Biochimica et Biophysica Acta, 1068 (1991) 179–188

ADONIS 000527369100345O

BBAMEM 75370

A zein signal sequence functions as a signal-anchor when fused to maize alcohol dehydrogenase

Robert G. Shatters, Jr. * and Jan A. Miernyk

Seed Biosynthesis Research Unit, USDA, Agriculture Research Service, National Center for Agricultural Utilization Research, Peoria, IL (U.S.A.)

(Received 7 May 1991)

Key words: Alcohol dehydrogenase; Chimeric protein; Protein translocation; Signal sequence; Signal anchor; Zein

A chimeric gene, preZad, was constructed encoding a zein signal sequence fused precisely to the amino terminus of maize alcohol dehydrogenase 1. Translocation and processing of this chimeric preZad protein were assayed *in vitro* using a rabbit reticulocyte lysate translation system supplemented with canine pancreatic microsomes. PreZad was cotranslationally translocated across the vesicular membranes. Unexpectedly, the signal sequence was not removed although a suitable cleavage site was preserved and presented within the vesicle lumen. Failure to cleave the signal sequence was apparently not due to the lack of a β-turn near the processing site. When a β-turn was introduced near the cleavage site through site-directed mutagenesis, no processing was observed. PreZad was not solubilized by alkaline treatment of the microsomes, indicating an integral membrane association. Resistance to proteolysis, in the absence of detergent, indicates that preZad is associated with the membranes in a type II orientation (C-terminus in and N-terminus outside the vesicles). Analysis of truncated versions of preZad showed that it is the uncleaved signal sequence that functions as a signal-anchor. Changing the ratio of net charge flanking the signal sequence to < 1 (N-terminal:C-terminal) did not alter the type II membrane orientation, as would have been predicted by the 'positive-in rule'. Our results provide additional insight into the role of the passenger protein and signal sequence-flanking regions in recognition of a signal peptidase processing site, and the orientation of insertion of a signal-anchor sequence into the endoplasmic reticulum membrane.

Introduction

Targeting of secreted and endomembrane resident proteins occurs via the same pathway in eukaryotes. Proteins recognized by this pathway are initially translocated into or across the ER membrane [1,2]. The proteins are then secreted, plasma membrane associated, or located in the lumen or membrane of the various compartments of the endomembrane system. The ultimate location of a protein depends upon the sorting information indigenous to that protein.

Entry of a protein into the secretory pathway involves translocation across the ER membrane, and is initiated by recognition of a signal sequence. A signal sequence, generally 15–30 N-terminal amino acids, is believed to function by virtue of its physicochemical properties. Although comparison of various secretory proteins revealed little primary sequence homology, all typical signal sequences share a common tripartite structure (reviewed in Ref. 3); an amino terminal region often containing one or more positive charges, a central hydrophobic core, and a C-terminal proteolytic cleavage site defined by the '−1, −3 rule' [4,5].

Residues of the signal sequence other than those at the −1 and −3 positions are also important in determining precursor processing in eukaryotes [6], as are residues within the passenger protein. Additionally, Duffaud and Inouye [7] have shown a strong correla-

* Present address: Agronomy Seed Laboratory, University of Florida, IFAS, Gainesville, FL 32611, U.S.A.

Abbreviations: ADH1, alcohol dehydrogenase 1 (EC 1.1.1.1, alcohol:NAD^+ oxidoreductase); CPM, canine pancreatic microsome; ER, endoplasmic reticulum; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

Names of vendors are included for the benefit of the reader and do not imply endorsement or preferential treatment by the United States Department of Agriculture.

Correspondence: J.A. Miernyk, Seed Biosynthesis Research Unit, USDA, ARS, NCAUR, 1815 North University St., Peoria, IL 61604, U.S.A.

tion between predictions of a β-turn near the signal peptidase processing site and efficient cleavage at that site in *Escherichia coli*. A requirement of this 2° structure feature has not been studied in eukaryotes. It is therefore apparent that, although a potential cleavage site can be identified according to the '$-1,-3$ rule', other residues surrounding this site function in its presentation to signal peptidase.

Integral association of translocated proteins within the ER membrane can occur either through the interaction between a stop-transfer sequence and the membrane or as the result of an uncleaved signal sequence functioning as a signal anchor [8–11]. The classification of membrane orientation is based on the type of anchor and the terminus of the protein that is translocated (for review see Ref. 3). Type I membrane proteins have a cleavable signal sequence and a stop-transfer sequence within the protein primary sequence. These proteins have the N-terminus within the ER lumen and the C-terminus in the cytoplasm. Type II and III proteins are associated with membranes by a signal anchor. If the N-terminus of a signal anchor is exposed on the cytoplasmic surface of the ER membrane, the protein is anchored in a type II orientation (N_{cyt}/C_{exo}). If the signal anchor is membrane-associated with its amino terminus directed toward the lumen of the ER, the protein will be anchored in a type III orientation (N_{exo}/C_{cyt}).

Some signal anchors contain potential signal peptidase processing sites [3]. The reason for the lack of cleavage by signal peptidase remains unclear, however deletion of the cytoplasmic tail from a type II oriented membrane protein resulted in the conversion of a signal anchor to a cleavable signal sequence [10]. Mutant or chimeric proteins have been constructed where a signal sequence has been converted to a signal anchor and *vice versa*, or the orientation of a signal anchor within the membrane was reversed [10,12–15]. In each case, the altered function of the signal sequence/signal anchor could be explained by changes in the flanking net charge and adherence to the 'positive-in rule'. Recently, however, there have been several reports where mutations or chimeric constructions have resulted in membrane associations not predicted by the 'positive-in rule' [16–18]. Our results support the suggestion that membrane orientation does not necessarily follow the 'positive-in rule', and additional features must be considered.

As a first step in analysis of the mechanisms of plant protein secretion, we have developed a chimeric gene to study signal sequence function. This gene contains the signal sequence from zein, a maize endosperm storage protein, fused to the N-terminus of a normally cytoplasmic protein, maize ADH1. *In vitro* synthesized RNA from the chimera was used for *in vitro* translation and translocation analyses. In this mammalian cell-derived *in vitro* system the uncleaved zein signal sequence functions to anchor preZad in a type II membrane orientation. This result is discussed in relation to the effects of the mature protein sequence on signal sequence processing and membrane orientation.

Materials and Methods

Reagents

Nucleic acid modifying enzymes, pGEM plasmids, the helper phage M13K07, and rabbit reticulocyte lysates were from Promega Biotechnology. Competent *E. coli* XL-1 Blue cells were supplied by Stratagene. Translation grade [^{35}S]methionine at > 800 Ci/mmol was from ICN. CPMs were from Amersham. Proteinase K was supplied by Boehringer Mannheim. m^7GpppG was from Pharmacia. Reagents for SDS-PAGE were from Bio-Rad. Oligonucleotides were synthesized using the reagents supplied by Applied Biosystems. DNA was sequenced using the Sequenase kit from US Biochemicals and the fluorescent-labeled primers from Applied Biosystems.

Plasmid construction

The preZad gene was initially constructed and inserted into pGEM-7Zf(+) to create the plasmid pZAD-20. Both strands of DNA encoding the zein Z4 signal sequence [19] contiguous with the first seven N-terminal residues of ADH1 were synthesized with an Applied Biosystems Model 381A DNA synthesizer us-

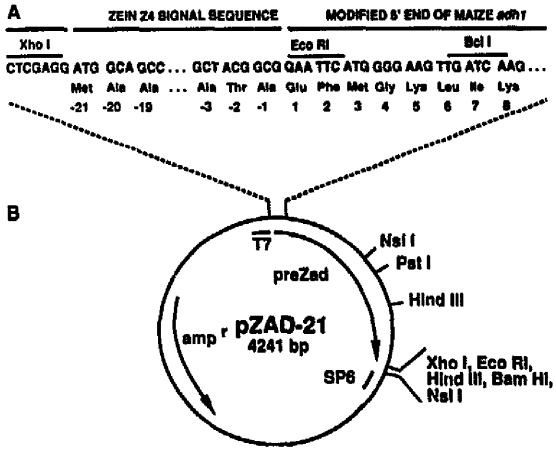

Fig. 1. Representation of pZAD-21. (A) Amino acid residues encoded by the preZad gene for part of the signal peptide and the amino terminus of the ADH1 protein. (B) Location and orientation of the preZad and the β-lactamase genes are indicated by the arrows. T7 and SP6 indicate the location of the respective RNA polymerase binding sites.

ing the phosphoramidite 'trityl-on' method. Amino acid residues 1, 2, 3, and 6 of the ADH1 sequence were altered as shown in Fig. 1A: an *Eco*RI site was inserted at the border between the sequence encoding the signal peptide and ADH1, a methionine residue was introduced at position 3 for improved detection of [^{35}S]methionine labeled preZad, and a leucine residue was introduced at position 6 to aid in future radiosequencing of the processed protein. The (+) strand was synthesized as an 81mer and the (−) strand as an 89mer. These were purified using Applied Biosystems oligonucleotide purification cartridges. The 5'-ends were phosphorylated using polynucleotide kinase. The strands were annealed yielding a double stranded molecule with an *Apa*I-ligatable end 5' to the signal sequence-encoding region and a *Bcl*I-ligatable 3'-end. This was combined in a ligation reaction with *Apa*I × *Xba*I restricted pGEM7Zf(+) and an 1180 bp *Bcl*I × *Nhe*I ADH1-containing fragment from pZML793 [20]. This mixture was used to transform *E. coli* XL-1 Blue cells to ampicillin resistance when plated on Luria Broth containing 200 μg/ml ampicillin, X-gal and IPTG. Plasmid DNA was purified from isolated colonies as described in [21], and the desired construct was identified by restriction mapping and sequence analysis. Single-stranded DNA was isolated by the procedure from Promega, using the helper-phage M13K07. Sanger dideoxynucleotide sequencing of single stranded DNA was done using the Sequenase protocol as modified for analysis using an Applied Biosystems 370A automated DNA sequencer.

Efficient translation initiation requires the initiator ATG codon to be within the consensus G/ANNATGG [22,23]. The sequence surrounding the prezad start ATG was modified to AGGATGG by isolating the prezad-containing 1305 bp *Apa*I × *Bam*HI fragment from pZAD-20 and inserting it into *Eco*RI × *Bam*HI restricted pGEM-7Zf(+). This was accomplished by digesting pZAD-20 with *Apa*I and pGEM-7Zf(+) with *Eco*RI, and removing the 5' single-stranded DNA from the ends of each linearized plasmid with S1 nuclease. Both plasmids were then digested with *Bam*HI and the 1305 bp fragment from pZAD-20 was isolated and combined with the digested pGEM-7Zf(+). These were ligated and used to transform competent *E. coli* XL-1 Blue cells. Plasmid DNA was isolated from single ampicillin resistant colonies and characterized by restriction endonuclease mapping and DNA sequencing. The desired construct was named pZAD-21 (Fig. 1).

Deletion derivatives of pZAD-21 were constructed using standard recombinant DNA techniques. pZAD 21-Δ*Pst*I was constructed by inserting the 699 bp *Xho*I × *Pst*I fragment from pZAD21 into *Sal*I × *Pst*I digested pGEM-3Z. pZAD21-Δ*Nsi*I was constructed by deleting an 808 bp fragment from pZAD-21.

Site-directed mutagenesis

The mutants preZad-B and preZad-C1 were developed using the Muta-gene kit and protocol supplied by Bio-Rad. Single-stranded pZad-21 DNA was prepared using the MK1307 helper phage. The primers used: 5'-GCAAGTGCTGCTACGGCGAGCTT-CATGGGGAAGTTGATC-3', for preZad-B; and 5'-GCTGCTSCGGCGTCAATCCCGCCGAAGTGCAG-TAAGTGCAAAGCTGCG-3', for preZad-C1, were synthesized with an Applied Biosystems Model 381A DNA synthesizer using the phosphoramidite 'trityl-on' method. Potential mutants were verified by sequence analysis.

In vitro transcription, translation and translocation

In vitro synthesized, m^{7}GpppG-capped RNA was produced from the pZAD-21 and pZAD21-Δ*Nsi*I using T7 RNA polymerase and from pZAD21-Δ*Pst*I using SP6 RNA polymerase, following the protocol from Promega. *In vitro* translations were done using a minus-methionine rabbit reticulocyte lysate kit and [^{35}S]methionine. Non-linearized plasmids were generally used as templates since minor translation products, presumably arising from plasmid transcripts, functioned as controls for proteinase K digestion treatments. Cotranslational translocation was assayed using *in vitro* translation reactions supplemented with CPMs added to one third the original volume. Proteinase sensitivity was determined by adding proteinase K at 0.3 mg/ml to completed, prechilled translation reactions and incubating on ice for 45 min. Where indicated, Triton X-100 was added to a concentration of 0.5% (v/v) before addition of proteinase K. Following proteinase K treatment, PMSF was added to 0.6 mM.

Analysis of membrane integration

Membrane association of preZad was determined using the alkaline sodium carbonate procedure of Fujiki *et al.*. [24]. 50 μl *in vitro* translation translocation reactions were layered onto a 200 μl 10 mM Tes (pH 7.5), 500 mM sucrose cushion and centrifuged at 100000 × *g* for 1 h in a Beckman TLA 100.2 rotor. The pellet was then sodium carbonate extracted by resuspending the membranes in 100 μl of 100 mM Na_2CO_3, (pH 11.5). After incubation at 4°C for 30 min, the extracted membranes were pelleted by centrifugation at 100000 × *g* for 1 h. Proteins in the supernatants from both the sucrose wash and the sodium carbonate extraction were precipitated with an equal volume of ice-cold 20% TCA and pelleted by centrifugation. Both the sodium carbonate membrane pellet and the TCA-insoluble material were resuspended in a solution containing equal volumes of (1) 3.0 M Tris (pH 8.8), 190 mM glycine, 0.1% SDS (w/v) and (2) 8 M urea, 4% SDS (w/v), 4% 2-mercaptoethanol (v/v), then analyzed as described in the figure legends.

Analysis of translation and translocation products

Proteins synthesized in translation and cotranslational translocation reactions were treated as described by Miernyk [25] and separated by SDS-PAGE using the procedure of Fling and Gregerson [26]. The results were visualized using an AMBIS Radioanalytical Imaging System. Quantitation was accomplished using the AMBIS software and is based on total cpm detected per protein band minus the sample background chosen for each lane.

Results

In vitro translation and translocation of preZad

The proteins produced in a rabbit reticulocyte lysate translation reaction in the presence of *in vitro* synthesized preZad RNA were analyzed by SDS-PAGE (Fig. 2A, lane 2). The major product was a protein of 44000 M_r. This is the size expected of the preZad protein, and the level of the translation product was proportional to the concentration of RNA added (data not shown). The protein band in lane 1 of Fig. 2A represents *in vitro* synthesized ADH1, and is included to show where processed Zad should migrate if the signal sequence is removed. Immunoprecipitation of *in vitro* translation reactions containing preZad RNA, with polyclonal anti-ADH1 antiserum, resulted in the selective precipitation of the 44000 M_r protein (Fig. 2A, lane 3). These data indicate that preZad is the major *in vitro* translation product of pZAD-21-encoded RNA.

To determine if preZad is translocation competent, CPMs were added to *in vitro* translation reactions. Analysis by SDS-PAGE showed no apparent processing of the preZad protein to the mature Zad form (Fig. 2B, lanes 4,5). The smaller molecular weight mature Zad band should be discernible in our gel system as *in vitro* synthesized ADH1 migrated detectably faster than preZad (Fig. 2A, lanes 1,2). Glycosylation can alter the electrophoretic migration in a SDS-PAGE system such that the processed and glycosylated protein migrates at the same rate as the non-glycosylated pre-protein. This is apparently not the case with preZad. The only potential N-glycosylation site (Asn_{148}-Pro-Ser_{150}) is very poorly recognized by oligosaccharyltransferase [27], and endoglycosidase H treatment did not alter the migration of preZad synthesized in the presence of microsomes (data not shown).

Although preZad is not processed by signal peptidase, it is translocated into the microsomes, as determined by proteinase K protection. Intact CPMs protected approx. 73% of the preZad molecules from proteinase K digestion (Fig. 2B, lanes 5,6). However, when the membranes were solubilized with 0.5% Triton X-100, all preZad was digested (lane 7). Evidence that protection from proteolysis is not due to general membrane association was tested by replacing the microsomes with maize endosperm mitochondrial membranes purified as described by Fang *et al.* [28] (data not shown). No proteinase K protection was observed in the presence of the mitochondrial membranes.

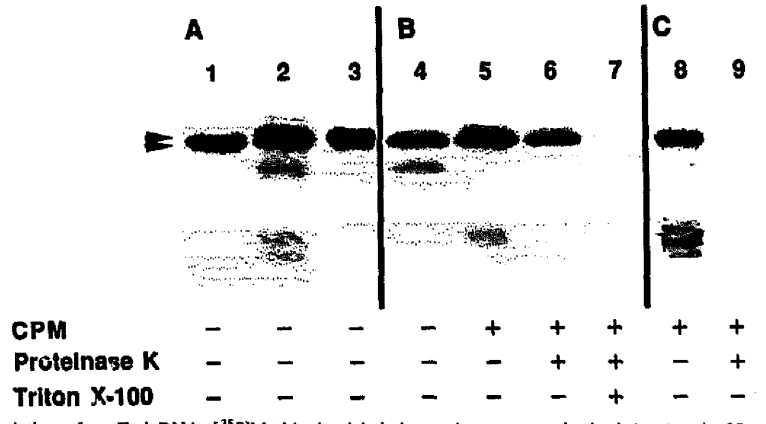

Fig. 2. *In vitro* translation of preZad RNA. [^{35}S]Methionine-labeled proteins were synthesized *in vitro* in 25 μl rabbit reticulocyte lysate reactions supplemented with 0.5 μg of *in vitro* synthesized RNA. Products were separated by SDS-PAGE using a 12.5% (T) acrylamide gel, and ^{35}S-labeled proteins were visualized using an AMBIS Radioanalytical Imaging System. The symbols below the lanes indicate the presence or absence of canine pancreatic microsomes (CPMs), or the post-translational incubation of the reactions with proteinase K in the presence or absence of Triton X-100. (A) Comparison of *in vitro* synthesized ADH1 and preZad. Lane 1, Translation of maize ADH1 RNA; lane 2, translation of preZad RNA; lane 3, immunoprecipitation of an *in vitro* translation reaction, containing preZad encoding RNA, with anti-ADH1 antiserum. (B) Proteinase protection of preZad by cotranslational addition of CPMs. Lane 4, translation of preZad RNA; lanes 5–7, cotranslational addition of CPMs followed by the indicated treatments. (C) Lanes 8 and 9, post-translational addition of CPMs.

Therefore preZad is translocated into the CPMs, but not processed by signal peptidase.

Cotranslational translocation of PreZad

Although all translocation experiments discussed thus far were cotranslational, they do not show that in our *in vitro* system translocation of preZad is obligately cotranslational. Post-translational translocation can occur in some instances [29,30]. To analyze this, translation reactions containing prezad RNA were incubated for 60 min prior to the addition of microsomes. Membranes were then added and the translation reactions were incubated for an additional 60 min. More than 90% of translation was complete within the first 60 min incubation (data not shown). Therefore, if translocation is obligately cotranslational, only a small portion of preZad should be translocated into the membrane vesicles. In these reactions no detectable preZad was protected from subsequent proteinase K treatment by the posttranslational addition of intact membranes (Fig. 2C, lanes 8,9). Thus, under our conditions, translocation of preZad must be cotranslational.

PreZad membrane association

Lack of signal sequence cleavage indicates that unprocessed preZad molecules are associated with the CPMs. Since the uncleaved zein signal sequence has characteristics similar to a signal anchor, experiments were conducted to determine if preZad is free within the CPM lumen or associated with the membranes. CPMs present during *in vitro* translation of preZad were centrifuged through a 500 mM sucrose cushion in order to separate proteins not associated with the microsomes (proteins remaining in the supernatant) from those that were (proteins present in the membrane pellet). The membrane fraction was then extracted with alkaline sodium carbonate to separate soluble (lumenal) from membrane associated proteins. The results showed that approx. 62% of preZad remained associated with the alkaline sodium carbonate extracted membrane fraction, and was therefore stably integrated into the membranes, whereas 35% of preZad was in the alkaline sodium carbonate supernatant that represents the CPM lumenal contents (Fig. 3A, lanes 1–3). As an internal control, pre-β-lactamase and preZad RNAs were included in the same cotranslational translocation assay. Mature β-lactamase was only detectable in the lumenal fraction and neither mature nor pre-β-lactamase was detected in the membrane fraction. At the same time, however, the majority of preZad was associated with the membrane fraction (data not shown). This indicates that membrane association of preZad is not an artifact of our experimental analysis.

As there were two pools of preZad associated with the CPMs (one associated with the membranes, and one soluble within the CPM lumen), it was important to determine which of these pools was protected from proteolysis by intact CPMs. If the membranes used for cotranslational translocation were proteinase K treated prior to alkaline sodium carbonate extraction, preZad was still present in the alkaline sodium carbonate supernatant and membrane pellet, in the same proportions as found without proteinase K treatment (Fig. 3,

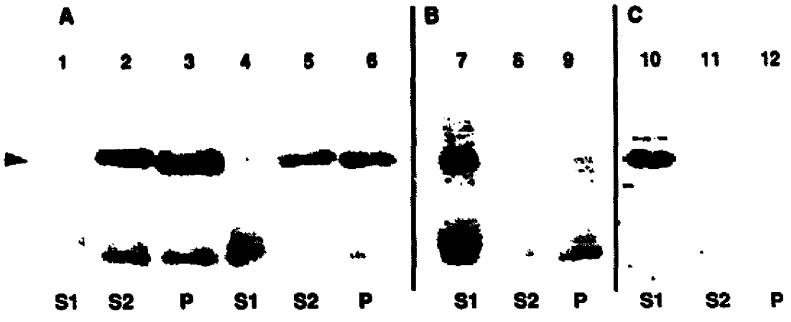

Fig. 3. Membrane association of preZad. *In vitro* synthesized RNA was translated *in vitro* using a rabbit reticulocyte lysate and [^{35}S]methionine as described for Fig. 2. (A) Lanes 1–3: membranes from a 50 μl cotranslational translocation reactions were centrifuged through a cushion of 10 mM Tes (pH 7.5), 500 mM sucrose. The membrane pellet was resuspended and extracted in 100 mM sodium carbonate (pH 11.5) and pelleted by centrifugation through a Tes-sucrose cushion. Soluble material from the 500 mM sucrose wash (S1) and the post-sodium carbonate centrifugation (S2) were TCA precipitated and resuspended (lanes 1,2, respectively). The post-sodium carbonate membrane pellet (P) was resuspended directly (lane 3). Lanes 4–6: a 50 μl cotranslational translocation reaction was incubated with proteinase K and then treated as described for lanes 1–3 above. (B) Post-translational addition of CPMs. preZad RNA was translated in a 50 μl reaction devoid of CPMs. After 60 min CPMs were added and the reaction was incubated for an additional 60 min. This sample was then sodium carbonate fractionated as described for lanes 1–3 above (lanes 7–9). (C) Cotranslational translocation of ADH1. RNA encoding ADH1 was translated in a 50 μl reaction and the sample was fractionated as described for lanes 1–3 above (lanes 10–12). [^{35}S]Methionine-labeled proteins were separated by SDS-PAGE on 12.5% gels and visualized using an AMBIS Radioanalytical detector.

lanes 1–3 versus 4–6). This indicates that the entire sequence of both CPM-associated pools of preZad is protected from proteolysis by intact CPMs.

We have previously shown that translocation of preZad, as determined by proteolysis protection, is cotranslational (Fig. 2). The results in Fig. 3A suggest that since both the membrane associated and the lumenal pools are protected from proteolysis by intact CPMs, both are the result of cotranslational translocation. To analyze this further, cotranslational membrane association of ADH1 and post-translational membrane association of preZad were analyzed. Results from alkaline carbonate fractionation of *in vitro* cotranslational translocation reactions containing ADH1-encoding RNA demonstrated that all detectable ADH1 was in the S1 fraction (Fig. 3C). Thus, without the zein signal sequence, ADH1 cannot associate with the membranes. When CPMs were added post-translationally to *in vitro* translation reactions containing preZad-encoding RNA, less than 5% of the total preZad was associated with the membrane fraction (Fig. 3B). Therefore, the majority of the membrane associated preZad is targeted to the membranes cotranslationally.

For preZad to be an integral membrane protein, a part of the sequence must be acting as a membrane anchor. To determine potential membrane spanning regions, the preZad amino acid sequence was analyzed using the method of Rao and Argos [31] as presented in the IntelliGenetics RAOARGOS program (Fig. 4). Potential membrane spanning regions are defined by the physicochemical properties of contiguous blocks of amino acids. PreZad has two regions that could potentially form a membrane spanning helix. The first, H1, extends from residue 1 to residue 25 and includes the uncleaved signal sequence. The second, H2, extends from residue 217 to residue 233. Membrane anchor regions of transmembrane proteins are often flanked by basic residues on the cytoplasmic side of the membranes (outside the CPMs) [14,32]. Basic residues flank both H1 and H2 (Fig. 4).

Plasmids containing C-terminal deletions of the preZad gene were constructed to analyze the importance of the H2 region in membrane association (Fig. 4). The plasmid encoding the first deletion, pZAD-*ΔPst*I was constructed by deleting all the ADH1 sequence downstream from a *Pst*I restriction site at base 693 of the preZad gene. This removed the residues C-terminal to the H2 region, including the basic amino acid residues immediately adjacent to H2. Translation of preZad-*ΔPst*I RNA produced a protein with a molecular weight of 24635. The second deletion construct, pZAD21-*ΔNsi*I, was prepared by removing the H2 and all the sequence C-terminal to it. The preZad sequence in pZad21-*ΔNsi*I was terminated at a *Nsi*I site 808 bp downstream from the start codon. PreZad-

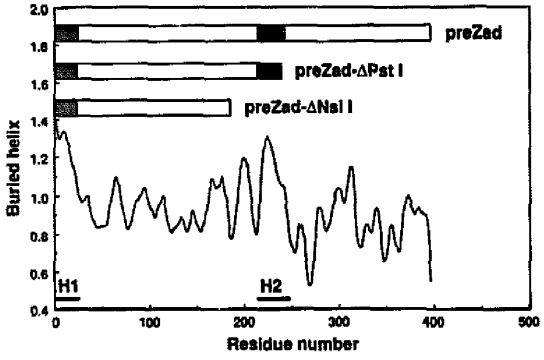

Fig. 4. Analysis of potential membrane-spanning regions within preZad and prezein. The amino acid sequences of preZad and prezein were analyzed for potential membrane spanning regions by the method of Rao and Argos [31] as presented in the RAOARGOS program from IntelliGenetics. A weighted, buried-helix value is assigned for each amino acid residue and plotted as a function of the residue number. The analysis was done using a minimal peak value for a transmembrane helix of 1.13, a minimal value for residues within a transmembrane helix of 1.05, and a minimum length for a transmembrane helix of 16 residues. Location of potential transmembrane helices is indicated by a bar above the *x*-axis. H1 and H2 represent the two potential transmembrane helices observed within preZad. A diagram of preZad and its C-terminal truncated derivatives preZad-*ΔPst*I and preZad-*ΔNsi*I is presented above the graph. The stippled area represents H1 and the black area represents H2.

*ΔNsi*I encodes a protein with a molecular weight of 19587. This truncated version of preZad contains only one potential membrane spanning region, H1 (Fig. 4).

In vitro synthesized RNA from these constructs was added to *in vitro* cotranslational translocation reactions. Neither preprotein was processed to a lower molecular weight mature form when CPMs were present (Fig. 5, lanes 1,2 and 7,8). The entire length of both truncated proteins was protected from proteinase K treatment by the cotranslational inclusion of CPMs (lanes 3 and 9). However, if the CPMs were disrupted prior to proteinase K treatment by the addition of Triton X-100, both truncated proteins were completely labile to proteolysis (lanes 4 and 10). Thus, both preZad-*ΔPst*I and preZad-*ΔNsi*I are translocated into CPMs.

Alkaline extraction of the membranes from *in vitro* translocation reactions of these truncated proteins showed that a significant portion of each was membrane associated, as was found with the full-sized preZad (Fig. 4, lanes 5,6 and 11,12). The only potential membrane spanning region of preZad-*ΔNsi*I is the uncleaved signal sequence (H1), therefore this hydrophobic region must be acting as the signal anchor. These results, along with the finding that the membrane associated preZad was protected from proteolysis (Fig. 2), indicate that the ADH1 sequence extends into the lumen of the CPMs resulting in a type II membrane orientation for preZad.

PreZad membrane orientation

The orientation of almost all known integral membrane proteins can be determined by the distribution of charged residues flanking the signal anchor [16]. Analysis of the charged residues flanking preZad indicate a net +1 on the N-terminal side and a net +2 charge on the C-terminal side. Therefore applying the 'positive-in rule' to the preZad signal anchor predicts a type III orientation for preZad, not the observed type II orientation. However, if the N-terminal methionine NH_2 group is unblocked the contribution of a +1 charge would balance the flanking net charges. Using site-directed mutagenesis, the glutamate at the +1 amino acid position with respect to the signal peptidase processing site was converted to a serine (the amino acid present at this position in the original zein sequence). The resulting preZad mutant, preZad-C1, has a net charge ratio of +2:+3 (N-terminus:C-terminus). *In vitro* translocation analysis showed that this mutant was protected from proteolysis by intact CPMs that were present cotranslationally (Fig. 6, lanes 1–4) and that 68% of this protected fraction was membrane associated after alkaline sodium carbonate extraction (Fig. 6, lanes 5–7). This indicates a type II orientation for the preZad-C1 mutant, and not the type III orientation that would be predicted based on the net charge distribution flanking the signal anchor. It is therefore apparent that factors in addition to simple charge distribution are influencing membrane orientation.

Protein secondary structure and signal sequence processing

Since membrane associated preZad is in a type II orientation, the potential processing site of signal peptidase should be located within the lumen of the CPMs for both membrane associated and soluble, lumenal-localized preZad. The active site of signal peptidase is also located within the lumen. Therefore it was surprising that processing of preZad to Zad was not observed. In *E. coli* it has been shown that decreasing the probability of a β-turn near a signal peptidase processing site results in a decrease in processing efficiency [7]. Fig. 7 shows the probability of β-turn structure near the signal peptidase processing site for the zein signal sequence when fused to zein (prezein) or ADH1 (preZad). These predictions were determined using the BETATURN program from IntelliGenetics based on a method developed by Chou and Fasman [33]. There is a predicted β-turn 5 residues after the processing site in prezein, however, preZad has no predicted β-turn near the potential cleavage site. The absence of a β-turn in preZad might therefore be preventing signal peptidase recognition.

To test this, site-directed mutagenesis was used to introduce a β-turn just after the predicted processing site in preZad (Fig. 7). This was done by introducing proline residues at positions +3 and +4 with respect to the processing site in order to disrupt the region of predicted α-helical structure that spans the processing site, and converting residues +1, +2, +7, and +8 to

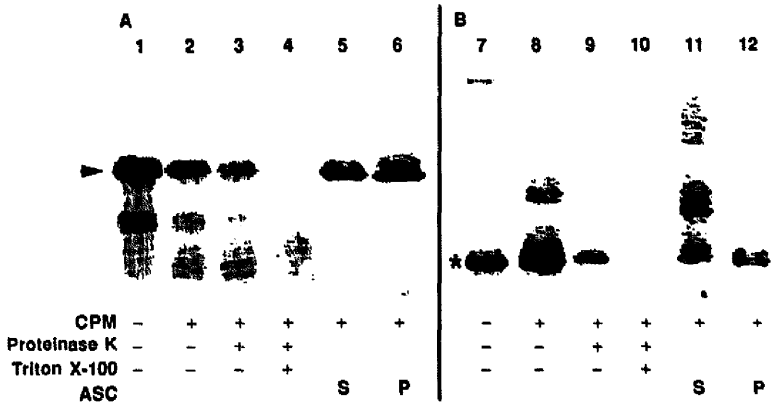

Fig. 5. Translocation and membrane association of the truncated preZad proteins. [^{35}S]Methionine-labeled proteins were synthesized *in vitro* in the absence (1,7) and presence (2–6 and 8–12) of canine pancreatic microsomes (CPM) as described in Fig. 2. A represents the analysis of preZad-Δ*Pst*I and B represents the analysis of preZad-Δ*Nsi*I. Lanes 2–4 and 8–10 were treated with proteinase K in the absence (3 and 9) and presence (4 and 10) of Triton X-100. Membranes from cotranslational translocation reactions of [^{35}S]methionine-labeled preZad-Δ*Pst*I and preZad-Δ*Nsi*I were extracted with 100 mM sodium carbonate (pH 11.5), and pelleted immediately after incubation. The supernatants (S) (5,11) and membrane pellets (P) (6,12) were treated as described in the legend for Fig. 4. The arrow indicates the preZadΔ*Pst*I protein while the star indicates the preZadΔ*Nsi*I protein. [^{35}S]Methionine-labeled proteins were separated by SDS-PAGE on 12.5% gels and visualized using an AMBIS Radioanalytical detector.

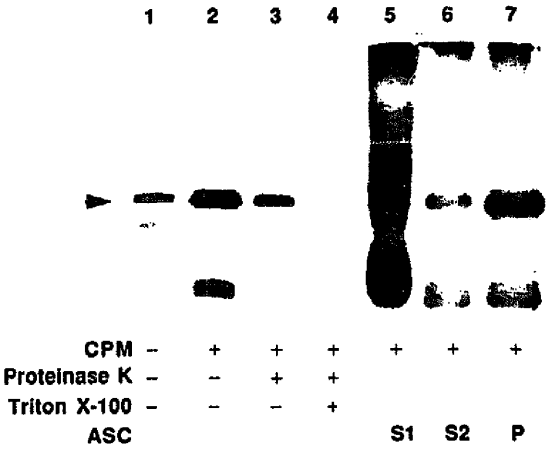

Fig. 6. Cotranslational translocation and membrane association of preZad-C1. [^{35}S]Methionine-labeled proteins were synthesized *in vitro* as described in Fig. 2. Cotranslational translocation was analyzed by comparing the products synthesized in the absence (lane 1) or presence of CPMs (lanes 2–7). Lumenal localization of preZad was determined by analysis of post-translational proteinase K treatment in the absence (lane 3) or presence (lane 4) of Triton X-100. Membrane association of preZad-C1 was analyzed by sodium carbonate fractionation as described for Fig. 5. [^{35}S]Methionine-labeled proteins were separated by SDS-PAGE on 12.5% gels and visualized using an AMBIS Radioanalytical detector.

the amino acids present at those positions in the native zein sequence. *In vitro* synthesized RNA produced from this mutant, designated preZad-B, was used for *in vitro* translations with and without added CPMs (Fig. 8). The addition of CPMs to the translations resulted in a full size preZad-B that was protected

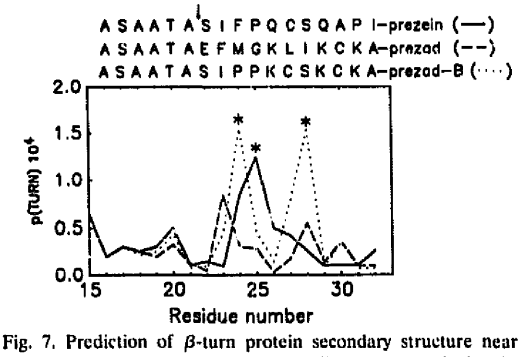

Fig. 7. Prediction of β-turn protein secondary structure near the signal sequence C-terminus. β-turn predictions were calculated using the BETATURN program from the IntelliGenetics PC/Gene software. The probability of a β-turn occurring at each residue, *p*(turn), is multiplied by 10000 and this value is plotted as a function of the residue number. A β-turn is predicted at any residue with a $p(\text{turn})10^4 \geq 0.75$. Predicted β-turns are labeled with an asterisk. Plots are: prezein (solid line); preZad (dashed line); and preZad-B (dotted line). The single letter amino acid code for residues 16–32 of prezein, preZad, and preZad-B is provided above the graph. The potential signal sequence cleavage site is indicated with an arrow.

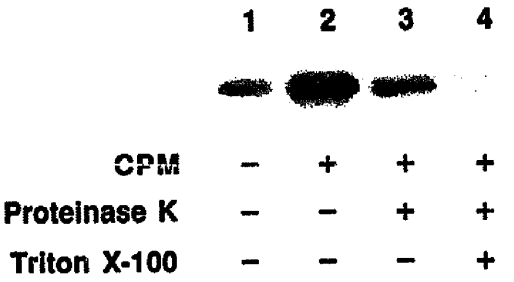

Fig. 8. Cotranslational translocation of preZad-B. *In vitro* synthesized RNA encoding preZad-B was translated in rabbit reticulocyte translation reactions. Treatment and analysis are as described for Fig. 2. Translocation was analyzed by comparing translations performed in the absence of CPMs (lane 1) with those performed in the presence of CPMs (lanes 2–4). Lumenal localization of preZad-B was determined by post-translational incubation with proteinase K in the absence (lane 3) or presence of Triton X-100 (lane 4). [^{35}S]Methionine-labeled proteins were separated by SDS-PAGE on 12.5% gels and visualized using an AMBIS Radioanalytical detector.

from proteinase K digestion by intact microsomes (Fig. 8, lanes 1–4). Approx. 61% of preZad-B was protected from proteolysis by intact membranes. This is similar to the 73% observed with preZad. Therefore introducing a β-turn near the signal peptidase processing site did not result in processing of the preZad signal sequence.

Discussion

A normally cleaved signal sequence from zein, a maize storage protein, functioned as a membrane anchor when fused to the N-terminus of maize ADH1 and expressed in a heterologous *in vitro* cotranslational translocation system. The ADH1 portion of the chimeric protein was translocated into the CPM lumen, however, the signal sequence was not cleaved and as a result it anchored a majority of the protein to the membrane in a type II orientation. Since zein is a plant storage protein, it is conceivable that this plant signal sequence is not recognized by the mammalian signal peptidase. However, it was previously reported that prezein is translocated and processed normally by *in vitro* systems containing either maize microsomes [34,35] or CPMs [35]. Thus, there are two significant aspects of our results. First, a potential signal peptidase cleavage site, as defined by the '−1,−3 rule', is present within the lumen of the CPMs but is not cleaved to produce the mature Zad protein. Second, the type II membrane orientation of preZad is not influenced by altering the ratio of net charge flanking the signal anchor.

The lack of signal sequence cleavage resulted in an integral association of the CPM-localized preZad with the membranes in a type II orientation. This association occurred only as a result of cotranslational translocation. Similar experiments using only the ADH1 gene

showed that translocation and membrane integration required the presence of the zein signal sequence. Although preZad contained a potential membrane spanning region within the ADH1 sequence, preZad-*ΔNsi*I, which has this region deleted, was still associated with the membranes in a type II orientation.

There have been several published reports of the conversion of signal sequences to signal anchors and *vice versa*, by changing their context. Lipp and Dobberstein [10,12], Szczesna-Skorupa *et al.*. [13], and Szczesna-Skorupa and Kemper [15] have shown that a type II membrane anchor can function as a cleavable signal sequence as a result of altering the hydrophilic sequence adjacent to the apolar anchor domain. In each case achieving cleavage can be explained by changes, either N- or C-terminal to the signal anchor, that allowed the proper presentation of a previously unexposed cleavage site to signal peptidase. These signal anchor to signal sequence conversions appear to result from one of two types of changes in the translocation process. The first is changing the membrane orientation to allow a potential signal peptidase processing site that normally remains on the cytoplasmic surface of the membranes to be translocated across the membrane [13]. The second type of change is when a potential cleavage site within the passenger sequence is moved adjacent to the apolar membrane spanning domain [10,12]. Haeuptle *et al.* [14] showed that a normally cleaved signal sequence could be converted into a type III signal anchor by altering the hydrophilic sequences either on the N- or the C-terminal sides of the signal sequence. The lack of signal sequence cleavage was apparently the result of the stop-transfer function of the signal sequence when the membrane orientation was altered from N_{cyt}/C_{exo} to N_{exo}/C_{cyt}. This resulted in the potential signal peptidase cleavage site remaining on the cytoplasmic side of the membrane.

Our results with preZad suggest that signal peptidase cleavage was prevented without altering either membrane orientation or the spatial relationship between the signal sequence/signal anchor apolar domain and the potential cleavage site. Both the soluble preZad within the CPM lumen and the type II oriented membrane-bound preZad should present the signal peptidase cleavage site within the CPM lumen, the location of the signal peptidase active site. The fact that an intact and complete zein signal sequence was used in constructing preZad indicates that the spatial relationship between the apolar membrane-spanning domain and the cleavage site was not modified.

Shaw *et al.* [36] converted a normally cleaved signal sequence into a signal anchor that anchored the passenger protein in a type II orientation within the membrane. This was accomplished by adding an N-terminal extension and altering the −1 amino acid within the signal sequence to a residue incompatible with cleavage by signal peptidase. Loss of signal peptidase cleavage in this instance was due to the lack of an appropriate signal peptidase recognition sequence as defined by the '−1,−3 rule'.

Duffaud and Inouye [7] have shown that a high probability for a β-turn near the processing site is necessary for efficient signal sequence cleavage in *E. coli*. Introduction of a β-turn near the processing site of preZad was accomplished through site-directed mutagenesis. The resulting mutant protein, preZad-B, was translocated, however the signal sequence remained uncleaved. This suggests that residues downstream from the signal sequence may have a more complicated role in determining the processing by signal peptidase than previously considered.

The finding that CPM-associated preZad exists in two pools, membrane associated and soluble within the lumen, may indicate that this signal sequence, in this context, is a relatively poor signal anchor. There have been several reports of chimeric or mutant membrane proteins that are found in both type I and type II orientations [37–39]. It seems likely that if signals determining membrane orientation are near the threshold between allowing N_{cyt}/C_{exo} or N_{exo}/C_{cyt}, two populations of the protein could exist distinguished by their membrane orientation. For preZad it may be that the anchor function of the zein signal sequence is weak, and as a result it is poorly recognized by factors that stabilize a protein within the membrane.

In vitro analysis has been used to show that the type II membrane orientation of preZad is not determined solely by the net charge ratio flanking the signal sequence as would be predicted by the 'positive-in rule'. This was shown for the mutant preZad-C1 that has a net charge ratio flanking the SA of +2:+3 (N-terminal:C-terminal) (Fig. 6). Given this ratio, a type III membrane orientation would be predicted. Noncompliance with this rule has been shown for several naturally occurring and constructed proteins [16–18]. All but one of these proteins have N-terminal extensions of 16 to 61 amino acids preceding the SA. It was suggested that secondary structure or the high total number of basic residues in this region may prevent protein translocation across the ER membrane. The one exception to this is the cytoplasmic tail-deletion mutant of the hemagglutinin-neuraminidase glycoprotein [18]. This mutant had the signal anchor within three residues of the N-terminus as the result of a 23 residue deletion, and was found in both type II and type III membrane orientations. These results are similar to our data that show a protein with a type II orientation despite its type III prediction. In both these cases there is only a very short amino-terminal extension containing a single basic residue. It should be noted that a small portion of preZad could be oriented within the membrane in a type III orientation and would not be detected by our

analysis. Often dual membrane orientations of a protein have been observed by analyzing glycosylation of the membrane associated proteins. Genes encoding glycosylated preZad are being constructed to analyze this aspect.

The results of Parks and Lamb [39] suggest that the presence of a positive net charge in the N-terminal flanking region can be dominant over a higher net positive charge in the C-terminal flanking region. It will be interesting to test this hypothesis with our chimeric protein, since preZad and the mutants presented in this paper contain a net +1 charge near the N-terminus.

Acknowledgments

We thank W.E. Hekman, J.C. Rogers, R.N. Trelease and M.J. Chrispeels for helpful suggestions, M.M. Sachs for providing pZML793, and D. Schwartz for providing the anti-ADH1 antiserum. We acknowledge S.M. Sturch for technical assistance and L. Tjarks for operation of the DNA synthesizer and DNA sequencer.

References

1 Walter, P., Gilmore, R. and Blobel, G. (1984) Cell 38, 5–8.
2 Wickner, W.T. and Lodish, H.F. (1985) Science 230, 400–407.
3 Von Heijne, G. (1988) Biochim. Biophys. Acta 947, 307–333.
4 Von Heijne, G. (1983) Eur. J. Biochem. 133, 17–21.
5 Perlman, D. and Halvorson, H.O. (1983) J. Mol. Biol. 167, 391–401.
6 Nothwehr, S.F. and Gordon, J.I. (1989) J. Biol. Chem. 264, 3979–3987.
7 Duffaud, G. and Inouye, M. (1988) J. Biol. Chem. 263, 10224–10228.
8 Blobel, G. (1980) Proc. Natl. Acad. Sci. USA 77, 1496–1500.
9 Spiess, M. and Lodish, H.F. (1986) Cell 44, 177–185.
10 Lipp, J. and Dobberstein, B. (1986) Cell 46, 1103–1112.
11 Von Heijne, G. and Gavel, Y. (1988) Eur. J. Biochem. 174, 671–678.
12 Lipp, J. and Dobberstein, B. (1988) J. Cell Biol. 106, 1813–1820.
13 Szczesna-Skorupa, E., Browne, N., Mead, D. and Kemper, B. (1988) Proc. Natl. Acad. Sci. USA 85, 738–742.
14 Haeuptle, M.-T., Flint, N., Gough, N.M. and Dobberstein, B. (1989) J. Cell Biol. 108, 1227–1236.
15 Szczesna-Skorupa, E. and Kemper, B. (1989) J. Cell Biol. 108, 1237–1243.
16 Hartmann, E., Rapoport, T.A. and Lodish, H.F. (1989) Proc. Natl. Acad. Sci. USA 86, 5786–5790.
17 Hong, W. and Doyle D. (1990) J. Cell Biol. 111, 323–328.
18 Wilson, C., Gilmore, R. and Morrison, T. (1990) Mol. Cell Biol. 10, 449–457.
19 Hu, N.-T., Peifer, M.A., Heidecker, G., Messing, J. and Rubenstein, I. (1982) EMBO J. 1, 1337–1342.
20 Dennis, E.S., Gerlach, W.L., Pryor, A.J., Bennetzen, J.L., Inglis, A., Llewellyn, D., Sachs, M.M., Ferl, R.J. and Peacock, W.J. (1984) Nucl. Acids Res. 12, 3983–4000.
21 Kahn, M.L., Kolter, R., Thomas, C., Figurski, D., Meyer, R., Remaut, E. and Helinski, D.R. (1979) Methods Enzymol. 68, 268–280.
22 Kozak, M. (1984) Nucl. Acids Res. 12, 857–872.
23 Kozak, M. (1986) Cell 44, 283–292.
24 Fujiki, Y., Hubbard, A.L., Fowler, S. and Lazarow, P.B. (1982) J. Cell. Biol. 93, 97–102.
25 Miernyk, J.A. (1987) J. Plant Physiol. 129, 19–32.
26 Fling, S.P. and Gregerson, D.S. (1986) Anal. Biochem. 155, 83–88.
27 Bause, E. (1983) Biochem. J. 209, 331–336.
28 Fang, T.K., David, N.R., Miernyk, J.A. and Randall, D.D. (1987) Curr. Top. Plant Biochem. Physiol. 6, 175.
29 Caulfield, M.P., Duong, L.T. and Rosenblatt, M. (1986) J. Biol. Chem. 261, 10953–10956.
30 Waters, M.G. and Blobel, G. (1986) J. Cell Biol. 102, 1543–1550.
31 Rao, J.K.M. and Argos, P. (1986) Biochim. Biophys. Acta 869, 197–214.
32 Von Heijne, G. (1986) EMBO J. 5, 3021–3027.
33 Chou, P.Y. and Fasman, G.D. (1979) Biophys. J. 26, 367–384.
34 Burr, F.A. and Burr, B. (1981) J. Cell Biol. 90, 427–434.
35 Campos, N., Palau, J., Torrent, M. and Ludevid, M.D. (1988) J. Biol. Chem. 263, 9646–9650.
36 Shaw, A.S., Rottier, P.J. and Rose, J.K. (1988) Proc. Natl. Acad. Sci. USA 85, 7592–7596.
37 Parks, G.D., Hull, D.J. and Lamb, R.A. (1989) J. Cell Biol. 109, 2023–2032.
38 Beltzer, J.P., Fiedler, K., Fuhrer, C., Geffen, I., Handschin, C., Wessels, H.P. and Spiess, M. (1991) J. Biol. Chem. 266, 973–978.
39 Parks, G.D. and Lamb, R.A. (1991) Cell 64, 777–787.