Bacterial proteins carrying twin-R signal peptides are specifically targeted by the ΔpH-dependent transport machinery of the thylakoid membrane system

Dirk Halbig[a], Bo Hou[b], Roland Freudl[a], Georg A. Sprenger[a], Ralf Bernd Klösgen[b,*]

[a] *Institut für Biotechnologie 1, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany*
[b] *Institut für Pflanzen- und Zellphysiologie, Martin-Luther-Universität Halle-Wittenberg, Am Kirchtor 1, D-06108 Halle, (Saale), Germany*

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Abstract Glucose-fructose oxidoreductase (GFOR), a periplasmic protein of *Zymomonas mobilis*, is synthesized as a precursor polypeptide with a twin-R signal peptide for Sec-independent protein export in bacteria. In higher plant chloroplasts, twin-R signal peptides are specific targeting signals for the Sec-independent ΔpH pathway of the thylakoid membrane system. In agreement with the assumed common phylogenetic origin of the two protein transport mechanisms, GFOR can be efficiently translocated by the ΔpH-dependent pathway when analyzed with isolated thylakoid membranes. Transport is sensitive to the ionophore nigericin and competes with specific substrates for the ΔpH-dependent transport route. In contrast, neither sodium azide nor enzymatic destruction of the nucleoside triphosphates in the assays affects thylakoid transport of GFOR indicating that the Sec apparatus is not involved in this process. Mutagenesis of the twin-R motif in the GFOR signal peptide prevents membrane translocation of the protein emphasizing the importance of these residues for the transport process.

Key words: Glucose-fructose oxidoreductase; Protein transport; Bacterial export; Thylakoid membrane; ΔpH-dependent pathway; Twin-arginine motif

1. Introduction

Protein export into the periplasmic space of Gram-negative bacteria has for a long time been considered to rely almost completely on the Sec system (e.g. [1]). In recent years however, compelling evidence for the existence of an additional, Sec-independent transport machinery for the transport of proteins across the cytosolic membrane has accumulated [2–4]. Many of these studies have been initiated by the finding that most bacterial proteins carrying complex redox cofactors have signal peptides with a twin pair of arginine residues (twin-R motif) preceding the hydrophobic core region and it has been suggested that this motif is a specific targeting signal for a Sec-independent export route [2].

In higher plant chloroplasts four independent pathways have so far been characterized for protein transport into or across the thylakoid membrane (summarized in [5]). As was already shown several years ago, at least two of these pathways operate in a Sec-independent manner. One of these, the ΔpH-dependent pathway was originally assumed to be a phylogenetic gain of the chloroplast, since its mechanism was unknown in any other membrane system. It operates independently from stromal factors and nucleoside triphosphates and requires solely, a transthylakoidal proton gradient in addition to the membrane-bound translocase [6–8]. Proteins are targeted to this pathway by signal peptides which carry twin-R motifs resembling those described above. Mutation analyses could show that both arginine residues within such twin-R motifs are essential for efficient thylakoid transport [9].

The structural similarity of the targeting signals suggests that the two twin-R transport pathways in the prokaryote and the prokaryote-derived organelle are of common phylogenetic origin. Indeed, homologous genes to *hcf106*, a maize locus which encodes the only component of the thylakoidal ΔpH-dependent transport machinery identified so far [10,11], can be found in most bacterial genomes sequenced to date. In *Escherichia coli* it was shown that both genes with homology to *hcf106* are required for the Sec-independent export of proteins carrying twin-R signal peptides [12]. However, it is difficult to demonstrate whether the two twin-R pathways also operate with similar mechanisms, due to the lack of established in vitro transport systems in bacteria. Swapping experiments analyzing bacterial proteins in chloroplast derived transport systems might provide valuable information about the degree of functional similarity between the two transport pathways, and it has been shown recently that twin-R signal peptides derived from *E. coli* proteins are capable of targeting a thylakoidal passenger protein by the ΔpH pathway into the thylakoid lumen [13]. On the other hand, an authentic cyanobacterial precursor protein carrying a twin-R signal peptide, CtpA, did not specifically interact with the ΔpH-dependent transport machinery of chloroplasts in such assays [14], suggesting a possible influence also of the passenger protein on pathway specificity.

In order to study the homology between the two twin-R pathways, we have analyzed glucose-fructose oxidoreductase (GFOR, gene: *gfo*), a periplasmic protein from *Zymomonas mobilis*, using in vitro transport assays based on isolated thylakoid vesicles from higher plant chloroplasts. GFOR is a relatively small protein which can easily be discerned from its larger precursor and which carries a complex cofactor (NADP) that is ubiquitous in both prokaryotic and eukaryotic cells. Export of GFOR has been extensively studied in both *Z. mobilis* and *E. coli*, and various alleles of the *gfo* gene are available encoding GFOR-proteins with altered signal peptides or cofactor binding sites [15–18].

2. Materials and methods

2.1. Materials

E. coli strain JM109 [19] was used for cloning and mutagenesis of

*Corresponding author. Fax: (49) (345) 5527 095.
E-mail: klosgen@pflanzenphys.uni-halle.de

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the various *gfo* alleles. Thylakoids were isolated from seedlings of *Pisum sativum*, var. Feltham First, which were grown for 8–9 days under a 12 h photoperiod before harvesting.

2.2. Plasmid construction

In order to obtain plasmid pGE*gfo*01 carrying the wild-type *gfo* gene under the control of the T7 promoter, a 1.6 kb *Eco*RI/*Sal*I fragment containing the complete *gfo* gene was recovered from plasmid pBAD*gfo*01 (our unpublished results) and cloned with plasmid pGEM-3Z (Promega, Mannheim). The generation of GFOR mutants carrying modifications in the twin-R motif of the signal peptide is described elsewhere [18].

2.3. Import into isolated thylakoids

Wild-type and mutant precursor proteins of GFOR from *Z. mobilis* were synthesized by in vitro transcription followed by in vitro translation in cell-free rabbit reticulocyte lysates in the presence of [^{35}S]methionine. Chloroplasts were isolated from young pea leaves by Percoll gradient centrifugation and used for thylakoid import experiments following essentially the protocol of Michl et al. [20]. Inhibitor studies as well as competition experiments were performed as described earlier [14,20]. Samples were analyzed by SDS-PAGE [21], and visualized after exposure to screens for a Fuji BAS-1500 phosphoimager using the software package TINA, v. 2.0 (Raytest, Straubenhardt, Germany).

3. Results

3.1. GFOR from Z. mobilis can be efficiently transported across thylakoid membranes from higher plant chloroplasts

In order to determine the compatibility of a bacterial protein carrying a twin-R signal peptide with the thylakoidal protein transport machinery, radiolabelled precursor protein of GFOR from *Z. mobilis* was synthesized by in vitro translation and incubated with thylakoids which had been isolated from pea chloroplasts. Although isolated thylakoid vesicles are known to be restrictive with respect to the proteins accepted, refusing even a subset of authentic thylakoid proteins [22,23], the precursor of bacterial GFOR turned out to be an excellent substrate in such experiments. After the import reaction, almost 50% of the protein present in the assay was resistant to externally added protease (Fig. 1) which is indicative of transport into the thylakoid vesicles. The efficiency of the transport process is comparable to that of authentic thylakoid lumen proteins, such as plastocyanin or the 23 kDa subunit of the oxygen-evolving system which were analyzed in parallel (Fig. 1). GFOR accumulating in the thylakoid lumen is quantitatively processed to its mature size in-

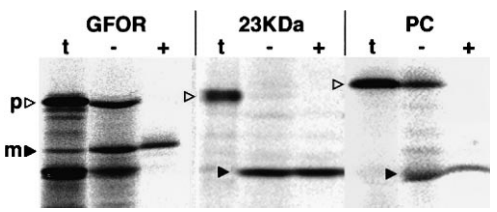

Fig. 1. Transport of GFOR across thylakoid membranes from pea chloroplasts. Precursor proteins of GFOR, the 23 kDa subunit of the oxygen-evolving system, or plastocyanin (PC) were incubated for 20 min at 25°C with isolated pea thylakoids that were resuspended in stromal extract at 0.75 μg chlorophyll/μl. After the import reaction, samples of the assays were analyzed on SDS-polyacrylamide gels either directly (lanes −) or after treatment of the thylakoids with thermolysin (lanes +). The positions of the precursor (p) and mature proteins (m) are indicated by open and closed arrowheads, respectively.

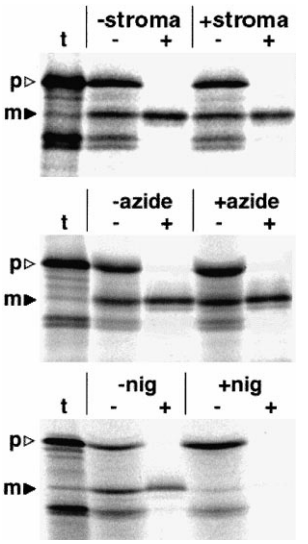

Fig. 2. Thylakoid transport of GFOR requires a transthylakoidal proton gradient but is independent of stromal factors and azide-sensitive components. In vitro synthesized GFOR precursor was incubated with isolated thylakoids in the presence and absence of 10 mM sodium azide (azide) or 2 μM nigericin (nig). In the upper panel incubation was additionally performed also in the absence of stromal extracts (−stroma), i.e. with thylakoids that were washed twice and then resuspended in [10 mM HEPES-KOH, pH 8.0, 5 mM $MgCl_2$] rather than in stromal extract (+stroma). For further details see the legend to Fig. 1.

dicating that also the thylakoidal processing [24] accepts the bacterial precursor polypeptide as a substrate.

3.2. Thylakoid transport of GFOR takes place by the ΔpH-dependent pathway

In order to characterize the mechanism of GFOR transport across the thylakoid membrane, the experiments were repeated under various physiological conditions that are indicative of the different thylakoidal translocation routes. In the first set of experiments, the influence of stromal factors and translocation inhibitors was studied (Fig. 2). It turned out that the transport of GFOR does not depend on the addition of stromal extracts to the thylakoid vesicles and that it is also not prevented by sodium azide, a potent inhibitor of SecA function in prokaryotes and chloroplasts [25–27]. In contrast, the transthylakoidal proton gradient apparently plays an important role in this process since GFOR translocation is abolished if the import assays are supplemented with nigericin, a protonophore which dissipates the ΔpH across the thylakoid membrane (Fig. 2).

In line with these findings, enzymatic destruction of the nucleoside triphosphates in the assays by apyrase does not affect thylakoid transport of GFOR to a significant extent (Fig. 3A): plastocyanin, on the other hand, which is targeted by the Sec pathway and which was included as a control, is no longer translocated into the thylakoid lumen under these conditions (Fig. 3B). Together with the azide insensitivity of the translocation process and the lack of requirement for stromal factors (Fig. 2), an involvement of either the Sec or the SRP machineries of the chloroplast can be ruled out.

Instead, the requirement for the transthylakoidal proton gradient (Fig. 2) suggests transport by the ΔpH-dependent pathway. Therefore, competition experiments were performed in which the ΔpH-dependent translocation machinery was

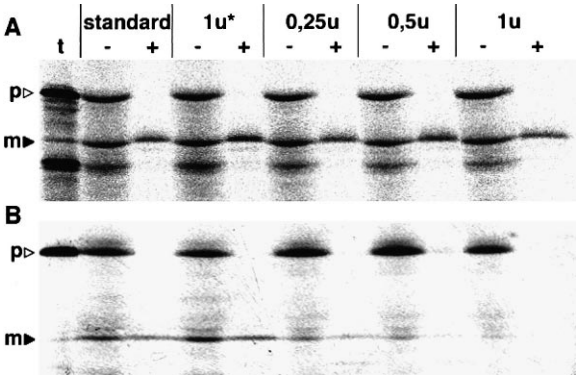

Fig. 3. Thylakoid transport of GFOR is independent of nucleoside triphosphates. Isolated pea thylakoids were incubated with the precursors of GFOR (A) or plastocyanin (B) in the presence of stromal extract. Before starting the incubation, the mixtures were preincubated with apyrase for 10 min on ice. The activity of apyrase added to each incubation mixture is indicated above the lanes. Into the standard assay, no apyrase was added. The control denoted 1u* contained apyrase that was inactivated by boiling for 5 min. For further details see the legend to Fig. 1.

saturated by a high concentration of unlabelled precursor of the 23 kDa subunit from the oxygen-evolving system [20], a specific substrate for this pathway [22]. With increasing amounts of competitor, GFOR is significantly inhibited in its thylakoid transport and accumulates in its unprocessed precursor form in the incubation mixture (Fig. 4). The degree of competition is similar to that observed for the authentic 23 kDa precursor protein which was analyzed in parallel (Fig. 4). These results unambiguously prove that GFOR from *Z. mobilis* utilizes specifically and exclusively the ΔpH-dependent pathway for its transport across the thylakoid membrane.

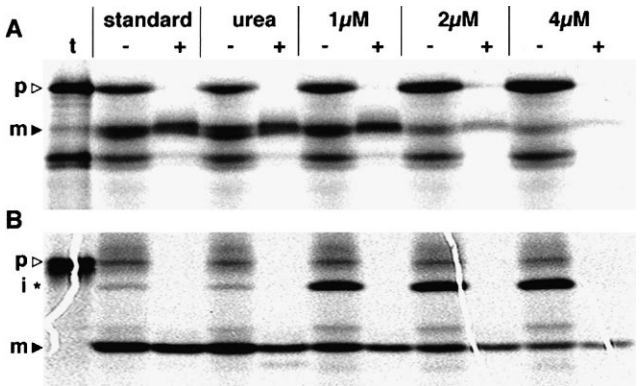

Fig. 4. Thylakoid transport of GFOR is inhibited if the ΔpH-dependent transloction machinery is saturated. In vitro synthesized, radiolabelled precursors of GFOR (A) or the 23 kDa subunit of the oxygen-evolving system (B) were incubated with isolated thylakoids in the presence of increasing amounts of unlabelled competitor protein at the concentrations indicated above the lanes. As competitor, the precursor of the 23 kDa protein was utilized which was obtained from overexpression in *E. coli* and prepared by solubilization of inclusion bodies in urea buffers as detailed in Michl et al. [20]. All assays except for the standard control contained equivalent amounts of urea. In the urea assay, no competitor protein but only solubilization buffer was added. For further details see the legend to Fig. 1.

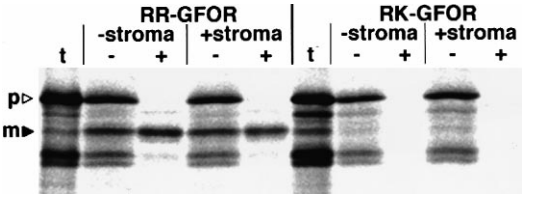

Fig. 5. Mutagenesis of the twin-R motif in the GFOR signal peptide prevents transport of the protein across the thylakoid membrane. In vitro synthesized precursor of wild-type GFOR (RR-GFOR) or of a mutant in which one of the arginine residues of the twin-R motif was replaced by lysine (RK-GFOR) was incubated with thylakoids in the presence or absence of stromal extracts. For further details see the legends to Figs. 1 and 2.

3.3. *Mutagenesis of the twin-R motif in the signal peptide abolishes thylakoid transport of GFOR*

The most significant feature of all thylakoidal signal peptides triggering ΔpH-dependent transport is a twin pair of arginine residues upstream of the hydrophobic core segment. As was shown for the precursor of the 23 kDa protein, conservative replacement of either of these arginines by lysine prevents almost completely the membrane transport of this protein [9]. In order to determine whether the corresponding twin-R motif in the signal peptide of GFOR is of comparable importance for membrane transport, mutant derivatives were analyzed in which these arginine residues were replaced by lysines either together or each one separately. It turned out that neither of the resulting twin-R mutants (RK-GFOR, KR-GFOR, and KK-GFOR) could be imported into isolated thylakoids, irrespective of whether stromal extract was included in the assays or not (Fig. 5 and data not shown). Thus, also in the heterologous substrate the twin-R motif is an essential element for the interaction with the ΔpH-dependent transport machinery. Neither of the mutants was instead targeted by the Sec- or SRP-dependent pathways, in line with the results described for the twin-R mutants of the 23 kDa protein which likewise could not utilize any of the other pathways for thylakoid translocation [9]. Remarkably though, the GFOR signal peptide lacks charged residues in its C-terminal segment [15,28], which are discussed as operating as 'Sec avoidance' signals in thylakoidal twin-R signal peptides [29].

4. Discussion

In this study we have aimed to examine the transport mechanism of a full bacterial precursor protein carrying a twin-R signal peptide into thylakoid vesicles isolated from higher plant chloroplasts. The principle idea of using this heterologous combination was to characterize the specificities, and possibly homologies, of bacterial and thylakoidal membrane transport processes that are engaged by twin-R signal peptides.

The results show that the precursor of GFOR from *Z. mobilis* is efficiently and selectively transported by the ΔpH-dependent translocation system across the thylakoid membrane which indicates the close relationship of the thylakoidal ΔpH-dependent pathway and the twin-R pathway of bacteria and further emphasizes their putative common evolutionary origin. Furthermore, it shows that not only bacterial twin-R signal peptides but also full precursors are compatible with the thylakoidal ΔpH-dependent transport machinery. While the former could already be deduced from studies analyzing

chimeric polypeptides that are composed of bacterial signal peptides and thylakoidal passenger proteins [13,30], the latter could not necessarily be expected: the only bacterial twin-R protein analyzed so far with thylakoid membranes, CtpA from *Synechocystis* PCC6803, did not specifically utilize the ΔpH-dependent pathway but was instead also targeted by the thylakoidal Sec pathway suggesting that it was not capable of distinguishing the two translocation routes in chloroplasts [14].

In spite of the overall homology between the two twin-R pathways, their mechanisms differ in at least one aspect. While transport of GFOR across thylakoid membranes is insensitive to sodium azide (Fig. 2), export of the protein into the periplasm of *Zymomonas* is sensitive to this inhibitor [15]. It cannot be distinguished as yet whether this is due to involvement of SecA in the bacterial transport process, or whether azide-sensitive component(s) other than SecA are responsible for the observed effect.

Interestingly, GFOR from *Z. mobilis* cannot be exported into the periplasmic space of *E. coli* when expressed with its genuine signal peptide [16]. Only after fusion to Sec-targeting signal peptides like those derived from OmpA or PhoA, export can be observed which notably takes place by the Sec pathway. However, GFOR does not accumulate in the periplasm in these instances but is rapidly degraded in this compartment. It was concluded that proteolysis in the periplasm is due to the lack of cofactor insertion which probably takes place in the bacterial cytosol. Consequently, export of GFOR in *Zymomonas* is assumed to be conducted by a pathway which, in contrast to the Sec pathway, is capable of handling folded proteins [16]. As yet, we do not know whether GFOR also binds its cofactor NADP in our import assays and if so, whether binding takes place prior to the membrane transport step. Experiments to examine these points are under way.

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