PREREQUISITES FOR TERMINAL PROCESSING OF THYLAKOIDAL TAT SUBSTRATES

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Running title: Terminal processing of Tat substrates
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In both, bacteria and chloroplasts, the Tat\(^1\) (twin arginine translocation) system is capable of translocating folded passenger proteins across the cytoplasmic and thylakoidal membranes, respectively. Transport depends on signal peptides that are characterised by a twin pair of arginine residues. The signal peptides are generally removed after transport by specific processing peptidases, namely the leader peptidase and the thylakoidal processing peptidase (TPP), respectively. In order to gain insight into the prerequisites for such signal peptide removal, we mutagenised the vicinity of TPP cleavage sites in several thylakoidal Tat substrates. Analysis of these mutants in thylakoid transport experiments showed that the amino acid composition of both, the C-terminal segment of the signal peptide and the N-terminal part of the mature protein, plays an important role in the maturation process. Efficient removal of the signal peptide requires the presence of charged or polar residues within at least one of those regions, whereas increased hydrophobicity impairs the process. The relative extent of this effect varies to some degree depending on the nature of the precursor protein. Unprocessed transport intermediates with fully translocated passenger proteins are found both in membrane complexes of high molecular weight, which presumably represent Tat complexes, as well as free in the lipid bilayer. This seems to indicate that the Tat substrates can be laterally released from the complexes prior to processing and that membrane transport and terminal processing of Tat substrates are independent processes.

The biogenesis of many proteins involves their transport across membranes. Such transport is mediated by specific targeting signals present in the primary translation products which are generally removed after transport by specific processing peptidases. Targeting signals operating at the cytoplasmic membrane of bacteria and at the thylakoid membrane of chloroplasts mostly belong to a group of structurally conserved signal peptides which are characterised by an N-terminal basic region, a hydrophobic central core and a polar C-terminal region ending with the consensus Ala-X-Ala motif at the terminal processing site (1). These signal peptides are removed after transport by membrane-bound peptidases addressed as leader peptidase or thylakoidal processing peptidase (TPP), respectively (2,3).

In both, bacteria and chloroplasts, one class of signal peptides that is characterised by a twin-pair of arginine residues in the N-terminal region (4,5) specifically mediates protein transport by the so-called Tat pathway (Tat = Twin arginine translocation) (6). The most remarkable feature of this protein transport pathway is its property to translocate folded passenger proteins across the membrane (7-11), and it is even a matter of debate whether folding of the passenger protein is a prerequisite for Tat-dependent transport (8,12,13). Mechanistically, protein transport by the Tat pathway is initiated by the direct insertion of the transport substrate into the target membrane (14,15), followed by its interaction with the oligomeric Tat B/C receptor complex (13,16) which in thylakoids has an apparent size of approximately 560-700 kDa (16-18). Subsequent membrane translocation of the passenger protein depends on the TatA component of the Tat transport apparatus (18,19). The final step in the translocation process is the removal of the signal peptide. In thylakoids, such processing is performed by the thylakoidal processing peptidase, TPP (20). The analysis of a chimeric substrate
showing both, slower transport kinetics and delayed processing by TPP, suggested an influence of this processing step on the kinetics of membrane transport (17). On the other hand, removal of the transport signal is not obligatory for Tat-dependent thylakoid transport, as indicated by the Rieske Fe/S protein which carries an uncleaved Tat-specific membrane anchor signal at the N-terminus of the mature polypeptide chain (21).

In order to examine the processing step following Tat-dependent protein transport in more detail, we have analysed mutant derivatives of both, authentic and chimeric Tat substrates, with thylakoid transport experiments. It was our goal to identify structural features within precursor proteins that are responsible for the efficiency and velocity of the maturation of thylakoidal Tat substrates.

**EXPERIMENTAL PROCEDURES**

**Site-directed mutagenesis**

Mutageneses were carried out using the Stratagene QuickChange® Site-Directed Mutagenesis Kit and confirmed by DNA sequencing. The following primers were used (a) 16 V137M: 5’gcagcccaaagacaaatgtcagctgtcattc3’ and 5’gatctttcagcgcctctgaaatcc3’; (b) 16 E84M: 5’gcgctgttcttgctatgg ccaggcccatcgttg3’ and 5’caacgatgggcctggccatagcaagaacagcc3’; (c) 23 D80L: 5’ggtttcccctgcacttgcagcctatggagaagc3’ and 5’gcttctccataggctgcaagtgcaggggaaacc3’; (d) 23 S77F,D80L: 5’gttggctctaaggttttccctgcacttgcagcctatggagaagc3’ and 5’ctccataggctgcaagtgcaggggaaacctagagcc3’; (e) 16/23 L82D: 5’ggttggctctaaggttttccctgcacttgcagcctatggagaagc3’ and 5’gcttctccataggctgcaagtgcaggggaaacctagagcc3’.

The 16 kDa precursor was furthermore modified by replacing a poorly conserved valine residue in the mature part of the 16 kDa protein (V137) by methionine in order to permit labelling of the protein with [35S] methionine. This mutation did not show any influence on the entire transport process.

**Sequence analysis**

Analysis of the TPP cleavage regions was performed with the following thylakoidal Tat substrates (22-24): OEC 16 kDa subunit (P16059), PSI-N (P49107), HCF136 (O82660), polyphenol oxidase A (Q08303), PSI-T (Q39195), thylakoid luminal 16.5 kDa protein (O22773), FtsH-like protein PfIF (AAD17230), protein of unknown function (AU070407), protease Do-like 8 (Q9LU10), FKBP-type peptidyl-prolyl cis-trans isomerase 3 (Q95CY2), protease Do-like 5 (Q9SEJ7), hypothetical protein (Q9LMX5), unknown thylakoid lumen protein (Q9S720), probable FKBP-type peptidyl-prolyl cis-trans isomerase 2 (O22870), protein similar to ascorbate peroxidase (AI490846), predicted protein of unknown function (AAC78263), unknown protein (AW201127), putative L-ascorbate peroxidase 4 (P82281), peptidyl-prolyl cis-trans isomerase (Q9LYR5), peptidyl-prolyl cis-trans isomerase (Q9M222), probable FKBP-type peptidyl-prolyl cis-trans isomerase 1 (Q9LM71).

**Protein transport experiments**

Isolatation of chloroplasts and thylakoids was carried out as described (14). Protein transport experiments with radiolabelled precursor proteins followed published protocols (25).

**Miscellaneous**

Gel electrophoresis of proteins under denaturing conditions was carried out according to (26). Blue Native gel electrophoresis (27) was carried out according to (17). The gels were exposed to phosphorimager screens and analysed with the Fujifilm FLA-3000 (Fujifilm, Düsseldorf, Germany) using the software packages BASReader (version 3.14) and AIDA (version 3.25; Raytest, Straubenhardt, Germany). Hydrophobicity plots were generated according to (28). All of the other methods followed published protocols (29).

**RESULTS**

**Terminal processing of the 16/23 precursor is inhibited at low temperature**

The analysis of the chimeric Tat substrate 16/23 which is composed of the transit peptide of the 16 kDa subunit and the mature part of the 23 kDa subunit, both of the oxygen evolving system associated with photosystem II, has led to the identification of distinct transport steps during Tat-dependent protein transport across the thylakoid membrane (14,17). This was facilitated...
by the retarded translocation of the chimeric precursor protein which led to the detection of two consecutive translocation intermediates that were termed Ti-1 and Ti-2, respectively (Fig. 1A and B). The degradation products indicative for Ti-1 and Ti-2 cannot be detected upon protease treatment in the absence of thylakoid vesicles demonstrating that they indeed represent translocation intermediates and not merely protease resistant domains of the precursor protein (Fig. 1C). The accumulation of Ti-2 which assumes a bitopic transmembrane topology with fully translocated passenger protein (Fig. 1B) indicates that for the 16/23 protein not only the actual membrane translocation process but also the terminal maturation step, i.e. the removal of the signal peptide, is affected. This finding was unexpected, because the amino acid sequences of both, the signal peptide and the mature part of the chimera, match perfectly to their counterparts in the respective authentic Tat substrates, notably the precursors of the 16 kDa and 23 kDa subunits of the oxygen evolving system of spinach (Fig. 2), which are quantitatively processed to the mature proteins in the course of thylakoid transport experiments (Fig. 1A).

Processing of the 16/23 protein by TPP is not abolished but only impaired, because significant amounts of mature 23 kDa protein accumulate with time in standard transport experiments performed at 25°C (Fig. 1A and (17)). However, if thylakoid transport is carried out at 4°C, only minute amounts of mature 23 kDa protein can be detected (Fig. 1A). One reason is that the membrane translocation process is affected at such temperatures leading to reduced amounts of Ti-2 and higher levels of Ti-1 in these assays. Yet, the decrease in Ti-2 accumulation is not sufficiently pronounced that it alone could fully account for the almost complete lack of mature protein demonstrating that also the terminal processing step is significantly inhibited under these conditions.

In order to distinguish whether the observed inhibition of processing at 4°C is due to reduced TPP activity at low temperature or instead a consequence of altered substrate properties caused by the chimeric cleavage site in the 16/23 precursor, the authentic precursors of the 16 kDa and 23 kDa subunits of the oxygen evolving system were analogously analysed. As expected, membrane translocation of the two proteins is significantly affected at 4°C leading to reduced levels of mature protein in both instances (Fig. 1A). This demonstrates reduced translocaction activity at low temperature which might be due to a specific demand for lipid fluidity of protein transport by the Tat pathway, in line with recent results concerning protein transport across thylakoid membranes with reduced levels of unsaturated lipids (30). Yet, terminal processing of the two authentic precursor proteins is not impaired at low temperatures, because bitopically translocated intermediates corresponding to Ti-2 cannot be detected for either of them. Instead, both proteins are quantitatively cleaved by the processing enzyme even if thylakoid transport is performed at 4°C which strongly suggests that TPP activity is not rate limiting at such temperatures. The delayed maturation of the 16/23 chimera is hence presumably a consequence of the chimeric processing site.

**The 16/23 precursor lacks polar residues in the vicinity of the TPP cleavage site**

To get a first hint on the possible reason for the impaired maturation of the 16/23 chimera, the amino acid sequences in the vicinity of the TPP cleavage sites of known thylakoidal Tat substrates were compared. Remarkably, from 22 independent thylakoidal Tat substrates analysed, 21 possess either charged (15) or at least polar (6) residues within the region from -3 to +3 of the terminal processing site (data not shown) suggesting that the level of polarity in this region plays a role in the cleavage process. The charged or polar residues can either be provided by the C-terminal region of the signal peptide, like in the precursor of the 23 kDa protein, or by the N-terminal segment of the mature polypeptide, as found for example in the 16 kDa protein (Fig. 2A). In contrast, the corresponding segment of the 16/23 chimera lacks any such residue due to the combination of the 16 kDa signal peptide comprising a non polar C-terminus and the mature 23 kDa protein which possesses an uncharged N-terminus (Fig. 2A). Consequently, the TPP cleavage site of the 16/23 chimera is more hydrophobic than those of the corresponding authentic precursor proteins (Fig. 2B).

**Removal of polar residues from the terminal processing sites affects maturation of authentic Tat substrates**
In order to examine whether a polar cleavage region is required for the terminal processing step, the charged residues flanking the TPP cleavage sites of the authentic 16 kDa and 23 kDa precursor proteins were removed by site directed mutagenesis. Analysis of the resulting polypeptides (16 E84M and 23 D80L, respectively, Fig. 2A and B) in time-course experiments showed that indeed one of them, notably 16 E84M, exhibits transport characteristics resembling those of the 16/23 chimera. It binds efficiently as the precursor protein to the thylakoid vesicles but it is only slowly converted to the mature 16 kDa protein, as indicated by the transient accumulation of a proteolytic fragment of approximately 22 kDa upon thermolysin treatment of the thylakoid vesicles after import (Fig. 3A and C). Both, the size and the kinetic features of this proteolytic fragment suggest that it corresponds to the bitopic translocation intermediate Ti-2 described for the 16/23 chimera (Fig. 1, and (17)). Furthermore, a second degradation product of approximately 8 kDa can be detected in protease-treated thylakoids at early time-points of the transport process (Fig. 3A). It might represent a translocation intermediate corresponding to Ti-1 which is inserted into the membrane in a loop conformation but not yet translocated (Fig. 1B). Like with the 16/23 chimera, these degradation products are not observed in the absence of thylakoid membranes (Fig. 1C).

In case of the 23 kDa precursor protein, the negative charge in the vicinity of the TPP cleavage site is apparently not of similar importance for maturation. The mutant derivative 23 D80L in which the negatively charged aspartic acid residue within the Ala-X-Ala motif of the signal peptide was replaced by leucine (Fig. 2A) shows no significant differences to the authentic precursor protein when analysed in time-course thylakoid transport experiments (Fig. 3B and C). Binding of the mutant protein to the thylakoid vesicles appears slightly stronger at early time points, but there is no indication for the accumulation of translocation intermediates like Ti-1 or Ti-2. Remarkably though, the vicinity of the TPP cleavage site within the 23 D80L mutant is still significantly more hydrophilic than the corresponding regions of the 16/23 or 16 E84M proteins (Figs. 2B and 3D). Considering the possible impact of polarity in this region on the maturation process, this degree of polarity might well be sufficient to facilitate efficient processing by TPP. Therefore, polarity in this region was further reduced by an additional mutagenesis in which a polar serine residue at position -5 from the processing site was replaced by phenylalanine. When analysed with time-course thylakoid transport experiments, the resulting precursor protein (23 S77F,D80L; Fig. 2A) shows membrane transport and processing characteristics similar to those observed for 16 E84M (Fig. 3B and C). It binds significantly better to the thylakoid membrane than the corresponding authentic precursor protein, but it is converted to the mature polypeptide only slowly. This leads to the transient accumulation of a proteolytic fragment of approximately 28 kDa upon thermolysin treatment of the thylakoid vesicles after import (Fig. 3B) which presumably represents the bitopic translocation intermediate Ti-2. However, when compared to the results with the 16/23 and 16 E84M proteins, the level of Ti-2 accumulation is relatively low indicating that maturation is still only mildly affected in the double mutant, and a degradation product corresponding to Ti-1 is not detected at all.

Charged residues in the vicinity of the processing site improve maturation of the chimeric 16/23 protein

Thus far, the results obtained with the mutants are in close agreement with the assumption that a polar TPP cleavage region is required for efficient processing. In order to examine this further with a reciprocal approach, a negatively charged aspartic acid residue was introduced into the Ala-X-Ala motif of the chimeric 16/23 protein, in analogy to the situation found in the authentic 23 kDa precursor protein (Fig. 2A). According to our assumption, the resulting increase in polarity within this region (Fig. 4C) should lead to more efficient processing of the chimeric precursor and, possibly, even to higher translocation rates.

Analysis of the mutant derivative 16/23 L82D in time-course thylakoid transport experiments shows that the precursor is indeed converted to the mature 23 kDa protein with significantly higher efficiency than the original 16/23 chimera. Consequently, the transient accumulation of Ti-2 is shifted towards earlier time-points of the transport experiment (Fig. 4A and B). Yet, the mutant derivative 16/23 L82D is still not as efficiently processed as either of the two
corresponding authentic precursor proteins, since the accumulation of Ti-2 is diminished but not prevented. The insertion of an additional charged residue at the N-terminus of the mature part of the 16/23 chimera could not further improve maturation (data not shown) indicating that besides polarity of the cleavage region other features of the substrate, e.g. secondary structure (31), may additionally influence this process.

In contrast to precursor processing, the membrane translocation step is apparently not affected by the polarity of the TPP cleavage region. The 16/23 and 16/23 L82D precursors show largely similar membrane transport rates if both, Ti-2 and mature polypeptide, are considered as the protein fraction that was translocated across the thylakoid membrane (Fig. 4A, and data not shown). Instead, the two proteins show significantly different thylakoid insertion properties, because accumulation of Ti-1 is hardly detectable for the 16/23 L82D chimera.

Translocation intermediates can occur free in the lipid bilayer

The observation that removal of polar or charged residues from the TPP cleavage site of authentic precursor proteins leads to a delay of the terminal processing step and thus to the accumulation of bitopic translocation intermediates raises the question whether these proteins are still associated with the protein translocase in the membrane or whether they have been released into the lipid phase of the membrane bilayer. In case of the 16/23 chimera, accumulation of translocation intermediates correlates with prolonged association of the precursor protein to two membrane complexes of approximately 560 kDa and 620 kDa, respectively, (17) which presumably represent thylakoidal TatB/C complexes (16). Time-course experiments show that complex association of the 16/23 precursor protein takes place only transiently (Fig. 5A) which is indicative for an active translocation process.

Similar results were obtained when the authentic 16 kDa and 23 kDa precursors as well as their mutant derivatives 16 E84M and 23 S77F,D80L respectively, were analysed in parallel by SDS-PAGE and Blue Native-PAGE. In all these instances, the accumulation of translocation intermediates is accompanied with their association to two membrane complexes of high molecular weight (Fig. 5B and C). These membrane complexes show mobilities upon native electrophoresis that are similar to those observed in transport experiments analysing the 16/23 chimera. Remarkably, even for the authentic 23 kDa precursor protein binding to such membrane complexes was detected in some experiments (Fig. 5C and data not shown) demonstrating that binding to these membrane complexes is not restricted to chimeric or mutagenised proteins.

However, due to technical constraints it is not possible to determine if all or only a fraction of the bitopic translocation intermediates accumulating in the membrane are actually bound to the membrane complexes. In fact, the mutant derivative of the 16/23 chimera with increased polarity in the TPP cleavage region (16/23 L82D) is not at all found associated with either of the two presumed Tat complexes during thylakoid transport (Fig. 5C), in spite of the fact that the delayed maturation of this polypeptide leads to the accumulation of significant amounts of the bitopic translocation intermediate within the membrane (Fig. 5B). This observation strongly suggests that the bitopic translocation intermediates do not necessarily remain associated with the translocase until cleavage by the thylakoidal processing peptidase takes place but that they might instead be released from the membrane complex prior to terminal processing.

DISCUSSION

In the present work, we have aimed to characterise the structural requirements for efficient maturation of thylakoidal precursor proteins after Tat-dependent membrane transport.

Charged or polar residues flanking the TPP site are required for efficient maturation

Thylakoid transport experiments performed with mutant derivatives of authentic and chimeric precursor proteins demonstrate that the efficiency of maturation depends on the composition of the amino acids flanking the TPP cleavage site. In addition to the well known dependence of processing on small and non polar residues at positions -3 and -1 (1,32), polar or even charged residues within this region are required for efficient processing. Unexpectedly, these residues may not only be supplied by the C-terminal part of
the signal peptide, but can likewise be provided by the N-terminal segment of the mature polypeptide. Lack of polar residues in either position which results in increased hydrophobicity within this region significantly reduces the efficiency of the maturation process (Figs. 3 and 4). This in turn leads to the accumulation of bitopic transport intermediates in which the translocated passenger protein remains anchored to the membrane from the luminal side by its signal peptide (Fig. 1B). Thus, depending on the signal peptide in a given precursor protein, the passenger polypeptide can take an active role in the transport process by influencing the efficiency of terminal processing by TPP.

**Need of polarity or problems with hydrophobicity?**

What is the reason for the requirement of polarity within the TPP cleavage region? It appears unlikely that the charged or polar residues are needed for direct interaction of the substrate with the processing enzyme, because the amino acid sequence flanking the cleavage site is not significantly conserved except for the Ala-X-Ala motif (Fig. 2 and data not shown), and neither the exact position of the charged residues nor the degree of polarity seems strictly fixed. Reduced polarity in this region might instead prevent the exposure of the cleavage site to the luminal face of the thylakoid membrane which in turn presumably affects its accessibility by the thylakoidal processing peptidase exerting its activity on the luminal side of the membrane (3). A similar conclusion has previously been drawn from the analysis of mutant derivatives of spontaneously inserting thylakoid proteins which likewise show reduced processing rates if polar residues in the vicinity of the TPP cleavage site are replaced by non polar amino acids (33).

**What comes first, cleavage by TPP or release from the translocase?**

Yet, in view of the results obtained with the 16/23 L82D protein an alternative or complementing scenario appears suitable. Considering the possibility that processing of the substrate by TPP may not take place at the translocase complex but instead within the lipid bilayer, charges and/or polarity in the region connecting the signal peptide with the passenger protein might be required to facilitate the lateral release of the translocated protein from the translocase into the lipid bilayer. The mutant 16/23 L82D accumulates as bitopic translocation intermediate Ti-2 already at early time points in kinetic thylakoid transport experiments (Fig. 4A). At later time points, this intermediate is almost quantitatively processed to the mature 23 kDa protein demonstrating that essentially all of the protein accumulating in the Ti-2 conformation is recognised as substrate by the processing enzyme. However, native gel electrophoresis shows that this translocation intermediate is not associated with either of the presumed Tat membrane complexes (Fig. 5C). Instead, it seems to accumulate freely in the lipid bilayer which strongly suggests that processing by TPP takes place in this instance after release of the protein into the membrane. It remains yet unsolved whether such release of a translocated protein from the Tat translocase is a prerequisite for processing. Considering however, spontaneously inserting thylakoid proteins, like CFo-II or PsbW which likewise carry cleavable signal peptides but do not depend on either Sec or Tat translocase for membrane transport (34,35), a thylakoidal processing peptidase not associated with protein transport machinery but floating freely within the membrane bilayer is even more likely.

The signal for lateral opening of the translocase after membrane transport is presumably based on conformational changes in the Tat substrate upon transition from Ti-1 to Ti-2 (Fig. 1). Yet, a further signal might be provided by the polarity of the TPP cleavage region. Considering the accumulation of the uncharged 16/23 protein at the translocase (Fig. 5A and C), any raise in hydrophobicity within this region seems to affect its lateral release into the membrane and thus result in stronger binding to the Tat complex. This assumption is in line also with a recent study demonstrating that increased hydrophobicity results in stronger Tat complex association (36).

Supporting evidence for this model can furthermore be obtained from the analysis of the Rieske protein which is targeted by the Tat pathway across the membrane. Transport is mediated by an N-terminal Tat-targeting signal which is not removed after transport but instead anchors the protein from the luminal side to the thylakoid membrane (21,37). The topology of the mature Rieske protein thus corresponds to that of
the bitopic translocation intermediates described here. It is obvious that at some point of the translocation process the protein has to be released laterally from the translocase into the lipid bilayer but it is yet unknown which signal triggers this process. According to our hypothesis, an explicit release signal for membrane anchor proteins would not be required, because release of the bitopic translocation intermediates could well be the “default pathway” for all proteins targeted by the Tat translocase. In case of the Rieske protein, no further processing would take place, whereas in the other cases TPP activity would be needed.

Further support comes from a recent study analysing the maize homolog of the 16 kDa precursor protein presented here (38). In that study, the mature part of the precursor is efficiently translocated across the thylakoid membrane despite the fact that its signal peptide was covalently cross-linked to the TatC protein prior to transport, i.e., the precursor assumes the bitopic Ti-2 conformation with a stroma exposed N-terminus and the C-terminus exposed to the lumen. Remarkably, processing of this protein to the mature polypeptide is not observed demonstrating that TPP does not have access to its processing site. Although it cannot be excluded that the cross-link leads to reduced flexibility of the protein preventing its accessibility by TPP, it might as well be a consequence of the inability of the protein to dissociate from the Tat complex due to the cross-link to TatC. This scenario would be in line with a model in which release from the Tat complex into the lipid bilayer is even a prerequisite for terminal processing, although it should be emphasised that TPP activity associated with the Tat complex cannot be excluded at this point.

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**FOOTNOTES**

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1The abbreviations used are: Tat, twin arginine translocation; Ti-1/Ti-2, translocation intermediate 1/2; TPP, thylakoidal processing peptidase

**FIGURE LEGENDS**

Fig. 1. Temperature dependence of Tat-dependent protein transport.
(A) The precursor proteins 16 kDa, 23 kDa, and 16/23 were obtained by *in vitro* transcription/translation and incubated with isolated pea thylakoids for 15 min in the light at either 25°C or 4°C. After the import reaction, the thylakoids were washed with HM buffer and either treated with thermolysin (200 µg/ml, 30 min on ice, lanes +), or mock-treated (lanes -). Stoichiometric amounts of each fraction, corresponding to 15 µg of chlorophyll, were separated on 10-17.5% SDS-polyacrylamide gradient gels and visualised by phosphorimaging. In lanes t, 1 µl of the *in vitro* translation assay was loaded. The positions of the precursor (p) and mature protein (m) are indicated by filled arrowheads, while open arrowheads point to the positions of the two integral translocation intermediates, Ti-1 and Ti-2. (B) Schematic representation of the topology of Ti-1 and Ti-2 during the transport process. The open ellipsoid indicates the hydrophobic central core within the thylakoid targeting signal peptide and RR points to the position of the twin-arginine motif. Ti-1 and Ti-2 represent the early and late translocation intermediates, respectively, that are observed during thylakoid membrane transport of the chimeric 16/23 protein. (C) Authentic and mutant precursors obtained by *in vitro* transcription/translation were subjected to thermolysin treatment, or mock treated, as described in (A) but in the absence of thylakoid vesicles.

Fig. 2. Amino acid sequence of TPP cleavage sites.
The amino acid sequences flanking the TPP cleavage sites of the proteins used in this study are shown. The RR-motif as well as the residues that were mutagenised are typed in bold. Charged residues (+/-) and polar amino acids (*) are labelled on top, hydrophobic regions are underlined and the TPP cleavage sites are denoted by a dashed line. The region from -3 to +3 is shaded. (B) Hydrophobicity plots of the 16/23 protein and authentic precursors, generated according to (28).

Fig. 3. Increased hydrophobicity in the TPP cleavage region leads to delayed processing.
(A) In thylakoido time-course experiments performed with the authentic 16 kDa precursor and its mutant derivative 16 E84M. The incubation time at 25°C in the light is given on top of the lanes. (B) In thylakoido time-course experiments performed with the authentic 23 kDa precursor protein and its mutant derivatives 23 D80L and 23 S77F,D80L. (C) Quantification of the amounts of Ti-2 and mature protein, respectively, from the import reactions shown in (A) and (B). For each time-point, the relative amounts of Ti-2 and mature protein (in terms of percentage of the sum of these products at the final time-point) were calculated taking into account the number of [35S] methionine residues in each protein species. (D) Hydrophobicity plots of the authentic and mutant precursors, generated according to (28). For further details, see the legend to Figure 1.

Fig. 4. A negative charge in the TPP cleavage region of the chimeric 16/23 protein leads to accelerated processing.
(A) In thylakoido time-course experiments performed with the 16/23 chimera and its mutant derivative 16/23 L82D. (B) Quantification of the amounts of Ti-1, Ti-2 and mature protein, respectively, from the import reactions shown in (A). For each time-point, the relative amounts of Ti-1, Ti-2 and mature protein (in terms of percentage of the sum of these products) were calculated taking into account the number of [35S] methionine residues in each protein species. (C) Hydrophobicity plots of the 16/23 and 16/23 L82D proteins, generated according to (28). For further details, see the legends to Figures 1 and 3.

Fig. 5. Association of translocation intermediates with Tat complexes in the thylakoid membrane.
(A) In thylakoido time-course experiment performed with the 16/23 chimera. Thylakoid transport reactions were washed twice with HM buffer, solubilised with digitonin (1.7 %), separated on a 5-13.5 % Blue Native polyacrylamide gradient gel, and visualised by phosphorimaging. The apparent sizes of the detected complexes are indicated. (B) In thylakoido experiments were performed with authentic and mutant precursor proteins for 5 min at 25°C and analysed by SDS-PAGE. (C) Thylakoid transport reactions from (B) were treated as in (A), separated on a Blue Native polyacrylamide gradient gel, and visualised by phosphorimaging. For further details, see the legend to Figure 1.
Figure 1

A

| temp [°C] | 16/23 | 23 kDa | 16 kDa |
|-----------|-------|--------|--------|
| thermolysin t | - | + | - | + |
| 25        | - | + | - | + |
| 4         | - | + | - | + |

B

C

thermolysin

| 16 kDa | /16 E84M | /23 kDa | /23 D80L | /23 ST77F | /D80L | /16/23 L82D | /16/23 |
|--------|----------|---------|----------|-----------|-------|-------------|--------|
| -      | +        | -       | +        | -         | +     | -           | +      |
**Figure 2**

**A**

|       | Sequence                          |
|-------|-----------------------------------|
| 16 kDa | SAEAE**T**RAMLGFVAAGLASGSFVKAVLA EARP**I**VGPPPLS |
| 16 E84M| SAEAE**T**RAMLGFVAAGLASGSFVKAVLA MARP**I**VGPPPLS |
| 23 kDa | VLN**G**VSRR**L**ALT**V**L**I**G**A**A**AVGSKVS**P**DA AYGEAANVF**G**PKK |
| 23 D80L| VLN**G**VSRR**L**ALT**V**L**I**G**A**A**AVGSKVS**P**LA AYGEAANVF**G**PKK |
| 23 S77F,D80L | VLN**G**VSRR**L**ALT**V**L**I**G**A**A**AVGSKVF**P**LA AYGEAANVF**G**PKK |
| 16/23  | SAEAE**T**RAMLGFVAAGLASGSFVKAVLA AYGEAANVF**G**PKK |
| 16/23 L82D | SAEAE**T**RAMLGFVAAGLASGSFVKAVDA AYGEAANVF**G**PKK |

**B**

![Graph showing hydrophobicity](image)

- **16 kDa**
- **23 kDa**
- **16/23**

**amino acid**  
(relative to TPP cleavage site)
Figure 4

A

| time [min] | 0 | 1 | 2 | 5 | 10 | 20 | 40 |
|---|---|---|---|---|----|----|----|
| thermolysin | - | + | - | + | - | + | - |
| 16/23 | + | - | + | - | - | + | - |
| 16/23 L82D | + | - | + | - | - | + | - |

B

16/23

Ti-1 [●] Ti-2 [○] mature [▼]

relative amount [%]

0 20 40 60 80 100

0 10 20 30 40 t [min]

16/23 L82D

Ti-1 [●] Ti-2 [○] mature [▼]

relative amount [%]

0 20 40 60 80 100

0 10 20 30 40 t [min]

C

hydrophobicity

3 2 1 0 -1 -2 -3

-31 -21 -11 -1 1 2 30

amino acid (relative to cleavage site)
Figure 5

A

| time [min] | 0 | 1 | 2 | 5 | 10 | 20 | 40 |
|------------|---|---|---|---|----|----|----|
| 620 kDa    |   |   |   |   |    |    |    |
| 560 kDa    |   |   |   |   |    |    |    |

B

|       | 16       | 23       | 16/23    |
|-------|----------|----------|----------|
| 16 kDa| E84M     | S77F D80L| L82D     |
| t     | -        | +        | -        |
| t     | -        | +        | t        |
| t     | -        | +        | t        |
| t     | -        | +        | t        |

C

|       | 16       | 23       | 16/23    |
|-------|----------|----------|----------|
| 16 kDa| E84M     | S77F D80L| L82D     |
|       |          |          |          |
Prerequisites for terminal processing of thylakoidal Tat substrates
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