Structure and Folding of Nascent Polypeptide Chains during Protein Translocation in the Endoplasmic Reticulum*

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To investigate the role of protein folding and chaperone-nascent chain interactions in translocation across the endoplasmic reticulum membrane, the translocation of wild type and mutant forms of preprolactin were studied in vitro and in vivo. The preprolactin mutant studied contains an 18-amino acid substitution at the amino terminus of the mature protein, eliminating a disulfide-bonded loop domain. In COS-7 cells, mutant prolactin accumulated in the endoplasmic reticulum as stable protein-protein and disulfide-bonded aggregates, whereas wild type prolactin was efficiently secreted. In vitro, wild type and mutant preprolactin translocated with equal efficiency although both translation products were recovered as heterogeneous aggregates. Studies with translocation intermediates indicated that aggregation occurred co-translationally. To evaluate the contribution of lumenal chaperones to translocation and folding, in vitro studies were performed with native and reconstituted, chaperone-deficient membranes. The absence of lumenal chaperones was associated with a decrease in translocation efficiency and pronounced aggregation of the translation products. These studies suggest that chaperone-nascent chain interactions significantly enhance translocation and indicate that in the absence of such interactions, aggregation can serve as the predominant in vitro protein folding end point. The ramifications of these observations on investigations into the mechanism of translocation are discussed.

Current models of protein translocation across the mammalian endoplasmic reticulum (ER)1 depict translocation as a process in which vectorial transport accompanies formation of a tight junctional complex between the ribosome and the protein conducting channel with the free energy for translocation provided by passive diffusion (1, 2). In alternative models, vectorial transport may also be driven through interaction of the nascent chain with lumenal molecular chaperones (3–6), as well as structural modifications of the nascent chain, i.e. protein folding, disulfide bond formation and, in many cases, addition of N-linked oligosaccharides, that occur coincident with translocation (7–11). In the latter model, interactions between the nascent chain and lumenal molecular chaperones are thought to prevent retrograde transport through the translocation pore and thus bias movement of the nascent chain into the luminal compartment (3–6, 12).

Recent reconstitution experiments have identified the minimum subset of ER proteins necessary for in vitro protein translocation in the mammalian ER (13, 14). In the minimal system, the translocation machinery is comprised of the signal recognition particle receptor which functions in the targeting of ribosome/nascent chain complexes to the ER, the Sec61p complex, which is thought to serve as a ribosome receptor and translocation channel, and, in some instances, the integral membrane protein TRAM, which participates in signal sequence recognition (13–15). The identification of the Sec61p complex as the primary ribosome receptor and translocation channel suggests that ribosome association with Sec61p could provide the aqueous pathway for nascent chain transit into the lumen (15, 16).

It is clear from molecular genetic and biochemical studies that hsp70 proteins perform an essential function in protein translocation across the yeast ER and the mitochondrial inner membrane (17–22). From these studies, it has been proposed that hsp70 proteins promote unidirectional transport by binding to the nascent chain as it emerges into the lumen (matrix). It has not yet been established whether the interaction of the hsp70 proteins with the nascent chain drives vectorial transport by a thermal ratchet mechanism (3–5), or, alternatively, by a conformationally driven, motor process (23, 24). Furthermore, there is disagreement as to whether the role of hsp70 proteins is limited to translocation events that occur post-translationally (20), or alternatively, whether hsp70 proteins are required for co- and post-translational translocation (25). Interestingly, it has been demonstrated that BiP is necessary for the complete translocation of prepro-α-factor, a precursor known to translocate post-translationally (26). In the absence of BiP function, pro-α-factor is unable to fully transit to the ER lumen (26). This defect, referred to as stalling, is quite similar to a translocation defect observed in mammalian microsomes that have been depleted of their luminal contents (5). In mammalian microsomes, the loss of luminal proteins causes a disruption of the translocation reaction at a point subsequent to signal sequence cleavage, and results in the accumulation of the signal-cleaved nascent chains that are unable to efficiently transit to the vesicle lumen (5).

The analysis of the energetics of protein translocation is made difficult by the fact that protein translation, translocation, and folding are coincident processes and furthermore, that protein folding occurs in an environment, the ER lumen, which is highly enriched in molecular chaperones and protein folding enzymes. To study the contribution of protein folding and luminal chaperone-nascent chain interactions to translo-
cation, the translocation behavior of wild type and mutant forms of preprolactin (pPPL) were analyzed in vivo and in vitro. The mutant preprolactin used in this study contains an 18-amino acid substitution at the NH₂ terminus of the mature protein, eliminating a small disulfide-bonded loop domain (27, 28). Whereas wild type (WT) prolactin was efficiently secreted in vivo, the folding mutant (FA) accumulated in the ER as large protein-protein and disulfide-bonded aggregates. In vitro, both WT and FA forms of prolactin were translocated with similar efficiencies but were recovered as mixed aggregates. Furthermore, aggregation was apparent co-translationally. In the absence of lumenal chaperones, aggregate formation was markedly enhanced and was accompanied by a reduction in translocation efficiency. On the basis of these data, we propose that lumenal protein-nascent chain interactions are paramount to efficient translocation and in their absence, irreversible protein-protein aggregation may serve as the predominant protein folding end point.

MATERIALS AND METHODS

Cell Culture/Transfection—COS-7 cells (29) were maintained in Dulbecco's modified Eagle's medium for 20 min at 37 °C. The supernatant was removed and the cells harvested by scraping into ice-cold PBS. Cells were washed 2 times in PBS and lysed by addition of 1 ml of lysis buffer, and radiolabeled prolactin translation products isolated by immunoprecipitation.

Pulse-Chase/Immunoprecipitation—pulse-chase studies were performed as described above and incubations quenched by addition of ice-cold PBS supplemented with 20 μM cell lysates were prepared as described (35) and cleared of large aggregates by centrifugation for 15 min at 15,000 × g (4 °C). Lysates were overlaid onto 8–35% sucrose gradients (Buccher Instruments, Lexington, KY). Gradients were centrifuged for 16 h at 39,000 × g in the Beckman SW-40 rotor (4 °C). 850-M fractions were collected, and direct prolactin immunoprecipitations performed as described above. Results

Generation of Preprolactin Structural Mutant FA—The preprolactins are members of a family of hormones that include the growth hormones and placental lactogens (27, 28). These proteins share extensive amino acid sequence homology and general, three-dimensional structure (27, 40). The mammalian prolactins can be readily distinguished from the growth hormones by a small, disulfide-bonded loop (amino acid residues 4–11) present at the amino terminus (27, 28). The disulfide-bonded loop immediately precedes one of the four a-helical segments characteristic of this family of hormones and is diagrammatically illustrated in Fig. 1. By PCR, mutants, the sequence encompassing this disulfide-bonded domain was exchanged with a random amino acid sequence, lacking the residues 4–11 disulfide bond pair, to yield a mutant referred to as pPL-FA (Fig. 1).

It has been established that disulfide bond formation can occur immediately upon the appearance of a relevant cysteine-cysteine pair in the ER lumen (41). In the prolactin mutant FA,
A structural model of prolactin: localization of the mutant disulfide-bonded loop domain. A structural model of prolactin was developed using the molecular coordinate data base for growth hormone, a prolactin homolog. The amino acid coordinates were obtained from the Brookhaven National Laboratories Protein Data Bank and the ribbon model developed using the RAS Mol (V2.6) molecular graphics program (63) obtained at http://hydrogen.cchem.berkeley.edu:8080/Rasmol. The amino acid sequence of the wild type and mutant sequence are illustrated, as is the location of the four prominent a-helical domains and the NH2-terminal disulfide bond present in the mature, folded protein.

The NH2-terminal domain lacks the cysteine pair present in the wild type protein, and therefore a protein folding event which likely occurs very early in translocation, perhaps immediately upon access of the nascent chain to the ER lumen, cannot occur. It was postulated that mutations within this discrete structural domain would significantly disrupt the protein folding pathway of prolactin, and thereby prove useful in investigations on the contributions of protein folding and lumenal protein interactions to protein translocation.

FA Is Neither Secreted Nor Degraded—Exit of secretory proteins from the ER occurs coincident with structural maturation and is the underlying basis for the variations in the rate of secretion observed between proteins in a given cell (42–45). To assay for disruptions in the folding behavior of pPL-FA, pulse-chase studies were performed in transfected COS-7 cells expressing either pPL-WT or pPL-FA. The results of these studies are depicted in Fig. 2. Under the described conditions, prolactin-WT is rapidly secreted with a half-time of 25 min (n = 4) (Fig. 2A, WT). Prolactin-FA, in contrast, remained predominately cell associated throughout the 90-min chase time (Fig. 2A, FA), indicating that prolactin-FA displays a significant defect in protein folding.

Proteolytic degradation via a non-lysosomal protein degradation pathway is a common fate of transport-incompetent proteins (46, 47). To assess the stability of the secretion incompetent prolactin-FA, extended pulse-chase studies were performed. In these experiments, it was observed that at chase periods of up to 8 h, the vast majority of the prolactin-FA was neither secreted nor degraded (Fig. 2B, compare WT versus FA). These results suggest that prolactin-FA is either not a substrate for the relevant proteases or, alternatively, has not gained access to the degradation compartment.

FA Is Retained in a Membrane Compartment—Recent studies on ER-associated protein degradation have provided evidence of a novel pathway which functions to transport malfolded proteins from the ER to the cytosol, for subsequent degradation by the proteasome complex (48–50). To determine if prolactin-FA was retained within a membrane compartment, presumably the ER, nascent chains were pulse-labeled and a microsomal fraction prepared from the labeled cells. The protease accessibility of prolactin-FA and prolactin-WT in the microsomal fraction was then ascertained by digestion with exogenous proteases. The results of these experiments are shown in Fig. 3. Both WT and FA forms of prolactin were equivalently protected from digestion with exogenous proteases in the absence (lanes 2 and 5) but not the presence (lanes 3 and 6) of detergent. The absolute degree of protease protection varied from 40 to 70% between experiments although in all cases the relative degree of protease protection of prolactin-WT and prolactin-FA was nearly identical. These data, in combination with those presented in Fig. 2, indicate that the described mutation in the NH2-terminal disulfide-bonded loop domain yields a form of prolactin that is efficiently translocated yet remains in a structural state that is unsuitable for trans-

\[ \text{WT} \]
\[ \text{FA} \]

2 R. L. Haynes and C. V. Nicchitta, unpublished observations.
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port through the secretory pathway or retrograde transport to the cytosol.

**FA Forms Protein-Protein and Disulfide-bonded Aggregates**—It has been reported that nascent chains may form protein-protein, as well as disulfide-bonded aggregates, during protein folding in the ER (44, 45, 51). To assess the structural status of translocated WT and FA forms of prolactin, transfected cells were metabolically labeled, chilled, treated with 20 mM NEM to block artificial disulfide bond formation (35), and detergent lysates prepared for analysis by velocity sedimentation (Fig. 4A). In cells expressing prolactin-FA, metabolic labeling was performed in the presence (FA + DTT), or absence (FA - DTT) of 20 mM DTT. It has previously been demonstrated that in the presence of DTT, disulfide bond formation in the ER is blocked, in many cases leading to a reversible dis-

ruption in protein folding (35, 44). Following centrifugation and harvesting of the gradients, prolactin was recovered by immunoprecipitation and analyzed by SDS-PAGE. As shown in Fig. 4A, prolactin-WT was recovered at the top of the gradient, consistent with a monomeric status for the export competent protein. In contrast, prolactin-FA was present as heterogeneous aggregates, widely dispersed through the gradient fractions and displaying sedimentation coefficients of 10–40 S (Fig. 4A; data not shown). The relative distribution of FA was unaffected when labeling was performed under reducing conditions, indicating that protein-protein interactions contribute substantially to aggregate formation. These results clearly indicate that the structural state of the mutant prolactin differs markedly from the wild type. In further studies, in which cells were treated with cross-linking reagents prior to lysis, the ER chaperone BiP was identified in the FA aggregate pool. The use of cross-linking reagents was, however, necessary to identify such interactions.

As prolactin-FA lacks the cysteine pair necessary for formation of the disulfide-bonded NH2-terminal loop, we postulated that protein folding would necessarily be disrupted at the level of disulfide bond formation. To ascertain the state of disulfide bond formation, WT and FA transformants were subjected to a pulse-chase study, and the cell-associated prolactin recovered at 0, 30, and 60 min. Following immunoprecipitation, samples were run on reducing and non-reducing gels. Disulfide bond formation stabilizes protein 3° protein structure, resulting in faster migration of the oxidized proteins on SDS-PAGE (35). This phenomenon was clearly evident with respect to WT-prolactin, in which oxidized prolactin was observed to migrate faster than reduced prolactin on SDS-PAGE gels (Fig. 4B, lanes 1–3 versus lanes 7–9). Prolactin-FA rapidly formed large disulfide-bonded aggregates and, under non-reducing conditions, was preferentially recovered in the stacking gel and stacking gel interface (Fig. 4B, lanes 4–6 versus lanes 10–12). In these experiments, large, disulfide-bonded prolactin-FA aggregates was observed at the zero time point (15-min labeling period), indicating that the formation of disulfide-bonded aggregates was quite rapid.

**Translocation Behavior of pPL-WT and pPL-FA in Vitro**—*In vitro* translocation systems have proven valuable in the identification and analysis of the molecular stages of translocation (5, 31, 36, 52). Having defined the structural basis of the *in vivo* secretion defect observed with prolactin-FA, the translocation and folding behavior of prolactin-WT and prolactin-FA were investigated *in vitro* using both native rough microsomes and reconstituted vesicles lacking luminal proteins.

Depicted in Fig. 5, are the results of an *in vitro* translocation study of the WT and FA forms of pPL. In *in vitro*, secretory protein translocation is commonly assessed by two criteria, signal sequence cleavage and insensitivity of the mature protein to digestion by exogenous proteases. By these criteria, pPL-FA behaves identically to pPL-WT. Thus, when translated in the presence of rough microsomes, both forms are subject to signal sequence cleavage (Fig. 5A, lanes 1, 2, and 4) and mature prolactin, but not the precursor, are protected from digestion with exogenous proteases (Fig. 5A, lanes 3 and 5). Clearly, both *in vivo* and *in vitro*, the protein folding defect associated with prolactin-FA was without effect on translocation.

To determine the structural state of the *in vitro* translocated WT- and FA-prolactin, completed translocation reactions were treated with NEM, solubilized, and the structural status of the nascent chains analyzed by velocity sedimentation. In contrast to the *in vivo* results (Fig. 4A), in which the WT-prolactin was recovered in a fully folded, monomeric state, both WT- and FA-prolactin formed large, heterogeneous aggregates *in vitro*.
fraction number is shown.

of translation product distribution (PhosphorImager PSL units)

distribution. Gels were quantitated by PhosphorImager analysis; a plot

type preprolactin; FA

SDS-PAGE gels. Digital images of the dried gels are depicted.

trated by trichloroacetic acid precipitation and separated on 12.5%

fuged on 8–35% sucrose gradients. Gradient fractions were concen-

fraction recovered by centrifugation through a 0.5 M sucrose cushion.

The membrane fractions were solubilized in detergent, briefly centri-

ated (∼lanes 3 and 5), proteinase K was added to a final concentration of

100 μg/ml and digestions performed for 30 min on ice. Samples were

processed as described under "Materials and Methods" and separated

on SDS-PAGE gels. A digital image of the gel, obtained by Phospho-

rImager analysis, is depicted. p, precursor form; m, mature, signal
cleaved form. B, WT and FA forms of pPL were synthesized in vitro

in the presence of canine pancreas rough microsomes. Following transla-

tion, reactions were treated with 20 mM NEM and the membrane frac-

tion recovered by centrifugation. Membrane fractions were solubilized in detergent and processed on 8–35% sucrose gradients as described in the legend to Fig. 5. Samples were resolved on 12.5% Tris-Tricine gels and digital images of the dried gels, obtained by PhosphorImager analysis, are depicted. A, WT pPL-86-mer, treated with puromycin; B, WT pPL 169-mer, without puromycin treatment; C, pPL 169-mer following puromycin treatment. D, plot of data depicted in A-C.

FIG. 6. Analysis of the structural state of preprolactin translation intermediates. Truncated mRNAs, encoding wild type preprolactin constructs of 86 and 169 amino acids, were translated in reticu-

loge lysate in the presence of RM for 30 min at 25 °C. Where indicated, puromycin was added to 0.5 mM and the reaction continued for 5 min at 25 °C. All reactions were subsequently treated with 20 mM NEM and the membrane fraction recovered by centrifugation. Membrane frac-

tions were solubilized in detergent and processed on 8–35% sucrose gradients as described in the legend to Fig. 5. Samples were resolved on 12.5% Tris-Tricine gels and digital images of the dried gels, obtained by PhosphorImager analysis, are depicted. A, WT pPL-86-mer, treated with puromycin; B, WT pPL 169-mer, without puromycin treatment; C, pPL 169-mer following puromycin treatment. D, plot of data depicted in A-C.

54). For this reason, it was important to determine whether the aggregation process observed in the in vitro system occurs co-translationally or post-translationally, and thus whether aggregation could contribute a driving force to translocation. It is clear from recent studies that many precursor proteins form reversible protein-protein aggregates during early stages of protein folding in the ER (44, 45, 51), although to date, co-

translational aggregation of nascent chains has not been demonstrated.

To evaluate the structural status of translocation intermediates, a series of stable, truncated translocation intermediates was studied. In these experiments, nascent pPL chains of 86 and 169 amino acids were translated from truncated mRNA transscripts. Such transcripts, because they do not possess a termination codon, direct synthesis of the nascent chain, but remain in stable association with the ribosome (55, 56). As depicted in Fig. 6, panels A and D, greater than 80% of mem-

brane-associated 86-amino acid pPL precursor (pPL 86-mer), either in association with the ribosome (not shown) or upon puromycin-induced release into the ER lumen (+ Puro), was recovered at the top of the sucrose gradients, and thus, by these criteria, does not undergo extensive aggregation. pPL 86-mer, when bound to the ribosome, is not a substrate for the signal peptidase and does not extend into the ER lumen (31, 36, 52). pPL 169-mer, in contrast, is of sufficient length to undergo signal peptide cleavage while remaining in association with the

Co-translational Aggregation of Translation Intermediates—The observation that WT-prolactin undergoes substantial aggregation under the experimental conditions used for in vitro translation/translocation prompted immediate concern. Aggregation of incompletely folded nascent polypeptide chains can be a thermodynamically favorable process, and is a commonly observed phenomena in in vitro folding studies (51, 53,
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Consistent with previous results (Fig. 5), pPL-WT, when translated in the presence of RM, is recovered throughout the gradient, with the predominant fraction present at the top of the gradients. When translated in the presence of reconstituted RM, WT-prolactin was preferentially recovered in the aggregate pool, with a distribution markedly similar to that observed for the prolactin-FA folding mutant in vitro (Fig. 4) and in vivo (Fig. 5). In point, when translated in the presence of reconstituted membranes, the translocation behavior and structural state of the WT- and FA-prolactin were very similar, indicating that in the absence of luminal proteins, the protein folding and translocation pathways are markedly altered from the in vivo state (data not shown).

ribosone, and thus has gained access to the ER lumen (34, 57). By comparing the relative migration in sucrose gradients of full-length prolactin, synthesized in vivo (Fig. 4A), with the signal cleaved pPL 169-mer translocation intermediate (Fig. 6, B-D), it is clear that at an early stage of translocation, in vitro translocation nascent chains can enter a heterogeneous aggregate pool. We have so far been unable to identify stable complexes of the translocation intermediates with luminal chaperones, such as BiP or PDI (data not shown). It thus appears that aggregates are forming with partially folded precursors present in the ER lumen during translocation (see “Discussion”). When pL 169-mer intermediates are released from the ribosome prior to solubilization, aggregation was exacerbated, and the protein was recovered in fractions throughout the gradient (Fig. 6, C and D). As expected from previous results, the FA form of pL 169-mer behaved similarly, although displaying a higher propensity for aggregation (data not shown).

Structural State of Nascent Chains in Chaperone-depleted Membranes—The results presented in Figs. 5 and 6 make evident an important point. Although the in vitro translation/translocation system can accurately reproduce the signal cleavage and translocation events that accompany translocation, the structural status of the translocated proteins, be they wild-type or mutant, are significantly different from that observed in vivo. In light of experimental evidence indicating that luminal proteins support both vortical translocation and efficient protein folding, the structural state of nascent chains synthesized in the presence of native RM or vesicles depleted of luminal chaperones was compared. Given the propensity toward aggregation seen in native membranes, we postulated that in the absence of chaperones, aggregation would be exacerbated, and thus might contribute the predominant driving force for translocation. Previously we reported that reconstitution of translocation competent vesicles from detergent-solubilized ER membranes results in the near complete loss of the luminal contents and a marked reduction in the efficiency with which signal-cleaved precursors are fully transported (39, 58). Similar findings are illustrated in panels A and B of Fig. 7. In comparing native and reconstituted RM, both vesicle populations were observed to efficiently mediate translocation to the point of signal sequence cleavage (Fig. 7A, compare lanes 1, 2, and 5). Protease protection studies, a measure of net translocation, demonstrate, however, that the rRM do not translocate the signal-cleaved precursors as efficiently as RM (Fig. 7A, lanes 2 and 3 versus 5 and 6). This observation is further substantiated in the sedimentation studies detailed in Fig. 7B. In the experiment illustrated in Fig. 7B, membrane fractions were isolated from the completed translation reactions by centrifugation, and the pellet and supernatant samples analyzed for the recovery of associated translation products. In the absence of membranes (Fig. 7B, lanes 1, 2, 5, and 6), the nascent precursor (p) is recovered in the supernatant fraction, whereas in the presence of native membranes, the signal cleaved, or mature form (m) co-sediments with membrane fraction (lanes 3 and 4). In contrast, when translations are performed in the presence of reconstituted membranes, substantial quantities of the signal-cleaved, mature protein are recovered in the supernatant fraction (Fig. 7B, lanes 7 and 8). The recovery of the signal-cleaved form of prolactin in the supernatant fraction indicates that the precursor had undergone targeting and translocation to the point of signal sequence cleavage. A substantial fraction of the signal-cleaved precursor, however, transited free from the translocon to the cytoplasm and was thus recovered in the supernatant. The structural state of the prolactin synthesized in the presence of control and reconstituted membranes was further analyzed by velocity sedimentation (Fig. 7C and D).
DISCUSSION

Substantial progress has been made in identifying the protein components that mediate protein translocation in the mammalian ER. It is, however, presently uncertain how the process is energetically driven. Current models suggest either of two mechanisms. In one model, the direct association of the translationally active ribosome with the protein conducting channel is thought to provide a topologically restricted pathway for the nascent chain such that the nascent chain has no topological alternative other than transfer into the ER lumen, or, in the case of membrane proteins, insertion into the ER bilayer (1, 2). In this model, the random, thermal motion of the nascent chain within the protein conducting channel would serve as the driving force. Alternatively, although not exclusively, protein translocation in the mammalian ER may be energetically driven in a manner similar to that proposed for translation in yeast ER as well as for protein import into mitochondria (3, 5, 6, 12, 25). In the latter model, the free energy for transport is derived primarily through transient physical interactions of the nascent chain with luminal, or matrix, molecular chaperones and structural alterations in the nascent chain that accompany translocation in the ER. It has been proposed that such interactions would perform a thermal ratchet function and thereby bias the random motion of the nascent chain to yield vectorial transport (3–5).

Protein folding in the ER occurs coincident with translocation, is accompanied by a significant free energy change, can be readily modified through alterations in the primary protein sequence, and occurs in an environment, the ER lumen, which is highly enriched in molecular chaperones (7, 9–11, 42). It is considered that protein aggregation, as a thermodynamically favorable process, could contribute a driving force to protein translocation. Indeed, extremely rapid heteroaggregate formation, occurring at or prior to chain termination, has been previously reported in cells synthesizing the Semliki Forest virus proteins E1 and p62 (51). For protein-protein aggregation to directly contribute to the energetics of translocation, however, it must occur co-translationally. Through use of truncated preprolactin precursor proteins synthesized in the presence of canine pancreas rough microsomes it was observed that indeed co-translational aggregation can occur. In these experiments, truncated forms of preprolactin, previously demonstrated to comprise defined translocation intermediates (31, 36, 52, 57), were assembled into rough microsomes and the structural state of the nascent chains subsequently determined by velocity sedimentation of a chemically alkylated, detergent-solubilized extract. Because homotypic, co-translational protein-protein interactions are unlikely to occur (7) (although there may be exceptions to this postulate (62)), it is most likely that the aggregate state observed for the truncated prolactin intermediates reflects interactions of the newly synthesized nascent chains with luminal proteins, and/or partially folded or incompletely assembled native proteins which would remain in the microsomal vesicle lumen during tissue isolation and membrane preparation. That we have as yet been unable to identify substantial interactions with the ER luminal proteins suggests that the latter possibility is likely.

Two additional noteworthy observations were obtained in the in vitro system. In studies with the full-length WT and FA translation products, it was observed that translocation proceeded normally, however, a significant fraction of the WT, and the majority of the FA form of prolactin, were recovered as large, heterogeneous aggregates. Thus, although the FA folding defect could be reproduced in vitro, it was also clear that in vitro the WT protein underwent substantial misfolding. Second, in luminal protein-depleted membranes, the efficacy of translocation and folding were markedly reduced (Fig. 7). As previously reported, the loss of the luminal chaperones had little effect on the efficiency of the early stages of translocation, i.e., translocation up to and including signal sequence cleavage (12, 58). When translated in the presence of rough microsomes lacking luminal proteins, the vast majority of the signal-cleaved WT translation products were present as large aggregates. The observation that aggregated, signal-cleaved prolactin could be recovered in the supernatant fraction following sedimentation of the membranes indicates that a significant fraction of the prolactin underwent retrograde transport from the translocon and subsequent aggregation. It should also be noted that the structural state of WT prolactin, synthesized in the luminal protein-depleted membranes, was remarkably similar to that of the folding mutant, FA, synthesized in the presence of native membranes. These data suggest that the luminal proteins likely perform two functions: (i) through interactions with the nascent chain early in translocation, luminal proteins may support unidirectional transport and (ii) interactions of the luminal chaperones with the nascent chains suppress irreversible aggregation reactions and enhance the efficiency of protein folding.

The data presented herein have significant ramifications on investigations into the molecular mechanism of protein translocation. To accurately identify the molecular basis of translocation, it is critical that the experimental system accurately mimic the in vitro scenario. In many regards, the in vitro translation/translocation systems admirably achieve this goal. Analysis of the structural state of the nascent chains indicates, however, that in vitro, translocated proteins may undergo highly aberrant folding processes and accumulate as protein-protein and disulfide-bonded aggregates. Furthermore, such disruptions in protein folding are exacerbated under conditions, such as detergent reconstitution, in which the luminal complement of chaperones and protein folding enzymes are
lost. This is especially problematic for studies of the energetic basis of translocation for which direct roles for luminal proteins in translocation have been identified or implicated. On the basis of these data, we suggest that the criteria for accurate protein translocation be extended to include proper protein folding and/or assembly.

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