Disulfide bonds and the translocation of proteins across membranes

(secretion/mitochondrial protein import/cysteine residues)

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ABSTRACT We are concerned with the mechanisms whereby hydrophilic proteins synthesized in the cytoplasm are translocated across one or two membranes into different cellular organelles. On the basis of a model of the translocation process to be described elsewhere, we propose an explanation of previous findings that the in vitro translocation across the endoplasmic reticulum of secretory proteins of higher eukaryotic cells appears to be obligatorily co-translational (i.e., occurs only while the polypeptide chain is being synthesized on the ribosome). We suggest that in vitro the intrachain disulfide bonds of the polypeptide rapidly form after it is released from the ribosome; the three-dimensional conformation of the chain is thereby stabilized and cannot undergo the unfolding that is required for post-translational translocation. In accord with this proposal, we show that the secretory preprotein human preprolactin, after translation and release from the ribosome, can indeed undergo translocation across endoplasmic reticulum membranes in vitro if the medium is sufficiently reducing. Those polypeptides that, in the absence of reducing agents, can be post-translationally translocated in vitro across bacterial, mitochondrial, and other types of membranes may generally lack intrachain disulfide bonds.

Many of the membrane-bounded organelles in eukaryotic cells obtain most or all of their internal proteins from the cytoplasm. This generally involves the translocation of a wide range of large hydrophilic protein molecules from the cytoplasm across one or two membranes of the organelle. The mechanisms of such translocations have received a great deal of attention in the last decade, but they are still not well understood. In particular, while it would seem plausible that these mechanisms might be fundamentally the same in all such cases, the evidence until recently appeared to indicate that they were significantly different. Translocation of proteins across the endoplasmic reticulum (ER) of eukaryotic cells seemed to occur only while the polypeptide chain was being synthesized on ribosomes attached to the ER (i.e., translocation was obligatorily co-translational), whereas in other cases (bacterial, mitochondrial, peroxisomal, and chloroplast membranes) the polypeptide chain, after being completed and released from the ribosome, could generally be transported into the organelle (i.e., translocation could be post-translational). Of special significance have been in vitro studies demonstrating these differences, for example, between protein import into the ER of higher eukaryotic cells (co-translational) as compared to import into mitochondria (post-translational) (for reviews, see refs. 1 and 2).

We have developed a general picture of how protein translocation might work (3). This picture utilizes current ideas (4–7) about how the polypeptide chains of soluble proteins fold into their equilibrium tertiary structures. Briefly, our proposal involves the sequential translocation across the membrane of successive folded "subdomains" of the polypeptide chain until finally the entire chain is translocated. This process is initiated by the binding of the signal peptide, which is generally present at the amino terminus of the polypeptide chain, to a receptor in the appropriate membrane, which "seeds" (7) the formation of the first folded subdomain of the chain. As this subdomain is translocated across the membrane, the next stretch of the chain is folded into a second subdomain and is translocated. This energy-dependent process continues until the entire chain is transported across the membrane. For convenience, we refer to this mechanism as "subdomain translocation." In principle, this mechanism can function either co-translationally or post-translationally. In the latter case, the completed polypeptide chain that is released into the cytoplasm, still bearing its signal peptide, is presumably first rapidly folded into some tertiary conformation in the aqueous solution. If this polypeptide is subsequently to be transferred across a membrane by subdomain translocation, the tertiary conformation must be capable of being unfolded so that the initial and successive subdomains can be sequentially formed at the membrane and translocated. It occurred to us that if the released polypeptide chain, upon acquiring its tertiary conformation, also became rapidly cross-linked by intrachain cystine disulfide bridges, it would subsequently be incapable of unfolding to its subdomains; its post-translational translocation might thereby be blocked.

Let us pursue this suggestion further. The polypeptides that are translocated across the membrane of the ER in higher eukaryotic cells are mainly the precursors of secretory proteins, and it is known that most of these proteins contain multiple intrachain disulfide bridges. The cytoplasm of eukaryotic cells is highly reducing, because of its high concentration of glutathione (8); it is altogether likely that, after synthesis in vivo, secretory proteins do not become disulfide-bridged until after they are translocated across the ER membrane. In in vitro translocation experiments, however, such reducing conditions are normally not duplicated. If, in the absence of ER membranes, a secretory protein precursor was translated in vitro and released under oxidizing conditions, the intrachain disulfide bridges might form and, on subsequent addition of ER membranes, translocation would be blocked. On the other hand, if ER membranes and the required soluble factors were present during in vitro translocation, the nascent polypeptide chain, while being translocated across the membrane at its amino terminus, would still be attached to the ribosome towards its carboxyl terminus. Therefore, the complete tertiary conformation characteristic of the released molecule could not form as long as the nascent polypeptide was simultaneously attached and partly engulfed at its two ends; as a consequence, even under oxidizing conditions, the cysteine residues would probably be sufficiently separated from one another to remain reduced while synthesis and translocation of successive subdomains proceeded. The disulfide bonds would form only after most

Abbreviations: ER, endoplasmic reticulum; mGMP, 7-methylguanosine 5'-phosphate.
or all of the chain had been co-translationally translocated. In other words, under such circumstances co-translation but not post-translational translocation would be observed in

_\textit{in vitro}_ experiments. These observations can therefore explain the experimental findings that _\textit{in vitro}_ translocation across ER membranes appears to be obligatorily co-translational.

A prediction arising from these considerations is that in all those instances (e.g., with mitochondria) in which _\textit{in vitro}_ post-translational translocation has been observed experimentally, the proteins involved must lack stable disulfide bridges that would inhibit unfolding of the polypeptide chain. The data required to test this prediction are fragmentary and limited. However, we are not aware of any results that contradict this rather stringent prediction (see Discussion).

Another prediction is that, for a disulfide-containing secretory protein, the _\textit{in vitro}_ translocation of whose precursor polypeptide across ER membranes has previously been found to be obligatorily co-translational, the same experiments carried out in a suitable reducing milieu might allow post-translational translocation to occur. This prediction has been borne out by the experiments described in this paper. We have translated mRNA for human preprolactin (9) in a reticulocyte cell-free system, with or without added dog pancreas microsomes, and with or without added dithiothreitol. In the absence of dithiothreitol, translocation of the preprolactin across the microsomal membranes was obligatorily co-translational (9), but in the presence of sufficient dithiothreitol, post-translational translocation was observed.

**MATERIALS AND METHODS**

The procedure used to examine post-translational translocation was essentially that of Rothman and Lodish (10). The basic translation mixture contained 30 µl of reticulocyte lysate, 16.5 µl of translation cocktail, 15 µCi (1 Ci = 37 GBq) of \[^{35}\text{S}\]methionine; 4 µl of human placental lactogen (prolactin) mRNA (all as obtained from New England Nuclear), 1.25 mM Mg(OAc)\(_2\), and 80 mM KOAc in a total volume of 75 µl. In some experiments freshly prepared 200 mM or 500 mM dithiothreitol in H\(_2\)O was added to this translation mixture to give final concentrations of 20 mM or 50 mM dithiothreitol, respectively. The mixtures were incubated at 37°C for 2 min. At this time, 20 mM 7-methylguanosine 5'-phosphate (m\(_7\)GMP) in H\(_2\)O was added (1:10, vol/vol) to stop further polypeptide chain initiation. After 3, 6, 10, 15, and 30 min at 37°C, 15-µl aliquots were removed and added to 0.5 µl of dog pancreas microsomal membranes (New England Nuclear). All mixtures were incubated for a total of 60 min and then brought to 4°C. The 15-µl samples were then treated with ribonuclease A (1 µg) (Sigma) for 10 min at 37°C with or without a prior treatment with trypsin as described below.

Control experiments were performed by adding the microsomal membranes at the start of translation, by omitting membranes entirely, or by omitting the mRNA.

To test for the inaccessibility of translocated polypeptide chains to protease digestion, to each 15-µl sample was first added 3 µl of 10 mM CaCl\(_2\) in 20 mM Hepes buffer, pH 7.5. After 10 min at 4°C, 9-µl portions were treated with 0.5 µg of toslylphenylalany1 chloromethyl ketone-treated trypsin (Worthington, Freehold, NJ) for 30 min at 4°C. Proteolysis was stopped by the addition of 10 µg of soybean trypsin inhibitor (Sigma), followed by another 10-min incubation at 4°C before the treatment with ribonuclease A.

For analysis by NaDodSO\(_4\)/PAGE, all samples were diluted with 5 vol of sample buffer (0.03 M Tris-HCl, pH 8.0/5% NaDodSO\(_4\)/4% 2-mercaptoethanol/16% glycerol, wt/vol/vol) containing 0.004% bromphenol blue and were boiled for 5 min. Equivalent amounts were then separated electrophoretically on 15% polyacrylamide gels (11). After electrophoresis, the gels were fixed in 25% isopropanol alcohol/10% acetic acid (vol/vol) and treated with EN-3\(^{\text{HANCE}}\) (New England Nuclear). The dried gels were then exposed to pre-flashed Kodak Blue Brand film for 48–96 hr at -70°C. The autoradiograms were scanned quantitatively with an LKB Ultrascan XL laser densitometer.

**RESULTS**

In the following experiments, m\(^3\)GMP was added 2 min after the initiation of translation of the mRNA for preprolactin, to block further initiation of polypeptide chains without affecting the rate of elongation of chains that had already been initiated (10). It is important for our purposes that the presence of 20 or 50 mM dithiothreitol during translation was found not to change the time course of incorporation of \[^{35}\text{S}\]methionine into protein after addition of m\(^3\)GMP (not shown). At 100 mM dithiothreitol, translation was markedly inhibited.

The translocation of preprolactin across ER membranes is accompanied by the cleavage of the signal peptide from the polypeptide chain (9), resulting in a change in apparent molecular weight in PAGE experiments from 25,000 to 22,000. Polypeptide chains that are not translocated are not cleaved (9, 12). These findings were confirmed (Fig. 1, lanes A and B). Translation in the absence of dog pancreas microsomal membranes resulted in intact preprolactin molecules (Fig. 1, lane A). In the presence of membranes during translation, a large fraction of the preprolactin chains underwent signal peptide cleavage (Fig. 1, lane B). When, after translation had occurred in the absence of dithiothreitol, the microsomes were added 3, 10, or 15 min later (Fig. 1, lanes C, F, and I, respectively), no signal peptide cleavage was observed; these results are similar to those previously reported (9). However, when translation was carried out in the presence of 20 mM dithiothreitol and was followed by the addition of microsomes 3, 10, or even 15 min later (Fig. 1, lanes D, G, and J, respectively), a significant percentage of the preprolactin (36%, 34%, and 37%, respectively) underwent cleavage. At 50 mM dithiothreitol, the extent of cleavage at different times after addition of membranes was even more pronounced (Fig. 1, lanes E, H, and K, respectively, corresponding to 68%, 67%, and 51% cleavage, respectively). These results indicate that translocation, as detected by signal peptide cleavage, could occur post-translationally if sufficient dithiothreitol was present during translation and translocation.

A second criterion for translocation of a polypeptide across ER membranes is the protection conferred on the chain against digestion by added protease (13). Thus, preprolactin translated in the absence of membranes was completely digested by trypsin (Fig. 2, lane B), and when it was translated in the presence of membranes but the absence of dithiothreitol, a substantial fraction of the polypeptide after signal peptide cleavage was unaffected by trypsin (Fig. 2, lanes C and D). These results were as expected (13). When, however, preprolactin was synthesized in the presence of 50 mM dithiothreitol and microsomal membranes were then added 3, 10, or 15 min later, essentially the same fraction of the pro lactin was protected from trypsin (Fig. 2, lanes F, H, and J, respectively) as was protected after co-translational translocation in the absence of dithiothreitol (Fig. 2, lane D). In the absence of membrane integrity, the pro lactin is fully digested by the trypsin treatment.

**DISCUSSION**

The translocation of the precursors of secretory proteins of higher eukaryotic cells across ER membranes _\textit{in vitro}_ has
The post-translational translocation of the polypeptide chain across dog pancreas microsomal membranes is recognized by the cleavage of the signal peptide from the chain and the conversion of preprolactin (band with downward-pointing arrow in lane K and corresponding broader bands in other lanes) to prolactin (upward-pointing arrow, lane K). The effect of dithiothreitol present during translation and post-translational translocation is investigated in this figure. Lanes A and B are controls: in A, translation was performed without membranes or dithiothreitol; in B, in the presence of membranes but without dithiothreitol. In lanes C–K, further polypeptide chain initiation beyond 2 min was blocked by the addition of mGMP, and membranes were then added 3 min (C–E), 10 min (F–H), or 15 min (I–K) after the start of translation. Translation and translocation were carried out in the presence of 0 mM dithiothreitol (C, F, I), 20 mM dithiothreitol (D, G, J), or 50 mM dithiothreitol (E, H, K). Signal peptide cleavage did not occur post-translationally in the absence of dithiothreitol (C, F, I), but it did occur in the presence of 20 mM dithiothreitol and to an even greater extent in 50 mM dithiothreitol.

It appears that secretory proteins, lysosomal proteins, and the extracellular domains of integral membrane proteins of higher eukaryotic cells may be unusual in their relatively high cysteine content (up to 10 mol %) and particularly in the involvement of their cysteine residues in disulfide bond formation (8). Proteins that are secreted by most bacteria, or that are imported into mitochondria or chloroplasts, generally have low cysteine contents (often less than 1 mol %), and even those with substantial numbers of cysteine residues may generally not form disulfide bonds. A preliminary examination of an updated version of the NEWAT amino acid sequence data bank (19), for example, reveals a significant number of exported bacterial proteins with no cysteine residues: e.g., the Escherichia coli periplasmic binding proteins for d-ribose, maltose, and phosphate; the sulfate-binding protein of Salmonella typhimurium; colicin E1; the E. coli outer membrane protein I (porin); protein A of Staphylococcus aureus; and thermolysin of Bacillus thermo- proteolyticus are among such proteins. Several other bacterial and mitochondrial proteins contain only one cysteine residue per molecule. All such proteins obviously have no intrachain disulfide bonds. On the other hand, a significant number of exported bacterial proteins, and imported mitochondrial and chloroplast proteins, contain two or more cysteine residues per molecule. Without additional information beyond the amino acid sequences of these proteins it is not clear whether any of these cysteines form disulfide bridges.

One source of that information would be the complete x-ray crystallographic structure of the molecule; however, this is available for only a very limited number of relevant proteins. One such protein is the mitochondrial enzyme

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**Fig. 1.** Autoradiograms of NaDodSO4/PAGE gels of the in vitro translation products of the mRNA for human preprolactin. The polypeptide has incorporated [35S]methionine. The post-translational translocation of the polypeptide chain across dog pancreas microsomal membranes is recognized by the cleavage of the signal peptide from the chain and the conversion of preprolactin (band with downward-pointing arrow in lane K and corresponding broader bands in other lanes) to prolactin (upward-pointing arrow, lane K). The effect of dithiothreitol present during translation and post-translational translocation is investigated in this figure. Lanes A and B are controls: in A, translation was performed without membranes or dithiothreitol; in B, in the presence of membranes but without dithiothreitol. In lanes C–K, further polypeptide chain initiation beyond 2 min was blocked by the addition of mGMP, and membranes were then added 3 min (C–E), 10 min (F–H), or 15 min (I–K) after the start of translation. Translation and translocation were carried out in the presence of 0 mM dithiothreitol (C, F, I), 20 mM dithiothreitol (D, G, J), or 50 mM dithiothreitol (E, H, K). Signal peptide cleavage did not occur post-translationally in the absence of dithiothreitol (C, F, I), but it did occur in the presence of 20 mM dithiothreitol and to an even greater extent in 50 mM dithiothreitol.
FIG. 2. Similar autoradiograms as in Fig. 1. Post-translational translocation of preprolactin across microsomal membranes is here assayed by the protection that translocation confers on the polypeptide against digestion by trypsin. Lanes A–D are controls without added dithiothreitol. Lanes A and B, translation in the absence of microsomal membranes, without and with subsequent trypsin treatment, respectively. Lanes C and D, translation with membranes present, without and with subsequent trypsin treatment, respectively. In lanes E–J, translation was carried out in the presence of 50 mM dithiothreitol, and after 2 min initiation was terminated with mGMP. Microsomal membranes were then added 3 min (E, F), 10 min (G, H), or 15 min (I, J) later. This was followed by treatment with trypsin (F, H, J) or no treatment (E, G, I). The preprolactin (band with downward-pointing arrow in lane I) was digested with trypsin in lanes F, H, and J but the prolactin (band with upward arrow in lane I) was not.

aspartate aminotransferase (mAAATase) of chicken. Its polypeptide chain contains five cysteine residues, and the x-ray crystallography of mAAATase (20) shows that none of these is disulfide-bridged to any other (J. N. Jansoniush, personal communication). Other data supporting the probable absence of disulfide bonds in cysteine-containing mitochondrial or chloroplast proteins are less direct, and in view of space limitations, will not be discussed here. Suffice it to say that there is at present insufficient information to test the proposal adequately, but we know of no information that contradicts the absence of disulfide bonds in any secreted bacterial protein, or in any imported mitochondrial or chloroplast protein, that has been shown to undergo post-translational translocation in vitro under the usual oxidizing conditions.

There are several unusual polypeptides whose in vitro translocation should provide interesting further tests of our proposals. Among the secretory proteins of higher eukaryotes, for example, several apolipoproteins have no cysteine (21, 22). The corresponding pre-apolipoproteins, which must lack disulfide bonds, may be found to undergo post-translational translocation across dog pancreas microsomes in vitro under nonreducing conditions, unlike most other higher eukaryotic secretory proteins. Conversely, certain unusual bacterial secretory proteins resembling the eukaryotic serine proteases (23, 24) do contain disulfide bonds, and we predict that their preproteins will not be capable of post-translational translocation across bacterial membranes in vitro, except under reducing conditions.

Similar considerations apply to the insertion of integral proteins into membranes. Whether such insertion in vitro appears to be obligatorily co-translational, as in the case of the G protein of vesicular stomatitis virus (9), or can occur post-translationally, as with the human glucose transporter (25), may depend on whether or not the translocated and intercalated regions of the integral proteins contain disulfide bridges.

The proposition that disulfide-bonded polypeptide chains cannot be translocated across membranes except in the reduced state does not, however, imply that its converse is always true; namely, that every polypeptide chain (with a proper signal peptide) lacking disulfide bridges will be post-translationally translocated across the appropriate membrane. If translocation occurs by subdomains, then any completed and released polypeptide chain that, for whatever reason, forms too stable a three-dimensional conformation to undergo unfolding and successive subdomain translocation at an adequate rate would not be expected to be translocated in vitro. For example, the binding of heme to apoproteins that lack disulfide bridges may produce a compact, stable three-dimensional conformation in the protein molecule that resists unfolding. This may in part explain the facts that apocytochrome c can be post-translationally translocated across mitochondrial membranes in vitro but the holoenzyme, although lacking disulfide bridges, cannot (26).

The considerations of in vitro protein translocation bear on the mechanism by which the same process occurs in vivo. The cytoplasm of such cells is highly reducing because of its high glutathione concentration (8), and newly synthesized pre-secretory proteins would probably remain in the reduced state if released from the ribosome in vivo. It follows that in vivo translocation across the ER membrane could in principle be either co-translational or post-translational. Indeed, there is suggestive evidence that the translocation of preprolactin into the ER of intact pituitary cells can occur post-translationally: a transient accumulation of preprolactin was observed in experiments in vivo (9). However, co-translational translocation across the ER membrane may generally be favored kinetically if the subdomain translocation mechanism is basically correct. A nascent polypeptide chain of a secretory protein, which is directed to the ER membrane by a bound signal recognition particle (13), may then form and translocate its successive subdomains continuously as the polypeptide chain is progressively synthesized. On the other hand, if the entire preprotein is first completed and released and then becomes folded into a three-dimensional conformation, it may have to unfold partially to form sequential subdomains that can translocate across the membrane. Post-translational translocation across a membrane in vivo may therefore be substantially slower than the corresponding co-translational process.

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