Influence of Specific Signal Peptide Mutations on the Expression and Secretion of the α-Amylase Inhibitor Tendamistat in Streptomyces lividans*

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The Streptomyces α-amylase inhibitor tendamistat is secreted by a signal peptide with an amino-terminal charge of +3. To elucidate the influence of the charged residues on protein secretion in Streptomyces, the amino-terminal charge was varied from +6 to neutral net charge. The effects of charge variation were analyzed in combination with three Streptomyces promoters and two transcriptional terminators. Introduction of additional positive charges significantly decreased the amount of secreted tendamistat. On the contrary, a charge reduction to +2 resulted in the doubling of inhibitor production. After exclusion of transcriptional effects, the observed alterations of inhibitor secretion by the mutants with a charge of +6 to +2 were attributed to a modulation of precursor synthesis. Furthermore, a tight coupling of synthesis and export was stated. Charge reduction to +1 or neutral charge generally reduced the yield of secreted tendamistat, yet remarkable differences were found for mutants with identical net charge. Elimination of the positive charge at a defined position resulted in the release of tendamistat precursor protein, which suggested a specific uncoupling of synthesis and translocation.

The majority of secreted proteins are synthesized in the cytoplasm in a precursor form with an N-terminal extension termed the signal peptide. Although little homology in the overall amino acid sequence is found, signal peptides show a common tripartite structure irrespective of their origin. A central core of hydrophobic residues is flanked by an amino-terminal region, also termed the N-domain, which is characterized by its content of basic residues, and a C-terminal part with an appropriate recognition site for a signal peptidase. Excellent reviews on signal peptide structure and its function in bacterial protein secretion have been submitted by several authors (1–5).

The impact of charge variation in the N-domain was studied essentially in signal peptides of Escherichia coli secretory proteins; the results are reviewed by Gennis et al. (3). Concurring experimental data (6–8) indicate that signal peptides with neutral or even negative N-terminal charge are still functional, but reduced in their ability to promote the translocation of a given protein. For maximal efficiency of translocation, a positive net charge is necessary, although this dependence may be influenced by length and overall hydrophobicity of the core region (9). From the observation that signal peptides with reduced but still positive net charge function as efficiently as the wild-type leader, it was concluded that multiple charges represent a functional redundancy (10). One assignment of the charged region is the definition of the signal peptide orientation during insertion into the membrane in accordance with the “positive-inside” rule proposed by von Heijne (11), which has been amply documented during the last years (12–15), and the “loop model” of secretion (16, 17). On the other hand, involvement of the charged domain in the interaction of the signal peptide with components of the secretory machinery like the E. coli SecA protein (18) is proposed.

In contrast to the variety of information on signal peptide mutations in E. coli, few publications are available concerning Gram-positive bacteria with emphasis on members of the Bacillus genus (reviewed in Ref. 19). A systematic study of the influence of signal peptide variations on the secretion of Bacillus amyloidoliquefaciens levansucrase in Bacillus subtilis (20, 21) is in general agreement with the results obtained for E. coli. However, the dependence on positive charges in the N-terminal region was more pronounced; signal peptides with neutral net charge significantly impaired the export of the precursor protein.

Comparison of signal peptide sequences from different organisms indicated that variations in length and amino acid composition of the three parts are species-specific (22). N-domains from Gram-positive bacteria are longer and higher charged than the respective parts of signal peptides from Gram-negative bacteria, human or plant origin. Streptomyces signal peptides occupy a special position because of their extraordinary long and highly charged N-domains as well as the strong preference of arginine for lysine in this segment.

The obvious capacity of the Streptomyces secretory machinery, demonstrated by the variety of proteins that are secreted at high levels into the culture medium, raised growing interest among bioscientists and biotechnologists (23–25). A valuable model of Streptomyces secretory protein is tendamistat, a polypeptide of 74 amino acids, that specifically and almost irreversibly inhibits α-amylases of the mammalian type (26). The tendamistat gene was cloned from an amplified genomic sequence of the original producer Streptomyces tendae 4158 and was successfully expressed in Streptomyces lividans 66 (27).

Here we describe the influence of N-terminal charge modifications of the tendamistat signal peptide on tendamistat secretion in combination with the original tendamistat promoter.
and terminator as well as with two other Streptomyces promot- ers, the mel promoter of Streptomyces antibiotics and the ermEup promoter of Sarcaphagus erythrea, and an addi- tional terminator derived from the gene of Streptomyces fradiae. Charge variations have a significant effect on tendam- istat expression and secretion in S. lividans, thus indicating that the N-terminal region of a signal peptide plays an impor- tant role in the synthesis of the precursor. Our results suggest that secretion of tendamistat occurs essentially cotranslation- ally. The coupling of translation and translocation, however, can be partially relieved by specific signal peptide mutations. Moreover, a hypothesis is developed in order to understand the modulation of precursor synthesis due to signal peptide variation.

**MATERIALS AND METHODS**

**Bacterial Strains—For site-specific mutagenesis, E. coli strains BMH71-18 and MK30-3 as well as the M13 phagemids M13mp18 and M13mp18ve (28) were used. Further cloning was done in E. coli XL1-Blue (Stratagene, La Jolla, CA). The host for expression plasmids with the tendamistat and the mel promoter was S. lividans 66 TK24, which was kindly provided by K.-P. Koller (Hoechst AG, Frankfurt, Germany). Shuttle vector plasmids were expressed in S. lividans 66 TK23, con- tributed by the group of W. Piepersberg (Wuppertal, Germany). TK23 equals TK24 with respect to expression of tendamistat.

**Plasmid Construction—Oligodeoxynucleotide-oligos were synthesized on an Applied Biosystems Model 380B DNA synthesizer and purified by reversed-phase chromatography. Restriction endonucleases and DNA-modifying enzymes were from New England Biolabs Inc. (Beverly, MA). DNA sequencing according to Sanger et al. (68) was carried out with the T7 sequencing kit (Pharmacia Biotech Inc.).

**Tendamistat Promoter and Terminator Constructions—Derivatives of the Streptomyces vector pT7 (29) with the tendamistat promoter, terminator, and signal sequence were constructed by oligonucleotide-directed site-specific mutagenesis (28). In this regard, the tendamistat gene was cloned into phagemid M13mp18 via EcoR I and Pst I. The signal sequence mutations were introduced by oligodeoxynucleotide R5R9 for the R5 (5) mutation, oligodeoxynucleotide R5 for the R5 (5) mutation, and oligodeoxynucleotide A2 for the A2 (2) mutation. The oligodeoxynucleotide sequences are as follows: R5R9, 5'-GCCAT-GCCTAGGGGCACTTTGCACATTCCCGCATGGCGGTTCACCTTCCTGAGA-3' and R5, 5'-GCCATGCCTAGGGGCACTTTGCACATTCCCGCATGGCGGTTCACCTTCCTGAGA-3'.

**Assemblies for Inhibitor Production—Selection of inhibitor-secreting bacteria was carried out on agar plates as described (32). Se- creted inhibitor in liquid cultures was determined by the modified dinitrosalicylic acid assay (33) and by a modified α-amylase assay purchased from WAK Chemie GmbH (Bad Soden, Germany). Reference α-amylase from porcine pancreas was purchased from Boehringer Mannheim (Mannheim, Germany). Purified tendamistat was a generous gift of Hoechst AG.

**SDS-PAGE of Proteins and Western Blotting—For SDS-PAGE according to Schägger and von Jagow (34), gels with 16.5% T, 6% C in the separating gel, 10% T, 3% C in the spacer, and 4% T, 3% C in the stacking gel were cast. Samples were resuspended in sample buffer (4 × urea, 0.1 M Tris, 1 M NaOH, 1% SDS, 0.05% bromophenol blue, 0.2% mercaptoethanol). Samples were boiled and loaded on the gel for 5 min prior to loading. Extended boiling is not recommended, for it will cause the appearance of partially or totally oxidized tendamistat. Samples were either stained with Coomassie Brilliant Blue R-250 according to Densley or electroblotted on Immobilon P membranes (Millipore Corp., Bedford, MA) according to the protocol of Plough et al. (35). The molecular weight markers were visualized with Coomassie brilliant blue R-250 and were included in all gels as part of the membrane. Tendamistat-specific polyclonal antiserum prepara- tions developed in goat and an anti-goat IgG-alkaline phosphatase conjugate were used for immunodetection of tendamistat. The tendam- istat antiserum was a generous gift of K.-P. Koller. The coloring reaction was carried out with 5-bromo-4-chloro-3-indolyl phosphate substrate and p-nitro blue tetrazolium according to standard procedures.
Signal Peptide Mutations Influence Tendamistat Secretion

RESULTS

Tendamistat Expression in plJ 702 Derivatives with the Tendamistat or the mel Promoter and with the Tendamistat or the aph Terminator—On the basis of the tendamistat gene, a cassette for transport expression in Streptomyces was developed in our group (Fig. 1). The construction of the signal peptide variants and the Streptomyces expression plasmids is described under "Materials and Methods"; the amino acid sequences of wild-type and mutant tendamistat signal peptides are shown in Fig. 2. The chosen codons for arginine (5'-GCC-3'), alanine (5'-GCC-3' and 5'-GGC-3'), and aspartate (5'-GAC-3') substitutions obey the preferences of the Streptomyces codon usage (37).

First, mutant signal peptides with an N-terminal charge ranging from +2 to +6 (Fig. 2) were tested for tendamistat expression in combination with the native tendamistat promoter and terminator as well as with another Streptomyces promoter (mel) and terminator (aph). The tendamistat promoter is located on a 194-base pair HindIII/SspI fragment of the expression vector. Downstream from the coding region, a sequence was identified that represents a preferred transcriptional termination site as proven by Northern blots (data not shown) and is therefore termed the tendamistat terminator. This terminator is not very efficient because a remarkable portion of extended transcripts are found.

According to a report of Pulido and Jiménez (38), an increase of secretory protein expression in Streptomyces is possible by the introduction of the strong transcriptional terminator derived from the neomycin resistance (aph) gene from S. fradiae. Introduction of the aph terminator resulted in the occurrence of uniform tendamistat transcripts with an approximate length of 520 bases on Northern blots (data not shown).

The promoter of the melC gene of S. antibioticus is present on the well known Streptomyces plasmid plJ 702 (39, 40). The construction of an expression vector with the tendamistat gene under the control of the mel promoter resulted in enhanced expression of the inhibitor as described by Schmitt-John and Engels (30). Transcripts of the tendamistat gene that initiate at the mel promoter and terminate at the aph terminator have an approximate length of 550 bases (data not shown).

Monitoring tendamistat secretion by activity assays yields a sigmoid production curve (Fig. 3A); saturation of inhibitor concentration is obtained on late days of culture. Variation of the signal peptide N-terminal charge from +6 to +2, however, severely affects the mean saturation concentration of inhibitor as demonstrated in Fig. 3 and Table I. The R5R9 signal peptide with an N-terminal charge of +5 reduces the amount of secreted inhibitor to ~25%. The R5 signal peptide with a charge of +4 results in a decrease to ~65% of the wild-type level. On the contrary, the yield of active secreted tendamistat is doubled...
by the use of the A2 signal peptide with an N-terminal charge reduced to +2.

Substitution of the tendamistat terminator for the aph terminator has no significant influence on inhibitor secretion as shown in Fig. 3B and Table I. On the other hand, the combination of the mel promoter and the described signal peptide variants causes an increase of tendamistat secretion by a factor of −4 (Fig. 3B and Table I). However, the effect of the signal peptide variation is not significantly influenced by promoter substitution.

The results of the activity assays were confirmed by SDS-PAGE and Western blotting of supernatant samples. Secreted tendamistat is totally active and migrates as a homogeneous protein band with an apparent molecular mass of 9 kDa on denaturing SDS gels (26).

The only mutant signal peptide apparently unable to promote the secretion of tendamistat in each construction (Table I) is the chimeric Xtend signal peptide, which was built by the fusion of the N-terminal domain of the XP55 signal peptide of S. lividans (Fig. 2) with the hydrophobic and C-terminal part of the tendamistat leader mutant R5R9 at the DNA level as described under “Materials and Methods.” In the case of this +6 leader, the absence of tendamistat in culture supernatants was confirmed by SDS-PAGE and Western blotting. Furthermore, no inhibitor was found in total protein from cell disruption. Hence, the introduction of the Xtend leader totally abolished the expression of tendamistat.

To test the assumption that signal peptide variations do not interfere with transcription, we attempted to quantify the amount of tendamistat mRNA in all strains. RNA isolation from many culture samples was achieved by development of a minipreparation method (see “Materials and Methods”) that provides total RNA in a quality suitable for qualitative and quantitative analysis of mRNA. Successful RNA isolations were possible only from logarithmic growing Streptomyces cultures on days 3–5, with a distinct optimum in yield and quality of the prepared RNA. The results of the Northern slot blot experiments are shown in Fig. 4. Signals obtained after stringent hybridization and washing conditions were solely caused by hybridization of a tendamistat-specific oligonucleotide with tendamistat mRNA.

At the level of transcription, no influence of the signal sequence variations is observed. The amount of tendamistat mRNA is only dependent on the given promoter. Moreover, the amount of tendamistat mRNA is not affected in the case of the nonproducing Xtend mutant. The level of tendamistat mRNA was estimated between 0.2 and 0.4% of total mRNA for constructions with the tendamistat promoter. Substitution for the mel promoter raises this portion −5-fold, which is coincident with the observed increase of secreted inhibitor (Table I); the aph terminator has no influence on the amount of mRNA. The results of the Northern slot blot experiments definitely rule out that the signal peptide variations have an effect on the transcription of the mutant tendamistat genes.

Expression of the Signal Peptide Variants with Charges of +6 to 0 in an E. coli–S. lividans Shuttle Vector with the ermEup Promoter Region of S. erythraea—The cloning of the entire set of signal sequence variants (Fig. 2) comprising a charge variation −6 leader, the absence of tendamistat in culture supernatants was confirmed by SDS-PAGE and Western blotting. Furthermore, no inhibitor was found in total protein from cell disrup-

Fig. 3. Secreted tendamistat in the culture supernatant of S. lividans strains transformed with plj 702 derivatives. A, comparison of typical production curves determined by the activity assay. Tendamistat was expressed by its native promoter and terminator; the N-terminal charge of the given signal peptide (SP) is indicated. The exponential increase of secreted inhibitor until the 5th day is correlated with an increase of cell mass. After the 6th day, the cell mass is reduced by autolytic processes within the mycelium. Because the extremely stable inhibitor is almost not proteolytically degraded, tendamistat reaches a saturation concentration, B, influence of promoter and terminator substitutions on tendamistat secretion. The columns represent the mean saturation concentration of three independent cultures per strain. The exact values are given in Table I. Tend/Tend, tendamistat promoter, tendamistat terminator; Tend/aph, tendamistat promoter/aph terminator; mel/aph, mel promoter/aph terminator.
found in the case of the variant with an elimination of all charged residues (A2A4A7, charge 0). The results were confirmed by SDS-PAGE of the supernatants (Fig. 6) and Western blotting.

Analysis of Intracellular Tendamistat by Western Blotting and by Activity Assay—The investigation of tendamistat expression was completed by immunological detection of tendamistat in the soluble protein fraction of cell homogenates. The results for strains transformed with a shuttle vector are shown in Fig. 7. The major immunoreactive protein has an apparent molecular mass of 9 kDa and is therefore identical to the mature inhibitor from supernatants as shown by comparison with the controls of purified secreted tendamistat. Two bands attributed to degradation of the mature form appear below. These observations were also made for the constructions with the tendamistat and the mel promoter.

To rule out that the detected mature tendamistat is just a remnant of the secreted inhibitor from the supernatant, the host strain TK23 was grown in the presence of 200 mg/liter purified tendamistat, and samples were subjected to Western blot analysis. In fact, a small amount of mature inhibitor was present in the soluble protein fraction of the control (Fig. 7, lanes 25 and 26). But comparison with the fraction found in the case of the R5 variant (lanes 9 and 10), which secretes ~200 mg/liter inhibitor, reveals that the remnant from the supernatant represents ~5% of the processed tendamistat detectable in a cell disruption sample from a producing strain. In addition, the proportionality of cell-associated mature inhibitor to secreted inhibitor is striking if Figs. 6 and 7 are compared.

The soluble protein fractions from disrupted cells were also subjected to the activity assays. An α-amylase inhibiting activity, which has to be attributed to correctly folded tendamistat, was detected in samples from each producing strain, except the A2A4A7 variant, which is characterized by an extremely low inhibitor secretion, too. In accordance with the course of inhibitor secretion (Fig. 3A), the cell-associated inhibitor activity increased with time and reached saturation after ~1 week. A comparison of the saturation concentrations is given in Fig. 8. The relative values clearly resemble the saturation concentrations of secreted inhibitor compared in Fig. 5. Hence, we suggest that the active cell-associated inhibitor represents a measure for the amount of translocated tendamistat.

If the cell-associated inhibitor in fact resides in the cytoplasm or if it is attached to the cell wall or the membrane has not yet been proven without doubt. The appearance of cell-associated mature inhibitor is probably a tendamistat-specific phenomenon due to extremely fast folding characteristics because tendamistat adopts its active conformation in ~20 ms.3

In accordance with the results for the constructions with tendamistat and the mel promoter, no tendamistat precursor protein (104 amino acids) is observed intracellularly in the case of the variants with the wild-type, R5R9, R5, or A2 signal peptide expressed by the ermEup promoter (Fig. 7). This may indicate that tendamistat synthesis and export are normally tightly coupled. On the contrary, in the soluble protein fraction of cell disruptions from strains with a +1 or neutrally charged signal peptide, precursor protein with an apparent molecular mass of 11.5–12 kDa is observed. The tendamistat precursor is subject to accelerated degradation as indicated by multiple degradation products below its major immunoreactive band. The appearance of the precursor protein provides evidence that the assumed coupling of synthesis and translocation can be impaired by specific signal peptide mutations.

The A2A4 signal peptide reduces tendamistat secretion to 54% of the wild-type level. However, a remarkable portion of precursor protein is found in the cytoplasm, thus indicating that the reason for the reduction of secreted inhibitor is an intracellular release of the precursor, which has no or negligible possibility to translocate post-translationally. Significantly less precursor protein is found in the case of the A2A7 variant, yet the amount of overall intracellular tendamistat protein is reduced. Therefore, the decrease of tendamistat secretion to 25% seems to be essentially due to a reduction of precursor synthesis.

An increased amount of free precursor protein in the case of a mutation at position 4 is also found for the signal peptide variants with a neutral net charge, if the results for mutants A2D4 and A2D7 are checked in Fig. 7. In addition, a comparison of the results for mutants A2A4 and A2D4 indicates that an acidic aspartate significantly raises the intracellular precursor fraction over a neutral alanine (Fig. 7). In accordance with the observations made for the +1 variants, it is assumed that the reduction of inhibitor secretion to 24% in mutant A2D4 is in principle caused by the release of the precursor due to the amino acid substitution at position 4, whereas the major effect responsible for the decrease to 9% in mutant A2D7 is a reduction of synthesis connected with the exchange at position 7. In the case of variant A2A4A7, which combines the substitutions of the A2A4 and A2A7 variants, a combination of both effects (which means a reduction of precursor synthesis and a release of the already synthesized precursor) is obtained, which rea-

TABLE I

| Signal peptide | N-terminal charge | Tendamistat promoter/tendamistat terminator | mel promoter/aph terminator |
|----------------|------------------|-------------------------------------------|----------------------------|
| Wild-type      | +3               | 38.6 ± 2.9 mg/l (100%)                    | 125.8 ± 10.4 mg/l (100%)  |
| A2             | +2               | 85.1 ± 1.3 mg/l (220%)                    | 227.2 ± 6.8 mg/l (181%)  |
| R5             | +4               | 23.8 ± 0.2 mg/l (62%)                     | 86.3 ± 4.5 mg/l (69%)    |
| R5R9           | +5               | 7.7 ± 0.2 mg/l (20%)                      | 39.4 ± 2.3 mg/l (33%)    |
| Xtend          | +6               | 0 mg/l (0%)                               | 0 mg/l (0%)               |

* Table I: Signal Peptide Mutations Influence Tendamistat Secretion

† T. Kiehhaber, personal communication.
reasonably explains the pronounced reduction of inhibitor secreted to the supernatant (<2% of the wild type).

**DISCUSSION**

This study presents the first results obtained for the influence of a systematic charge variation in the amino-terminal region of a signal peptide on protein secretion in *Streptomyces*. The chosen model system was the secretory expression of the *S. tendae* α-amylase inhibitor tendamistat in *S. lividans*. Ten tendamistat signal peptide variants with an N-terminal charge ranging from +6 to neutral net charge (Fig. 2) were examined. With the exception of the +6 variant, all signal peptides were able to promote the transport expression of tendamistat (Figs. 3 and 5). This tolerance for charge variation is in general agreement with the results obtained for *E. coli* and *B. subtilis* (see the Introduction).

However, significant effects on the amount of secreted inhibitor were observed (Figs. 3 and 5 and Table I). An increase of the positive charge resulted in a gradual decrease of tendamistat secretion from 100% for wild type (+3) to ~65% for mutant R5 (+4), to ~25% for mutant R5R9 (+5), and to a total inhibition of expression in the case of mutant Xtend (+6). On the other hand, a significant increase of secreted inhibitor was obtained by charge reduction to +2 in mutant A2. The presented signal peptide effects were found in combination with three different promoters, the tendamistat promoter, the *mel* promoter, and the *ermE* promoter, as well as with two transcriptional terminators, the tendamistat and *aph* terminators (Table I). Introduction of the strong transcriptional terminator of the *aph* gene of *S. fradiae* had no effect on the yield of secreted tendamistat (Fig. 3B and Table I) and on the amount of tendamistat mRNA (Fig. 4). A stabilization of tendamistat mRNA is therefore not supposed to be a key to optimization of inhibitor expression. Substitution of the tendamistat promoter for the *mel* promoter from the *melC* gene of *S. antibioticus*.

| Total RNA | Tendamistat Promoter, Tendamistat Terminator | Tendamistat Promoter, *mel* Terminator | Signal Peptide Variant | Standard: in vitro synthesized Tendamistat mRNA |
|-----------|--------------------------------------------|--------------------------------------|------------------------|---------------------------------------------|
| 2.5 μg    | ![Image](https://example.com/image1)         | ![Image](https://example.com/image2)    | Wild Type +3           | ![Image](https://example.com/image3)         |
| 1 μg      | ![Image](https://example.com/image4)         | ![Image](https://example.com/image5)    | Mutant +5              | ![Image](https://example.com/image6)         |
| 500 ng    | ![Image](https://example.com/image7)         | ![Image](https://example.com/image8)    | Mutant +4              | ![Image](https://example.com/image9)         |
| 250 ng    | ![Image](https://example.com/image10)        | ![Image](https://example.com/image11)   | Mutant +2              | ![Image](https://example.com/image12)        |
| 2.5 μg    | ![Image](https://example.com/image13)        | ![Image](https://example.com/image14)   | Mutant +6              | ![Image](https://example.com/image15)        |
| 1 μg      | ![Image](https://example.com/image16)        | ![Image](https://example.com/image17)   | Negative Control TK24  | ![Image](https://example.com/image18)        |
increased the amount of inhibitor by a factor of 4 (Fig. 3B and Table I) as a consequence of an ~5-fold increase of tendamistat mRNA (Fig. 4). A 10-fold increase of inhibitor expression was found for constructions with the ermEup promoter of S. erythreus (Table I).

Concerning the biotechnological aspect of this study, the construction of the A2 signal peptide variant, which doubled the yield of secreted inhibitor, demonstrates that an optimal production of a given secretory protein may not be reached with its native signal peptide. Hence, signal peptide mutagenesis has to be considered as a suitable approach to the evaluation of secretory protein expression. The fact that only few comparable examples are published (for example, the 1.9-fold increase of the secretion of human lysozyme in yeast by engineering of the hydrophobic segment (43)) suggests, however, that the prospective success of signal peptide variation is limited. On the other hand, the effects of an optimized signal peptide and a strong promoter can be combined as shown by the expression of tendamistat with the A2 signal peptide and the mel or ermEup promoter. Moreover, the increase in yield of secreted protein attributed to the A2 signal peptide is not limited to the tendamistat model protein, but was also transferred to a quite different protein, the α-amylase of Streptomyces limosus (44), comprising 538 residues.

In addition to the +6, +5, +4, +3, and +2 variants, two mutants with an N-terminal charge of +1 and three mutants with a neutral net charge were constructed and expressed in S. lividans in combination with the ermEup promoter in a novel shuttle vector system. Charge reduction below +2 again decreased the yield of secreted inhibitor (Fig. 5), indicating that a certain positive net charge is necessary for maximal efficiency of tendamistat secretion. However, net charge is not the crucial factor because the secretion of variants with identical net charge significantly differed. The results suggest that charged residues in distinct locations are of special importance for signal peptide function. In the case of tendamistat secretion, the need of a positively charged residue in the vicinity of the hydrophobic core is assumed.

Substitution of arginine at position 4 or 7 with an acidic residue more severely impaired the yield of secreted inhibitor than the introduction of a neutral amino acid. These results may hint at an involvement of the positively charged residues in ionic protein-protein interactions.

To understand how signal peptide variations give rise to such pronounced differences in inhibitor secretion, several efforts were made. The independence of the signal peptide effect from the promoter used (Table I) as well as the results of the quantitation of tendamistat mRNA (Fig. 4) strongly suggest that signal peptide mutations do not act on transcription. Concerning the mRNA structure of the signal sequence variants, no significant changes were found with a current program for secondary structure prediction (Fold, Version 7, Genetics Computer Group, Madison, WI). The mutations at position 7 of the signal peptide that resulted in a gradual decrease in the amount of expressed inhibitor from a basic arginine via a neutral alanine to an acidic aspartate residue also indicate that not the sequence of the mRNA, but the nature of the polypeptide is responsible for the observed effects.

A blocking of the general secretion pathway by export-deficient precursors was not taken into consideration because the pattern of exoproteins was not significantly altered in all variants (Fig. 6). That the export-deficient tendamistat precursors remain in a membrane-bound form is unlikely as well because no influence on viability and growth was observed for strains with reduced inhibitor secretion. Thus, we assume that the signal peptide variations already influence the synthesis of the mutant tendamistat precursor, which was investigated by analysis of tendamistat inside the cells.

The detection of cell-associated tendamistat revealed that mature inhibitor is present in the case of each producing strain (Fig. 7) and at least partially adopts a native conformation as proven by the determination of an α-amylase inhibiting activity (Fig. 8). The amount of cell-associated processed tendamistat is proportional to the amount of secreted inhibitor (Figs. 5 and 8) and thus represents a measure for the amount of translocated inhibitor.

The variants with wild-type and mutant R5R9 (+5), R5 (+4), and A2 (+2) signal peptides lack any detectable free precursor protein (Fig. 7). Therefore, the differences in the amount of both cell-associated mature tendamistat and secreted inhibitor

![Image](http://www.jbc.org/)

**Fig. 5.** Effect of the signal peptide variation on the amount of secreted tendamistat in the culture supernatant of S. lividans strains transformed with shuttle vectors containing the ermEup promoter. The mean saturation concentrations of three independent cultures per strain were determined by activity assay: mutant A2A4, 205.4 ± 13.4 mg/liter; mutant A2A7, 95.5 ± 13.7 mg/liter; mutant A2A4A7, 69.0 ± 0.9 mg/liter; mutant A2D4, 91.6 ± 16.9 mg/liter; and mutant A2D7, 32.8 ± 4.4 mg/liter. For other values, see Table I. wt, wild type.

![Image](http://www.jbc.org/)

**Fig. 6.** Coomassie Blue-stained SDS-polyacrylamide gel of culture supernatants. Lyophilized 20-μl aliquots of culture supernatant from strains transformed with shuttle vectors containing the ermEup promoter were sampled from days 6 to 8 and were separated on SDS gels (see Ref. 34). Lane 1, 2 μg of purified tendamistat from S. lividans; lane 2, 5 μg of molecular mass markers (Sigma); lanes 3–5, mutant R5R9 (+5); lanes 6–8, mutant R5 (+4); lanes 9–11, wild type (wt) (+3); lanes 12–14, mutant A2 (+2); lanes 15–17, mutant A2A4 (+1); lanes 18–20, mutant A2A7 (+1); lanes 21–23, mutant A2A4A7 (0); lanes 24–26, mutant A2D4 (0); lanes 27–29, mutant A2D7 (0). Tend designates the tendamistat band.

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4. S. Nußbaum and J. W. Engels, unpublished results.
Fig. 7. Western blot analysis of the soluble protein fraction from cell disruptions. The strains were transformed with shuttle vectors containing the ermEup promoter. Experimental procedures are given under "Materials and Methods." Soluble protein fractions were isolated from cells on days 7 and 8 of an expression culture. Lane 1, 5 μg of molecular mass markers (Sigma) transferred to the membrane and stained with Coomassie Brilliant Blue R-250; lanes 2–4, immunodetection of purified secreted tendamistat from S. lividans (100, 50, and 25 ng, respectively); lanes 5 and 6, negative control TK23; lanes 7 and 8, mutant R5R9 (+5); lanes 9 and 10, mutant R5 (+4); lanes 11 and 12, wild type (wt) (+3); lanes 13 and 14, mutant A2 (+2); lanes 15 and 16, mutant A2A4 (+1); lanes 17 and 18, mutant A2A7 (+1); lanes 19 and 20, mutant A2A4A7 (0); lanes 21 and 22, mutant A2D4 (0); lanes 23 and 24, mutant A2D7 (0); lanes 25 and 26, control. TK23 grown in the presence of 200 mg/liter purified tendamistat (compare with lanes 9 and 10, because strain R5 secretes 200 mg of inhibitor/liter of culture fluid). Tend indicates the band of mature tendamistat; proTend denotes the tendamistat precursor protein.

Fig. 8. Comparison of cytoplasmic inhibitor activity. α-Amylase inhibiting activity in the soluble protein fractions from disrupted cells of strains transformed with the ermEup promoter—containing shuttle vector was determined by activity assay on days 5–8 of a selected culture. The calculated saturation concentrations, representing the portion of active inhibitor with respect to the total amount of protein in the fraction, are compared: Mutant R5R9 (+5), 0.50 mg of inhibitor/g of total protein; mutant R5 (+4), 1.20 mg/g; wild type (wt) (+3), 2.57 mg/g; mutant A2 (+2), 5.84 mg/g; mutant A2A4 (+1), 1.63 mg/g; mutant A2A7 (+1), 0.68 mg/g; mutant A2D4, 0.78 mg/g; and mutant A2D7, 0.19 mg/g. Inhibitor activity in the soluble protein fraction from samples of mutant A2A4A7 was below the detection limit.

have to be attributed to a modulation of tendamistat synthesis. On the other hand, the absence of precursor protein suggests that synthesis and export of tendamistat are coupled in general.

However, in the case of the new mutants with a charge of +1 or 0, the situation is different because the tendamistat precursor is observed (Fig. 7). Substitution of the arginine residue at position 4 resulted in accumulation of a significant fraction of precursor protein. This effect is strongly pronounced if an aspartate residue with an acidic side chain is introduced instead of a neutral charged alanine. Thus, we suggest that the residue at position 4 interacts with a proteinaceous component of the secretory apparatus, which accounts for the coupling of translation and translocation of a secreted protein. The release of precursor protein seems to be essentially responsible for the reduction of inhibitor secretion by variants A2A4 and A2D4.

The intracellular appearance of the precursor was also obtained by substitution of the arginine residue at position 7, but to a significantly minor extent. Substitutions at position 7 are characterized by a reduction of synthesis, which is supposed to be the major reason for the decrease of inhibitor secretion observed for these mutants. Analogous to the variations at position 4, this reduction is more pronounced for an acidic amino acid than a neutral amino acid at position 7.

These results, as well as similar observations made for the influence of signal peptide charge variation on the synthesis of precursor protein in E. coli (6, 7, 45–47), are hardly explainable by the post-translational export mechanism represented by the Sec-dependent or even the Sec-independent translocation pathway (reviewed in Ref. 4). However, concuring evidence (48–52) strongly suggests the existence of a prokaryotic equivalent of the eukaryotic signal recognition particle (SRP)-dependent pathway determined for protein translation into the endoplasmic reticulum lumen of mammalian cells (53–55). On the basis of an essentially cotranslational pathway involving the prokaryotic homologue of the SRP, a conception may be developed. The tendamistat precursor (104 amino acids) meets the basic requirement for a SRP-dependent translocation because it exceeds the lower size limit, which may be somewhat different with respect to the particular protein (56, 57), but lies within the range of 64 (58, 59) to 74 (60) amino acids. For the secretion of proteins into the lumen of the endoplasmic reticulum, it was shown that the translocation arrest caused by the interaction of the mammalian SRP with the signal peptide of a nascent protein is only released if the complex of ribosome, nascent polypeptide chain, and bound SRP interacts with the SRP receptor on the face of the endoplasmic reticulum membrane. This interaction and the following release of the SRP are governed by the binding and hydrolysis of GTP, which is influenced by the signal peptide (51). Recent publications (51, 62) demonstrated that a similar GTPase cycle is found for the interaction of the E. coli homologue of the SRP54 subunit, the Ffh protein (63–66), with the membrane-associated FtsY protein, which is the bacterial homologue of the α-subunit of the mammalian SRP receptor (63). The Ffh protein forms a ribonucleotide particle (RNP) with the 4.5 S RNA, which is the E. coli equivalent of the 7 S RNA participating in mammalian SRP (67). Miller et al. (51) have shown that the interaction of the RNP and the Ffh protein is modulated in vitro by synthetic signal peptides derived from the E. coli LamB leader. The GTPase activity of the RNP–FtsY protein complex was inhibited by signal peptide variants, which also promoted protein export in vivo. An export-deficient signal peptide, however, failed in preventing GTP hydrolysis. The authors suggested that the stimulation of GTP hydrolysis caused by binding of the RNP–signal peptide complex to the FtsY protein represents the key step that leads to the release of the signal peptide.

Our hypothesis is that deficient signal peptides prevent binding of the RNP to the homologue of the E. coli FtsY protein by stabilization of a RNP conformation that tightly binds GTP or, more reasonably, retards dissociation of GDP. The latter model strongly resembles the GTPase cycle of the bacterial elongation factor Tu (61) as an example for GTP/GDP-binding proteins. Presuming that translation is also arrested by the prokaryotic
SRP equivalent interaction with the appropriate membrane receptor, the “inactivated” complex of a deficient signal peptide and the RNP with bound GDP may be released without completion of precursor synthesis. This hypothesis offers the possibility of explaining modulations of precursor synthesis by signal peptide mutations at the level of the polypeptide. According to this model, release of precursor protein into the cytosol is understandable by proposing an impairment of the interaction between the SRP homologue and the mutant signal peptide, which causes an uncoupling of translation and the SRP-dependent export pathway. Released precursors that are not able to translocate post-translationally will reside in the cytoplasm until they are proteolytically degraded.

Nevertheless, very little is known about the molecular mechanism of protein secretion in Streptomyces. Only cloning of the Streptomyces secretory apparatus proteins, which is recently in progress,5 mutational analysis, and the in vitro reconstitution of the translocation pathway will give a satisfying answer to the questions evoked by the presented effects that signal peptide mutations have on the secretion of tendamistat in S. lividans.

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Influence of Specific Signal Peptide Mutations on the Expression and Secretion of the α-Amylase Inhibitor Tendamistat in *Streptomyces lividans*

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