The Sec-independent Twin-arginine Translocation System Can Transport Both Tightly Folded and Malfolded Proteins across the Thylakoid Membrane*

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A subset of lumen proteins is transported across the thylakoid membrane by a Sec-independent translocase that recognizes a twin-arginine motif in the targeting signal. A related system operates in bacteria, apparently for the export of redox cofactor-containing proteins. In this report we describe a key feature of this system, the ability to transport folded proteins. The thylakoidal system is able to transport dihydrofolate reductase (DHFR) when an appropriate signal is attached, and the transport efficiency is almost undiminished by the binding of cofate analogs such as methotrexate that cause the protein to fold very tightly. The system is moreover able to transport DHFR into the lumen with methotrexate bound in the active site, demonstrating that the ΔpH-driven transport of large, native structures is possible by this pathway. However, correct folding is not a prerequisite for transport. Truncated, malfolded DHFR can be translocated by this system, as can physiological substrates that are severely malfolded by the incorporation of amino acid analogs.

The translocation of globular proteins across biological membranes is mediated by a variety of sophisticated multi-component systems, several of which have been characterized in some detail. These systems vary significantly in mechanistic terms, but one common theme has emerged in the majority of cases: the substrate proteins are usually translocated in a largely unfolded state. This applies to the transport of proteins across the endoplasmic reticulum (ER), the import of proteins across the outer and inner membranes of both mitochondria and chloroplasts, and the transport of proteins across the bacterial plasma membrane by the Sec machinery (1–5). With most proteins this necessitates the translocation of proteins that lack significant tertiary structure, and this is achieved in different ways by the various translocase systems. In some cases (for example the Sec61p complex in the ER and the Sec machinery in the bacterial plasma membrane), proteins are transported before being able to fold properly, whereas mitochondria and chloroplasts are able to physically unfold proteins once bound to the translocation machinery (6, 7). It should, however, be noted that this common theme does not apply to all membranes, since fully folded proteins can clearly be transported into peroxisomes (8).

Recent studies have pointed to the existence of a protein translocase that may use a very different mechanism, and which appears to be extremely widespread. In chloroplasts, a subset of proteins are translocated across the thylakoid membrane by an ATP-dependent Sec system that appears to be similar in most respects to prokaryotic Sec systems (9–11). Other proteins, however, are transported by a mechanism that does not require NTPs but which is completely dependent on the thylakoidal ΔpH (12–16). Surprisingly, substrates for the ΔpH-dependent pathway are synthesized with N-terminal targeting signals (transfer peptides) that resemble classical Sec-type “signal” peptides in overall structure but which nevertheless direct highly specific targeting by the ΔpH-dependent route (15, 17). An essential targeting determinant for this route is the presence of a twin-arginine motif in the N-terminal region of the transfer peptide (18).

There are now clear indications that a similar system operates in a wide range of bacteria. The first component of the ΔpH-dependent system has recently been identified (termed Hcf106 (19)) following the isolation of a maize mutant defective in this targeting pathway (20), and proteins homologous to Hcf106 are encoded in previously unassigned genes in most bacterial genomes sequenced to date. In Escherichia coli, Hcf106 homologs are encoded by two distinct genes, one of which is the first gene of a four-gene operon, whereas the other is unlinked. Both gene products have recently been shown by Sargent et al. (21) to be required for the Sec-independent export of a range of proteins bearing twin-arginine presequences. Sec-dependent export was shown to be unaffected in strains in which these genes were disrupted, demonstrating the operation of a separate translocation pathway in prokaryotes, and the genes were designated tatA (in a putative tatABCD operon) and tatE (for twin-arginine translocation pathway). Another recent study (22) confirmed the importance of the tatB gene in this export process.

The bacterial Sec-independent translocase has not been studied at the biochemical level, but considerations of its apparent substrate specificity, together with known details of the thylakoidal system, raise the possibility of an unprecedented translocation mechanism. The predicted substrates for the bacterial system include a variety of periplasmic proteins that bind complex redox cofactors, including FeS and molybdopterin centers (23, 24). These cofactors are probably inserted in the cytoplasm of bacteria, requiring the correct folding of at least some of the proteins and hence suggesting an unusual export mechanism in which a range of large globular proteins are transported in a fully folded state. Consistent with this possibility, it has been shown that the stromal substrate for the thylakoidal ΔpH-driven system comprises a monomeric polypeptide that is probably correctly folded (25). However, this study did not resolve whether the substrate was transported in

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1 The abbreviations used are: ER, endoplasmic reticulum; DHFR, dihydrofolate reductase; PCR, polymerase chain reaction; MTX, methotrexate.
a folded state or whether the ΔpH was harnessed to drive concomitant translocation and unfolding. It has furthermore been shown that bovine pancreatic trypsin inhibitor can be translocated into the lumen in a folded state by this pathway (26), although this protein is at the lower end of the size spectrum for globular proteins. In the present study we present evidence that an average size, tightly folded passenger protein can be transported by this pathway in a native state. We also show that malfolded proteins can be targeted by this system indicating that correct folding is not a prerequisite for translocation.

**EXPERIMENTAL PROCEDURES**

23K-DHFR Construct—23K-DHFR constructs were made by fusing the coding sequence of pea pre-23K (27) in front of that encoding a cytochrome b₆ linker together with mouse DHFR (28). The fusion of the entire coding region of pea 23K to a pre-existing fusion of cytochrome b₆ and DHFR was performed in two stages. First, cDNA for the pea 23K polypeptide of the oxygen evolving complex of photosystem II (GenBank™ accession number X15552) was amplified by tag PCR. Unique restriction sites were also introduced during the PCR. The upstream oligonucleotide (GAATTCATATGGCATCTACACAGGAGACTTTG) introduced an AvaI site over the 23K stop codon. This PCR fragment, representing the entire coding region of pea 23K except the stop codon, was introduced into the EcoRI/AvaI sites of the pGEM4Z expression vector. The cytochrome b₆-DHFR fusion was kindly supplied in pGEM-4Z by Dr. R. Stuart (Munich, Germany). cDNA for this fusion was cut upstream using a unique AvaI restriction site at position 52 in the cytochrome b₆ sequence. The downstream restriction site was the HindIII site, originally used to ligate this fragment into the pGEM vector. This restricted fragment of cytochrome b₆-DHFR was ligated into the AvaI/HindIII sites of the pGEM-4Z expression vector carrying the 23K fragment to form the 23K-DHFR fusion. The linkage regions of this fusion have been confirmed by DNA sequence analysis.

Construction of the Truncated 23K-DHFR Fusion Protein and and Expression of 33K-DHFR—The cDNA for the 23K-DHFR fusion protein was amplified using pwo PCR. The upstream primer (CATGCGCATG-GCATCTACACAAATTGTTTC) introduced a unique NcoI site across the start codon for the fusion protein. The downstream oligonucleotide (GAAGATCCACCTTTAAGAAGCTTCTCTCACA) introduced a BglII restriction site XhoI base pairs upstream of the DHFR stop codon. The PCR fragment was ligated into the NcoI/BglII sites of the pQE-60 overexpression vector (Qiagen). For *in vitro* transcription and translation, the entire overexpression cassette was removed from the pQE-60 vector by restriction with EcoRI/BamHI and ligated into pGEM-4Z (Promega). The resulting translation product represents the 23K-DHFR fusion protein with a 77-residue C-terminal truncation and a C-terminal hexa-His-tag. The presence of the 6xHis-tag has no effect on the stability or import competence of the protein (*data not shown*). The 33K-DHFR construct contained the presequence of wheat 33K, together with 13 residues of mature 33K protein, linked to mouse DHFR; its construction is described (29).

The constructs were synthesized *in vitro* by transcription using SP6 RNA polymerase followed by translation in a wheat germ cell-free system. Precursor proteins were imported into pea chloroplasts or isolated thylakoids as described (12, 30). Amino acid analogs (Sigma) were incorporated into nascent chains by including 10 mM analog in the translation system as described (31).

**RESULTS**

Construction of Chimeric 23K-DHFR Proteins—Our tests on the mechanism of the ΔpH-dependent system involved the use of a passenger protein, dihydrofolate reductase (DHFR), which has been used for numerous studies in other systems. Binding of the folate analogs methotrexate (MTX) or aminopterin causes DHFR to fold extremely tightly, to the extent that translocation by the mitochondrial import machinery was found to be blocked due to an inability to unfold the protein (7). The bacterial and thylakoidal Sec systems are similarly unable to transport DHFR that has been stabilized in this way (5, 32). In contrast, DHFR-containing constructs can be translocated across the chloroplast envelope in the presence of MTX due to the powerful unfolding action of this system, which leads to the removal of the MTX and subsequent translocation (6). For tests on the thylakoidal ΔpH-dependent system, we used a chimera in which the entire precursor of the pea 23 kDa oxygen-evolving complex protein (23K) was linked to mouse DHFR via a short linker derived from cytochrome b₆. Fig. 1A shows the overall structure of this 23K-DHFR construct, and Fig. 1B demonstrates that the DHFR moiety is indeed stabilized by the binding of these analogs. Binding of aminopterin results in a characteristic increase in protease resistance, and this figure shows that, whereas incubation of 23K-DHFR with a high concentration of trypsin normally results in complete digestion of the construct, the presence of low concentrations of aminopterin renders the DHFR domain completely resistant to digestion. The 56-kDa construct is digested quantitatively to a lower molecular mass polypeptide, and other tests (not shown) have confirmed that this polypeptide has an identical electrophoretic mobility to authentic DHFR. Identical results were obtained using MTX as ligand (not shown).

As well as testing the ability of the thylakoidal translocase to transport tightly folded proteins, we also sought to determine whether folding is in fact a prerequisite for translocation, for reasons detailed below. For this purpose we inserted a stop codon 77 residues from the C terminus of DHFR in the expectation that folding would be drastically affected. Fig. 1C shows this to be the case because the construct is totally digested by trypsin even in the presence of high concentrations of aminopterin.

**23K-DHFR Is Targeted Efficiently and Specifically by the ΔpH-dependent Pathway**—We first tested whether 23K-DHFR behaves as a typical substrate for the ΔpH-dependent system, and Fig. 2 shows chloroplast import experiments confirming that this construct is targeted efficiently and exclusively by this
pathway. In the control experiment, 23K-DHFR is imported into the organelles and converted to two polypeptides as follows: an intermediate size protein that is recovered in the stromal fraction (lane S), and a smaller protein found in the thylakoid fraction (T) that is resistant to protease digestion of the membranes (7+). Pre-23K thus directs correct targeting of the attached DHFR into the lumen, where the thylakoid transfer peptide is removed by the lumen-facing thylakoidal processing peptidase. The stromal intermediate presumably reflects the action of stromal processing peptidase, which removes the initial “envelope transit” domain of the bipartite 23K presequence (33). The remaining panels indicate that the DHFR is being targeted solely by the ΔpH-dependent pathway. Translocation across the thylakoid membrane is blocked by the proton ionophore nigericin, which dissipates the proton gradient (Fig. 2). The precursor, intermediate, and mature forms of the construct are indicated (pre, int, and mat).

Aminopterin Does Not Block the Targeting of 23K-DHFR into the Thylakoid Lumen—To test the effects of stabilizing 23K-DHFR we carried out chloroplast import experiments in the presence of 10 μM aminopterin. The DHFR domain is probably unfolded, and the aminopterin thus released, upon translocation into the chloroplast, but aminopterin is membrane-permeable, and 10 μM represents a massive excess since 5–10 nm was found to stabilize the DHFR domain as shown in Fig. 1. Fig. 3 shows that this compound does not prevent targeting into the thylakoid lumen, because mature size 23K-DHFR accumulates in the thylakoid lumen to approximately the same extent as in the absence of analog.

The above data suggest that DHFR can be transported into the lumen despite binding aminopterin, but we considered it possible (although unlikely) that these 23K-DHFR molecules may be transported across the thylakoid membrane before being able to bind the analog. We therefore used assays for the import of proteins by isolated thylakoids (30) as shown in Fig. 4A. After incubation with thylakoids in the absence of MTX, a significant proportion of 23K-DHFR is processed to the mature size (lane Im). The mature size protein is found predominantly in the thylakoid fraction where it is resistant to digestion by thermolysin (lane T+) indicating that import has taken place. Samples of the supernatant (Sn) show only a small quantity of mature size protein. It is, however, surprising that this sample should contain any mature protein since thylakoidal processing peptidase faces the luminal side of the thylakoid membrane. This polypeptide species may therefore result from translocation events that are not correctly completed or from degradation during the import incubation.

Significantly, import efficiency is essentially unaffected by preincubation of the 23K-DHFR with a vast excess of MTX, such that the DHFR domain is quantitatively stabilized. These data provide clear evidence that the thylakoidal ΔpH-dependent system is able to translocate proteins that are very tightly folded.

The above data raise the important question as to whether the proteins remain folded during the translocation process. To address this question we removed free MTX from the translation mixture prior to import into thylakoids, and we subsequently tested whether the imported luminal DHFR domain is resistant to digestion by trypsin. This would indicate that the MTX had been translocated across the thylakoid membrane while bound to the 23K-DHFR. The 23K-DHFR translation mixture was incubated with the minimum concentration of MTX required to stabilize the DHFR, after which it was passed down a desalting column (see “Experimental Procedures”) and the high molecular weight eluate used in an import experiment similar to that shown above. Control experiments (not shown) confirm that the DHFR is eluted with MTX still bound, since the 23K-DHFR is converted by trypsin to the characteristic DHFR polypeptide. However, this fraction does not contain a significant concentration of free MTX; in a separate control the procedure was repeated using unlabeled translation mixture after which the eluate was incubated with labeled 23K-DHFR. No stabilization of the DHFR was observed, indicating that the free MTX is effectively removed by the column (not shown).

Thylakoid import experiments using desalted translation products are shown in Fig. 4B. A significant proportion of precursor is imported and processed to the mature size after incubation with MTX, and a similar level of import is observed in the mock-treated control (−MTX). Samples of the thermolysin-treated thylakoids were then sonicated and centrifuged to release the lumen contents, and the supernatant fractions were incubated with 0.4 mg/ml trypsin (lane Th+T+). The figure shows that the luminal construct that had been preincubated with MTX is converted to DHFR, whereas “free” construct is quantitatively degraded. These samples were obtained from thylakoids equivalent to three times that used in the remaining lanes, and quantitation of the data reveals that 10.5% of the mature 23K-DHFR in the thermolysin-treated thylakoids is converted to mature DHFR in the “+MTX” sample. In the
control sample lacking MTX hardly any mature size DHFR is generated as expected. These data indicate that at least some of the 23K-DHFR crosses the thylakoid membrane with MTX bound in the active site. However, it has not proved possible to gauge the precise extent to which this takes place, due to technical problems inherent in this experiment. We have found that trypsin activity is altered in the presence of thylakoid membranes, such that the protease becomes more active and is able to digest even DHFR that is known to be complexed with MTX (not shown). The characteristics of the luminal 23K-DHFR could therefore only be tested after sonication of the thylakoids and subsequent centrifugation to remove the membranes. This procedure may result in the loss of some of the bound MTX and an underestimation of the extent to which the luminal 23K-DHFR is complexed with MTX. In addition, not all of the luminal 23K-DHFR is released by the sonication; about a third of the protein remains trapped in the vesicles. Nevertheless, the data show that the translocation of folded proteins is possible by this system.

Stabilization of DHFR Blocks Translocation by the Sec Pathway—The above data indicate that the ΔpH-driven translocase has the unusual ability to translocate DHFR even when stabilized by folate analogs. Previous studies (31) have shown that the binding of MTX prevents translocation of DHFR across the thylakoid membrane by the Sec pathway when a plastocyanin presequence is attached. However, we considered it important to determine whether this held true under our experimental conditions, since this would provide further evidence of the unusual characteristics of the ΔpH-dependent system. This was carried out using a construct, 33K-DHFR, comprising the presequence and first 13 amino acids of wheat 33K (another Sec substrate) linked to the DHFR protein used in the 23K-DHFR constructs. Fig. 5 shows that in the control tests this chimera is imported into chloroplasts and processed to a stromal intermediate form and a luminal “mature” form, as found in a previous study (29). In the presence of aminopterin, however, translocation across the thylakoid membrane is almost totally blocked, and the stromal form accumulates together with another smaller polypeptide that probably results from degradation of the stromal intermediate form (35). These data confirm that the DHFR protein is very tightly folded when complexed with aminopterin, and the data are consistent with the premise that the translocation capabilities of the Sec- and ΔpH-dependent systems are completely different.

Correct Folding Is Not a Prerequisite for Translocation by the ΔpH-dependent Pathway—The structure of the ΔpH-driven system remains to be characterized, but there is already clear evidence that an analogous system is used for the export of fully folded proteins in bacteria. Furthermore, there are suggestions that the bacterial systems possess a “proofreading” capability whereby the export of incompletely folded proteins is actively prevented (see “Discussion”). We therefore considered it possible that the thylakoid system may be incapable of efficiently transporting globular proteins that are malfolded. This possibility was raised in a previous study (36) following the observation that C-terminal truncations of pre-23K affected targeting into the thylakoid lumen. However, the data in this study were complicated by the apparent rapid turnover of the imported 23K mutants. We addressed this possibility in two ways. First, we used a truncated form of 23K-DHFR lacking the C-terminal 77 residues (see Fig. 1) that clearly contains a malfolded domain because the entire construct (23K-DHFRΔC) becomes highly sensitive to proteolysis even in the presence of high concentrations of MTX. Fig. 6 shows that this deletion has essentially no effect on the targeting of the construct into the lumen; the ratio of stromal intermediate to mature luminal form is very similar to those observed in import experiments using the full-length construct (see Fig. 2). Fortunately, the luminal form is not subject to rapid turnover, enabling us to conclude that severely malfolded proteins can be translocated by this pathway.

Although this experiment shows the transport of a malfolded protein, the construct used in this study does still contain a (presumably) correctly folded 23K domain in front of the DHFR domain, which may somehow enable an attached, malfolded DHFR domain to be translocated under unusual circumstances. We therefore sought to destabilize the entire construct, and this was achieved by the incorporation of amino acid analogs into the construct during translation. Several analogs have been found to be incorporated into nascent proteins in vitro in place of normal amino acids, and this procedure has been used in previous studies to study the importance of particular side chains at given positions (e.g. Ref. 31). Several analogs were tested, and Fig. 7 shows the effects of incorporating β-hydroxyornithine in place of threonine. Both the 23K and DHFR domains contain numerous threonine residues, and we...
reasoned that replacement of these would lead to a marked change in structure. This is confirmed by the upper panel of Fig. 7. In Fig. 7A, 23K-DHFR was preincubated in the presence of MTX and then incubated with thermolysin to test whether the DHFR domain is resistant and hence correctly folded. The “normal” construct is converted to the DHFR polypeptide, but the analog-containing version is digested to a series of low molecular mass products, confirming a substantial change in structure. This is confirmed by the formation of an 18-kDa degradation product which is then degraded further.

Fig. 7B shows chloroplast import experiments using analog-containing 23K-DHFR. In this experiment the “control” construct was imported and sorted with high efficiency. Some stromal intermediate form is apparent, suggesting that transport across the thylakoid membrane is inhibited to some extent. More importantly, these data show that the import efficiency is probably adversely affected by the incorporation of the analog but not to a marked extent. More importantly, these data show that the incorporation of the analog does not block transport into the thylakoid lumen. The similarity of the 23K-DHFR constructs to the analog-containing 23K-DHFR was synthesized in a wheat germ lysate as normal control or in the presence of 10 mM β-hydroxyvaline, a threonine analog. A, after synthesis, samples were incubated with thermolysin for 40 min on ice at the concentrations indicated. The mobility of mature size DHFR is indicated. B, samples of the translation mixtures were incubated with intact chloroplasts, after which samples were analyzed as described in Fig. 3. DP, degradation product; other abbreviations as in Fig. 3.

DISCUSSION

In this report we have sought to test the properties and preferences of the ΔpH-driven translocase using DHFR constructs that appear to be targeted into the lumen by the normal

FIG. 6. Translocation of a malfolded DHFR domain by the ΔpH-dependent pathway. A 23K-DHFR construct containing a stop codon 77 residues from the C-terminus (see “Experimental Procedures”) was expressed by transcription-translation and imported into chloroplasts. After incubation, samples were analyzed of the chloroplasts and subfractions as detailed in Fig. 3.

FIG. 7. 23K-DHFR is still targeted into the lumen despite structural destabilization by the incorporation of an amino acid analog. 23K-DHFR was synthesized in a wheat germ lysate as normal control or in the presence of 10 mM β-hydroxyvaline, a threonine analog. A, after synthesis, samples were incubated with thermolysin for 40 min on ice at the concentrations indicated. The mobility of mature size DHFR is indicated. B, samples of the translation mixtures were incubated with intact chloroplasts, after which samples were analyzed as described in Fig. 3. DP, degradation product; other abbreviations as in Fig. 3.

FIG. 8. Destabilization of the 23K mature protein conformation slows down but does not block import into thylakoids. Wheat pre-23K was synthesized under control conditions or in the presence of 10 mM β-hydroxyvaline (+analog) as described in Fig. 7 for the 23K-DHFR construct. A, samples of the translation products (T) were incubated with trypsin at the indicated concentrations for 40 min on ice. DP denotes degradation product. B, the translation products were incubated with isolated pea thylakoids and samples transferred to ice after 1, 5, and 10 min. These samples were analyzed directly or after treatment with thermolysin (+therm.).

have shown that incubation of wheat pre-23K with appropriate concentrations of trypsin or thermolysin leads to the rapid formation of an ~22-kDa degradation product which is then highly resistant to further degradation unless the protein is deliberately unfolded. These data suggested that pre-23K comprises an extended presequence together with a tightly folded mature protein. Fig. 8A shows that this degradation product is generated by 5 μg/ml trypsin in the control experiment, whereas analog-containing pre-23K is quantitatively digested. These data confirm that the analogs markedly destabilize the 23K protein, and essentially identical results were obtained using pea pre-23K (not shown). Since the 23K-DHFR construct contains the same pea pre-23K sequence, we can conclude that both the 23K and DHFR domains in the 23K-DHFR construct were destabilized in the previous experiment (Fig. 7), but as a final test on the preferences of the translocase, we tested whether pre-23K could be imported when destabilized as shown in Fig. 8A. Normal and analog-containing wheat pre-23K were incubated with isolated thylakoids, and samples were analyzed at early time points as shown in Fig. 8B. The data show rapid import in the control experiment, to the extent that import is almost complete after 5 min. Importantly, the analog-containing pre-23K is also imported despite being severely malfolded as shown in Fig. 8A. The import efficiency appears to be lowered because some precursor remains evident in this time course analysis. However, most of this residual precursor remains import-incompetent even after 60 min (not shown), which suggests that it might be aggregated. We therefore conclude that the import efficiency is probably adversely affected by the incorporation of the analog but not to a marked extent. More importantly, these data show that this system is able to transport proteins that are severely malfolded.
The Substrate for This Translocase Can Be Fully Folded Prior to Translocation—Previous studies on the import of pre-23K into thylakoids suggested that the substrate for this system comprises an extended targeting signal together with a correctly folded mature protein (25). Whereas the construct used in this study is not a physiological substrate, our data provide strong evidence that this premise is correct, because the system is able to import DHFR that has bound a substrate analog in the active site, and which is thus almost certainly correctly folded prior to translocation. These data represent the most conclusive evidence to date that soluble factors or chaperone molecules play no role in the normal translocation mechanism and that the ΔpH-dependent system is able to import substrates in which the mature proteins are fully folded prior to the translocation process. Other studies (12, 13) have shown that stromal extracts are not required for the operation of this system, but these studies did not demonstrate unequivocally that the passenger protein was correctly folded or that soluble chaperone molecules in the translation system played some role. We should, however, point out that soluble factors may well play a role in the targeting of some substrates for this system, possible examples being those proteins that bind redox cofactors (see below).

The ΔpH-dependent System Is Capable of Translocating Tightly Folded Proteins—The ΔpH-dependent translocase has the ability to transport a passenger protein that is tightly folded. The binding of folate analogs essentially blocks the transport of DHFR across the ER, bacterial plasma membrane, and mitochondrial membranes, and although this protein can be imported into chloroplasts, it has been shown that the import process is slowed to a significant extent (35), presumably while the import machinery unfolds the DHFR domain. In contrast, thylakoid import assays have shown that the 23K-DHFR construct is imported with very similar efficiencies irrespective of whether the DHFR domain is stabilized with folate analogs.

Our data furthermore indicate that at least some of the DHFR molecules remain in a correctly folded conformation throughout the entire translocation process. Some of the luminal form is resistant to trypsin treatments that totally degrade DHFR that is not complexed with aminopterin, indicating that the aminopterin has remained attached to the DHFR during translocation across the thylakoid membrane. Any significant unfolding of the DHFR would almost inevitably result in the loss of bound aminopterin, as found in studies on chloroplast protein import where the aminopterin or methotrexate is lost from the DHFR prior to or during transport across the envelope membranes (35). We therefore believe that this system is capable of transporting fully folded proteins, although we reiterate the point made earlier that we cannot exclude the possibility that a significant proportion of the transported molecules are in fact unfolded during the translocation process. It remains to be determined whether proteins are normally transported in a folded state, but our data nevertheless provide a rationale for the existence of a separate, Sec-independent system: the ability to transport proteins that are too tightly folded for the Sec system to handle or which require folding in the stroma.

This is not the first demonstration that the ΔpH-dependent system can transport a folded protein. Clark and Theg (26) also addressed this point using a construct in which a ΔpH-specific presequence was attached to bovine pancreatic trypsin inhibitor, which could be folded using internal cross-links. This protein was clearly transported across the thylakoid membrane in a folded state. However, the construct was also efficiently transported across the chloroplast envelope membranes, and several previous studies have shown that this is normally achieved in an unfolded state (3, 35). It is possible that bovine pancreatic trypsin inhibitor is not an ideal subject for this type of study because it is simply too small (6.5 kDa, with a diameter of approximately 2.3 nm), and “typical” protein translocases may therefore be able to accommodate this size of folded domain during the translocation process. Our data therefore serve to confirm and extend the findings of Clark and Theg (26), in that the DHFR passenger protein is significantly unfolded.

Correct Folding Is Not a Prerequisite for Transport by the ΔpH-dependent Pathway—The question of substrate folding is particularly relevant in the case of the ΔpH-dependent translocase. The available evidence suggests that chloroplasts and bacteria possess twin-arginine-dependent protein translocases that are similar in both structural and mechanistic terms (19, 21, 22). The primary substrates in bacteria appear to be precursors of periplasmic proteins that bind various redox cofactors such as FeS or molybdopterin centers (23, 24). These cofactors are apparently inserted in the cytoplasm (19, 21, 22), necessitating folding of the mature protein and, by implication, the export of a fully folded protein. Furthermore, there is evidence that export is blocked when cofactor insertion is prevented (reviewed in Ref. 24), raising the fascinating possibility that correct folding is not only allowed but is in fact a prerequisite for export by this pathway. This of course makes sense because export could otherwise proceed before the cofactor was inserted, but such a proofreading ability would be unique among known translocation mechanisms. Nevertheless, we have shown that correct folding is not a prerequisite for the transport of at least some proteins by the thylakoidal system because both 23K and DHFR can be efficiently transported when the mature proteins are destabilized by the introduction of an amino acid analog or by a large C-terminal truncation. We speculate that any proofreading system may be linked with the insertion of cofactors.

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