The Amino-terminal Charge and Core Region Hydrophobicity Interdependently Contribute to the Function of Signal Sequences*

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We have constructed a series of signal sequence mutants that contain negatively charged amino termini and simplified core regions of varying hydrophobicity levels. This series provides a means of exploring the relative roles of the amino terminus and the hydrophobic core region during transport. The signal peptides with highly hydrophobic core regions support a rapid rate of transport in the presence of a negatively charged amino terminus. We have found that these negatively charged mutants are secreted in a manner similar to the wild-type signal sequence; sodium azide and carbonyl cyanide 3-chlorophenylhydrazone treatments indicate that the negatively charged mutants depend on SecA and the protonotive force, respectively. These same mutants also demonstrate reduced competition with co-expressed β-lactamase, reflecting the lower overall affinity for the transport pathway due to the net negative charge at the amino terminus. In addition, the pronounced effects of introducing three negative charges support the conclusion that the two regions function in a concerted manner.

Extractoytoplasmic proteins in Escherichia coli often require a signal peptide to direct proper localization. As a group, signal peptides display similar physical properties, including a short positively charged amino terminus, a highly hydrophobic core region, and a more polar carboxyl terminus, which are potentially important for their role in secretion (for a review, see Izard and Kendall (1994)).

The importance of the central hydrophobic region has been demonstrated extensively by mutational analyses that test the limits of the hydrophobic requirement (for a review, see Izard and Kendall (1994)). The helical potential, which has been both predicted (Austen, 1979) and observed by physical analyses of synthetic signal peptides (Gierasch, 1989; Izard et al., 1995), further defines the core as a unique physical structure that might serve as a recognition element for a proteinaceous component in the export pathway.

The positive charge at the amino terminus has been proposed to play a direct role in association of the signal sequence with the membrane due to an electrostatic attraction to the negatively charged phospholipids. Anionic phospholipids are essential for efficient protein transport both in vivo (de Vrije et al., 1988) and in vitro (Phoenix et al., 1993) and have been shown to promote the interaction of a precursor protein with phospholipids in a model membrane system (Keller et al., 1996). The positively charged amino terminus may also be regarded as a contributing factor to the formation of a net dipole along the signal sequence (von Heijne (1986); for a review, see Geller (1991)). Such a dipole may facilitate inner membrane insertion complementary to the energetics of the protonotive force, i.e., the positively charged residues remain anchored on the cytoplasmic side while the negatively charged residues are readily transported.

The positive charge may also enhance a specific interaction between the signal sequence and SecA during the export process. In vitro cross-linking studies of the OmpF-Lpp fusion protein with SecA showed an interaction that was enhanced with increasing net positive charge of the signal sequence (Akita et al., 1990). Furthermore, a secretion deficiency due to the presence of a negative charge at the amino terminus was partially suppressed by mutations in the prlD (SecA) gene (Puzzi et al., 1989).

There have been some indications that the amino terminus and hydrophobic core regions may have overlapping functions. One study revealed that the defect caused by the introduction of a negative charge in the amino terminus was more pronounced when the core region was truncated (Puzzi et al., 1989). In Bacillus subtilis, an unusually long wild-type core region was shown to tolerate the presence of negatively charged residues in the amino terminus (Chen and Nagarajan, 1994). Likewise, a core composed of nine leucine residues supports in vitro translocation of a model secretory protein with zero charge, whereas shorter hydrophobic cores require positive charge (Hikita and Mizushima, 1992).

We have investigated the interdependence of these two regions using a systematic series of mutant signal sequences composed of at least one negatively charged residue amino-terminal to a series of simplified core regions with increasing hydrophobicity. We observe very efficient export of mutants containing a negatively charged amino terminus when the core region is sufficiently hydrophobic. However, even a highly hydrophobic signal peptide can be made less efficient if three negative charges are introduced, demonstrating the disruptive influence of excessive negative charge. Interestingly, the negatively charged signal sequence mutants still rely on the protonotive force and functional SecA, the former suggesting that the role of the membrane potential is not exclusively electrostatic and the latter indicating that a positively charged amino terminus is not the only requirement for engaging SecA.

The most efficient negatively charged mutants even demonstrate a competition for the transport pathway by preventing normal levels of secretion of coexpressed wild-type β-lactamase, yet the level of competition is lower than for the corresponding positively charged signal peptide. Our results suggest that the amino terminus and hydrophobic core of the signal peptide function in a concerted manner.

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A MATERIALS AND METHODS

Bacterial Strains and Media—E. coli strain AW1043 (ΔlacgalU galK Δ(leu-ara) pho-E15 proC::Tn5) was used for mutant construction and in all transport experiments. For general propagation of bacteria and for production of mutants, cells were grown in LB medium (Miller, 1972) containing 250 μg/ml ampicillin and 50 μg/ml kanamycin. Transport studies were conducted in MOPS1 (Neidhardt et al., 1974) under no-phosphate conditions to induce alkaline phosphatase expression.

Construction of Mutants—All of the mutants were produced by cassette mutagenesis by using plasmids that contain unique restriction enzyme sites flanking specific regions of the wild-type and mutant alkaline phosphatase signal peptides. The construction of the WT-Afl vector, which displays an AflII site at the initial methionine of alkaline phosphatase, was described by Izard et al. (1995). The AflII site was introduced into vectors containing various alkaline phosphatase signal peptide core region mutants as follows. After digestion of the WT-Afl vector with Scal and Sall, the intervening BSS-base pair DNA segment that included the AflII site was isolated and inserted into mutant vectors from which the Scal-Sall fragment had been removed. These new vectors were then digested with AflII and Sall and subjected to gel electrophoresis to remove the DNA coding for the wild-type amino terminal sequence of the signal peptide; synthetic oligonucleotides encoding the desired amino-terminal sequence, MEQST, were annealed and ligated with the prepared vectors. Due to interference with the Sall restriction enzyme sites, the amino-terminal mutant containing three glutamic acid residues and a 10-leucine core region, 3E10L, was constructed by replacement of the entire signal peptide using the flanking AflII and BsHII sites as described by Izard et al. (1995). The mutant sequences were confirmed by restriction enzyme analysis and direct DNA sequencing (Sanger et al., 1977).

Pulse-Chase Analysis—To determine the rate of precursor processing, cells were grown to the logarithmic phase, labeled at 37°C with 40 μCi of L-[35S]methionine and a 100 Ci of [3H]Ci of Ci-lactamase. The rate of precursor processing for each negatively charged mutant was determined by pulse-chase analysis. As shown in Fig. 2, the presence of a glutamic acid residue in tandem with a highly hydrophobic core, E10L, was processed very rapidly, in spite of the negative charge at the amino terminus. To determine the degree of hydrophobicity in the core required to overcome the effect of a single negative charge, we reduced the hydrophobicity (through a series of mutants) by incrementally increasing the ratio of alanine to leucine residues from 0:10 to 6:4. Interestingly, little effect of one negative charge is observed for the simplified mutants that have a net hydrophobicity similar to (i.e. E4A6L) or above that of the wild-type. It is not until the core region hydrophobicity is dropped to a level corresponding to six alanines and four leucines that substantial inhibition of precursor processing occurs; E6A4L exhibits only 17% processing in a 30-s chase in comparison to 48% in 30 s for the same mutant with the wild-type amino terminus (Doud et al., 1993). However, the introduction of three negative charges adjacent to the 10-leucine core, 3E10L, inhibits secretion. The EWT, E6A4L, and 3E10L mutants process further during a 10-min chase, but do not achieve the extent of processing observed for the other mutants in just 30 s. Lysosome-EDTA cell fractionation (Lafont and Kendall, 1991) was used to confirm the correct localization of the mature form of alkaline phosphatase to the periplasm (data not shown).

One question to be addressed was whether these negatively charged mutants followed the same pathway as wild-type signal peptides or if they utilized a different, perhaps less efficient, pathway. Since the amino-terminal positive charge has

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1 The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; CCCP, carbonyl cyanide 3-chlorophenylhydrazone.

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B Pulse-Chase Experiment—To determine the rate of precursor processing, cells were grown at 37°C in MOPS, to an OD of 0.6 to 0.7, and labeled with 40 μCi of L-[35S]methionine and 100 μCi of [3H]Ci of Ci-lactamase. The pulse-chase experiment was performed as described above, followed by immunoprecipitation of the cell sample with antiserum to β-lactamase and SDS-Polyacrylamide Gel Electrophoresis and Quantitation of Protein. Immunoprecipitated proteins were separated using 7.5% to 20% gradient gels and visualized by fluorography. For quantitation of the precursor and mature forms of alkaline phosphatase or β-lactamase, the radioactive protein was solubilized from the gel by the SOLVABLE protocol (DuPont NEN), followed by scintillation counting (Rusch et al., 1994).

RESULTS

In this study, a systematic series of signal sequences for alkaline phosphatase was constructed to investigate the functional interrelationship between the amino terminus and the hydrophobic core of the enzyme. The simplified core regions were composed of varying ratios of alanine and leucine residues, producing a range of hydrophobicities with minimal variation in amino acid content (Doud et al., 1993; Rusch et al., 1994). Furthermore, these sequences have the potential for forming uniform α-helices that may effectively produce a more hydrophobic surface (Izard et al., 1995). By substituting a glutamic acid residue for the lysine in the wild-type amino terminus of signal peptides containing select core regions, this well-defined series provides a basis for examining the effect of a negative charge on the association of precursors with the transport pathway. An additional mutant was also generated in which we introduced three negatively charged residues amino-terminal to the 10-leucine core (Fig. 1).

The rate of precursor processing for each negatively charged mutant was determined by pulse-chase analysis. As shown in Fig. 2, the presence of a glutamic acid residue in tandem with the wild-type core region resulted in a decrease in the amount of alkaline phosphatase processed within a 30-s chase, from 95 ± 0.2 to 67 ± 3.6%. In contrast, a similar mutant containing a highly hydrophobic core, E10L, was processed very rapidly, in spite of the negative charge at the amino terminus. To determine the degree of hydrophobicity in the core required to overcome the effect of a single negative charge, we reduced the hydrophobicity (through a series of mutants) by incrementally increasing the ratio of alanine to leucine residues from 0:10 to 6:4. Interestingly, little effect of one negative charge is observed for the simplified mutants that have a net hydrophobicity similar to (i.e. E4A6L) or above that of the wild-type. It is not until the core region hydrophobicity is dropped to a level corresponding to six alanines and four leucines that substantial inhibition of precursor processing occurs; E6A4L exhibits only 17% processing in a 30-s chase in comparison to 48% in 30 s for the same mutant with the wild-type amino terminus (Doud et al., 1993). However, the introduction of three negative charges adjacent to the 10-leucine core, 3E10L, inhibits secretion. The EWT, E6A4L, and 3E10L mutants process further during a 10-min chase, but do not achieve the extent of processing observed for the other mutants in just 30 s. Lysosome-EDTA cell fractionation (Lafont and Kendall, 1991) was used to confirm the correct localization of the mature form of alkaline phosphatase to the periplasm (data not shown).

One question to be addressed was whether these negatively charged mutants followed the same pathway as wild-type signal peptides or if they utilized a different, perhaps less efficient, pathway. Since the amino-terminal positive charge has
been suggested to play a role in SecA interaction (Puziss et al.,
1989; Akita et al., 1990), we used a SecA inhibitor to evaluate
the extent to which our mutants still interact with this com-
ponent. Sodium azide has been shown to specifically inhibit the
function of the SecA-ATPase, usually resulting in precursor
accumulation (Oliver et al., 1990). In Fig. 3, the wild-type
alkaline phosphatase shows a substantial decrease in precursor
processing when treated with azide as compared with an
untreated control. In addition, the EWT, E4A6L, and E2A8L
mutants exhibit precursor accumulation as a consequence of
azide treatment, indicating that the negatively charged mu-
tants still depend on SecA. However, the extent to which azide
affects each mutant diminishes as the net hydrophobicity of the
core region increases, with the most hydrophobic mutant,
E10L, remaining unaffected. The pattern is striking even when
the differences in processing among the untreated samples are
taken into account; the amounts of mature protein that are
affected by azide treatment were calculated to be 73, 53, and
10% for E4A6L, E2A8L, and E10L, respectively. Comparison of	
the differences in processing among the untreated samples is
reported. The data represent the average of at least three experiments; error bars show S.E. WT, WT-Afl.

In this study, the effect of introducing a negative charge into
the amino terminus of a signal sequence was titrated by de-
creasing the level of hydrophobicity in the core region. Our
analysis suggests that high hydrophobicity, coupled with an
interrupted α-helical structure, in the core region can com-
penstate for an altered amino-terminal segment and is consist-
ent with findings using B. subtilis signal peptide mutants
(Chen and Nagarajan, 1994). The transport of wild-type
β-lactamase, coexpressed from the same plasmid as each alka-
line phosphatase mutant, provides an indicator of whether the
two proteins use the same pathway and their relative affinities
for that pathway. As shown in Fig. 5, some of the negatively
charged alkaline phosphatase mutants cause inhibition of
β-lactamase processing, and the amount of β-lactamase precur-
sores with increasing hydrophobicity of the alkaline
phosphatase signal sequence. Interestingly, the E10L mutant
causes somewhat less β-lactamase precursor accumulation
than the 10L mutant, indicating less competition due to the
presence of the negative charge, and β-lactamase is completely
processed in cells harboring the less efficient mutants, EWT
and 3E10L.

DISCUSSION

In this study, the effect of introducing a negative charge into
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ent with findings using B. subtilis signal peptide mutants
(Chen and Nagarajan, 1994). The results suggest that the
amo terminus and core region act together as a unit; when
the entire unit is less than ideal, the precursor has a lower
affinity for the transport machinery, and export is inefficient.
The recognition of the two regions as a unit is significant
because it implies that their functions overlap.

Treatment of cells with azide and CCCP demonstrated that
the negatively charged mutants require both the protonmove
force and SecA for transport. These results are contrary to
proposals that the amino-terminal positive charge provides an
alignment with the protonmove force or that it solely drives
the interaction with SecA. Several studies using membrane proteins have indicated that the membrane electrochemical potential promotes the transfer of negative charges and inhibits the transfer of positive charges across the bacterial inner membrane (Andersson and von Heijne, 1994; Cao et al., 1995). Thus, the presence of the proton motive force might be expected to inhibit the processing of our precursors containing negatively charged amino termini because of the tendency to be inserted into the membrane in the “inverted” orientation (especially since the carboxyl terminus contains a positive charge). However, the efficient processing and localization to the periplasm in the presence of the proton motive force and the loss of processing in its absence show that these mutants are affected in the same way as the wild type.

Mutations in the prlD (SecA) gene partially suppressed the transport defect caused by a negatively charged amino terminus in the maltose-binding protein signal peptide (Puziss et al., 1989). The suppression may not actually be allele-specific; alternatively, the suppression may merely reflect an increased interaction with the hydrophobic core region such that the presence of the negative charge is tolerated. This idea parallels the results of our evaluation of the negatively charged series presented here.

We observe a graded response of the mutants described here to CCCP and azide treatment; those with the most hydrophobic cores and fewest negative charges were least sensitive. The diminishing influence of azide on the transport of the more hydrophobic mutants indicates that these require lower levels of SecA. One interpretation of this finding is that the most highly hydrophobic signal peptides simply do not utilize SecA. Alternatively, we propose that the diminishing sensitivity may actually reflect an increasing affinity of these signal sequences for SecA; since the inhibition of SecA by azide treatment may not be complete, signal sequences having a high affinity are successfully secreted via the few sites that remain unaffected by the azide. This is in line with our findings using other mutants (Rusch et al., 1994) and those described by Sääf et al. (1995). Likewise, the impact of CCCP is consistent with the notion that sensitivity strongly depends on the number of sites that remain uninhibited and on the affinity of each mutant for these sites. A similar trend was observed for the dependence of the in vitro translocation of LamB, OmpA, and Skp on SecA and the proton motive force (Ernst et al., 1994). That the SecA requirements for several different precursors (wild type and mutant) parallel the proton motive force requirements (Rusch et al., 1994; Ernst et al., 1994; this study) likely points to a link between SecA and the proton motive force (Geller, 1991).

Ernst et al. (1994) suggested that the precursor-dependent level of sensitivity may reflect the kinetics of folding or aggregation of the precursors in the cytoplasm, with those that fold least readily more frequently remaining in a translation-competent state and thus requiring the lowest levels of SecA and the proton motive force. Our data suggest several complementary possibilities. 1) The most hydrophobic signal peptides may help retard folding of the precursor into a translocation-competent state (Park et al., 1988). 2) The highly hydrophobic signal peptides may remain more accessible to the secretion pathway. 3) The more hydrophobic signal peptides may possess an inherently higher affinity for the pathway. The increase in precursor accumulation of coexpressed β-lactamase in cells harboring the most highly hydrophobic signal peptides is most easily interpreted in view of the latter possibility.

Here, we have shown that very hydrophobic core regions have a high affinity for the transport apparatus, yet the contribution of the positive charge at the amino terminus for less hydrophobic sequences remains evident. The positive charge in tandem with a hydrophobic core may provide a functional unit, possibly for recognition by a proteinaceous component analogous to the recognition of polypeptide sequences with a similar combination of features by the heat shock protein hsc70 (Takenaka et al., 1995). The binding energy for any interacting proteins will be the sum of the energy derived from several interactions including the free energy of hydrophobic interactions and electrostatic interactions. For the negatively charged signal peptides, maximizing the binding energy derived from the hydrophobic-hydrophobic interaction could overcome an unfavorable electrostatic repulsion involving the negative charge both in the protein and the amino terminus of the signal peptide. This study highlights the importance of combining the physical features of both regions in order to provide the functional units of natural targeting sequences.

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