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Signal Peptide Hydrophobicity Is Finely Tailored for Function

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Abstract In order to titrate the dependence of individual steps in protein transport on signal peptide hydrophobicity, we have examined a series of mutants which involve replacement of the hydrophobic core segment of the *Escherichia coli* alkaline phosphatase signal peptide. The core regions vary in composition from 10:0 to 0:10 in the ratio of alanine to leucine residues. Thus, a nonfunctional polyalanine-containing signal peptide is titrated with the more hydrophobic residue, leucine. Analysis of this series identified a midpoint for rapid precursor processing between alanine to leucine ratios of 6:4 and 5:5 [Doud et al. (1993): Biochemistry 32:1251–1256]. Examination of precursors that are processed more slowly indicates a lower limit of signal peptide hydrophobicity that permits membrane association and translocation. Analysis of precursors that are processed rapidly defines an intermediate range of hydrophobicity that is optimum; above this level precursors become insensitive to transport inhibitors such as sodium azide and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) in parallel with substantial inhibition of β-lactamase processing. Our data indicate that there is a surprisingly narrow range of signal peptide hydrophobicity which both supports transport of the protein to which it is attached and which does not have such a high affinity for the transport pathway that it disrupts the appropriate balance of other secreted proteins.

Key words: alkaline phosphatase, β-lactamase, carbonyl cyanide 3-chlorophenylhydrazone, membrane insertion, membrane translocation, protein transport, sodium azide

Secretory proteins in *Escherichia coli* are generally synthesized in the cytoplasm and transported to their final cellular location with an attached leader sequence or signal peptide, which is cleaved upon translocation across the cytoplasmic membrane. Although signal peptides lack primary sequence homology [Watson, 1984], they do share common structural features including a basic amino terminus, a central hydrophobic core, and a more polar carboxyl-terminal region which contains the signal peptidase cleavage site.

The central hydrophobic core is often regarded as the hallmark of the signal peptide. This region is shared by most signal sequences in both prokaryotic and eukaryotic systems. Even mitochondrial presequences, which include charged residues in the core segment, adopt a substantial hydrophobic surface in an α-helical conformation [Roise et al., 1988]. Although the primary sequence of the core regions varies extensively among different signal peptides [von Heijne, 1981, 1985], the overall level of hydrophobicity is remarkably constant [von Heijne, 1981; Doud et al., 1993].

The cumulative effort of numerous studies in this area has brought about a good understanding of the characteristics of the core which are required for a functional signal peptide. These characteristics include a propensity to form an α-helix, particularly in a membrane environment [Briggs and Gierasch, 1984; Bankaitis et al., 1984; Kendall et al., 1986; Ryan et al., 1986]. The α-helical conformation may contribute to the optimization of a hydrophobic surface [Engelman and Steitz, 1981; Engelman et al., 1986] and, in turn, provide an effectively higher overall hydrophobicity. Reduced hydrophobicity in the core region by the introduction of charged residues results in impaired processing [Lin et al., 1978; Lee et al., 1983; Sung et al., 1992] and although the length of the core region is somewhat flexible [Freudl et al., 1988], there is a lower and upper limit on what constitutes a functional core [Chou and Kendall, 1990; Ribbe

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and Nagarajan, 1992] and large deletions are not allowed [Bankaitis et al., 1984; Lehnhardt et al., 1987; Lunn and Inouye, 1987].

Fewer studies have successfully examined why these elements are important; what protein and/or lipid interactions are involved with a given core region feature and at what step(s) of the protein transport process? Using synthetic peptides, a correlation between signal peptide hydrophobicity and insertion into model membranes has been observed. Interestingly, it was found that peptides corresponding to nonfunctional OmpA signal sequence mutants may retain high helical content but not penetrate deeply into the acyl chain region of bilayers owing to seemingly small changes in hydrophobicity [Hoyt and Gierasch, 1991a,b]. Analysis of the in vivo transport properties of nonfunctional precursors has further suggested that hydrophobic interactions are crucial for the transfer of the polypeptide through the membrane. In these studies mutant precursors were identified that were membrane associated but not translocated [Thom and Randall, 1988; Chou and Kendall, 1990; Rusch and Kendall, 1992]. There is no direct evidence that the signal peptide hydrophobic core interacts with any of the protein components of the transport pathway. However, several studies suggest such interactions [Puziss et al., 1989; Lill et al., 1990; Oliver et al., 1990; Ito, 1992]. In fact, mutations in both the *prl*D (SecA) gene [Fikes and Bassford, 1989] and the *prl*A (SecY) gene [Puziss et al., 1992] have been found which suppress mutations in the hydrophobic core of the signal peptide.

In order to titrate the dependence of individual steps in protein transport on signal peptide hydrophobicity, we have designed a series of mutants which vary systematically in the ratio of alanine to leucine residues. Previously this series was characterized with regard to precursor processing to "titrate" the degree of hydrophobicity required for protein transport overall [Doud et al., 1993]. While precursor processing is a readily quantifiable parameter and an indicator of the penultimate step of transport, it is likely that the degree of signal peptide hydrophobicity is actually critical for one or more earlier steps prior to the cleavage event. Here we use the series of alanine-leucine mutants to assess the involvement of signal peptide hydrophobicity in individual steps in transport in vivo.

Our data indicate that there is a surprisingly narrow range of signal peptide hydrophobicity which supports transport of the protein to which it is attached and which does not interfere with the transport of other proteins. Examination of precursor proteins that are processed slowly indicates a lower limit of signal peptide hydrophobicity that permits membrane association and translocation. Analysis of precursors that are processed rapidly reveals an intermediate range of hydrophobicity that optimally defines a signal peptide; above this level precursors become apparently insensitive to transport inhibitors in parallel with substantial competition with the transport of β-lactamase. These findings suggest a reason why natural signal sequences are not even more hydrophobic than they are. We propose that highly hydrophobic signal sequences have such a strong affinity for the transport pathway that they outcompete other precursors and may compromise opportunities for regulation and maintenance of the appropriate repertoire of different secreted proteins.

MATERIALS AND METHODS

Bacterial Strains and Media

Escherichia coli strain AW1043 [Δlac galU galK Δ(leu-ara)phoA-E15 proC::Tn5] was used. Bacteria were grown in 4-morpholinepropanesulfonic acid (MOPS) low phosphate (100 μM KH_2PO_4) medium [Neidhardt et al., 1974] containing 250 μg/ml ampicillin and 50 μg/ml kanamycin as described previously [Rusch and Kendall, 1992].

Construction of Mutants

All of the mutants were constructed from the CASS3 plasmid [Kendall and Kaiser, 1988] as described previously by Doud et al. [1993].

Sodium Hydroxide Cell Fractionation

The method used by Russel and Model [1982] was employed. This method discriminates between proteins which are tightly membrane-associated and soluble proteins. Cells in logarithmic growth phase were washed and resuspended in MOPS medium containing 20 μg/ml amino acids minus methionine. Cells were radiolabeled with 40 μCi L-[^{35}S]methionine for 40 s, chased with 4 mg/ml cold methionine for 30 s, and removed to an Eppendorf tube on ice containing 0.2 N NaOH. Soluble proteins were separated from membrane-associated proteins by microcentrifugation at 4°C for 15 min. Each fraction was precipitated by the addition of ice cold tri-

chloroacetic acid (TCA) and immunoprecipitated as described by Kendall et al. [1986].

Protease Accessibility Experiment

This experiment discriminates between proteins which are cytoplasmic or cytoplasmically oriented (untranslocated protein) and proteins which are periplasmic or periplasmically oriented (translocated protein). Cells were labeled with 40 μCi L-[^{35}S]methionine for 60 s and three portions were removed to ice. To the first, TCA was added to a final concentration of 5% after a 35 min incubation. The other two portions were washed with 30 mM Tris (pH 8). Following microcentrifugation, cells were resuspended in 0.5 M sucrose, 30 mM Tris (pH 8) containing 20 μg/ml lysozyme and 1 mM EDTA, and incubated on ice for 20 min. Proteinase K (25 μg/ml) was then added, after which one portion also received 0.2% CHAPS. Samples were incubated on ice for 10 min, then 25°C for 5 min and precipitated with 5% TCA. Cells were washed with acetone and immunoprecipitated. Proteins which are cytoplasmic or membrane bound but facing the cytoplasm are degraded by proteinase K in the presence of detergent which solubilizes the inner membrane; periplasmic proteins are resistant to protease degradation.

CCCP Analysis

To examine the involvement of the protonmotive force in transport of mutant precursors, the method described by Kuhn and Wickner [1985] was used. Cells were harvested, diluted 1:10 in MOPS medium supplemented with 20 μg/ml amino acids minus methionine, and incubated for 1 min at 37°C with 0.4 mM CCCP in dimethylsulfoxide (DMSO) or an equal volume of DMSO alone prior to labeling with 64 μCi L-[^{35}S]methionine for 1 min. Total protein was then TCA-precipitated and alkaline phosphatase was immunoprecipitated as described by Kendall et al. [1986].

Sodium Azide Analysis

To examine the requirement for the SecA component of the export pathway, the procedure of Oliver et al. [1990] was used. Sodium azide (1 mM) was added for 2 min at 37°C prior to 30 s labeling with 40 μCi L-[^{35}S]methionine and 30 s chase with 4 mg/ml cold methionine. Control samples were treated similarly but sodium azide was not added prior to radiolabeling. Sodium azide is a potent and specific inhibitor of SecA in vivo. Alkaline phosphatase was immunoprecipitated as described above.

Western Blot Analysis of β-Lactamase

Whole cell samples were run on a 10% Laemmli SDS-PAGE [Laemmli, 1970]. Electrophoretic transfer to polyvinylidene difluoride membranes (PVDF) was followed by development as described in the protocol provided by Bio-Rad (Rockville Centre, NY).

SDS-PAGE and Densitometry

Immunoprecipitated alkaline phosphatase was run on 7.5% Laemmli SDS-PAGE [Laemmli, 1970], subjected to autoradiography as described by Kendall and Kaiser [1988], and analyzed by densitometry.

RESULTS

The *E. coli* alkaline phosphatase signal peptide, like most signal peptides, is composed of a basic amino-terminus, a central hydrophobic core, and a polar carboxyl-terminus. Figure 1 shows the hydrophobic core segments of a series of signal peptide mutants in which this region has been replaced with polymers of varying ratios of alanine and leucine residues. Both alanine and leucine have strong propensities for forming α-helices [Ferretti and Paolillo, 1969; Arfmann et al., 1977; Zhang et al., 1992], have alkyl side chains and are nonpolar, although to very different degrees. This series of mutants allows us to "titrate" the hydrophobicity of the core region. Previously, precursor processing of this series of mutants was evaluated to determine the threshold level of hydrophobicity which supports transport [Doud et al., 1993]. While precursor processing may be diagnostic of the overall export process, it is likely that hydrophobicity directly affects one or more earlier steps in transport.

One such step is membrane insertion. To determine the level of hydrophobicity required for membrane insertion, the series of mutants was analyzed by the fractionation method of Russel and Model [1982]. The relative extent to which the mutants partitioned with membrane and soluble fractions is shown in Figure 2. A clear correlation between signal peptide hydrophobicity and membrane association of precursors is observed. Precursor 8A2L is mainly in the soluble fraction with only ~10% membrane as-

		Processing (%)[a]	
		30"	20'
WT:	Ile Ala **Leu** Ala **Leu Leu** Pro **Leu Leu** Phe	84	99
10A0L:	Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala	9	0
9A1L:	Ala Ala Ala Ala Ala Ala Ala Ala **Leu** Ala	2	13
8A2L:	**Leu** Ala Ala Ala Ala Ala Ala Ala **Leu** Ala	8	8
7A3L:	**Leu** Ala Ala Ala Ala Ala **Leu** Ala **Leu** Ala	16	67
6A4L:	**Leu** Ala **Leu** Ala Ala Ala Ala **Leu** Ala **Leu**	35	89
5A5L:	**Leu** Ala **Leu** Ala **Leu** Ala **Leu** Ala **Leu** Ala	82	94
4A6L:	**Leu** Ala **Leu** Ala **Leu Leu Leu** Ala **Leu** Ala	88	96
3A7L:	**Leu** Ala **Leu Leu Leu Leu Leu** Ala **Leu** Ala	93	96
2A8L:	**Leu** Ala **Leu Leu Leu Leu Leu Leu Leu** Ala	92	100
1A9L:	**Leu** Ala **Leu Leu Leu Leu Leu Leu Leu Leu**	98	99
0A10L:	**Leu Leu Leu Leu Leu Leu Leu Leu Leu Leu**	96	99

Fig. 1. Amino acid sequences of the hydrophobic core segment of mutant and wild type alkaline phosphatase signal peptides. The natural amino-terminal (MKQST) and cleavage region (TPVTKA) sequences are maintained in all mutants. All results pertaining to wild type in this paper refer to WT CASS3 which contains unique *Sal*I (GTCGAC) and *Bss*HII (GCGCGC) sites that were introduced into the wild type alkaline phosphatase signal peptide without changing the native amino acid sequence [Kendall and Kaiser, 1988]. The leucine residues of the hydrophobic core regions are boldfaced. [a]The extent of precursor processing of each mutant following 30 s and 20 min chase periods is provided for comparison.

sociated. A more substantial proportion of precursor 7A3L is associated with the membrane (~50%). The 6A4L signal peptide is sufficiently hydrophobic that ~65% of the precursor has achieved stable membrane association including that portion which proceeds to become processed to the mature form. At hydrophobicities equivalent to the 5A5L mutant or above, the protein efficiently undergoes translocation and processing. Since translocation and processing must be preceeded by membrane insertion, all of the mutant precursors, from 5A5L to 0A10L, must be sufficiently hydrophobic to readily undergo membrane interaction. The results indicate that the 7A3L mutant represents the minimum hydrophobicity necessary for stable membrane association.

Proteolysis studies were used to determine if translocation follows membrane association concomitantly. Detergent was used to permeabilize the cytoplasmic membrane, and cytoplasmic or cytoplasmically oriented proteins were accessible to added protease. Alkaline phosphatase is sensitive to protease when it is not translocated because it is in a partially unfolded state; on the other hand, the translocated protein is resistant to protease because it is tightly folded into a more compact form. In this context the term translocated is used to describe protein for which the mature region has traversed the membrane and is periplasmically exposed; this may include periplasmically oriented precursors which are not cleaved as well as processed alkaline phosphatase that is released into the periplasm. Figure 3 shows that with weakly hydrophobic signal peptides, like that of mutant 8A2L, translocation is not accomplished and here the alkaline phosphatase is entirely protease sensitive. Some translocation is observed (~50%) with mutant 7A3L and when the hydrophobicity of the core region is increased to the equivalent of mutant 6A4L, most of the alkaline phosphatase is translocated (and processed). A comparison of the extent of membrane association (Fig. 2) and membrane translocation (Fig. 3) reveals that both processes are enhanced by increases in signal pep-

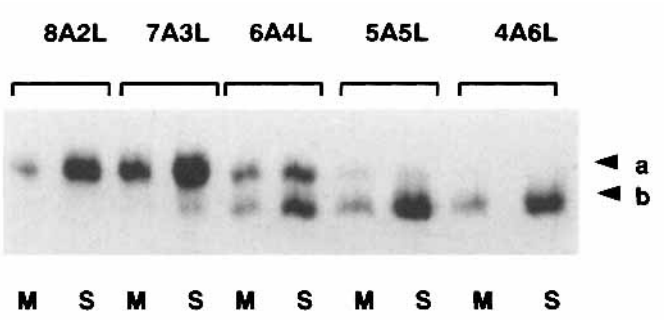

Fig. 2. Relative membrane partitioning of mutant alkaline phosphatase. Cells were radiolabeled, treated with NaOH, and separated into a soluble (cytoplasmic and periplasmic) fraction and a membrane fraction by microcentrifugation. Migration of precursor is indicated by arrowhead a; mature enzyme is indicated by b. S, soluble fraction; M, membrane fraction.

tide hydrophobicity. For both processes, mutant 7A3L represents a critical transition point. Interestingly, pulse-chase analysis of this mutant indicated that precursor processing of 7A3L was highly time dependent with about 16% mature present after a 30 s chase and 67% observed after a 20 min chase period; i.e., the hydrophobicity of its signal peptide is just below the threshold value for rapid processing, but over time productive interactions at critical steps do occur, leading to transport [Doud et al., 1993]. Our findings here indicate that those critical steps include membrane insertion and translocation.

The above studies demonstrate that specific minimum levels of hydrophobicity are essential for different stages of protein transport. Interestingly, further examination of this mutant series also reveals that signal peptides that are too highly hydrophobic may be, by some criteria, overly efficient or even counterproductive. For example, precursors composed of natural signal sequences require the protonmotive force to achieve translocation [Kuhn and Wickner, 1985; Randall et al., 1987; Geller, 1990]. Experimentally, the protonmotive force can be dissipated by treating cells with the ionophore, CCCP [Daniels et al., 1981]. As shown in Figure 4, the series of mutants exhibit a graded response to CCCP treatment. Mutants that are weakly hydrophobic through mutant 2A8L show precursor accumulation as a result of CCCP treatment; the proportion of the normally mature protein (in untreated cells) which is sensitive to CCCP (observed as additional precursor in treated cells) diminishes as the hydrophobicity of the signal peptide increases. Transport of the highly hydrophobic mutants, 1A9L and 0A10L, is apparently insensitive to CCCP treatment, with no accumulation of precursor observed.

A similar pattern is observed when the effect of sodium azide is examined. Previous studies have established that azide is a rapid and potent inhibitor of protein transport in vivo and mutations conferring azide resistance are mapped to SecA [Oliver et al., 1990]. SecA is typically required for the initial binding of precursors to the inner membrane and the subsequent translocation step [Oliver, 1993]. Figure 5 shows the response of the series of mutants when cells harboring these are treated with azide. Like CCCP treatment, precursors accumulate as a consequence of the presence of azide for the weakly hydrophobic mutants through 2A8L. The effect becomes less substantial as the hydrophobicity of the signal peptide increases, and mutants 1A9L and 0A10L show no effect. Furthermore, we confirmed that in the presence of azide, 10L is translocated and released into the periplasm (data not shown).

Experiments involving the treatment of cells with inhibitors such as CCCP and sodium azide can be expected to be leaky; that is, the affinity of these reagents for their targets may not be sufficient to completely block all sites at concentrations which are experimentally feasible. The "apparent" decrease in sensitivity to sodium azide and CCCP, observed with increasingly hydrophobic signal peptides, may actually reflect an increase in affinity for components such as SecA, and these higher affinity signal peptides may more efficiently utilize available target sites which remain unaffected by the inhibitors. If this is the case, and the properties of the 1A9L and 0A10L signal peptides do reflect an unusually high affinity interaction for the transport pathway, we would expect these to compete so well as to retard the transport of precursors with less hydrophobic signal peptides.

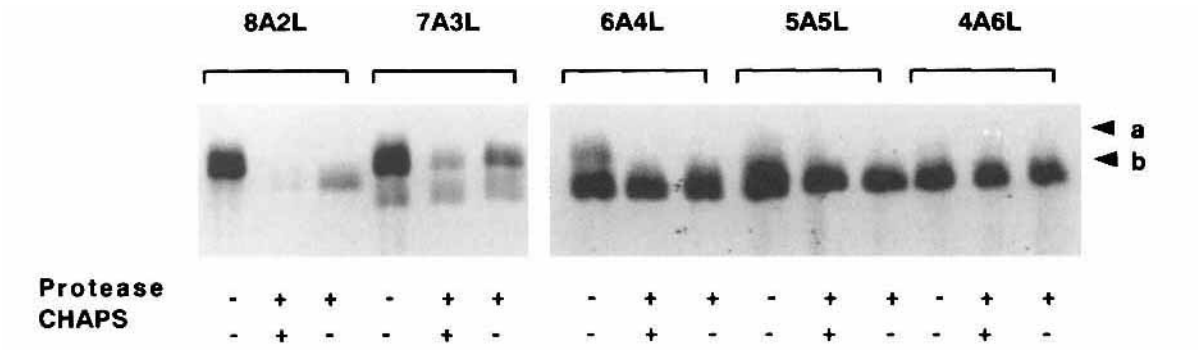

Fig. 3. Cellular orientation of mutant precursors. Cells were radiolabeled, lysozyme treated, and proteolyzed with proteinase K in the presence or absence of detergent. An untreated whole cell fraction was also taken for comparison. Migration of precursor is indicated by arrowhead a; mature enzyme is indicated by b.

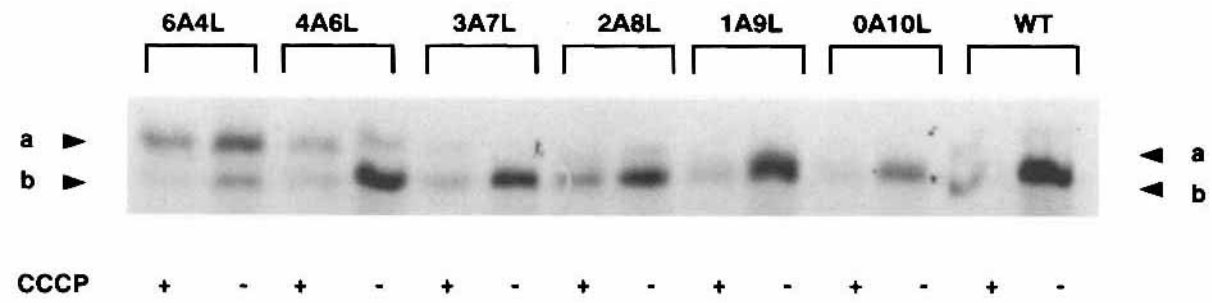

Fig. 4. Effect of an uncoupler on mutant precursor processing. Cells were treated with DMSO in the presence or absence of an uncoupler, CCCP, prior to radiolabeling. Migration of precursor is indicated by arrowhead a; mature enzyme is indicated by b.

Indeed, in parallel with the trend observed for the effect of CCCP and sodium azide, the mutants with highly hydrophobic signal peptides interfere with the transport of wild type β-lactamase. Figure 6 shows a Western blot of total β-lactamase accumulation during the exponential growth of cells coexpressing the various alkaline phosphatase mutants. The signal peptide mutants of lowest hydrophobicity have no apparent affect on the transport of β-lactamase, whereas the mutants of higher hydrophobicity, 1A9L and, especially, 0A10L, interfere with the processing of β-lactamase. It should further be noted that this type of analysis reveals total β-lactamase accumulation over more than 3 h of cell growth during which time some precursor may slowly convert to the mature form or degrade in the cytoplasm. The magnitude of the effect is more pronounced if the transient inhibition of β-lactamase processing is analyzed by a pulse-chase study. Following a 40 s radiolabeling period, 96% of β-lactamase is in the precursor form when coexpressed with the 0A10L mutant in contrast to 17% when coexpressed with wild type alkaline phosphatase. Thus this highly hydrophobic signal peptide competes so effectively for the transport pathway that the rate of β-lactamase processing is severely retarded.

DISCUSSION

A large collection of mutations in the core region of a variety of signal sequences has been generated and analyzed [for review, see Gennity et al., 1990]. While these have been useful for identifying amino acid compositions which give rise to functional and dysfunctional cores, it is difficult to rank them with respect to their relative degree of hydrophobicity, and consequently to correlate levels of hydrophobicity with specific signal peptide roles. The analysis is complicated by the variety of different amino acids in these signal sequences, the contribution of varying types of conformation as well as side chain hydrophobicity, and the bias of the experimental basis of any given hydropathy table. We have circumvented several of these problems by analyzing a set of signal peptides, each of which differs by only one alanine to leucine conversion but as a series spans a wide range of hydrophobicities. By using polymers of only two different

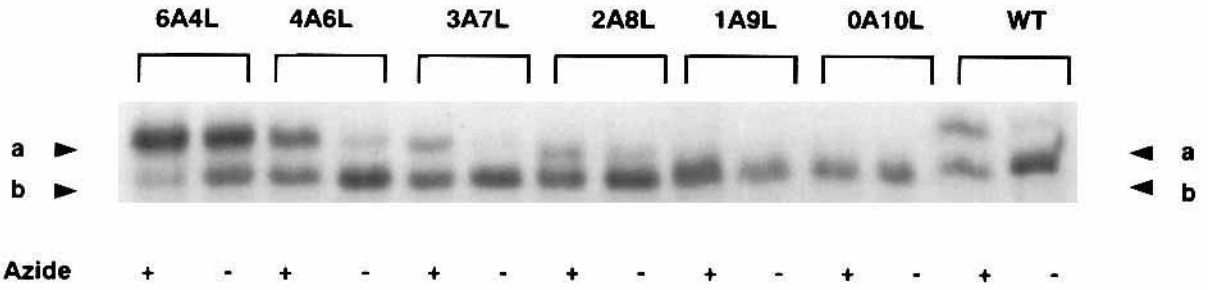

Fig. 5. Effect of SecA inhibitor on mutant precursor processing. Cells were treated with sodium azide prior to radiolabeling and then chased with cold methionine. Migration of precursor is indicated by arrowhead a; mature enzyme is indicated by b.

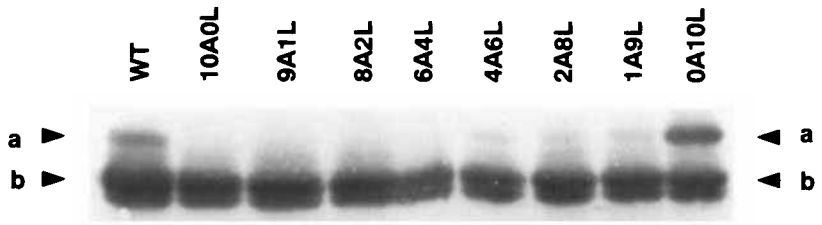

Fig. 6. Precursor processing of β-lactamase in the presence of mutant alkaline phosphatase signal peptides. Shown are Western blots of whole cell samples developed using anti-β-lactamase antiserum. Migration of precursor is indicated by arrowhead a; mature enzyme is indicated by b. The appearance of the additional lower molecular weight band is typically observed with this anti-β-lactamase and is consistent with the presence of a contaminant in the original gel slabs used to raise the antisera.

residues which are closely related with regard to their chemical structure and their strong propensity for α-helix formation, the variables involved in our analysis are greatly reduced.

Analysis of the alanine-leucine series of precursors which are slowly processed suggests that the requirements for the membrane association and translocation steps are just slightly shifted relative to those for rapid signal peptide cleavage. We find that the 7A3L signal peptide represents the minimum degree of hydrophobicity necessary for significant levels of membrane association and translocation. Analysis of rapid (30 s chase) precursor processing levels defines the midpoint as equivalent to between 6A4L and 5A5L, while that for longer times (20 min chase) is equivalent to about 7A3L [Doud et al., 1993]; i.e., mutant 7A3L undergoes substantial post-translational processing. Furthermore, the processing data suggested that for 7A3L the equilibrium between unprocessed and processed states lies just to the left. The results presented here suggest that the data actually reflect the equilibrium between unproductive and productive membrane association and/or translocation.

While the NaOH treatment used to analyze for membrane association is a rapid and potent inhibitor of all cellular processes, we recognize that the protease sensitivity assay employed here does not provide a good kinetic analysis of translocation because some precursor processing occurs during the incubation with protease. Thus the data are likely somewhat of an overestimate of the extent of translocation for a given mutant. We are currently developing methods to examine the level of rapid translocation and to provide a kinetic comparison of the membrane association and translocation steps.

At the other end of the spectrum, analysis of mutants that are processed rapidly reveals that the transport of these becomes less affected by sodium azide and CCCP as the signal peptide hydrophobicity is increased. Mutants 1A9L and 0A10L provide examples of signal peptides which efficiently support protein transport in vivo in the presence of sodium azide or CCCP. Interestingly, Yamada et al. [1989] have found that a precursor protein could be translocated in vitro in the absence of the protonmotive force if a high concentration of SecA was added. In our experiments, sodium azide treatment, or indirectly CCCP treatment, may leave only a small population of SecA functional. However, if the 1A9L and 0A10L precursors have an unusually

high affinity for SecA, they may effectively compete for the limited amount of the active form available, ensuring their transport. Consistent with this notion is the accumulation of β-lactamase precursor when coexpressed with the highly hydrophobic mutants in the absence of cellular inhibitors.

We cannot rule out the possibility that the highly hydrophobic signal peptides facilitate direct lipid partitioning in such a way that requirements for SecA and the protonmotive force are obviated. Nevertheless, Hikita and Mizushima [1992] have shown that in vitro translocation directed by signal peptides incorporating polyleucine does require SecA, and the hydrophobic region of the signal peptide in addition to the amino-terminal charged region has been implicated in SecA interactions [Oliver, 1993]. Furthermore, a similar model for mammalian signal sequences and SRP interactions has been described which suggests that different signal sequences have different binding affinities for SRP and that the more hydrophobic peptides are among those that bind best [Rapoport et al., 1987; Bird et al., 1990].

A comparison of the alanine-leucine signal peptides with several natural signal sequences indicates that the hydrophobicity of the latter is typically on the order of the 4A6L mutant [Doud et al., 1993]. This level of hydrophobicity is more than sufficient to achieve membrane association, translocation, and rapid processing (88% in 30 s), yet retain sensitivity to sodium azide and CCCP. We are not aware of any natural signal sequences which are more hydrophobic than the equivalent of 3A7L. This indicates that the 1A9L and 0A10L are excessively hydrophobic signal peptides. Although such a highly hydrophobic signal peptide enhances the secretion efficiency of the protein to which it is attached, and can overcome a transport defect in the mature portion of the precursor [Rusch and Kendall, 1994], the interference with β-lactamase transport that we observe suggests that such a strong affinity for the transport pathway by one signal peptide may be disruptive to the appropriate balance of other secreted proteins. This would not be the case if all signal sequences were equally highly hydrophobic but there is no obvious driving force for evolving signal peptides to be any more efficient than they already are. Furthermore, few combinations of amino acids could produce the equivalent of 0A10L. Isoleucine and phenylalanine are at least as hydrophobic as leucine but both may pose structural problems if used repeatedly and the disproportionate use of a small subset of amino acids would undoubtedly tax the components used in their synthesis. The possible combinations of amino acids that give rise to signal peptides that are somewhat less hydrophobic, on the other hand, is tremendous; indeed, this is evident from the large array of combinations used in natural signal peptides [Watson, 1984].

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REFERENCES

Arfmann H-A, Labitzke R, Wagner KG (1977): Conformational properties of L-leucine, L-isoleucine, and L-norleucine side chains in L-lysine copolymers. Biopolymers 16: 1815–1826.

Bankaitis VA, Rasmussen BA, Bassford PJ Jr (1984): Intragenic suppressor mutations that restore export of maltose binding protein with a truncated signal peptide. Cell 37: 243–252.

Bird P, Gething M-J, Sambrook J (1990): The functional efficiency of a mammalian signal peptide is directly related to its hydrophobicity. J Biol Chem 265:8420–8425.

Briggs MS, Gierasch LM (1984): Exploring the conformational roles of signal sequences: Synthesis and conformational analysis of λ receptor protein wild-type and mutant signal peptides. Biochemistry 23:3111–3114.

Chou MM, Kendall DA (1990): Polymeric sequences reveal a functional interrelationship between hydrophobicity and length of signal peptides. J Biol Chem 265:2873–2880.

Daniels CJ, Bole DG, Quay SC, Oxender DL (1981): Role for membrane potential in the secretion of protein into the periplasm of *Escherichia coli*. Proc Natl Acad Sci USA 78:5396–5400.

Doud SK, Chou MM, Kendall DA (1993): Titration of protein transport activity by incremental changes in signal peptide hydrophobicity. Biochemistry 32:1251–1256.

Engelman DM, Steitz TA (1981): The spontaneous insertion of proteins into and across membranes: The helical hairpin hypothesis. Cell 23:411–422.

Engelman DM, Steitz TA, Goldman A (1986): Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. Ann Rev Biophys Biophys Chem 15:321–353.

Ferretti JA, Paolillo L (1969): Nuclear magnetic resonance investigation of the helix to random coil transformation in poly-α-amino acids. I. Poly-L-alanine. Biopolymers 7:155–171.

Fikes JD, Bassford PJ Jr (1989): Novel *sec*A alleles improve export of maltose-binding protein synthesized with a defective signal peptide. J Bacteriol 171:402–409.

Freudl R, MacIntyre S, Degen M, Henning U (1988): Alteration of the signal peptide of an outer membrane protein (OmpA) of *Escherichia coli* K-12 can promote either the cotranslational or the posttranslational mode of processing. J Biol Chem 263:344–349.

Geller B (1990): Electrochemical potential releases a membrane-bound secretion intermediate of maltose-binding protein in *Escherichia coli*. J Bacteriol 172:4870–4876.

Gennity J, Goldstein J, Inouye M (1990): Signal peptide mutants of *Escherichia coli*. J Bioenerg Biomembr 22:233–269.

Hikita C, Mizushima S (1992): Effects of total hydrophobicity and length of the hydrophobic domain of a signal peptide on in vitro translocation efficiency. J Biol Chem 267:4882–4888.

Hoyt DW, Gierasch LM (1991a): Hydrophobic content and lipid interactions of wild-type and mutant OmpA signal peptides correlate with their in vivo function. Biochemistry 30:10155–10163.

Hoyt DW, Gierasch LM (1991b): A peptide corresponding to an export-defective mutant OmpA signal sequence with asparagine in the hydrophobic core is unable to insert into model membranes. J Biol Chem 266:14406–14412.

Ito K (1992): SecY and integral membrane components of the *Escherichia coli* protein translocation system. Mol Microbiol 6:2423–2428.

Kendall DA, Kaiser ET (1988): A functional decaisoleucine-containing signal sequence. J Biol Chem 263:7261–7265.

Kendall DA, Bock SC, Kaiser ET (1986): Idealization of the hydrophobic segment of the alkaline phosphatase signal peptide. Nature 321:706–708.

Kuhn A, Wickner W (1985): Conserved residues of the leader peptide are essential for cleavage by leader peptidase. J Biol Chem 260:15914–15918.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lee N, Yamagata H, Inouye M (1983): Inhibition of secretion of a mutant lipoprotein across the cytoplasmic membrane by the wild-type lipoprotein of the *Escherichia coli* outer membrane. J Bacteriol 155:407–411.

Lehnhardt S, Pollitt S, Inouye M (1987): The differential effect on two hybrid proteins of deletion mutations within the hydrophobic region of *Escherichia coli* OmpA signal peptide. J Biol Chem 262:1716–1719.

Lill R, Dowhan W, Wickner W (1990): The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. Cell 60:271–280.

Lin JJC, Kanazuwa H, Ozols J, Wu H (1978): An *Escherichia coli* mutant with an amino acid alteration within the signal sequence of outermembrane prolipoprotein. Proc Natl Acad Sci USA 75:4891–4895.

Lunn CA, Inouye M (1987): Effects of prolipoprotein signal peptide mutations on secretion of hybrid prolipo-β-lactamase in *Escherichia coli*. J Biol Chem 262:8318–8324.

Neidhardt FC, Bloch PL, Smith DF (1974): Culture medium for enterobacteria. J Bacteriol 119:736–747.

Oliver DB (1993): SecA protein: Autoregulated ATPase catalysing preprotein insertion and translocation across the *Escherichia coli* inner membrane. Mol Microbiol 7:159–165.

Oliver DB, Cabelli RJ, Dolan KM, Jarosik GP (1990): Azide-resistant mutants of *Escherichia coli* alter the SecA protein, an azide-sensitive component of the protein export machinery. Proc Natl Acad Sci USA 87:8227–8231.

Puziss JW, Fikes JD, Bassford PJ Jr (1989): Analysis of mutational alterations in the hydrophilic segment of the maltose-binding protein signal peptide. J Bacteriol 171: 2303–2311.

Puziss JW, Strobel SM, Bassford PJ Jr (1992): Export of maltose-binding protein species with altered charge distribution surrounding the signal peptide hydrophobic core in *Escherichia coli* cells harboring *prl* suppressor mutations. J Bacteriol 174:92–101.

Randall LL, Hardy SJS, Thom JR (1987): Export of protein: A biochemical view. Ann Rev Microbiol 41:507–541.

Rapoport TA, Heinrich R, Walter P, Schulmeister T (1987): Mathematic modeling of the effects of the signal recognition particle on translation and translocation of proteins across the endoplasmic reticulum membrane. J Mol Biol 195:621–636.

Ribbe J, Nagarajan V (1992): Characterization of the secretion efficiency of a plant signal peptide in *Bacillus subtilis*. Mol Gen Genet 235:333–339.

Roise D, Theiler F, Horvath SJ, Tomich JM, Richards JH, Allison DS, Schatz G (1988): Amphiphilicity is essential for mitochondrial presequence function. EMBO J 7:649–653.

Rusch SL, Kendall DA (1992): Signal sequences containing multiple aromatic residues. J Mol Biol 224:77–85.

Rusch SL, Kendall DA (1994): Transport of an export defective protein by a highly hydrophobic signal peptide. J Biol Chem 269:1243–1248.

Russel M, Model P (1982): Filamentous phage precoat is an integral membrane protein: Analysis by a new method of membrane preparation. Cell 28:177–184.

Ryan JP, Duncan MC, Bankaitis VA, Bassford PJ (1986): Intragenic reversion mutations that improve export of maltose-binding protein in *Escherichia coli malE* signal sequence mutants. J Biol Chem 261:3389–3395.

Sung CY, Gennity JM, Pollitt NS, Inouye M (1992): A positive residue in the hydrophobic core of the *Escherichia coli* lipoprotein signal peptide suppresses the secretion defect caused by an acidic amino terminus. J Biol Chem 267:997–1000.

Thom JR, Randall LL (1988): Role of the leader peptide of maltose-binding protein in two steps of the export process. J Bacteriol 170:5654–5661.

von Heijne G (1981): On the hydrophobic nature of signal sequences. Eur J Biochem 116:419–422.

von Heijne G (1985): Signal sequences: The limits of variation. J Mol Biol 184:99–105.

Watson MEE (1984): Compilation of published signal sequences. Nucl Acids Res 12:5145–5164.

Yamada H, Matsuyama S, Tokuda H, Mizushima S (1989): A high concentration of SecA allows proton motive force-independent translocation of a model secretory protein into *Escherichia coli* membrane vesicles. J Biol Chem 264:18577–18581.

Zhang H-J, Baase WA, Matthews BW (1992): Multiple alanine replacements within α-helix 126-134 of T4 lysozyme have independent, additive effects on both structure and stability. Protein Sci 1:761–776.