Titration of Protein Transport Activity by Incremental Changes in Signal Peptide Hydrophobicity†

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ABSTRACT: A systematic series of mutants has been generated which provides a means for titrating the dependence of protein transport activity on signal peptide hydrophobicity. These mutants involve replacement of the hydrophobic core segment of the *Escherichia coli* alkaline phosphatase signal peptide while maintaining the natural amino- and carboxyl-terminal segments and the overall length. The new 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. Using precursor processing to quantify transport activity, we observe a clear, nonlinear dependence on hydrophobicity. At ratios of alanine to leucine of less than or equal to 8:2, the signal peptide is essentially nonfunctional; at ratios greater than or equal to 3:7, the signal peptide functions efficiently. The midpoint is between alanine to leucine ratios of 6:4 and 5:5. Signal peptides with hydrophobicity just below the midpoint show substantial, additional precursor processing over time while the others do not. The data are consistent with a simple model involving a two-state equilibrium between the untransported and transported species and a change in the ΔG of –0.85 kcal/mol for every alanine to leucine conversion.

Many proteins that are synthesized in the cytoplasm of *Escherichia coli* ultimately reside in noncytoplasmic locations. The export of these proteins typically requires an amino-terminal extension sequence or signal peptide. As a group, signal peptides have very little primary sequence homology but they do share some common features. Most are about 20 residues long and are composed of a positively charged amino terminus, a central segment rich in hydrophobic residues, and a more polar region recognized by the signal peptidase (Watson, 1984). The conservation of this pattern suggests that it is vital for signal peptide function, and perhaps each region plays one or more specific roles during the transport process. For example, the positively charged amino terminus may interact with the negatively charged lipids of the inner leaflet of the inner membrane (Inouye et al., 1977; Inouye & Halegoua, 1980). Recent studies have also implicated an interaction of the amino terminus and SecA (Akita et al., 1990). The carboxyl terminus provides a recognition site for cleavage by the signal peptidase to yield the mature form of the protein (Zwizinski & Wickner, 1980). Several studies have suggested that this region may adopt a β-turn which is important for topological alignment and enzyme recognition (Inouye et al., 1986; Duffaud & Inouye, 1988) although this motif does not appear to be a necessity for efficient processing (Laforet & Kendall, 1991).

The hydrophobic core region is the hallmark of bacterial signal sequences and as such has been the focus of several studies aimed at probing its primary and secondary structural requirements and the roles this region plays during the transport process [for review, see Gennity et al. (1990)]. Evaluation of a variety of different mutations has indicated that signal peptide function is highly sensitive to the inclusion of charged residues in the core region which almost always results in severe impairment of function (Emr et al., 1978; Emr & Silhavy, 1980; Stader et al., 1986). Although some variation in core region length is readily tolerated, there are limits on the minimum and maximum number of hydrophobic amino acids that can constitute a functional signal peptide (Ryan et al., 1986; Lehnhard et al., 1987; Chou & Kendall, 1990). Several studies indicate that functional signal sequences, particularly the core segment, have a high propensity for α-helical formation although the conformation may vary depending on the environment (Briggs & Gierasch, 1984; Briggs et al., 1986; Bruch & Gierasch, 1990). Involvement of the hydrophobic core has been implicated in several different steps and interactions along the transport pathway. It may serve as a signal that initiates participation in protein export, it may help maintain the precursor protein in an unfolded state suitable for translocation, it may interact directly with the nonpolar region of the lipid bilayer to facilitate translocation, and it may bind membrane proteins such as Sec Y during translocation (Chen et al., 1987; Park et al., 1988; Killian et al., 1990; Oliver et al., 1990).

One of the goals of our laboratory has been to define the physical properties required for functional signal peptides and to explore the limits of these parameters. Our long-range aim is then to correlate these features with specific interactions and steps involved in protein transport. The principles which evolve from these should also be useful in the design of generic signal sequences useful for the transport of a variety of proteins including normally cytoplasmic proteins and eukaryotic proteins in *E. coli*. In previous studies, we have found it advantageous to explore these issues through utilization of polymeric sequences and cassette mutagenesis. Cassette mutagenesis permits the rapid construction of mutants with multiple residue substitutions and well-defined, parallel constructions are easily produced. The use of polymeric sequences reduces the variation in the interactions of different side chains and facilitates comparative analyses. Using this

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approach, we have demonstrated the importance of overall physical properties rather than primary sequence specificity in signal peptide function (Kendall et al., 1986), explored the role of conformation in the function of the signal peptide core (Kendall & Kaiser, 1988) and cleavage regions (Laforet & Kendall, 1991), and examined the interrelationship between the hydrophobicity and length of signal peptides (Chou & Kendall, 1990) and the suitability of aromatic amino acids for functional signal peptides (Rusch & Kendall, 1992). This type of sequence has now also been utilized to explore protein transport in *Saccharomyces cerevisiae* (Yamamoto et al., 1987, 1989) and with in vitro systems (Hikita & Mizushima, 1992).

One of the principles that emerges from these studies is the notion that the "hydrophobic density" or degree of hydrophobicity for a given number of residues is critical for efficient precursor translocation. By comparing the function of signal peptides composed of different homopolymers, we demonstrated that the loss in processing that accompanies a decrease in the hydrophobicity of the constituent residue, from leucine to valine to alanine, can be compensated for by increasing the length of (i.e., number of residues in) the core region. The compensation is only partial, however, and a high mean hydrophobicity per residue is necessary for complete, rapid processing and efficient translocation (Chou & Kendall, 1990; Rusch & Kendall, 1992).

Since our earlier experiments indicated that functional signal peptides must possess a threshold level of hydrophobicity in order to exhibit wild-type levels of processing and transport, we predicted that we ought to be able to "titrate" a weakly hydrophobic signal peptide with a more hydrophobic residue, in a clear, systematic way and determine that threshold level. For this purpose, we have now constructed a series of mutants in the hydrophobic core region of the *E. coli* alkaline phosphatase signal peptide. Each mutant contains a core segment of 10 residues, but the composition varies from 10:0 to 0:10 in the ratio of alanine to leucine residues. Precursor processing is used as an earmark of transport activity, and the extent of processing is quantified for each mutant. The results indicate a clear nonlinear relationship between hydrophobicity and transport activity with an apparent threshold requirement equivalent to the hydrophobicity of about six alanines and four leucines. Interestingly, the hydrophobicity of typical wild-type core segments is similar. Furthermore, signal peptides at or just below the midpoint are the only ones tested which exhibit substantial posttranslational processing.

Mutant	DNA Sequence of Core Region
10Ah	[GCA GCA GCT GCA GCT GCG GCT GCC GCG GCG]
9A1Lh	[GCA GCA GCT GCA GCT GCG GCT GCC **L**TG GCG]
8A2Lh	[**L**TG GCA GCT GCA GCT GCG GCT GCC **L**TG GCG]
7A3Lh	[**L**TG GCA GCT GCA GCT GCG **L**TG GCC **L**TG GCG]
6A4Lh	[**L**TG GCA **L**TG GCA GCT GCG **L**TG GCC **L**TG GCG]
6A4Lh-II	[**L**TG GCA GCT **L**TG **L**TG GCT GCG **L**TT GCT GCA]
6A4Lh-III	[**L**TG GCA **L**TG GCA GCT GCG GCT **L**TG GCG **L**TT]
5A5Lh	[**L**TG GCA **L**TG GCA **L**TC GCG **L**TG GCC **L**TG GCG]
4A6Lh	[**L**TG GCA **L**TG GCA **L**TC **L**TG **L**TG GCC **L**TG GCG]
3A7Lh	[**L**TG GCA **L**TG **L**TG **L**TC **L**TG **L**TG GCC **L**TG GCG]
2A8Lh	[**L**TG GCA **L**TG **L**TG **L**TC **L**TG **L**TG **L**TA **L**TG GCG]
1A9Lh	[**L**TG GCA **L**TG **L**TG **L**TC **L**TG **L**TG **L**TA **L**TG **L**TA]
10Lh	[**L**TG **L**TT **L**TG **L**TG **L**TC **L**TG **L**TG **L**TA **L**TG **L**TA]

FIGURE 1: DNA sequences of the core region of the various mutant signal peptides. Only the plus strand has been shown. Codons for leucine residues are indicated by a boldfaced L. All other codons are for alanine residues. The DNA sequences for the remainder of the protein is the same as that for WT CASS3. This plasmid encodes the wild-type *E. coli* alkaline phosphatase precursor, except that the DNA sequence is modified to produce unique *Bss*HII and *Sal*I sites (Kendall & Kaiser, 1988).

MATERIALS AND METHODS

Bacterial Strains and Media. *E. coli* strain AW1043 (Δ*lac galU galK* Δ(*leu–ara*) *phoA-E15 proC*::Tn5) was used in all experiments. Standard LB medium (Miller, 1972) was used for general propagation of cells and for mutagenesis. Transport experiments were carried out in MOPS[1] medium (Neidhardt et al., 1974). Kanamycin (50 μg/mL) and ampicillin (250 μg/mL) were used in all media.

Oligonucleotides and Plasmids. All of the mutants were produced using the plasmid CASS3, a pBR322-phoA derivative (Kendall & Kaiser, 1988). This plasmid incorporates unique restriction sites for *Sal*I (5′ end) and *Bss*HII (3′ end) just outside the hydrophobic segment of the signal sequence. While creation of these sites involved alterations in the DNA sequence, the amino acid sequence of the wild type was maintained. Mutant sequences were generated by digestion of CASS3 with *Sal*I and *Bss*HII, removal of the wild-type core-encoding region, and ligation of the remaining vector with oligonucleotides coding for the new region. Each insert involved four oligonucleotides, two per DNA strand. In order to be cost efficient, the design of the oligonucleotide sequences was based primarily on maximizing their versatility in producing the different mutants while trying to avoid producing any particular amino acid sequence patterns. In addition, the codon usage for alanine and leucine within the alkaline phosphatase gene and in *E. coli* in general (Konigsberg & Godson, 1983) was considered. Thus, 23 oligonucleotide sequences were used to generate 13 mutants. The nucleotide sequence of the wild-type and mutant signal peptides are shown in Figure 1. Verification of these was accomplished by direct DNA sequencing (Sanger et al., 1977).

Transport Studies. For pulse–chase analyses, cells in the logarithmic growth phase were washed and resuspended in MOPS medium supplemented with amino acids at 20 μg/mL minus methionine. The cells were labeled with 100 μCi of

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; TCA, trichloroacetic acid; Tris, tris-(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

[^{35}S]methionine for 40 s, and the chase was initiated by addition of unlabeled methionine to a concentration of 4 mg/mL. At various time points, an aliquot of cells was precipitated in 5% TCA and then the pellet was washed twice with ice-cold acetone. Protein was solubilized in 10 mM Tris, pH 8, 1% SDS, 1 mM EDTA by boiling for 3 min. Following dilution by addition of 50 mM Tris, pH 8, 150 mM NaCl, 0.1 mM EDTA, and 2% Triton X-100, the clarified supernatant was immunoprecipitated as previously described (Kendall et al., 1986). Samples were run on 7.5% Laemmli gels (Laemmli, 1970). To quantify the amount of precursor and mature alkaline phosphatase, the corresponding area of dried gels, visualized by autoradiography, was excised and rehydrated. The radioactivity was solubilized from the gel matrix in 2 mL of 80% Protosol (Du Pont) with gentle shaking at 55 °C for 18–20 h. Following the addition of 18 mL of scintillation mixture, the Protosol was neutralized with glacial acetic acid and the radioactivity was determined. The percentage of mature alkaline phosphatase for each sample was calculated, correcting for the additional radioactive methionine present in the precursor form.

RESULTS

Like other signal peptides, the *E. coli* alkaline phosphatase signal peptide is characterized by a polar (and positively charged) amino-terminal region, a hydrophobic core segment, and a more polar carboxyl-terminal region. For various structure–function studies, we have routinely identified the hydrophobic core region as the segment of 10 amino acid residues between the amino- and carboxyl-terminal threonines (see bracketed region in Figure 2). By replacing this region with various homopolymeric sequences, we have found that a high "hydrophobic density" is necessary to achieve efficient transport overall and, in particular, in the translocation step (Chou & Kendall, 1990; Rusch & Kendall, 1992). In order to determine the minimum level of hydrophobicity that supports transport, we have designed a series of mutants which allows us to "titrate" the hydrophobicity of the signal peptide core region. The sequences we utilized are shown in Figure 2. Alanine and leucine were chosen for this study because both have alkyl side chains which are nonpolar though to very different degrees. We knew from our previous work that a signal peptide incorporating 10 leucine residues was sufficiently hydrophobic to direct protein transport very efficiently while a signal peptide incorporating 10 alanine residues functioned poorly (Chou & Kendall, 1990). Consequently, these two mutant sequences were good candidates for the extremes of the titration curve and the intermediates were generated by changing the proportion of alanines to leucines in single amino acid increments. In addition, since polymers of alanine and of leucine both have a strong propensity to form α-helices (Ferretti & Paolillo, 1969; Arfmann et al., 1977; Zhang et al., 1992), varying the ratio of the two residues should not alter conformation and thus introduce another variable.

The specific sequence of alanine and leucine residues used for the mutant core regions evolved from our attempt to scramble the residues randomly and to avoid generating any specific sequence patterns. At the same time, however, we tried to design the core region-encoding DNA nucleotides such that they could be used to produce several of the different mutants in order to be most cost effective.

The extent to which each mutant signal peptide supported the transport process was evaluated by pulse–chase studies to determine the degree of precursor processing in various time intervals. Since the active site of the signal peptidase is oriented

Mutant	Amino Acid Sequence
WT	MKQST [I A L A L L P L L F] TPVTKA
10Ah	MKQST [A A A A A A A A A A] TPVTKA
9A1Lh	MKQST [A A A A A A A A L A] TPVTKA
8A2Lh	MKQST [L A A A A A A A L A] TPVTKA
7A3Lh	MKQST [L A A A A A L A L A] TPVTKA
6A4Lh	MKQST [L A L A A A L A L A] TPVTKA
6A4Lh-II	MKQST [L A A L L A A L A A] TPVTKA
6A4Lh-III	MKQST [L A L A A A A L A L] TPVTKA
5A5Lh	MKQST [L A L A L A L A L A] TPVTKA
4A6Lh	MKQST [L A L A L L L A L A] TPVTKA
3A7Lh	MKQST [L A L L L L L A L A] TPVTKA
2A8Lh	MKQST [L A L L L L L L L A] TPVTKA
1A9Lh	MKQST [L A L L L L L L L L] TPVTKA
10Lh	MKQST [L L L L L L L L L L] TPVTKA

FIGURE 2: Amino acid sequences of the wild-type and mutant alkaline phosphatase signal peptides. The natural amino-terminal and cleavage regions are maintained in all mutants. The hydrophobic core regions are bracketed, and the leucine residues are in boldface. Mutants are names by the number of alanine and leucine residues in the hydrophobic core region. Mutants 6A4Lh, 6A4Lh-II, and 6A4Lh-III share the same amino acid composition but differ in the core region sequence as described in the text.

toward the periplasm (Zimmerman et al., 1982), processing of the alkaline phosphatase precursor typically requires membrane insertion and translocation of the nascent polypeptide chain prior to signal peptide cleavage. Concomitant to signal peptide cleavage, the mature enzyme is released into the *E. coli* periplasm. Consequently, precursor processing provides a good earmark of signal peptide function. Furthermore, it is a readily quantifiable parameter which facilitates comparative analysis.

The extent of precursor processing as a function of the ratio of alanine and leucine residues in the signal peptide core region is shown in Figure 3. These data, obtained following a 30-s chase period, establish a clear pattern. At ratios of alanine to leucine of less than or equal to 8:2, the signal peptide is essentially nonfunctional (<10% processing) while at ratios greater than or equal to 3:7 the signal peptide functions efficiently (>90% processing). The midpoint is between alanine to leucine of 6:4 and 5:5. The data reveal that signal peptide activity is titratable; increasing the hydrophobicity of the core region while maintaining its natural length as well as the native sequence of the amino and carboxyl segments results in an increasingly more efficient signal peptide. In every instance examined, cleavage of the signal peptide resulted in release of the mature enzyme in the periplasmic space as expected (data not shown). Interestingly, the relationship between processing and core region hydrophobicity is not linear and the degree of processing increases dramatically over a narrow range of hydrophobicity change.

The results indicate that the 6A4Lh mutant deviated most notably from the titration pattern. Consequently, we used

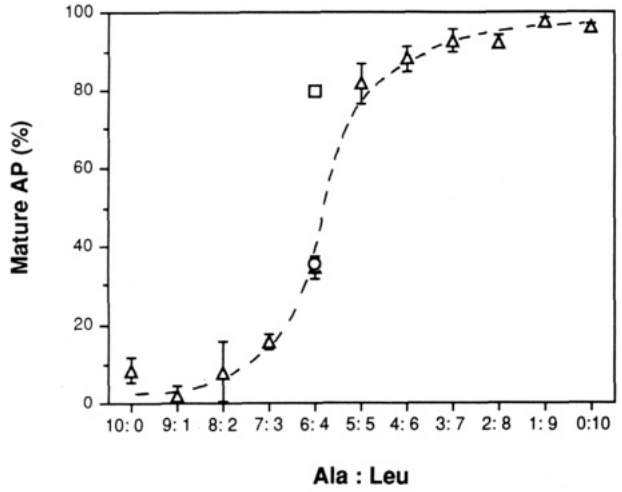

FIGURE 3: Extent of precursor processing as a function of the ratio of alanine to leucine residues in the signal peptide core region. The three mutants which possess six alanine and four leucine residues are as follows: 6A4Lh (□), 6A4Lh-II (△), and 6A4Lh-III (○). Pulse–chase studies were conducted as described in Materials and Methods. The percentage of total alkaline phosphatase present as the mature form after a 30-s chase was determined. Each point represents the average of at least three experiments. The error bars are shown for all points. The dashed line is meant to help visualize the overall pattern and does not represent a true best fit. Processing of the wild-type precursor under the same conditions is 84% complete.

CPK space-filling models to examine the 6A4Lh sequence, as well as others, to determine if we had inadvertently produced any sequence patterns that might inhibit or promote signal peptide function disproportionately. In general, no such patterns were observed. We did note that the 6A4Lh sequence, in an α-helical arrangement, is somewhat symmetrical with respect to the leucines. Two of the leucines are located on one face of the helix while the remaining two leucines are located on the opposite side of the helix producing a peptide with minimal hydrophobic moment. To examine the possibility that this motif played a role in the extent to which this mutant functioned, we designed two additional mutants which retain the same amino acid composition as 6A4Lh but which differ in sequence. Mutant 6A4Lh-II was designed to have a low hydrophobic moment like 6A4Lh but a different sequence, and in contrast, mutant 6A4Lh-III was designed to have a high hydrophobic moment. These sequences are included in Figure 2.

The data presented in Figure 3 indicate that the extent of processing exhibited by the 6A4Lh mutant (80%) is anomalous and not a reflection of any given sequence pattern. The other two mutants of the same composition, 6A4Lh-II and 6A4Lh-III, were designed to have very different spatial arrangements yet they were processed to a similar degree (34.6% and 35.0%, respectively), and the extent to which they are processed is consistent with the overall titration pattern. It is not surprising that there is some scatter in the region of the most dramatic activity change (alanine to leucine of 7:3 to 5:5), and thus the region most sensitive to hydrophobicity changes.

Analysis of the time dependence of precursor processing for the series of mutants reveals an interesting feature. As shown in Figure 4, for most of the mutants the extent of precursor processing does not vary substantially over time (e.g., <10% in 20 min); the amount of processing during the initial rapid phase is comparable to the total extent of processing. In contrast, the processing of the mutants 7A3Lh and 6A4Lh-II and 6A4Lh-III shows considerable time dependence. Processing proceeds from the 15.7% level (7A3Lh) and 34.6% level (6A4Lh-II) after a 30-s chase to a

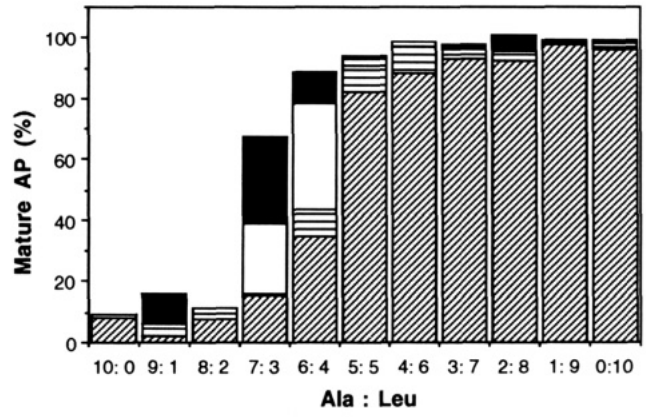

FIGURE 4: Time dependence of precursor processing as a function of the ratio of alanine to leucine residues in the signal peptide core region. Pulse–chase studies were conducted as described in Materials and Methods. The percentage of total alkaline phosphatase present as the mature form at various time intervals was determined. The increase in processing for successive time points from each mutant was then plotted in the stacked bar graph. The successive segments of each bar represent the percentage of mature alkaline phosphatase after a 30-s chase (diagonal lines) (i.e., the difference from the zero time point), then the additional processing after 1-min (horizontal lines), 5-min (no shading), 20-min (solid shading) chase. Only the 7A3Lh and 6A4Lh mutants show substantial processing after the initial 30-s time point. The data represent the average of at least three experiments.

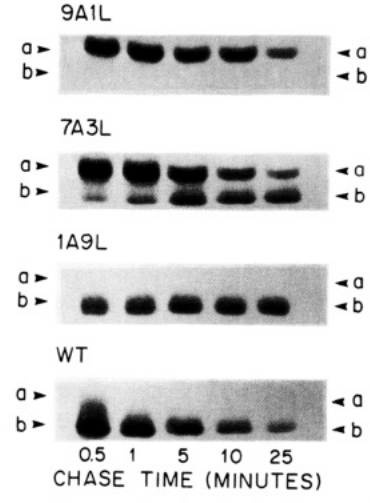

FIGURE 5: Autoradiogram showing the distribution of precursor and mature alkaline phosphatase during a pulse–chase study of representative mutants. Cells were radiolabeled with [^{35}S]methionine for 30 s and then chased with cold methionine for 30 s, 1 min, 5 min, 10 min, and 25 min, and immunoprecipitated as described in Materials and Methods. Migration of precursor is indicated by arrowhead a, and the mature enzyme is indicated by arrowhead b.

67.3% level (7A3Lh) and 88.9% level (6A4Lh-II) after 20 min. This point is further illustrated in Figure 5, which shows an autoradiogram of the pulse–chase data for a mutant at each extreme and the mutant 7A3Lh which exhibits intermediate behavior and substantial posttranslational processing over several minutes.

DISCUSSION

By varying the proportion of leucine and alanine residues in the signal peptide core region, we have been able to titrate the transport of *E. coli* alkaline phosphatase. Using precursor processing as an indicator of transport, a clear correlation with the core region hydrophobicity was observed. The relationship is nonlinear and, interestingly, transport activity is highly sensitive to small changes in hydrophobicity within a narrow range.

The data are consistent with a model of protein transport in which the series of events leading to transport can be represented by a simple two-state equilibrium between the unprocessed and processed species. The unprocessed state may include all cytoplasmic species and membrane-bound species which remain in the precursor form. Transfer to the processed state involves signal peptide cleavage and is accompanied by release of the mature protein. By representing the process as follows:

$$AP_{(unprocessed)} \overset{K_{eq}}{\rightleftharpoons} AP_{(processed)}$$

where K_{eq} is the global equilibrium constant, then $\Delta G = -RT \ln K_{eq}$. Using hydropathy tables which rank the amino acids on the basis of their free energies of transfer, every alanine to leucine conversion involves a change in ΔG of approximately -1 kcal/mol.[2] Consequently, the series of mutants from 10A0Lh to 0A10Lh spans a range of about 10 kcal/mol, assuming that no other changes are involved. Therefore, if the 10A0Lh mutant has an absolute ΔG that is very positive, the equilibrium will be far to the left and no transport will occur. Conversion of one residue to leucine changes the ΔG by only -1 kcal/mol leaving the ΔG for the overall process still positive. As the change in ΔG is incrementally changed by units of -1 kcal/mol, the equilibrium becomes favorable for processing and completion of transport. Using this model, the amount of product (processed protein) can be calculated from the logarithmic relationship between ΔG and K_{eq} to yield the sigmoidal relationship observed between transport activity and signal peptide hydrophobicity.

To illustrate this relation, we have generated a theoretical curve relating product formation to ΔG. The percentage of product at equilibrium was calculated from the ratio of product to reactant as given by the equilibrium constant at a given value of ΔG. An optimal agreement with the experimental data was obtained using least-squares analysis to determine the increment in ΔG. We used -1 kcal/mol as our initial estimate and then varied this to find the best fit to the experimental data. The least-squares difference was at a minimum at a value of $\Delta G = -0.85$ kcal/mol for each alanine to leucine conversion. The data shown in Figure 3 have been replotted with this theoretical curve in Figure 6. The agreement is quite good and the sharp transition from minimal to maximal activity is predicted by the model.

Furthermore, the data are consistent with the notion that for mutants 7A3Lh and 6A4Lh the equilibrium between the unprocessed and processed species lies just to the left. The hydrophobicity of these signal peptides is, however, sufficient that small changes in the concentration of protein which completes the transport process results in an observable shift of precursor from the unprocessed state. Consequently, these mutants show substantial conversion of the precursor to mature form over the course of a pulse–chase study.

We have chosen to consider the equilibrium between the unprocessed and processed states in our model because that is the parameter our experiments directly measure and that we could readily quantify (i.e., the extent of precursor processing in a given unit of time). It is, of course, likely that the degree of signal peptide hydrophobicity is actually critical

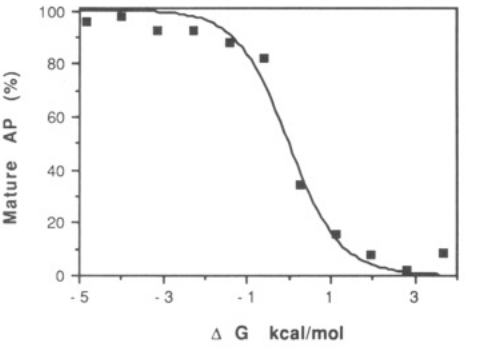

FIGURE 6: Best fit of alkaline phosphatase transport to changes in ΔG. The theoretical line (—) was derived assuming a two-state equlibrium at 37 °C. The amount of mature AP (■) is taken from the data in Figure 3. The incremental change in ΔG for each alanine to leucine conversion and the midpoint of the transition were treated as fittable parameters, and the optimal value for the change in ΔG of -0.85 kcal/mol was obtained by least-squares analysis. Consequently, the experimental points are plotted using these values in place of alanine to leucine ratios.

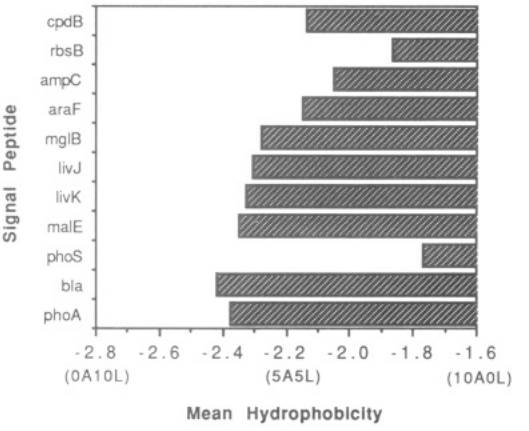

FIGURE 7: Mean hydrophobicity of the signal peptide core region from various periplasmic proteins. The sequences of the signal peptides were provided in Sjöström et al. (1987). The core regions were defined as beginning after the last amino-terminal charged residue (or in two cases after the last threonine to avoid an unusually short segment) and ending six residues from the cleavage site. The hydropathy table of Engelman et al. (1986) was used to determine the mean hydrophobicity per core region residue in kilocalories per mole. The scale used on the x-axis corresponds to the range of hydrophobicity for the alanine/leucine mutants in this study (listed in Figure 2), and three of these are identified for clarity.

for one or more steps during the transport process and prior to the processing event. These steps may involve binding to other components involved in the transport pathway such as Sec A, Sec Y, signal peptidase, or lipids. Previous studies have indicated that the membrane insertion process (Hoyt & Gierasch, 1991) and the subsequent translocation process (Thom & Randall, 1988; Chou & Kendall, 1990) are especially sensitive to the degree of signal peptide hydrophobicity. The data presented here indicate that at least one key step is highly dependent on signal peptide hydrophobicity and that if the signal peptide is sufficiently hydrophobic so as to promote a successful interaction at this critical step then processing and completion of the transport process follow concomitantly. Certainly, mutants have been produced which uncouple the latter processes, but for wild-type precursors and the mutants in this study this is a reasonable assumption. Mutants with hydrophobicity levels just below the threshold value for rapid processing are somewhat less efficient at the critical step, but over time productive interactions and subsequent processing

[2] For a discussion regarding different hydropathy tables and their applicability to various systems, see von Heijne (1985). Here we use -1 kcal/mol as an approximate value for the difference in the transfer free energies of alanine and leucine for the purpose of illustration. It is in the range of values derived from several hydropathy tables; e.g., the value is -1.2 kcal/mol using the treatment in Engelman et al. (1986).

do occur. For mutants that are weakly hydrophobic, however, the equilibrium lies so far to the left that observable changes in the forward direction are negligible.

Given the good correlation between the hydrophobicity of the polymeric sequences used here and transport activity, we wondered how wild-type signal sequences with their varied amino acid compositions would compare. Using the hydropathy scale from Engelman et al. (1986), the wild-type alkaline phosphatase signal peptide has a hydrophobicity ranking between the 4A6Lh and 3A7Lh mutants. Under the conditions used to generate the data for Figure 3, the level of wild-type processing is about 84%, comparable to that of mutant 4A6Lh. The hydrophobicity of other wild-type signal peptides falls in the same general range. Of 11 signal peptides for periplasmic proteins [sequences provided in Sjöström et al. (1987)], only two have core segments which are significantly less hydrophobic than the 5A5Lh mutant, which supports precursor processing to a level of about 80% in 30 s (Figure 7). None of the signal peptide core segments is more hydrophobic than that of the 3A7Lh mutant. Apparently, a higher degree of hydrophobicity is of no particular advantage to natural signal peptides. Indeed, signal peptides which are as highly hydrophobic as some of our mutants may bind components of the pathway too tightly, be degraded less well after cleavage, or perturb the bilayer too drastically. The hydrophobicity of natural signal sequences may be appropriate for a specific cell type, for regulation, and/or for maintaining the optimum concentration and balance of several different proteins that must compete for the same transport pathways [e.g., see Bird et al. (1990) and von Heijne et al. (1991)].

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