The Rieske Fe/S Protein of the Cytochrome b6/f Complex in Chloroplasts

MISSING LINK IN THE EVOLUTION OF PROTEIN TRANSPORT PATHWAYS IN CHLOROPLASTS?

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The Rieske Fe/S protein, a nuclear-encoded subunit of the cytochrome b6/f complex in chloroplasts, is retarded in the stromal space after import into the chloroplast and only slowly translocated further into the thylakoid membrane system. As shown by the sensitivity to nigericin and to specific competitor proteins, thylakoid transport takes place by the ΔpH-dependent TAT pathway. The Rieske protein is an atypical TAT substrate, however. It is only the second integral membrane protein shown to utilize this pathway, and it is the first authentic substrate without a cleavable signal peptide. Transport is instead mediated by the NH2-terminal membrane anchor, which lacks, however, the twin-arginine motif indicative of ΔpH/TAT-dependent transport signals. Furthermore, transport is affected by sodium azide as well as by competitor proteins for the Sec pathway in chloroplasts, demonstrating for the first time some cross-talk of the two pathways. This might take place in the stroma where the Rieske protein accumulates after import in several complexes of high molecular mass, among which the cpn60 complex is the most prominent. These unique features suggest that the Rieske protein represents an intermediate or early state in the evolution of the thylakoidal protein transport pathways.

of the mature polypeptide chain that comprises the membrane anchor (8).

To date, four independent pathways have been identified that are each specific for the transport of a subset of thylakoid proteins into or across the thylakoid membrane (summarized in Refs. 9 and 10). According to their transport mechanism, they are described as Sec-dependent, ΔpH/TAT1 (twin arginine translocation)-dependent, SRP-dependent, and spontaneous pathway, respectively. Among these, the ΔpH/TAT pathway has received particular attention in the past few years because of its unique mechanism; it does not require soluble factors nor nucleoside triphosphates but depends solely on the transthylakoidal proton gradient (11–13). In contrast to the other pathways, it is furthermore capable of translocating not only unfolded polypeptide chains but also folded protein domains across the membrane (14–16).

In bacteria, a phylogenetically related protein transport pathway exists that shows a strikingly similar substrate specificity (17–19). This pathway is responsible for the export of a group of periplasmic proteins carrying complex redox cofactors (20, 21). Assembly of these cofactors takes place in the cytoplasm, i.e. the proteins must fold at least partially prior to membrane translocation (22, 23). Since the thylakoidal Rieske protein also carries a redox cofactor in its native conformation, it is appealing to speculate that it likewise might be targeted in a folded conformation by the ΔpH/TAT-dependent pathway across the thylakoid membrane. However, the thylakoid transport signal of the Rieske protein does not resemble ΔpH/TAT-specific targeting signals. It is not a cleavable signal peptide but operates as a signal anchor domain (8), and furthermore, it lacks the indicative twin-R motif (24), which even accounts for the denomination of the entire pathway.

Here we show that the Rieske protein of higher plant chloroplasts is transported with a mechanism that is unique among all thylakoid proteins characterized so far. Although specifically translocated by the ΔpH/TAT-dependent pathway across the thylakoid membrane, the targeting process is characterized by a number of unusual features including the involvement of components from the Sec-dependent protein transport route. This suggests that the Rieske protein might represent an ancient or intermediate state in the evolution of transport pathways at the thylakoid membrane in chloroplasts.

EXPERIMENTAL PROCEDURES

Materials—Spinach (Spinacia oleracea var. Lina) was grown in hydroponic culture under constant temperature (18–22 °C) and light regime (8/16 h light/dark cycles) and harvested 2–3 months after sowing.

1 The abbreviation used is: TAT, twin arginine translocation.
Pea seedlings (Pisum sativum var. Feltham First) were grown for 8–10 days under a 16-h photoperiod.

**Protein Import into Isolated Chloroplasts**—Precursor proteins were synthesized by *in vitro* transcription of the corresponding cDNA clones and subsequent *in vitro* translation in cell-free rabbit reticulocyte lysates in the presence of [35S]methionine. Intact chloroplasts were isolated from pea or spinach leaves by Percoll gradient centrifugation essentially as described (25, 26). They were used in protein transport experiments following the protocol in Ref. 27. Competition experiments were performed with precursor proteins that were obtained by overexpression in Escherichia coli (28) and recovered from inclusion bodies by solubilization in a buffer containing 7 M urea, 30 mM HEPES, pH 8.0, and 2 mM EDTA. The solubilized proteins were included in the import assays at concentrations up to 4 μM, taking care that the concentration of urea in the assays never exceeded 300 mM. Control assays contained the same amount of buffer lacking any such solubilized protein.

**Miscellaneous**—Gel electrophoresis of proteins under denaturing conditions was carried out according to Ref. 29. Blue native gel electrophoresis (30, 31) was performed according to the protocol in Ref. 32. The gels were exposed to PhosphorImager screens (Molecular Dynamics) and analyzed using the software package ImageQuant (version 1.2). Western blot analysis was performed according to Ref. 33 except that the blue native gels were soaked for 5 min at 40°C in transfer buffer containing 1% SDS and 14 mM β-mercaptoethanol prior to transfer onto polyvinylidene difluoride membranes. Stromal protein complexes were separated under nondenaturing conditions (34) using a running buffer of 45 mM Tris and 45 mM boric acid. All other methods followed the protocols of Sambrook et al. (35).

**RESULTS**

**Thylakoid Targeting of the Rieske Protein Is Retarded in the Chloroplast Stroma**—A striking feature in the targeting process of the Rieske protein is the remarkably slow sorting of the protein within the chloroplast to its final destination, the thylakoid membrane system. In experiments with intact chloroplasts isolated from spinach, only about 30% of the Rieske protein that is imported into the organelle reaches the thylakoids during the incubation period of 20 min (Fig. 1). The majority of the protein accumulates in the stroma where it is processed to the mature polypeptide of ~20 kDa. This behavior is even more pronounced if pea chloroplasts are used in the experiment. In this instance, usually more than 90% of the imported Rieske protein remains in the stroma during the incubation process (Fig. 1). Such retardation in the intraorganelar sorting and targeting process is unique among all thylakoid proteins analyzed so far. Usually, these proteins arrive at their target membrane almost quantitatively under these conditions (e.g. Ref. 27).

After the Rieske protein has reached the thylakoids, it is to a large extent protected against the activity of proteases that are added externally to reisolated thylakoid vesicles, which indicates that the COOH-terminal hydrophilic domain has been completely translocated into the luminal space (Fig. 1). This fraction of the Rieske protein is furthermore correctly assembled into the cytochrome b$_{6f}$/complex after import, as demonstrated by its co-migration with the native cytochrome complex during blue native polyacrylamide gel electrophoresis (Fig. 2). Thus, whereas the protein is only slowly targeted to the thylakoid system after import into the organelle, its subsequent assembly into the cytochrome b$_{6f}$/complex apparently is an efficient process.

**The Rieske Protein Is Targeted by the ΔpH-dependent TAT Pathway to the Thylakoids**—Similar to a few other proteins, e.g. PSI-F (36, 37), the Rieske protein depends on intact chloroplasts for its thylakoid transport and is not able to translocate into isolated thylakoid vesicles obtained after osmotic lysis of the organelles (data not shown). However, it is possible in experiments with intact organelles to demonstrate that the Rieske protein is targeted across the thylakoid membrane by the ΔpH-dependent TAT pathway. In the presence of nigericin, an ionophore that dissipates the transthylakoidal proton gradient, thylakoid translocation of the Rieske protein is almost abolished, and only minute amounts are found associated with the thylakoid membrane (Fig. 3). Transport inhibition cannot be cured by supplementing the assays with increased amounts of ATP, in contrast to the findings for Sec-dependent transport (38), proving that it is the proton gradient itself (rather than its influence on ATP synthesis) that is required for the translocation process.

Transport of the Rieske protein is furthermore impaired by saturating amounts of TAT-specific competitor proteins such as the precursor of the 23-kDa subunit of the oxygen-evolving system. Raising the concentration of competitor in the assays decreases the thylakoid transport of the Rieske protein until at a competitor concentration of 0.5 μM, the protein accumulates almost quantitatively in the stromal space after import (Fig. 4). This demonstrates that the Rieske and the 23-kDa protein depend on the same translocation machinery for their transport across the thylakoid membrane. Sec-dependent protein transport (which was analyzed in parallel) is not affected under these conditions (Ref. 39 and data not shown), confirming that competition was pathway-specific.

**Transport of the Rieske Protein Involves Components of the Sec Pathway in Chloroplasts**—While the results so far clearly demonstrate transport of the Rieske protein by the ΔpH/TAT pathway, other features of the transport process suggest some deviation from this route. In the presence of sodium azide, a potent inhibitor of Sec-dependent protein transport in prokaryotes and chloroplasts (40–42), thylakoid transport of the Rieske protein is affected (Fig. 5A). The inhibitory effect is
moderate but still specific because transport of typical ΔpH/TAT substrates such as the 23-kDa subunit of the oxygen-evolving system is not disturbed by the antimetabolite (Fig. 5B). At first glance, this suggests the involvement of SecA in the transport process, the azide-sensitive translocation ATPase of the Sec pathway (40). However, it should be considered that sodium azide is not only an inhibitor of SecA function but is able to impair the activity of numerous other nucleotide-binding proteins (e.g. Ref. 43). This is particularly relevant here because spinach chloroplasts, which were used in our experiments, are known to harbor a SecA protein that is largely azide-resistant (44).

We therefore performed competition experiments as a complementary approach to examine the possible involvement of the Sec machinery in Rieske transport. In these experiments, the thylakoidal Sec pathway was saturated by excess amounts of the precursor of the 33-kDa subunit of the oxygen-evolving system (B). For further details, see the legends for Figs. 1 and 4. m, mature polypeptide; t, translation assay; s, stroma.

FIG. 5. The SecA inhibitor sodium azide affects the thylakoid transfer of the Rieske protein. Import experiments were performed in the presence or absence of 10 mM sodium azide with the precursors (p) of either the Rieske protein (A) or the 23-kDa subunit of the oxygen-evolving system (B). For further details, see the legend for Fig. 1. m, mature polypeptide; t, translation assay; s, stroma.

FIG. 4. Saturation of the ΔpH/TAT-dependent pathway totally inhibits transport of the Rieske protein across the thylakoid membrane. Import experiments were performed in the presence of increasing amounts of precursor (p) of the 23-kDa subunit of the oxygen-evolving system that were obtained by overexpression in E. coli. The concentration of competitor protein (in μM) present in each assay is indicated above the lanes. The portion of the Rieske protein that is translocated across the thylakoid membrane under these conditions (in terms of percentage of protein imported into the organelle) is given below each panel. For further details, see the legend for Fig. 1. m, mature polypeptide; t, translation assay; s, stroma.
expected, only the Sec pathway is saturated under these conditions, as demonstrated by the appearance of the stromal intermediate of the 33-kDa protein (Fig. 6B). In contrast, the ΔpH-dependent thylakoid transport route is not affected by the Sec competitor. Only transport of the 23-kDa protein across the chloroplast envelope is impaired by the competitor to a certain extent, which confirms that both proteins are imported by the same translocase into the organelle.

These results strongly suggest that the Rieske protein, although translocated across the thylakoid membrane by the ΔpH/TAT translocase, depends for its correct targeting on the availability of components also involved in Sec-dependent protein targeting. However, it appears unlikely that the whole process of thylakoid targeting and translocation can be mediated solely by the Sec machinery because in this case, the complete block of Rieske transport in the presence of the 23-kDa TAT competitor protein could not be explained.

**Both the Rieske Protein and the 33-kDa Polypeptide Interact with Several Protein Complexes during Their Passage through the Stromal Space**—As an initial step to identify the components involved in the intraorganellar targeting of the Rieske protein, we recovered the stromal fraction after the import experiment and analyzed it in nondenaturing gel systems. Unexpectedly, the Rieske protein was found in several distinct complexes in the chloroplast stroma ranging in size from ~130 to more than 700 kDa (Fig. 7). The size of the latter corresponds well with that of the stromal cpn60 complex (48, 49). Indeed, co-immunoprecipitation experiments could demonstrate that a substantial amount of the newly imported Rieske protein is associated with this chaperonine (Fig. 8), which corroborates earlier observations of Madueno et al. (50). It is particularly remarkable that not only the processed, mature size Rieske protein but also its unprocessed precursor is found associated with this chaperonine. Actually, the Rieske precursor shows even a much higher affinity to cpn60 than the mature protein, which is co-immunoprecipitated by the antisera only to a minor extent (<10%; Fig. 8). These results suggest that the Rieske protein interacts with the cpn60 complex very early in the translocation process, even before the removal of the transit peptide by the stromal processing peptidase. This interaction might even be mediated by the protein transport machinery of the inner envelope membrane in chloroplasts because some cpn60 was found in close contact with the translocase (51).

Cpn60 is probably not a binding partner for all the proteins imported into the chloroplast, however, because no interaction with the 33-kDa protein could be observed in import experiments, neither in native gel systems nor by co-immunoprecipitation experiments (Fig. 7 and data not shown). Instead, the stromal intermediate of the 33-kDa polypeptide, which was obtained by supplementing the import assays with nigericin...
of the Rieske protein, although to a different extent. In the presence of TAT competitor (23-kDa protein), transport of the Rieske protein is completely abolished (Fig. 4), demonstrating that a functional ΔpH/TAT pathway is essential for its membrane transport. Because the 23-kDa protein that was used as the competitor traverses the stromal space as a tightly folded monomer, probably without interacting with the stromal targeting and folding machinery (14), it is likely that its competition with the Rieske protein occurs at the thylakoid membrane, presumably at the receptor or at the translocase itself. The inhibitory effect observed with the Sec competitor (33-kDa protein), on the other hand, requires a significantly higher concentration of competitor protein and still does not lead to a complete block of the thylakoid transport process (Fig. 6A). This suggests that competition in this case is caused by saturating a relatively abundant targeting factor rather than the translocase at the membrane.

There are numerous candidates for such targeting factors in the chloroplast stroma. As shown by native gel electrophoresis, both the Rieske protein and the 33-kDa polypeptide interact after import into the organelle with several stromal complexes of high molecular mass. It remains elusive, however, which of these complexes represents true transport intermediates and could thus be responsible for the observed competition. Because of the size difference of the two proteins analyzed, co-migration even of corresponding protein complexes cannot be expected, so this point remains unsolved at the moment. However, it is already remarkable that there is such a multitude of interactions for proteins passing through the chloroplast stroma on their way to the thylakoids. This suggests a much higher degree of complexity in the recruitment of targeting factors than anticipated so far. Apparently, stromal factors in addition to SecA are involved in Sec-dependent protein targeting of the 33-kDa protein. Even more striking, the Rieske protein is the first example of a protein transported by the ΔpH-dependent pathway in chloroplasts that shows interaction with, and probably requirement for, stromal components in the targeting process. Thus, protein transport by the ΔpH/TAT pathway can no longer be strictly distinguished by its independence of stromal factors.

It will be interesting to examine whether the demand for stromal factors in the intraorganellar targeting of the Rieske protein is created by the transport signal or by its “passenger,” i.e. the hydrophilic domain that is translocated across the thylakoid membrane. Interaction of the hydrophilic domain with stromal components might, for example, be required for the assembly of the iron-sulfur cluster into the apoprotein if this process takes place in the chloroplast stroma. A precedent for such a scenario is found in yeast mitochondria, which house an essential part of the machinery for the biogenesis of Fe/S proteins in their matrix (52). Stromal assembly of the Fe/S cluster and, consequently, at least partial folding of the Rieske protein prior to membrane transfer would also be in line with transport by the ΔpH/TAT translocase, which is capable of translocating folded protein domains across the membrane (15, 16). It would furthermore explain why the Rieske protein but not the 33-kDa polypeptide interacts in the stroma with the cpn60 chaperonine (Fig. 7), the chloroplast homolog of the hsp60 folding machinery of mitochondria and prokaryotes (48, 53, 54). Sec-dependent targeting of the 33-kDa protein requires the unfolded polypeptide (55), and any folding of the protein in the stroma would probably impair its membrane translocation.

Stromal retardation of the Rieske protein to allow cofactor assembly and folding might also be the cause for the exceptional structure of its thylakoid transport signal. Not only is it the first membrane anchor signal known for thylakoid proteins,
but it furthermore lacks the twin-R motif, which is indicative for ΔpH/TAT-targeting signals. Instead, a KR-sequence is found at the corresponding position in all Rieske proteins from higher plant chloroplasts characterized to date, which should essentially prevent transport of the protein across the membrane (18, 24, 56). Interestingly, cyanobacteria, which are the closest relatives to the endosymbiotic ancestors of chloroplasts known today, possess Rieske proteins with perfect twin-R motifs in their presumed targeting signals. It is therefore appealing to speculate that the thylakoid transport signal of the Rieske protein was modified after the phylogenetic transfer of the gene into the nucleus in order to slow down the passage of the protein through the stromal space to the thylakoid membrane, thereby providing sufficient time for the assembly of the Fe/S cluster.

Taken together, the exceptional transport mechanism of the Rieske protein, which is the only chloroplast protein with cyanobacterial homologs known so far that is targeted by the thylakoidal ΔpH/TAT pathway, suggests that it is an ancient substrate of this pathway and thus can be considered as a missing link in the evolution of the protein transport pathways operating at the thylakoid membrane of chloroplasts.

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REFERENCES
