiments from Butow's laboratory have demonstrated that 80S ribosomes bound to mitochondrial outer membranes are not

artifacts caused by polysome relocation during cell fractionation. Bound 80S monosomes, released from mitochondria by nonionic detergent, are more resistant to dissociation into subunits by 0.4 M KCl than are 80S ribosomes isolated from post-mitochondrial supernates (80). These results implicate the occurrence of mRNA fragments and nascent chains which stabilize the bound monosomes. Also, the nature of the ribosome-membrane interaction in mitochondria is quite similar to that in rough microsomes (78). About 30% of the mitochondrial-bound 80S ribosomes are released by high-salt treatment alone whereas the remaining 70% are released only in the presence of high-salt and puromycin. The recovery of bound 80S ribosomes is enhanced by pre-incubation of cells with cycloheximide which prevents ribosome run-off. Thus, the 80S ribosomes are attached to the mitochondrial outer membrane not only by ionic interactions but also via their nascent chains. Butow and co-workers observed that starved cells possess mitochondria depleted of bound 80S ribosomes and that bound 80S ribosomes disappear in a mutant temperature-sensitive for initiation of protein synthesis when the mutant is incubated at restrictive temperatures (81). Together, these results indicate that binding to the mitochondrial outer membrane is specific for ribosomes engaged in protein synthesis.

The biochemical evidence discussed above is substantiated by morphological observations obtained with isolated mitochondria. Kellems et al. (79) prepared mitochondria from growing spheroplasts and found that the 80S ribosomes are attached specifically to regions in which the outer and inner mitochondrial membranes are in contact or apparent fusion. Such contact sites are particularly evident in de-energized mitochondria which have inner membranes in the condensed configuration (56). The restricted localization of bound ribosomes at the contact sites suggests a mechanism in which the growing polypeptide chains are transferred directly across two membrane barriers (21, 79).

If the bound 80S ribosomes are indeed engaged in the synthesis and vectorial discharge of mitochondrial proteins, it should be possible to demonstrate the association of nascent chains with intact mitochondria after puromycin treatment. Kellems et al. (78) labeled nascent chains of bound 80S ribosomes by incubating mitochondria in an amino acid-incorporating system containing pH 5 enzymes, [^{3}H]leucine, and chloramphenicol

to inhibit mitochondrial protein synthesis. They estimated that 50% of the nascent chains released from bound ribosomes by puromycin are vectorially discharged.

More recently, direct evidence for the unidirectional discharge of nascent chains of bound 80S polysomes has been presented by Ades and Butow (1). Nascent polypeptide chains on 80S polysomes bound to mitochondria were labeled with [^{35}S]Methionine (Met) in an in vitro system which allows chain extension, and then subjected to papain digestion in the presence or absence of deoxycholate. In the absence of deoxycholate, 80% of the nascent chains are resistant to proteolysis whereas in the presence of the detergent almost all of the labeled proteins are degraded. In contrast, labeled nascent chains on free polysomes are digested to an equal extent (85%) with or without deoxycholate. These experiments provide strong evidence that the nascent chains of bound 80S polysomes are preferentially segregated into the mitochondria during translation.

Although the occurrence of 80S polysomes bound to the outer membrane of yeast mitochondria has been well documented (21, 78–81), the functional significance of these findings is not fully known. If these ribosomes are engaged in the synthesis and segregation of mitochondrial proteins, their products should be quite different from those of free cytoplasmic polysomes. Bennett et al. (11) detected some differences between the polypeptide products synthesized by free and mitochondria-bound polysomes in an in vivo system which allows completion of nascent chains. Compared to the free polysomes, the bound polysomes synthesize polypeptides of higher molecular weights. Identification of some of these products with known mitochondrial proteins would provide supporting evidence for the suggested role of the mitochondria-bound polysomes.

According to the model of Butow and co-workers (21, 78–81), proteins are inserted into the mitochondria as they are synthesized. Thus, inhibition of cytoplasmic protein synthesis should result in an immediate cessation of transport. Also, there should be no pool of newly synthesized mitochondrial proteins outside the organelle. Neupert and co-workers have carried out a series of experiments using double-labeling and immunoprecipitation techniques in an attempt to ascertain whether the direct insertion model applies in N. crassa. To this end, whole cells of N. crassa were pulse-labeled with [^{3}H]leucine, and the ki-

netics of its incorporation into proteins of different subcellular fractions (free ribosomes, microsomes, mitochondria, and soluble proteins) were determined (57). It was found that the rate of labeling of total mitochondrial proteins lags behind that of the other subcellular fractions. Delayed labeling kinetics were also observed for individual mitochondrial protein fractions (mitochondrial matrix proteins, the CAT-binding protein, cytochrome c, and mitochondrial ribosomal proteins) which were immunoprecipitated from a detergent extract of mitochondria with specific antibodies. Significantly, Hallermayer et al. (57) found in pulse-chase experiments that the appearance of labeled proteins in mitochondria continues even when protein synthesis is blocked by cycloheximide. The synthesis and transport of mitochondrial proteins in N. crassa therefore appear to be separated in time. Hallermayer et al. (57) have cautioned that the occurrence of protein relocation during cell fractionation and cross-contamination of subcellular fractions might affect the interpretation of their results. With this reservation in mind, their results indicate the existence of an extramitochondrial pool of proteins that are transported into the mitochondria by a post-translational mechanism.

Harmey et al. (59) developed an interesting in vitro system to investigate transport of proteins from the cytosol into the mitochondria of N. crassa. [^{3}H]leucine was added to a cell-free homogenate in the presence of chloramphenicol. At subsequent intervals, the homogenate was fractionated to determine the distribution of labeled proteins in the various subcellular fractions. Because only 80S ribosomes are active in this system, it is assumed that any labeled proteins recovered in the mitochondrial fraction must have been first synthesized by cytoplasmic ribosomes and then transported into the mitochondria. In control experiments, prelabeled mitochondrial matrix proteins were not taken up by mitochondria, indicating that transport of the newly synthesized proteins into mitochondria in the in vitro system is not an adsorption artifact. Although protein synthesis in the in vitro system ceases after 10–15 min, labeled proteins continue to accumulate in the mitochondrial fraction for as long as 80 min. Similar labeling kinetics are obtained for the newly synthesized mitochondrial matrix proteins, the CAT-binding protein, and cytochrome c. Thus, cytoplasmically synthesized proteins appear to be transported into mitochondria in the absence of protein synthesis, as indicated previously by the in

vivo results of Hallermayer et al. (57).

In the cell-free homogenate system of Harmey et al. (59), >90% of the incorporation of [³H]leucine into proteins occurs in the first 10 min. However, addition of cycloheximide at 10 min inhibits transport of matrix proteins and the CAT-binding protein by ~50% during the subsequent 70 min of incubation. Because in control experiments, transport of these proteins continues after cessation of protein synthesis, the results suggest that cycloheximide may interfere with the transport process itself in vitro.

Recently, Zimmermann et al. (134) showed that newly synthesized apocytochrome c is present in the cytosolic fraction of the in vitro system of Harmey et al. (59). After cessation of protein synthesis, there is a decrease in the amount of apocytochrome c in the cytosol and an increase in the amount of cytochrome c in the mitochondria. Because the outer mitochondrial membrane is impermeable to cytochrome c (91, 133), it is possible that apocytochrome c is the transport form of the enzyme. However, whether the apocytochrome c synthesized in vitro is the primary translation product is not known.

Marra et al. (93) have claimed that rat liver mitochondria are capable of taking up mitochondrial aspartate aminotransferase but not the cytoplasmic isoenzyme. These results are in direct contradiction with those of Harmey et al. (59) who showed that proteins of the mitochondrial matrix are not transported into mitochondria in vitro. Because aspartate aminotransferase is a matrix enzyme, its transport into the mitochondria would be most convincingly demonstrated if it could be shown that presumably sequestered enzyme is insensitive to proteolytic digestion. Unfortunately, this stringent test was not applied in the experiments of Marra et al. (93).

Although the kinetic data of Hallermayer et al. (57) obtained in vivo, and of Harmey et al. (59) obtained in vitro, suggest the existence of extra-mitochondrial pools of newly synthesized mitochondrial proteins, the authors have prudently pointed out that the site of synthesis of these proteins and the precise subcellular location of the pools cannot be defined unequivocally because of possible artifacts that may have occurred during cell fractionation. For example, Harmey et al. (59) have suggested that the pools are located in the cytosolic compartment, but this could have been a consequence of protein leakage from microsomes or from vesicles derived from the ER.

In fact, the kinetic data do not rule out a model in which mitochondrial proteins are first synthesized on the rough ER, segregated into the ER cisternae, and then packaged into vesicles which fuse selectively with the mitochondria.

Ades and Butow (personal communication) analyzed the rate of pulse incorporation of labeled amino acids into yeast mitochondrial and cytosolic proteins in vivo. During the first 1-2 min of the pulse, the mitochondrial and cytosolic fractions show identical labeling kinetics but, at later time-points, the rate of incorporation into the former is almost twice that of the latter. Incorporation into both fractions stops within 90 s after the addition of cycloheximide. Thus, there is no apparent lag in the kinetics of labeling of the mitochondrial fraction as compared to the cytosol in either the presence or absence of cycloheximide. These results, indicating tight coupling of cytoplasmic protein synthesis and transport of proteins into the mitochondria, are in sharp contrast to those obtained with N. crassa (57) discussed earlier.

In pulse-chase experiments, Ades and Butow (personal communication) noted that the incorporation of label into the cytosolic fraction stops within 1 min while radioactivity continues to accumulate in the yeast mitochondrial fraction for as long as 5-6 min. According to the Signal Hypothesis (14-16), translation of mRNA specific for mitochondrial proteins begins first in the cytosol, and the translating polysomes become attached to the outer mitochondrial membrane only when the signal sequence is available for binding. Ades and Butow (personal communication) proposed that the continued incorporation of label into mitochondrial proteins in the pulse-chase experiments reflects the time required for transport of the polysomes to the outer membrane plus the time required to complete one round of translation.

Kellems et al. (79) reported that the 80S ribosomes are attached specifically to the contact sites of yeast mitochondria. It is difficult to see how this type of topological arrangement would be suitable for the synthesis of proteins in the intermembrane space and of peripheral proteins (e.g., cytochrome c) located on the C-side of the inner membrane. Perhaps, the bound 80S ribosomes are used exclusively for the synthesis of matrix proteins, integral proteins of the inner membrane, and peripheral proteins on the M-side of the inner membrane. Also, the occurrence of this subpopulation of bound 80S ribosomes appears to be restricted to yeast mitochondria; it has not been found in

mitochondria of *N. crassa* (57) or rat liver (126). Finally, there are no cytoplasmic ribosomes directly attached to the chloroplast outer envelope membrane either in higher plants (55) or in *C. reinhardtii* which has been treated with anisomycin to prevent possible polysomal run-off (Chua, unpublished results). Whether the bound 80S ribosomes represent a special formula for transport of proteins into yeast mitochondria remains to be established.

3. Synthesis on Free Polysomes

Although there is no direct evidence as yet to implicate the synthesis of mitochondrial proteins on free cytoplasmic ribosomes, some recent results with yeast cytochrome *c* and subunits IV-VII of cytochrome oxidase are consistent with the notion that these proteins may be synthesized on free polysomes.

Zitomer and Hall (135) translated wild-type yeast poly(A) RNA in a wheat germ cell-free system and detected, by immunoprecipitation, a product identical in electrophoretic mobility to the authentic cytochrome *c*. Furthermore, poly(A) RNA extracted from a chain-terminating ochre mutant, cyc1-72, directed the synthesis in vitro of a shorter polypeptide which has the expected size of the ochre fragment of cytochrome *c*. Because the wheat germ extract may contain the requisite processing enzymes, these results do not rule out the possibility of the synthesis of a larger precursor which is converted to apocytochrome *c* in the in vitro system. The N-terminal amino acid of yeast iso-1-cytochrome *c* has been identified as threonine (102). Through genetic analysis of yeast mutants affected in the cytochrome, Sherman and co-workers (cf. reference 125) have deduced that the codon for the threonine residue is preceded only by the AUG codon, specific for the initiating amino acid, Met, which is removed during or after completed synthesis of the apoprotein. Therefore, iso-1-cytochrome *c* is not synthesized with an N-terminal chain extension.

If yeast iso-1-cytochrome *c* were synthesized initially on membrane-bound ribosomes, it would be expected to contain an N-terminal signal sequence specific for the ER or outer mitochondrial membrane. Because the possibility of an N-terminal precursor sequence has been ruled out by the genetic considerations noted above (cf. reference 125), it may be argued that the function of the signal sequence is served by the N-terminal portion of cytochrome *c* itself. If this were true, there

would be a stringent sequence requirement for this portion of the molecule. However, in intragenic revertants of yeast, amino acid substitution of several positions near the N-terminus of iso-1-cytochrome *c* does not lead to a disappearance of this enzyme in the mutant mitochondria (cf. reference 125). Furthermore, mutant forms of iso-1-cytochrome have been isolated which are either two residues longer or four residues shorter at the N-terminus compared to the wild type enzyme (cf. reference 125). These results demonstrate that drastic alterations in amino acid sequence near the N-terminus of cytochrome *c* do not appear to impair the transport of this enzyme into mitochondria. Thus, it is unlikely that the enzyme is synthesized on membrane-bound ribosomes. By the process of elimination it appears, therefore, that cytochrome *c* is synthesized on free polysomes. However, it remains to be established whether the primary translation product of cytochrome *c* mRNA contains an extra sequence at the C-terminus. This may be difficult to ascertain especially if the in vitro translation systems contain the requisite processing activities.

Poyton et al. (114, 115) have presented interesting results which indicate the existence of a polyprotein precursor of the cytochrome *c* oxidase subunits made in yeast cytoplasm. Their novel approach explored effects of cytoplasmic products on protein synthesis in isolated mitochondria. In vitro yeast mitochondrial protein synthesis normally ceases after 30 min whereas mitochondria isolated from cycloheximide-treated cells do not engage in protein synthesis at all. However, Poyton and Kavanagh (114) discovered that yeast cell supernates contain heat- and trypsin-sensitive, nondialyzable components which, upon addition to incubation mixtures, stimulate mitochondrial protein synthesis in a stoichiometric manner. Cytochrome *c* oxidase subunits I-III are among the mitochondrial products subject to regulation by the cytoplasmic factors. Poyton and Kavanagh (114) found that in vitro synthesis of subunits I-III, unlike the bulk of mitochondrial products, was not stimulated if cell supernates were pretreated with antibodies against either cytochrome *c* oxidase or subunits IV or VI. The immunoreactive material has a mol wt of 55,000 and contains many tryptic peptides in common with those derived from a mixture of subunits IV-VII (114). Thus, the 55,000-dalton protein appears to be a polyprotein precursor to subunits IV-VII. Although it is not known whether the precursor is a

primary translation product, these results indicate that it occurs in a soluble pool in vivo and, therefore, is likely to be synthesized on free polysomes.

The nature of the stimulatory effect on mitochondrial protein synthesis is unclear but it would appear to require translocation of the precursor into apparently intact mitochondria in a post-translational fashion. Immunoprecipitates of solubilized mitochondrial membranes can be resolved in SDS gels into the full complement of cytochrome c oxidase subunits and also a substantial amount of the precursor. This indication that the newly transported 55,000-dalton component resides at a membrane site until it is processed is supported by in vivo pulse-labeling experiments. Appearance of newly synthesized precursor in mitochondrial membranes was found to precede that of mature subunits. Because a free pool of subunits is not detected, Poyton and McKemmie (115) suggested that processing into subunits and their assembly occur at the membrane site. It remains to be verified whether the soluble form of the precursor is identical to the membrane-associated counterpart, whether the soluble form can translocate through the outer membrane of intact mitochondria, and whether the protein is restricted to an inner membrane site and, if so, what orientation it assumes. The concept of a polyprotein precursor is attractive in that it provides a vehicle which most efficiently assures stoichiometric transport of the components of enzyme complexes into organelles.

Some progress has been made recently on the in vitro synthesis of chloroplast proteins and their transport into intact chloroplasts. Most of the work has concentrated on the small subunit of RuBPCase which was chosen as a model system because of its great abundance in photosynthetic tissues.

Dobberstein et al. (36) found that addition of polyadenylated RNA from C. reinhardtii to a wheat germ cell-free system resulted in the synthesis of numerous discrete polypeptides. Prominent among these was a polypeptide of 20,000 daltons which was specifically immunoprecipitated by antibodies to the RuBPCase small subunit (S) of the alga. Because the 20,000-dalton protein is immunochemically related to S but is larger than the latter by ~4,000 daltons, it was identified as a precursor. After its synthesis, the precursor (pS) could be converted, by an endoproteolytic activity present in postribosomal supernates of C. rein-

hardtii, to S and a small peptide fragment, which presumably represents the extra sequence in pS. When free polysomes of C. reinhardtii were employed to complete synthesis of nascent polypeptide chains in vitro, S instead of pS was immunoprecipitated. Subsequent experiments showed that the free polysome preparation contained the enzymatic activity for processing of pS to S and that this activity could be removed from the polysomes by high-salt wash. No physiological significance was ascribed to the association of the endoprotease with polysomes because the enzyme might have been relocated from either the cytosol or the chloroplast stroma during cell fractionation. Because the small subunit is synthesized on free ribosomes, it follows that the transport of pS into chloroplasts and its processing to S occur after translation. Moreover, it was suggested that the additional sequence in pS contains the necessary information for binding to a putative chloroplast envelope receptor and somehow facilitates the transport of pS into chloroplasts. However, the inability to prepare intact chloroplasts from C. reinhardtii has precluded the in vitro reconstitution of transport of the algal pS as well as the subcellular localization of the processing enzyme.

The finding of pS in C. reinhardtii (36) has stimulated the search for a similar precursor in higher plants. It has been reported recently that in vitro protein synthesis in the wheat germ cell-free system primed with polyadenylated RNA from pea (23, 28, 29, 65) and spinach (28, 29) results in the formation of putative precursors to the RuBPCase small subunits. The precursors are immunoprecipitated with antibodies against either the RuBPCase holoenzyme (65) or the purified small subunits (28, 29), contain tryptic peptides common to those of S (23), and exhibit mol wt 4,000–5,000 greater than S. As in C. reinhardtii, the higher plant precursors are synthesized in vivo by free polysomes (23, 118). The pea pS is converted to the size of S when postribosomal supernates of in vitro translation mixtures are incubated with crude preparations of pea chloroplasts obtained by differential centrifugation (65).

The occurrence of higher-plant small subunit precursors has afforded the opportunity to test the hypothesis that transport of pS into chloroplasts occurs in a post-translational manner (36). Thus, Highfield and Ellis (65) demonstrated that conversion of pS to S in the presence of crude chloroplast preparations apparently coincides with transport because the processed form is protected

from digestion with trypsin. Because processing could be carried out in the presence of cycloheximide, chloramphenicol, or with postribosomal supernates of translation mixtures, it is clear that the phenomenon occurs independent of translation.

Because in the chloroplast the small subunits are in association with the large subunits as RuBPCase holoenzyme, the transport of pS into isolated chloroplasts can be most convincingly demonstrated if the following two criteria are met. First, the isolated chloroplasts must contain a high proportion of intact plastids and be free from contamination by soluble proteins and other membrane-bounded organelles. Such contaminants may contain spurious proteolytic activities which complicate the interpretation of the results. Second, in vitro transport of pS into the chloroplast stroma is assured only if assembly of newly transported S with large subunits to form the RuBPCase holoenzyme can be demonstrated. Otherwise, it can be argued that the small subunit is lodged in the chloroplast envelope or trapped in the intermembrane space; in both cases, the apparent transport in vitro would have no physiological significance. The above two criteria were satisfied in the in vitro reconstitution experiments of Chua and Schmidt (28, 29). Highly purified, intact chloroplasts of pea and spinach were obtained by centrifugation on silica sol gradients by the procedure of Morgenthaler et al. (98, 99). Electron microscope studies established that these chloroplasts retain both inner and outer envelope membranes and are free of contamination by other cell membrane components. It was found that pS in postribosomal supernates from both spinach and pea mRNA in vitro translation products is transported interchangeably into intact chloroplasts of higher plants. Not only are the small subunits protected against protease digestion, but 80% of the newly transported S is assembled in the form of the holoenzyme. The precursor form is undetected in chloroplasts, suggesting that processing occurs during or soon after transport. The significance of heterologous and homologous transport, processing, and assembly of pea and spinach small subunits synthesized in vitro is enhanced by the demonstration that neither pS nor S from C. reinhardtii was taken up by chloroplasts from either higher plant. Because uptake of pea and spinach pS is unaffected by incubation of the chloroplasts in the light, dark, or light plus chloramphenicol, the transport process is not dependent on active chloroplast protein

synthesis or active photophosphorylation. This conclusion can be drawn also from the recent observations of Feierabend and Wildner (42). In their experiments, the mature small subunit is recovered from intact plastids of rye plants which are grown at 32°C and consequently do not contain either 70S chloroplast ribosomes or immunologically detectable RuBPCase large subunits.

The data of Feierabend and Wildner (42) may be taken as evidence that the protease which converts pS to S and presumptive envelope receptors from pS are not synthesized within the chloroplast. Furthermore, because the intact plastids from heat-bleached rye leaves contain the mature RuBPCase small subunit but not the large subunit (42), it follows that the latter is not required for the transport and processing of pS.

Although it is certain that the proteases specific for pS are associated with highly purified spinach and pea chloroplasts (28, 29), their precise location is still unclear. Highfield and Ellis (65) found that only 40% of the processing activity remained when pea chloroplasts were lysed in a hypotonic buffer. There was an additional 15% loss of activity when thylakoid membranes in the chloroplast lysate were removed by centrifugation (4,000 g, 5 min). Further centrifugation of the 4,000 g supernate at 30,000 g for 40 min to sediment chloroplast envelopes resulted in nearly complete loss of the processing activity in the supernate. From these results, they proposed that the processing enzyme resides in the chloroplast envelope. Unfortunately, the 30,000 g pellet, which presumably contained chloroplast envelopes, was not tested for pS processing. Therefore, the possibility remains that the pea protease, like that of C. reinhardtii, is soluble but deteriorates very quickly after chloroplast lysis.

By the use of microsequencing techniques, the peptide extension of the C. reinhardtii small subunit precursor has been shown to be located at the N-terminus and contains 44 mostly nonpolar amino acid residues (Schmidt, Devilliers-Thiery, Blobel, and Chua, unpublished results). After in vitro processing of pS, the resulting product possesses an N-terminal amino acid sequence identical to that of the mature small subunit. This is conclusive evidence that the translation product is not an in vitro artifact and that the processing of pS to S occurs with fidelity. Because the precursor extension is likely to be involved in the posttranslational transport mechanism, we have designated it as the "transit peptide" to distinguish it

from the "signal peptide" of precursors of secretory proteins. Transit peptides are defined as extensions found on precursors of organelle proteins synthesized by free cytoplasmic ribosomes. The transit peptide can occur at either the N- or C-terminus of the protein destined for post-translational transport across intracellular membranes.

It is also of interest whether other chloroplast proteins which are synthesized by cytoplasmic ribosomes are made as soluble precursors. The most abundant thylakoid membrane polypeptides synthesized by cytoplasmic ribosomes are those associated with the light-harvesting CP II (cf. reference 47). In both spinach and pea, at least two of these polypeptides share antigenic determinants, whereas in *Chlamydomonas* there are at least three immunologically related species (Chua and Blomberg, unpublished results). We have purified free polysomes from spinach and *Chlamydomonas* and employed them to complete polypeptide chains in vitro. The CP II antibody is immunoreactive with two and three products of the spinach and *Chlamydomonas* polysomes, respectively. These possess mol wt ~4,000 greater than the mature membrane polypeptides. Moreover, these putative precursors are also detected as major in vitro products of spinach, *Chlamydomonas*, and also pea polyadenylated RNA translation in the wheat germ cell-free system (Schmidt and Chua, unpublished results). Apel and Kloppstech (7) have employed antibody against CP II of *Acetabularia* to analyze the in vitro products of barley polyadenylated RNA. They identified a 29,000-dalton product as a precursor to the CP II apoprotein (mol wt, 25,000) on the basis of peptide mapping. Recently, Huisman et al. (67) showed that ferredoxin, a peripheral thylakoid membrane protein, is synthesized as a precursor ~4,000 daltons larger than the mature protein when polyadenylated RNA from *Chlamydomonas*, beans, and tobacco is translated in the wheat germ cell-free system. Thus, from these preliminary characterizations, we expect that transport of these thylakoid membrane proteins into chloroplasts is quite like that of the RuBPCase small subunit. Even in the case of the CP II polypeptides, which in their mature forms display an intense hydrophobicity commensurate with their roles as integral membrane proteins, we predict that all precursors of chloroplast proteins are soluble and are transported into chloroplasts in a post-translational fashion.

VII. CONCLUSIONS AND CONJECTURES

We have emphasized that the mechanism of transport of organelle proteins across membranes is determined primarily by whether the cytoplasmic ribosomes synthesizing these proteins are free or membrane-bound (cf. section V). The cytoplasmic ribosomes could be bound to either the ER membrane or the organelle outer membrane. In either case, the synthesis of organelle proteins and their transfer across membranes occur in the same time and space. Thus, the transport process is a co-translational event (cf. reference 22). On the other hand, if organelle proteins are synthesized by free cytoplasmic ribosomes, the site of synthesis is necessarily removed from the site of transport into the organelle. In this case, the transport process is a post-translational event, temporally separated from the synthesis of these proteins in the cytosol. Therefore, while there are three sites where cytoplasmic ribosomes may be localized, there are in fact only two basic mechanisms by which organelle proteins are transported across membranes.

Critical evaluation of the experimental evidence reveals no compelling reason to support the view that mitochondrial proteins are synthesized on the rough ER. Most of the experiments that were purported to have shown this site of synthesis have not ruled out rigorously possible cell fractionation artifacts. There is also no biochemical evidence so far to implicate the rough ER in the synthesis of chloroplast proteins. The paucity of rough ER in mesophyll cells of higher plants (55) is consistent with this notion. However, in certain groups of algae, the chloroplast appears to be completely surrounded by a cisterna of the rough ER, referred to as the chloroplast ER (cf. section II). If it can be established by serial sections that the chloroplast ER is uninterrupted, then it would be reasonable to assume that it is involved in synthesis and/or transport of proteins into the chloroplast.

The direct insertion hypothesis as proposed by Butow and co-workers (21, 78-81) is attractive because of its simplicity; it dispenses with the intervening steps required of the model implicating synthesis on rough ER. The biochemical and morphological observations presented thus far have established conclusively that the bound cytoplasmic ribosomes are not artifacts of cell fractionation. However, it remains to be shown that the bound cytoplasmic polysomes synthesize spe-

cific mitochondrial proteins and that the transport of these proteins into mitochondria is a co-translational process. Because the morphological equivalent of this subpopulation of bound ribosomes has not been found either on mitochondria of other organisms or on chloroplasts, the general applicability of the direct insertion model is uncertain.

The in vitro studies on the small subunit of RuBPCase have shown that the protein is synthesized on free ribosomes (23, 36, 118). Furthermore, the precursor, pS, is imported into isolated, intact chloroplasts in a post-translational manner (28, 29, 65), processed into the mature small subunit (28, 29, 65), and the latter is assembled into the RuBPCase holoenzyme (28, 29). The successful reconstitution of transport of RuBPCase small subunit in vitro raises the question of whether other chloroplast proteins are also transferred into the organelle by a similar post-translational mechanism. Because there are three compartments in the chloroplast, it is important to know whether proteins residing in different compartments are handled differently in terms of their transport. Based on their final localizations, chloroplast proteins may be divided into three classes:

Class 1: Outer envelope membrane proteins; proteins in the inter-membrane space; integral and peripheral membrane proteins facing the cytoplasmic side of the inner envelope membrane.

Class 2: Transmembrane proteins of the inner envelope membrane; integral and peripheral proteins facing the stromal side of the inner envelope membrane; matrix proteins; integral and peripheral thylakoid membrane proteins facing the stromal side.

Class 3: Transmembrane proteins of the thylakoids; peripheral and integral thylakoid membrane proteins facing the thylakoid space; proteins of the thylakoid space.

Class 1 and 2 proteins have to pass through one and two membranes, respectively, whereas Class 3 proteins have to traverse three membranes before reaching their functional sites.

On the basis of the morphological evidence discussed above, we would like to propose the working hypothesis that most, if not all, of these proteins, irrespective of their localizations, are synthesized on free rather than membrane-bound cytoplasmic ribosomes. Accordingly, their transport into the chloroplast is accomplished by a post-translational mechanism similar in principle

to the transfer of certain toxins across plasma membrane (cf. references 104 and 108). By analogy to the RuBPCase small subunit, these proteins are synthesized as larger precursors containing "transit peptides" at the N- or C-terminus or at both ends. The transit peptide(s) contain the sequence information to ensure not only specific transport into the chloroplast but also subsequent localization of the newly transported proteins at specific sites within the organelle. Therefore, we expect the transit peptide(s) within each class of precursors to possess similar chemical properties. Interaction of the transit peptide(s) with a specific envelope receptor or class of receptors somehow facilitates post-translational transport of the proteins into the chloroplast. The Chlamydomonas small RUBCase subunit is synthesized by free ribosomes (36) even though the precursor possesses an extension of 44 amino acids at the N-terminus (Schmidt et al., unpublished results). This seems paradoxical because one would expect that the transit peptide(s) would interact with chloroplast envelope receptors immediately upon its emergence from polysomes. This would result in binding of the translating polysomes to the envelope. As this is not the case, it is possible that envelope interaction may require post-translational modification(s) of the transit peptide(s) in the precursor. Alternatively, the binding activity of the precursor may be latent until the entire molecule is synthesized.

It is quite possible that some organellar proteins are not synthesized with polypeptide chain extensions. The extra-organellar form of such proteins may possess specific conformations necessary for binding to envelope receptors. Post-transport protein modifications, e.g., attachment of a prosthetic group, may change newly transported protein irreversibly, thereby "trapping" it within the organelle.

We propose that transport of proteins into their respective chloroplast compartments is determined by the localization of the receptors. In the case of Class 1 proteins, the receptors are localized on the outer envelope membrane. Class 2 proteins pose an additional problem because they are apparently transferred across not one but two membranes. If the transport process involves one membrane at a time, it would be necessary to invoke two sets of receptors, the additional set being located at the inner envelope membrane. To circumvent this difficulty, we

suggest that the receptors for Class 2 precursors are restricted to specific regions of the chloroplast envelope in which the outer and inner membranes are fused. Thus, the proteins would have to traverse only one membrane during transport. Preliminary electron microscope experiments show that regions of apparent contact between the envelope membranes can be seen in spinach chloroplasts suspended in a hypertonic medium (Chua and Schmidt, unpublished material). Such putative contact zones in the chloroplast envelope are morphologically similar to contact sites previously described in mitochondria (56). The envelope contact zones of mitochondria are reminiscent of the adhesion zones found in the cell envelope of *E. coli* (9). The latter are distinct areas in which the outer and inner bacterial membranes are attached to one another. Interestingly, the adhesion zones are known to contain specific receptors for F-pili insertion and for attachment of phage particles (9).

Because of the paucity of data on the topography of thylakoid membrane polypeptides, it is not known whether Class 3 proteins exist, and if they do, whether any of them are synthesized on cytoplasmic ribosomes. In the event that some Class 3 proteins are synthesized in the cytosol, we expect the precursors to enter the chloroplast at the putative contact regions also. However, in contrast to the Class 2 precursors, the Class 3 precursors are expected to contain yet another sequence which directs their insertion into the thylakoid membrane or transfer across the latter into the thylakoid space.

We suggest that most of the cytoplasmically synthesized mitochondrial proteins are also made as larger precursors and transported into the organelle by a post-translational mechanism similar to that used for the transport of chloroplast proteins. The recent results on the polyprotein precursor to subunits IV–VII of yeast cytochrome oxidase (114, 115) and labeling kinetics of *Neurospora* cytochrome *c* and the CAT-binding protein (57, 59) are consistent with this notion. The post-translational mechanism may also operate in the transfer of proteins from the cytosol into other cellular compartments, e.g., peroxisomes and glyoxysomes. A larger precursor to the glyoxysomal MDH has recently been reported (132).

Because mitochondria contain only two compartments, the proteins must traverse only one

or two membranes to reach their final destinations. Thus, mitochondrial proteins are equivalent to the Class 1 and 2 chloroplast proteins. By analogy with model of Butow et al. (21), we propose that the receptors for Class 2 protein precursors are localized specifically in the contact sites so that these proteins pass through only one membrane during transport. It is possible that some mitochondrial proteins of certain organisms may be synthesized by cytoplasmic ribosomes attached directly to the outer mitochondrial membranes (21, 78–81).

In our opinion, pulse-labeling and pulse-chase experiments provide only indirect evidence concerning the sites of synthesis of mitochondrial and chloroplast proteins and, consequently, the mechanisms of their transport. The most direct approach to distinguish between the two basic transport mechanisms (cf. section V, 2) is reconstitution in vitro.

However, we anticipate that some problems may be encountered when organelle proteins are synthesized in an in vitro system. For example, the apparent absence of a larger precursor might result from the presence of specific or nonspecific proteases in the cell-free translation system used. In this case, it is also necessary to verify that the immunoprecipitated protein is a primary translation product. In addition, another cell-free translation system should be tried to see whether the same product is synthesized. Possible N-terminal processing of precursors can be ruled out by specific labeling with $[^{35}S]$Met tRNA$_{fMet}$ (66).

To demonstrate unambiguously reconstitution of protein transport in vitro, it is important to use highly purified mitochondria (89) and chloroplasts (98–100) prepared by density gradient centrifugation. Organelles obtained by differential centrifugation alone (39, 65, 121), although suitable for routine biochemical experiments, are usually contaminated by cytosolic proteins and other subcellular membrane-bounded compartments. Spurious proteolytic activities in such preparations may lead to confusing results and erroneous interpretations. If an organelle protein is a subunit of an enzyme complex or a membrane component, it is essential to show that the newly transported protein is correctly assembled into the complex or assumes the right orientation in the membrane. Otherwise, the apparent reconstitution of transport in vitro would be of doubtful physiological significance.

Although we have proposed that most of the

organellar proteins are transported in a post-translational manner, we have refrained from postulating an explicit model to explain how these proteins are translocated across membranes. We feel that elucidation of the detailed mechanisms and identification of the driving force for transport depends on knowledge of the complete amino acid sequences of the precursor chain extensions and characterization of the envelope membrane receptors. It is fortunate that the transit peptide of the *Chlamydomonas* pS is located at the N-terminus and that therefore its amino acid sequence can be established by Edman degradation. If the transit peptides of other organelle protein precursors are similarly disposed, the analysis of their sequences will be greatly facilitated. Such sequence data are essential to establish whether the precursor chain extensions of proteins localized in the same organellar compartment are homologous and whether the precursor sequence of Class 1 proteins is distinct from that of Class 2 proteins. Nothing is known about the envelope receptors which presumably mediate protein transport. The receptor for the pS is likely to be a protein or group of proteins because intact chloroplasts lose their ability to take up this precursor after pre-treatment with proteases (28, 29).

The post-translational transport of organellar proteins may offer certain physiological advantages over the co-translational mode. Depending on the rate of translation and the size of the mRNA, a membrane-bound polysome may contain anywhere from 5 to 20 or more ribosomes. Therefore, not one but several membrane receptors must be engaged simultaneously for the transport of an organellar protein. If the number of receptors is limited, a problem might arise when rapid rates of protein synthesis are required, such as during organelle development and replication. Continued synthesis by polysomes, which are unable to attach to the membrane because of a limitation of the requisite receptors, would result in the release of organellar proteins into the cytosol. Proteins thus synthesized could no longer be sequestered into the organelle, as has been shown for secretory proteins in vitro (14, 15). In contrast, the post-translational mode does not commit a group of receptors at any given time for the exclusive transport of an organellar protein. Newly synthesized proteins may queue up in the form of cytosolic pools even if all receptors are engaged

and, consequently, abortive synthesis of organellar proteins will not result regardless of their rate of translation. It could be argued that proteins which are not immediately transported after synthesis will be rapidly degraded. However, this is inconsistent with the evidence for cytosolic pools of organellar proteins (57, 114). Finally, post-translational regulation of organellar protein transport, whenever necessary, may be accomplished by modulating the number and/or activity of the envelope receptors.

The technological advances that have developed in the last few years have made it possible now to analyze directly a hitherto vexing problem in cell biology, namely, the transport of proteins into mitochondria and chloroplasts. We believe that future analysis of this problem will generate results relevant not only to the regulatory aspects of organelle biogenesis but also mechanisms by which transfer of proteins into other membrane-bounded compartments in the cell is achieved.

Note Added in Proof: Recently, Korb and Neupert (*Eur. J. Biochem.* 1978. **91:**609–620) synthesized apocytochrome *c* in a cell-free homogenate from *N. Crassa*. They showed that in vitro synthesized apocytochrome *c* in the post-ribosomal supernate can be transferred to mitochondria where it is converted to the holoenzyme. Maccecchini, Rudin, Blobel, and Schatz (*Proc. Natl. Acad. Sci. U.S.A.* 1979. **76:**343–347) detected in vitro and in vivo forms of α, β, and γ subunits of yeast F_1-ATPase. They showed that the precursors are imported into mitochondria by a post-translational mechanism. Thus, the transport of these proteins into mitochondria does not follow the co-translational mechanism. Finally, a higher molecular weight precursor of the cytoplasmically synthesized subunit V of cytochrome bc_1 complex in yeast mitochondria has been reported (C. Côté, M. Solioz, and G. Schatz. 1979. *J. Biol. Chem.* **254:**1437–1439).

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