The Twin Arginine Consensus Motif of Tat Signal Peptides Is Involved in Sec-independent Protein Targeting in Escherichia coli*

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In Escherichia coli a subset of periplasmic proteins is exported through the Tat pathway to which substrates are directed by an NH₂-terminal signal peptide containing a consensus SRRXFLK “twin arginine” motif. The importance of the individual amino acids of the consensus motif for in vivo Tat transport has been assessed by site-directed mutagenesis of the signal peptide of the Tat substrate pre-SufI. Although the invariant arginine residues are crucial for efficient export, we find that slow transport of SufI is still possible if a single arginine is conservatively substituted by a lysine residue. Thus, in at least one signal peptide context there is no absolute dependence of Tat transport on the arginine pair. The consensus phenylalanine residue was found to be a critical determinant for efficient export but could be functionally substituted by leucine, another amino acid with a highly hydrophobic side chain. Unexpectedly, the consensus lysine residue was found to retard Tat transport. These observations and others suggest that the sequence conservation of the Tat consensus motif is a reflection of the functional importance of the consensus residues. Tat signal peptides characteristically have positively charged carboxyl-terminal regions. However, changing the sign of this charge does not affect export of SufI.

Proteins that are exported to the periplasm or periplasmic face of the bacterial cytoplasmic membrane are usually synthesized with an NH₂-terminal signal peptide that mediates targeting to the export apparatus. After translocation of the precursor protein to the periplasm the signal peptides are normally removed by the enzyme signal peptidase. The majority of periplasmic proteins are exported via the Sec apparatus. A major feature of the Sec apparatus is that proteins are translocated in an extended conformation and are often bound by SecB or other cytoplasmic chaperones to prevent folding prior to export (1–3). Sec pathway signal peptides are typically 18–26 amino acids in length. Although conserved amino acid sequences are absent, Sec signal peptides possess a common tripartite structural organization comprising a positively charged amino-terminal (n-)³ region, a central hydrophobic (h-) region with a high propensity for α-helix formation, and a carboxyl-terminal (c-) region that carries the cleavage site for the signal peptidase (4–6).

It has recently become clear that bacteria possess a second general protein export pathway that is quite distinct from the Sec apparatus (7–12). This Sec-independent pathway has been termed the Tat (for twin arginine translocation) system (12) because precursors are targeted to the pathway by signal peptides that, while preserving the tripartite structural organization of Sec signal peptides, bear a characteristic n-region sequence motif that includes two consecutive and invariant arginine residues. Studies in Escherichia coli suggest that the Tat system is minimally comprised of the four integral membrane proteins TatA, TatB (also termed MttA2; GenBank accession no. AF067848), TatC, and TatE (11–14). Remarkably and uniquely this translocon functions to transport folded proteins across the cytoplasmic membrane, a feat that must be achieved without rendering the membrane freely permeable to protons and other ions.

The thylakoid membrane of plant chloroplasts contains protein transport systems homologous to both the Sec and Tat pathways of bacteria (15). Targeting to the Tat-analogous thylakoid ΔpH-dependent transport pathway is, like its bacterial counterpart, mediated by signal peptides (“transfer peptides” or “lumen-targeting domains”) with paired arginine residues (16), and indeed bacterial Tat signal peptides have been shown to direct efficient thylakloid import specifically by the ΔpH-dependent system (17–19). In both bacteria (6, 20) and chloroplasts (21, 22) the signal peptide alone is capable of mediating mutually exclusive sorting of precursor proteins between the Tat and Sec pathways. Thus, even though they conform to the tripartite structure of Sec signal peptides, Tat signal peptides avoid functional interactions with the Sec apparatus. Recent studies in bacteria suggest that the low h-region hydrophobicity of Tat signal peptides relative to that of Sec signal peptides is the major structural feature precluding targeting of Tat substrates to the Sec pathway (6), and the same mechanism may apply in chloroplasts (23). In addition, basic residues commonly found in the c-region of bacterial and thylakoid Tat signal peptides, but characteristically absent from the c-region of Sec signal peptides, have been shown in thylakoids to operate as a “Sec-avoidance” determinant (24). A similar function is likely for these residues in bacterial Tat signal peptides given the longstanding observations that introduction of positive charges in the vicinity of the leader peptidase site slows transport of Sec-targeted precursors (e.g. Refs. 6 and 25).

Targeting to the Tat apparatus in chloroplasts exhibits an absolute dependence on the invariant twin arginine pair within the signal peptide (16, 23). Studies with bacterial Tat signal peptides suggest that the twin arginine residues are also important in targeting to the bacterial Tat pathway (6, 26–29). However, a major difference between bacterial and plant Tat...
Signal peptides is that the bacterial signal peptides contain additional conserved amino acids surrounding the paired arginines. A consensus for this extended twin arginine motif has been defined as SRRXFLK where the arginine residues are invariant, and the frequency of occurrence of the other amino acids exceeds 50% (7). The observation of an extended n-region motif immediately raises the question of the role and importance of the non-arginine consensus residues in the bacterial Tat transport process. A further obvious question is whether these additional potential targeting determinants reduce the importance of the arginine pair that is essential for thylakoid Tat transport. In an attempt to address these questions we have undertaken a systematic site-directed mutagenesis study of the extended bacterial Tat consensus motif and assessed the effect of these signal peptide alterations on protein export. In addition, we have investigated the importance of the c-region charge in targeting to the bacterial Tat apparatus.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Strain BUDDY (MC4100 ΔtatC::HiSpeeD) was constructed as follows. The interposon fragment carrying the srrXftrcE-srrxyftrcE resistance gene from pPH45 (30) was subcloned following EcoRI digestion into plasmid pFAT23, which carries an in-frame deletion in the tatC gene (13). The resultant plasmid, pFAT25, was digested with XbaI and EcoRI and the marked deletion allele of tatC cloned into the poly linker of pMKA705 (31) to give plasmid pFAT27. The marked deletion allele of tatC was subsequently recombined into the chromosome of MC4100 (F’ ΔlucU169 araD139 ρspL150 relA1 ptsF rbs flbB5301) (32) as described (31). Strain NES-1 was constructed by P1 transduction (33), of the ΔsrrxyftrcE allele into K88 (HfrC phoA4 pit-10 tonA22 ompF 627 relA1 spoT1 1) (34).

During all genetic manipulations E. coli strains were grown aerobically in Luria-Bertani medium (35). Concentrations of antibiotics were as described previously (12).

**Plasmid Construction**—Plasmids for the in vivo synthesis of pre-SufI and pre-Yack were constructed as follows. A 1.430-base pair polymerase chain reaction fragment covering the region from 18 nucleotides upstream of the start codon of sufI, including the Shine-Dalgarno sequence, to the stop codon was amplified using the primers 5’-GGCG-GAATTCCTTATGGAAACTATG-3’ and 5’-GC-GCTGTACTTGATCGTACCGATGTGACC-3’ with MC4100 chromosomal DNA as the template. The product was digested with EcoRI and XbaI and cloned into the poly linker of pT7.5 (36) to give plasmid pNR14, which was used as the template for all subsequent mutations in the signal peptide coding portion of sufI. A 1.464-base pair DNA fragment covering the region from 16 nucleotides upstream of the start codon of yack to the stop codon was amplified from MC4100 chromosomal DNA using the primers 5’-GGCG-GAATTCCAAATGGAATATAAGATTATG-3’ and 5’-GCGCATCCAAGACCCTAAC-3’. The polymerase chain reaction product was digested with EcoRI and XbaI and cloned into the poly linker of pT7.5 to give plasmid pNR15, which was used as the template for the introduction of mutations into the signal peptide-coding portion of yack. Site-specific mutations in pNR14 and pNR19 were constructed either by polymerase chain reaction methods or using the QuikChangeTM system (Stratagene). All mutations were verified by DNA sequencing using the Applied Biosystems Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

**Pulse-Only and Pulse-Chase Experiments**—An overnight culture of K88 containing both pGP1-2 (36) and the appropriate pT7 recombinant plasmid was grown aerobically in Luria-Bertani medium at 30 °C with the appropriate concentrations of antibiotics (12). The strain was subcultured at a 1:80 dilution in fresh Luria-Bertani medium and grown until the A600nm reached 0.2. At this point 1 ml of culture was harvested and washed once in M9 medium (35). The washed cells were resuspended in 5 ml of M9 medium supplemented with 0.002% thiamine, 2 mM MgSO4, 0.1 mM CaCl2, and a methionine- and cysteine-free amino acid mix (0.01%). The cells were grown for a further hour at 30 °C. During this period 0.5 ml of the experimental sample was then incubated in 5 ml of M9 plus supplements in a 25-ml universal tube. Samples of the plasmid-encoded Tat-dependent precursor proteins, which are under the transcriptional control of the T7 σ10 promoter, was induced by a temperature shift from 30 °C to 42 °C for 10 min and then transferred to 30 °C for 20 min prior to labeling. 0.05 mCi of [35S]methionine (NEN Life Science Products) was added to each 5-ml culture. For the pulse-only experiments the initial 0.5-ml sample was taken immediately. For the pulse-chase experiments the initial 0.5-ml sample was collected after a 5-min labeling period and the remaining 4.5 ml of the culture was used for the immediate addition of 750 μl unlabeled methionine. In both types of experiment additional 0.5-ml samples were removed at defined time points during the subsequent incubation. Samples were flash frozen in liquid nitrogen as they were collected. The samples were subsequently thawed and the cells pelleted by centrifugation. The pellets were resuspended in 75 μl of SDS loading buffer, and the samples were resolved by pulsed-gel electrophoresis following SDS-PAGE (37) on 12.5% polyacrylamide gels. Quantification of the gel bands was undertaken using a Fuji BAS-1000 PhosphorImager and the software package MacBAS version 2.0.

**Subcellular Localization of Radiolabeled Proteins**—The cells to be fractionated were prepared as for the pulse-chase experiments with the exception that only 2 ml of cell culture was labeled. After the appropriate chase period the cells were pelleted by centrifugation, resuspended in 1 ml of cell fractionation buffer (30 mM Tris-HCl, pH 8.0, 20% (v/v) sucrose, 1 mM Na2EDTA), and incubated at 20 °C for 10 min. The cells were centrifuged, the supernatant discarded, and the pellet resuspended in 133 μl of ice-cold 5 mM MgSO4. After 10 min on ice the cells were centrifuged, and the supernatant was retained as the periplasmic fraction.

**RESULTS AND DISCUSSION**

**Establishing a Semiquantitative Export Assay**—To assess the effect of mutagenesis of the twin arginine consensus region upon protein export it was first necessary to establish an appropriate assay. Since it has not so far proved possible to develop an in vitro transport assay for the bacterial Tat system we investigated methods for studying Tat translocation in whole cells. We chose the precursor of the E. coli protein SufI as the experimental substrate for our in vivo assay. SufI was originally identified on the basis that, when overproduced, it suppressed the cell division defect of an ftsI mutant (38). SufI is clearly homologous to the multicopper oxidase superfamily (39). Nevertheless, SufI is unlikely to bind copper cofactors because it contains only 2 of the 12 consensus copper-binding ligands, and neither of these is conserved in the SufI protein from the closely related organism Haemophilus influenzae. In addition, purified recombinant SufI does not contain detectable copper binding sites.2 It is thus reasonable to expect that the observed export kinetics of SufI will not be complicated by the kinetics of cofactor insertion processes. The use of SufI as the assay substrate has a number of additional practical advantages. SufI is a sufficiently small protein that the mass difference between precursor (51.9 kDa) and mature (49.1 kDa) forms is readily detectable by denaturing electrophoresis, and therefore export can be routinely and conveniently monitored using a SDS-PAGE-based assay. SufI, in contrast to the majority of E. coli Tat substrates, is a water-soluble rather than peripheral membrane protein, which allows export to be confirmed by subcellular fractionation. Use of an indigenous substrate of the E. coli Tat pathway helps ensure that the experimental data obtained in our studies are relevant to the export of physiological substrates.

The twin arginine region of the SufI signal peptide (Fig. 1, top) exhibits a close match to the Tat consensus sequence differing only in conservatively substituting an isoleucine for the consensus leucine (this is the most frequently observed replacement at this position in Tat signal peptides; see Ref. 7) and substituting a glutamine for the consensus lysine residue. Two additional sequence features typical of bacterial signal peptides (6), a proline at the end of the h-region (Pro-22) and a cysteine residue, to the stop codon was amplified using the primers 5’-GGCG-GAATTCCAAATGGAATATAAGATTATG-3’ and 5’-GCGCATCCAAGACCCTAAC-3’. The polymerase chain reaction product was digested with EcoRI and XbaI and cloned into the poly linker of pT7.5 to give plasmid pNR15, which was used as the template for the introduction of mutations into the signal peptide-coding portion of yack. Site-specific mutations in pNR14 and pNR19 were constructed either by polymerase chain reaction methods or using the QuikChangeTM system (Stratagene). All mutations were verified by DNA sequencing using the Applied Biosystems Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

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2 N. Stanley, B. C. Berks, and T. Palmer, unpublished observations.
by the addition of [35S]methionine and then chased from time zero with Pre-SufI, expressed in strain K38[pGP1-2], was pulse labeled for 5 min labeled with [35S]methionine, followed by the addition of excess nine motif, together with basic amino acids in the COOH-terminal of [35S]methionine at time zero. Precursor synthesis and processing strain K38[pGP1-2]. Labeling of pre-SufI was initiated by the addition (**Fig. 1** c)). (**lane 3**). (**Panel a**). (**Panel b**), SufI is exported exclusively via the Tat pathway. Pre-SufI was pulse labeled with [35S]methionine for 5 min and then chased with nonradioactive methionine. In lane 1 the precursor protein was expressed in the ΔtatC strain NRS-1[pGP1-2], and whole cells were analyzed at a chase time of 60 min. In lanes 2 and 3 the precursor was expressed in strain K38[pGP1-2], and labeled proteins were analyzed at a chase time of 10 min either in whole cells (lane 2) or in the isolated periplasmic fraction (lane 3). Panel c, in vivo pulse-only analysis of pre-SufI export in **E. coli** strain K38[pGP1-2]. Labeling of pre-SufI was initiated by the addition of [35S]methionine at time zero. Precursor synthesis and processing were assessed by SDS-PAGE of whole cells followed by autoradiography. (**Panel b**). (not shown), confirming that these peptides correspond to precursor and processed forms of SufI. Subcellular fractionation experiments showed that the mature form represents protein that has been exported to the periplasm (**Fig. 1b**, lanes 2 and 3). A strain bearing a deletion in the tatC gene has been shown previously to be completely defective in the correct localization of proteins with Tat signal peptides (13). When the pulse-chase experiment was repeated in this ΔtatC background only the precursor protein was visible after a 60-min chase (**Fig. 1b, lane 1**), indicating that processing of pre-SufI is exclusively Tat-dependent and that no export of this protein occurs via the Sec pathway.

Although export of wild-type precursor is essentially complete within minutes (**Fig. 1a**), precursors with mutated signal peptides were anticipated to have slowed rates of export. It was therefore important to establish that export continued to occur for extended time periods under our assay conditions. In the experiments shown in **Fig. 1c**, labeling of newly synthesized pre-SufI was initiated by the addition of [35S]methionine and allowed to continue throughout the experiment. The mature protein continued to accumulate over the full 60-min time period, demonstrating that export occurs throughout the time scale of the experiment.

**Analysis of Site-specific Amino Acid Substitutions in the SufI Signal Peptide—** A range of site-specific amino acid substitutions was introduced into the signal peptide coding region of sufI. The resulting engineered precursors have been given shorthand designations made up of the substituted amino acid type and position followed by the replacement amino acid. Thus precursor SufI-R5K carries a lysine substitution of the arginine at position five in the SufI signal peptide. The effects of the various site-specific mutations on the export kinetics of pre-SufI were assessed by pulse-chase assays. The averaged export data for a minimum of three independent cultures expressing each engineered precursor are plotted in **Fig. 2**. Fractionation studies were used to verify that the mature protein observed in the pulse-chase experiments was located in the periplasm (data not shown). These experiments confirm that the slow processing exhibited by some of the signal peptide mutants represents a genuine export event and is not a consequence of nonspecific proteolysis of the unexported precursor protein. No processing was observed over a 60-min chase for any of the mutant precursors when the export experiments were repeated in a ΔtatC background (data not shown). Thus, none of the signal peptide mutations investigated in this study caused the precursor protein to default onto the Sec export pathway. This phenotype may not, however, reflect the true potential for interactions between the altered signal peptides and the Sec apparatus but may instead originate from an incompatibility between the Sec pathway and the mature SufI protein. Indeed, there is now clear evidence that some Tat pathway precursors are inherently refractory to transport by the Sec apparatus (20, 24).

**Mutations Affecting the Twin Arginine Residues—** The invariant arginine residues of pre-SufI were conservatively replaced both individually and in combination by the amino acid lysine. The individual arginine to lysine mutations severely retard export of pre-SufI, whereas export was blocked completely when both arginines were replaced (**Fig. 2a**). These results are in marked contrast to the behavior of Sec signal peptides where mutations that conserve the n-region charge have no effect on the transport process (40) and argue that both Tat consensus arginine residues play a specific and critical role in mediating the interaction between the signal peptide and the Tat apparatus. That the single lysine for arginine mutation in the SufI signal peptide permits transport by the Tat pathway was surprising because equivalent mutations had been found to block both the Tat-dependent export of Zymomonas mobilis glucose-fructose oxidoreductase (29) and the import of wheat pre-23K protein via the plant thylakoid Tat system (16). However, a further study using a Tat signal peptide:β-lactamase chimera expressed in **E. coli** suggested that

![Fig. 1. Establishing a semiquantitative assay for Tat protein transport using the protein pre-SufI as substrate. The SufI signal peptide sequence is shown at the top of the figure with the hydrophobic region underlined and residues of the consensus SRRXFLK twin arginine motif, together with basic amino acids in the COOH-terminal region, shown in bold type. Panel a, pulse-chase analysis of SufI export. Pre-SufI, expressed in strain K38[pGP1-2], was pulse labeled for 5 min by the addition of [35S]methionine and then chased from time zero with unlabeled methionine. Precursor synthesis and processing were assessed by SDS-PAGE of whole cells followed by autoradiography. Panel b, SufI is exported exclusively via the Tat pathway. Pre-SufI was pulse labeled with [35S]methionine for 5 min and then chased with nonradioactive methionine. In lane 1 the precursor protein was expressed in the ΔtatC strain NRS-1[pGP1-2], and whole cells were analyzed at a chase time of 60 min. In lanes 2 and 3 the precursor was expressed in strain K38[pGP1-2], and labeled proteins were analyzed at a chase time of 10 min either in whole cells (lane 2) or in the isolated periplasmic fraction (lane 3). Panel c, in vivo pulse-only analysis of pre-SufI export in **E. coli** strain K38[pGP1-2]. Labeling of pre-SufI was initiated by the addition of [35S]methionine at time zero. Precursor synthesis and processing were assessed by SDS-PAGE of whole cells followed by autoradiography. The mean measured counts in (i) precursor and (ii) mature bands from four independent experiments are plotted. The bars represent the standard error of the mean. A plasmid, pNR14, was constructed in which expression of sufI is under the control of the T7 φ10 promoter. Upon provision of T7 RNA polymerase, and in the presence of rifampicin to block transcription from **E. coli** promoters, this construct allows specific in vivo radiolabeling of the SufI precursor protein. A pulse-chase experiment using the sufI-bearing plasmid pNR14 is shown in **Fig. 1a**. After induction of T7 polymerase coded by the helper plasmid pGP1-2, the cells were pulse labeled with [35S]methionine, followed by the addition of excess unlabeled methionine. Over time a labeled protein with the apparent molecular mass of pre-SufI was converted to a smaller mature form. Neither form is present when a plasmid lacking the sufI insert is used (not shown), confirming that these proteins correspond to precursor and processed forms of SufI. Subcellular fractionation experiments showed that the mature form represents protein that has been exported to the periplasm (**Fig. 1b**, lanes 2 and 3).
The first consensus arginine residue could be replaced not only by lysine but also by glutamate, valine, or isoleucine without completely blocking export (26). It should be kept in mind though that there is no evidence that the transport observed in this early study was mediated by the Tat pathway. These variations in the severity of a single lysine for Tat consensus arginine substitutions observed in different studies could be

**FIG. 2.** Pulse-chase analysis of the export rates of SufI signal peptide mutants. Precursor proteins, expressed in strain K38[pGP1-2], were pulse labeled for 5 min by the addition of [35S]methionine and then chased from time zero with unlabeled methionine. Precursor synthesis and processing were assessed by SDS-PAGE of whole cells followed by autoradiography. The mean percentage of total SufI protein remaining in precursor form in whole cells at each of the indicated time points is plotted (n = 3–6). The bars represent the standard error of the mean. The export of wild-type pre-SufI (○) is compared with that of precursors with substitutions of the Tat signal peptide consensus phenylalanine residue: SufI-F8L (E), SufI-F8Y (f), and SufI-F8A (M).

**FIG. 3.** Analysis of pre-YacK export. The YacK signal peptide sequence is shown at the top of the figure with the hydrophobic region underlined and residues of the consensus SRRXFLK twin arginine motif, together with basic amino acids in the COOH-terminal region, shown in bold type. Panel a, YacK is exported exclusively via the Tat pathway. Pre-YacK was pulse labeled with [35S]methionine for 5 min and then chased with unlabeled methionine. In lane 1 the precursor protein was expressed in the ΔtatC strain NRS-1[pGP1-2], and whole cells were analyzed at a chase time of 60 min. In lanes 2 and 3 the precursor was expressed in strain K38[pGP1-2], and labeled proteins were analyzed at a chase time of 10 min either in whole cells (lane 2) or in the isolated periplasmic fraction (lane 3). Panel b, comparative pulse-chase analysis of export of pre-YacK with precursors possessing alterations in the Tat signal peptide consensus motif. Precursor proteins were expressed in K38[pGP1-2], pulse labeled for 5 min by the addition of [35S]methionine, and then chased from time zero with unlabeled methionine. Precursor synthesis and processing were assessed by SDS-PAGE of whole cells followed by autoradiography. The mean percentage of total YacK protein remaining in precursor form in whole cells at each of the indicated time points is plotted (n = 3–6). The bars represent the standard error of the mean. The export of wild-type pre-YacK (○) is compared with that of precursors with substitutions in the Tat signal peptide consensus motif: YacK-K8A (E), YacK-K8R (f), YacK-R3K (M), and YacK-R3K,R4K (E).
indicative of genuine differences in the behavior of disparate signal peptides. Alternatively, they could be a consequence of the variable sensitivity of the export assays employed by different investigators. In an attempt to distinguish between these two possibilities we produced an additional lysine for arginine mutation in another E. coli Tat substrate, pre-YacK, a multicopper oxidase (39) of unknown physiological function. YacK is homologous to SufI (37% amino acid sequence identity), and the two proteins have quite similar Tat signal peptides (Figs. 1 and 3). A pulse-chase export assay analogous to that used for pre-SufII was established for pre-YacK with the processed mature form of YacK shown to be located in the periplasm (Fig. 3a, lanes 2 and 3), and export demonstrated to be tatC-dependent (Fig. 3a, lane 1) and to continue for at least 60 min after labeling was initiated (data not shown). Since export of the YacK and SufII was analyzed by identical methodology, the phenotypes of equivalent mutations in the two proteins are directly comparable. Whereas single arginine to lysine mutations in pre-SufII permit export, it was found that an analogous mutation in YacK (YacK-R3K, Fig. 3b) abolishes transport. We conclude that in certain signal peptide contexts the presence of both arginine residues is not an obligate requirement for transport by the Tat pathway. Nevertheless, even in such a permissive signal peptide it is likely that an arginine can only be functionally replaced with another positively charged amino acid (that is lysine) because we found export to be blocked by an alanine for arginine substitution (SufII-R5A, Fig. 2a). This observation suggests that the Tat system recognizes the twin arginine residues in part via electrostatic interactions.

**Mutations Affecting the Consensus Serine Residue**—We have noted previously (7) that the residue that directly precedes the arginine pair is most frequently one of those amino acids (serine, threonine, asparagine, and aspartate) that is a preferred capping residue for the NH₂ termini of helices (41). As a consequence we speculated that this residue might stabilize the core of the signal peptide in an α-helical conformation. We tested the possible importance of the SufII Tat consensus serine as a helix cap by replacing this residue with alanine, an amino acid with a high helical propensity, but which is unable to stabilize the NH₂ termini of helices because it does not have a hydrogen bond-accepting side chain. This SufII-S4A mutation had no significant effect on export kinetics (Fig. 2b), suggesting that neither the functionality of the serine side chain nor the presence of a helix-capping residue at this position is critical for the export process. NH₂-terminal to the consensus serine the SufII signal peptide possesses an additional good helix-capping residue, Ser-2. This residue could potentially substitute for the function of the consensus serine in the SufII-S4A mutant. We were able to exclude this possibility by demonstrating that export of a mutant lacking both serine residues was not significantly impaired (SufII-S2A,S4A; Fig. 2b). The consensus serine residue in the SufII signal peptide was additionally mutated to cysteine, an amino acid that differs structurally from serine only in replacing the oxygen atom with a sulfur atom. The resultant SufII-S4C construct exhibited a substantially slowed rate of export (Fig. 2b). Given the unchanged transport phenotype of the SufII-S4A mutant where all side chain functionality is missing, it is most likely that it is the substantially increased hydrophobicity of the cysteine side chain in SufII-S4C which is interfering with the export process.

**Mutations at the Consensus “X,” Phenylalanine or Leucine Positions**—A feature of the consensus motif, noted here for the first time, is that the amino acid position immediately following the paired arginine residues (residue X in the consensus motif) is any polar residue or, rarely, the short side chain amino acids glycine or alanine. To test the importance of this amino acid to Tat transport we changed the glutamine found at this position in the SufII signal peptide to leucine, which has a similarly sized, but highly hydrophobic, side chain. This SufII-Q7L mutation had no significant effect on export rate (Fig. 2c), and we therefore conclude that the polar amino acid that follows the twin arginine residues is not critical for Tat transport.

The consensus phenylalanine residue is, after the invariant arginine residues, the most highly conserved amino acid of the bacterial Tat motif, being found in approximately 80% of twin arginine signal peptides. Remarkably, a phenylalanine is never found at this position in plant Tat transfer peptides. We found that replacing the consensus phenylalanine of pre-SufII with either tyrosine (SufII-F8Y), which differs from phenylalanine by having a hydroxyl group attached to the ω carbon of the aromatic ring, or with alanine (SufII-F8A), which effectively removes the side chain functionality, severely retards export (Fig. 2d). In contrast, a substitution by leucine, the amino acid found most frequently after phenylalanine at this position, had no discernible effect on export kinetics (SufII-F8L, Fig. 2d). The nature of the amino acid side chain at the phenylalanine consensus position is thus critical to export efficiency. The fact that leucine but not tyrosine can be substituted for the consensus phenylalanine argues against the aromatic nature of the phenylalanine side chain being a major arbiter of interactions with the Tat apparatus. Instead, we suggest that it is the high hydrophobicity (and possibly also high helical propensity) exhibited by phenylalanine and leucine, but not tyrosine, which is the critical structural determinant at this consensus position.

The amino acid position three residues after the arginine pair is always hydrophobic with a strong preference for a leucine residue. Plant Tat transfer peptides also contain a hydrophobic amino acid at the equivalent position, and this has been shown experimentally to have a weak influence on transport (42). The importance of a nonpolar side chain at the consensus leucine position in bacterial Tat signal peptides was tested by substituting an alanine residue for the isoleucine present in the signal peptide of pre-SufII. This SufII-I9A mutation showed a small decrease in the rate of export (Fig. 2c), suggesting that, as for thylakoid Tat signal peptides, a hydrophobic amino acid at the consensus leucine position contributes to efficient Tat transport.

**Mutations at the Consensus Lysine Position**—A lysine residue is normally present four amino acids after the arginine pair in bacterial Tat signal peptides. This is in marked contrast to thylakoid Tat transfer peptides where a charged amino acid is never found at this position. Nevertheless, bacterial Tat signal peptides containing the consensus lysine are capable of mediating thylakoid import via the chloroplast Tat pathway (17, 18). In the SufII signal peptide the consensus lysine is replaced by a glutamine residue. To investigate the significance of this substitution we mutated the glutamine to the consensus lysine. Surprisingly, export of the resultant SufII-Q10K mutant was severely retarded (Fig. 2e). We also investigated the effects of replacing the glutamine with alanine (SufII-Q10A) to remove the side chain functionality at this position, or leucine (SufII-Q10L), an amino acid with a hydrophobic side chain of similar bulk to that of glutamine. Both of these substitutions resulted in only small decreases in the rate of SufII export rather than the severe transport defect exhibited by the SufII-Q10K mutant (Fig. 2e). Thus for SufII the identity of the amino acid at the consensus lysine position appears to be functionally important, but, surprisingly, of the amino acids tested it was substitution with the consensus lysine residue which had the strongest inhibitory effect on export. We decided to investigate this unusual finding further by examining the effect of mutating the
Mutagenesis of the Tat Consensus Motif

The c-region of bacterial Tat signal peptides is not required for Tat targeting but act to prevent functional interaction of the precursor protein with the Sec apparatus (24). Bacterial Tat signal peptides, including that of pre-SufI, also commonly possess a positively charged c-region (18, 43). We found that mutation of the lysine found in this region of pre-SufI either to alanine or to aspartate had no discernible effect on the rate of export (SufI-K24A and SufI-K24D, Fig. 2c), while transport remained exclusively Tat-dependent. As noted above, the SufI mature region may well be Sec-incompatible, and this would mask our ability to assess the Sec compatibility of the signal peptide itself. Clearly, however, our results show that a c-region basic residue is not essential for transport of an authentic bacterial Tat substrate through the Tat apparatus.

Although the c-region of Tat signal peptides commonly contains basic amino acids, we note here that acidic residues are rarely found in the Tat signal peptides of either bacteria or plants and are in fact totally absent from E. coli Tat substrates. The net effect is that the c-regions of bacterial Tat signal peptides have an overall positive charge (the mean charge is +0.64 when calculated from the non-Rieske protein data set in Ref. 7). In our SufI-K24D mutant the signal peptide c-region has both an acidic residue and an overall negative charge (note also that the first charged residue in the mature protein is at residue 16). Nevertheless, this protein exhibits normal export rate with a single lysine for arginine substitution, although compatible with the deleterious effects of certain substitutions at other consensus positions, strongly supports the idea that the observed sequence conservation of the extended twin arginine motif found in bacterial, but not plant, signal peptides reflects an important role for these residues in mediating specific interactions with Tat pathway components.

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