How Hydrophobic Is Alanine?*

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By a number of measures, alanine is poised at the threshold between those amino acids that promote the membrane integration of transmembrane α-helices and those that do not. We have measured the preference of alanine to partition into the lipid-water interface region over the central acyl chain region of the endoplasmic reticulum (ER) membrane by its ability to promote the formation of so-called helical hairpins, i.e., a pair of transmembrane helices separated by a tight turn, and by mapping the position relative to the membrane of the lumenal end of a transmembrane α-helix that ends with a block of 10 alanines. Both measures show that Ala has a weak but distinct preference for the interface region, which is in agreement with recent biophysical measurements on pentapeptide partitioning in simple water-lipid or water-octanol systems (Jayasinghe, S., Hristova, K., and White, S. H. (2001) J. Mol. Biol. 312, 927–934). Considering the complexity of the translocon-mediated insertion of membrane proteins into the ER, the agreement between the biochemical and biophysical measurements is striking and suggests that protein-lipid interactions are already important during the very early steps of membrane protein assembly in the ER.

Most integral membrane proteins are composed of tightly packed bundles of transmembrane α-helices (1). The loops connecting the helices tend to be short (2), suggesting the possibility that pairs of helices with a short connecting loop, i.e., a helical hairpin (3), may be inserted en bloc into the membrane (4–6) rather than the helices being recognized and inserted one by one by the translocation/membrane insertion machinery (7). Using in vitro translation in the presence of rough microsomes (RM) of model membrane proteins containing engineered poly-Leu segments of various lengths, we have shown previously that the balance between forming a long, continuous transmembrane helix and a helical hairpin can be a delicate one; in constructs with 35–40-residue-long poly-Leu stretches, a single Leu → Pro mutation near the middle of the poly-Leu stretch is sufficient to convert a long transmembrane helix into a helical hairpin (8, 9). Other polar and charged residues also promote helical hairpin formation in this context (9–11). As an example of a similar effect in a wild type protein, an Asn-Pro → Leu-Leu mutation between two tightly spaced transmembrane helices in the Saccharomyces cerevisiae Ste14p protein has been shown to convert the helical hairpin into a single, long transmembrane helix (12).

In our previous studies (9, 10), Ala has not shown any tendency to promote the formation of helical hairpins. The question of whether Ala should be considered a “membrane-seeking” or a “water-seeking” residue is not an easy one, however, as Ala balances close to the hydrophobic/hydrophilic threshold in many well known hydrophobicity scales (13). Poly-Ala segments of ~20 residues or more can insert as transmembrane helices during membrane protein assembly into the endoplasmic reticulum (ER) membrane (14), but segments of a very similar composition do not form transmembrane helices during protein integration in the inner membrane of Escherichia coli (15). Thus, poly-Ala segments appear to be very close to the threshold for membrane integration.

Here, we have explored the ability of a block of Ala residues to induce a helical hairpin when placed near the middle of a long poly-Leu stretch. Using in vitro translation of model proteins in the presence of RMs, we show that a run of 3–4 Ala residues placed centrally in the poly-Leu stretch efficiently induces helical hairpin formation, thus suggesting that Ala, at least in this context, has a slight preference for the lipid-water interface region over the central acyl chain region. As additional support for this contention, we show that a transmembrane segment composed of 13 Leu followed by 10 Ala and one Val is partly cleaved by signal peptidase near its C terminus, that cleavage is prevented by the addition of a Leu residue to the C-terminal end of the Ala-stretch, and that a part of the Ala-stretch appears to extend well into the lipid-water interface region as determined by the glycosylation mapping technique (16). These results agree surprisingly well with recent biophysical studies on simple peptide-lipid systems (17), suggesting that the nascent polypeptide chain can interact with membrane lipids at a very early stage of the translocon-mediated insertion of membrane proteins into the ER.

MATERIALS AND METHODS

**Enzymes and Chemicals—**Unless otherwise stated, all enzymes, plasmid pGEM1, and rabbit reticulocyte lysate were from Promega (Madison, WI) or New England Biolabs (Boston, MA). T7 DNA polymerase, [35S]Met, [3H]methylated marker proteins, ribonucleotides, deoxyribonucleotides, deoxyribonucleosides, and the cap analogs m7G(5′)ppp(5′)G were from Amersham Biosciences. TaKaRa Ex Taq polymerase was from TaKaRa Biomedicals (Shiga, Japan). Dog pancreas rough microsomes were prepared as described (18). The competitive glycosylation inhibitor peptide benzoyl-Asn-Leu-Thr-methylamide was from Quality Controlled Biochemicals (Hopkinton, MA). The signal peptidase inhibitor N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone was from Sigma.

**DNA Manipulations—**For cloning into and expression from the pGEM1 plasmid, the 5′-end of the lep gene was modified in two ways as...
follows: 1) by the introduction of an XhoI site; and 2) by changing the context 5' to the initiation ATG codon to a "Kozak consensus" sequence (19, 20). Replacement of the H2 region in Lep was performed by first introducing the BclI and NdeI restriction sites in codons 59 and 80 flanking the H2 region and then replacing the BclI-NdeI fragment with the appropriate double-stranded oligonucleotides. Residues 59–80 in H2 were thus replaced by poly-Leu-based sequences of the general form

\[
\text{XXX-XXX-XXX-XXX-XXX-XXX-XXX-XXX-XXX}
\]

appropriately positioned codons with codons for the acceptor tripeptide sequence, 1.4 mM) or competitive glycosylation inhibitor peptide (final concentration, 0.2 mM) at 30 °C for 1 h. Expression in vitro—The DNA template for in vitro transcription of full-length Lep mRNA was prepared by transcription of the Lep-pGEM1 plasmid with SP6 RNA polymerase. All cloning steps were done according to standard procedures.

Transcription templates encoding constructs with deletions in the N-terminal H1-H2 region were prepared using PCR to amplify DNA fragments from the relevant pGEM1 plasmids. The 5′-primers all had the common sequence 5′-GATTATTTGTTGACATATAGGAAA-CAGCCCAATTG-(unique sequence)-3′ containing the SP6 promoter, a ribosome binding site, and an initiator codon (underlined). The unique sequence at the 5′-end was designed to hybridize at the position of the first residue after the intended deletion to make constructs encoding the sequence MVQQQP-P2 and AAAAVQQQP-P2. The 3′ primer was chosen to be located downstream of the stop codon in lep.

Expression in vitro— The DNA template for in vitro transcription of full-length Lep mRNA was prepared by transcription of the Lep-pGEM1 plasmid with SP6 RNA polymerase for 1 h at 37 °C. The transcription mixture was as follows: 1–5 μg of DNA template, 5 μl of 10× SP6 buffer (400 mM Hepes-KOH, pH 7.4, 60 mM magnesium acetate, and 20 mM spermidine HCl), 5 μl of bovine serum albumin (1 μg/μl), 5 μl of m7G(5′ppp5′G) (10 μM), 5 μl of dithiothreitol (50 mM), 5 μl of nTP mix (10 mM ATP, 10 mM CTP, 10 mM UTP, and 5 mM GTP), 15 μl of H2O, 1.5 μl of RNase inhibitor (40 units/μl), and 2 μl of SP6 RNA polymerase (20 units/μl) in a total volume of 50 μl. Translation of 1 μl of Lep mRNA in 9 μl of nuclelease-treated reticulocyte lysate, 1 μl of RNase inhibitor (40 units/μl), 1 μl of [35S]Met (10 μCi/μl), 1 μl of amino acid mix (1 mM each amino acid except Met), and 1 μl of RM (4 eq) was performed as described (27) for 30 °C for 1 h.

To demonstrate cleavage by signal peptidase and N-linked glycosylation by oligosaccharyl transferase, translation reactions were performed with the addition of signal peptidase inhibitor (final concentration, 1.4 mM) or competitive glycosylation inhibitor peptide (final concentration, 0.2 mM) at 30 °C in a total volume of 14 μl.

For protease treatment of rough microsomes, proteinase K was used at a final concentration of 150 μg/ml, and samples were incubated for 30 min on ice. Proteins were analyzed by SDS-PAGE, and gels were visualized using a Bio-Rad Molecular Imager FX using the Quantity One Quantitation software.

RESULTS

Model Proteins—As in our previous studies (8–11), we have used the well-characterized E. coli inner membrane protein Lep as a model protein. Lep contains two transmembrane helices (H1 and H2) and a large globular C-terminal domain (P2). When expressed in vitro in the presence of RM, Lep has been shown to insert into the microsomal membrane with both the N and C terminus on the lumenal side (25), i.e. in the same orientation as it normally inserts into the inner membrane of E. coli (26). Translocation of the P2 domain to the lumenal side of the RM membrane is conveniently assayed by the glycosylation of a unique acceptor site for N-linked glycosylation (Asn-Ser-Thr) situated 20 residues downstream of H2 and by protease treatment of RM-integrated Lep (see Fig. 1).

For the studies reported here, H2 was replaced by various poly-Leu-based segments flanked by four lysines at the N-terminal end and a Gln-Gln-Gln-Pro stretch at the C-terminal end. The P2 domain is glycosylated in constructs where the poly-Leu segment forms a single transmembrane helix (Fig. 1, left) and is not glycosylated in constructs where it forms a helical hairpin (Fig. 1, right) (27). In previous studies (8, 10), we have shown that the poly-Leu segment in both glycosylated and non-glycosylated constructs is properly inserted into the RM membrane as assayed by alkaline extraction.

Helical Hairpin Formation and Processing by Signal Peptidase— In the constructs L13A10L4V, L15A10L4V, L13A10L8V, and L15A10L8V—As a first test of the effect of Ala residues on helical hairpin formation, we made two series of constructs wherein the H2 segment had the composition L13A10LnV or L15A10LnV (Fig. 2A). When translated in the presence of microsomes, the longer versions of both series gave rise to a single product (band A). For shorter constructs, a new product with lower mobility also appeared (band B). Finally, for the shortest construct, L15A10V, a third product with higher mobility than the unmodified full-length protein was also seen (band C).

When the L13A10LnV construct was translated in the absence of microsomes only, band A (Fig. 2B) was seen. Band A thus represents unglycosylated products, and band B (Fig. 2B) represents molecules that have been glycosylated on the acceptor site in the P2 domain. The addition of a peptide inhibitor of the signal peptidease enzyme (SP-F in Fig. 2B) largely prevented the formation of band C (Fig. 2B), suggesting that this band represents molecules in which signal peptidease has cleaved in or near the H2 segment. Addition of an inhibitor of N-linked glycosylation (AP in Fig. 2B) led to the disappearance of bands B and C, while band A and a new band D appeared (Fig. 2B). Band C thus represents glycosylated molecules cleaved by signal peptidease, and band D represents the unglycosylated, cleaved species (Fig. 2B). Consistent with the glycosylation results, proteinase K treatment of intact microsomes carrying constructs L13A10L4V, L15A10L4V, L13A10L8V, and L15A10L8V gave rise to a protected H2-P2 fragment in the two former but not in the latter cases (data not shown).

A comparison of the gel mobilities of a L13A10V construct lacking a glycosylation acceptor site (L13A10 V1), a construct wherein the entire N-terminal H1-P1-H2 domain up to and including the L13A10V segment has been deleted (MVQQQP-P2; cf. Fig. 1), and the same construct but with an additional N-terminal A10V-segment (MA10VQ10-P2) shows that the size of
the signal-peptidase cleaved form of L13A10V (band D) is close to that of MVQ3P-P2 (Fig. 2C). Thus, the signal peptidase cleaves close to the C-terminal end of the A10 stretch in L13A10V.

From the quantification of gel data shown in Fig. 2D, we conclude that a stretch of 10 Ala residues promotes the formation of a helical hairpin (as shown by the absence of glycosylation of the P2 domain) in the L13A10LnV and L15A10LnV constructs when n ≥ 7 and ≥ 6, respectively. The overall length of the hydrophobic stretch must thus be ≥31 residues for efficient helical hairpin formation, exactly the same minimal length as found previously for the formation of a Pro-induced helical hairpin (9).

A Run of 3–4 Ala Residues Induces Helical Hairpin Formation in Poly-Leu Segments of Varying Lengths—To determine the minimum number of Ala residues required to induce a helical hairpin in a long poly-Leu segment, we made three further series of constructs with H2 segments of the general composition LnAmLnV. In the first series, n + m + k = 39 and n ≈ k, i.e. the Ala residues were placed centrally in a poly-Leu segment of a constant overall length. The composition of second and third series was L15A5L16V and L15A5L15V, respectively. As seen in Fig. 3, constructs with a run of four or more centrally placed Ala residues were poorly glycosylated and, hence, form helical hairpins in all three series. Thus, Ala can induce helical hairpin formation even when the overall length of the hydrophobic segment is only 34 residues (construct L15A3L15V).

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Ala Residues Extend Outside the Membrane in L13A10LnV Segments—Finally, we used the glycosylation mapping technique (16) to determine the location of the Ala10 stretch in the L13A10LnV constructs relative to the surface of the ER membrane. In this approach, an Asn-Ser-Thr glycosylation acceptor site is placed at different separations (d) from the luminal end of the H2 transmembrane segment, and the minimum number of residues between the end of the hydrophobic stretch and the Asn residue required for efficient glycosylation is determined.
L13A10V segment to be glycosylated in the full-length protein in constructs where the glycosylation site is too close to the signal peptidase cleavage product (band C) is glycosylated even (Fig. 3). The fraction of glycosylated molecules (band B in panel A) is given as a function of the number of alanines (m) in the poly-Leu stretch. Results for two additional series of constructs with the H2 compositions L15A10V (hatched bars) and L15A10V (white bars) are also shown.

Fig. 3. Four alanines placed in the middle of a long poly-Leu-based hydrophobic segment induce the formation of a helical hairpin. A, L13A10V constructs with n + m + k = 39, and the n, m, and k values as given below the lanes were translated in the presence of RM. Band A is the non-glycosylated form of the protein, and band B is the glycosylated form of the protein. B, quantification of the gel in panel A (black bars). The fraction of glycosylated molecules (band B in panel A) is given as a function of the number of alanines (m) in the poly-Leu stretch. Results for two additional series of constructs with the H2 compositions L13A10V (hatched bars) and L13A10V (white bars) are also shown.

For an H2 segment with the composition L20V that was studied previously, this minimal glycosylation distance (MGD) is 9.5 residues (27). As shown in Fig. 4, B and C, however, the MGD for the L13A10V segment is only 5.6 residues (counting from the first Gln after the hydrophobic segment), strongly suggesting that a part of the A10 stretch extends well into the lipid-water interface region at the time when glycosylation takes place. If we assume that these residues are in an extended conformation, the location in the membrane of the C-terminal end of the L20V segment would correspond roughly to the location of Ala10 in the L13A10V segment.

The addition of one or three Leu residues to the C-terminal end of the Ala10 stretch gives rise to a further decrease in the MGD (down to 3.2 for construct L12A10V), which is as expected if these extra residues also protrude outside the membrane and adopt a mostly extended conformation.

Strikingly, the MGD increases to 5.6 when three more Leu residues are added (construct L13A10V). A possible interpretation is that the C-terminal end of the L13A10V segment is now sufficiently hydrophobic to be at least partially pulled back into the membrane, thereby increasing the MGD. Indeed, the L12A10V construct is right at the threshold of being able to form a helical hairpin (Fig. 2D), and it may thus be expected that its C-terminal end should interact strongly with the membrane.

Parenthetically, it is interesting to note in Fig. 4B that the signal peptidase cleavage product (band C) is glycosylated even in constructs where the glycosylation site is too close to the L13A10V segment to be glycosylated in the full-length protein (d = 5). Thus, the mammalian oligosaccharyl transferase can modify glycosylation sites near the N terminus of the P2 domain after signal peptidase cleavage, as was demonstrated previously for S. cerevisiae (28).

DISCUSSION

In this study, we have analyzed the ability of Ala residues to partition between the acyl chain and lipid-water interface regions of the ER membrane during translocon-mediated assembly of membrane proteins into dog pancreas microsomes. Ala is found with roughly equal frequency in transmembrane helices and non-membrane-embedded parts of integral membrane pro-
proteins (29) and is poised on the threshold between hydrophobic and hydrophilic residues in most hydrophobicity scales (13). In vivo, poly-Ala segments are right at the border between segments that can and cannot integrate as transmembrane segments during membrane protein assembly (14, 15).

To address the question of whether Ala has a higher preference for the acyl chain or the interface region, we have placed runs of Ala residues in two different contexts, i.e. near the middle of 30–40-residue-long poly-Leu transmembrane segments and at the C-terminal end of L13 and L15 transmembrane segments. In all three cases, the poly-Leu segments were placed in the position of the H2 transmembrane helix (Fig. 1).

Using in vitro transcription/translation of the different constructs in the presence of RM and an engineered acceptor site for N-linked glycosylation in the C-terminal P2 domain as a topological reporter, we find that Ala has a slight tendency to prefer the lipid-water interface region over the central part of the membrane. Thus, a run of four or more Ala residues placed centrally in a long poly-Leu stretch is sufficient to promote the formation of a helical hairpin (Figs. 2 and 3). Given the lengths of the two helices in these hairpins (15–20 residues each), at least some of the Ala residues must be located in or close to the luminal lipid-water interface region.

These results should be compared with earlier results showing that a similar degree of helical hairpin formation as found for four Ala is induced by single Pro, Asn, Arg, Asp, Gln, His, Lys, and Glu residues (10) and by pairs of Gly, Trp, Ser, Tyr, and Thr residues (30). As expected, Ala is thus a considerably less strong promoter of helical hairpin formation than the more polar and/or helix-breaking residues.

Similarly, using the glycosylation mapping technique (16, 27), we find that the C-terminal Ala stretch in a transmembrane segment of the composition L12A10V is not fully embedded in the membrane but extends into or even through the lipid-water interface region (Fig. 4). Moreover, signal peptidase cleaves the L12A10V segment (albeit inefficiently) near the C-terminal end of the A10 stretch in this construct (Fig. 2). Because signal peptidase is thought to have its active site located in the lipid-water interface region, this further confirms the notion that the A10 stretch extends outside the core of the membrane. The hydrophobic L12A10V segment is a rather poor substrate for signal peptidase, possibly because it may be too long (23) and/or because the A10V part may have a tendency to form an α-helix rather than the extended conformation that is recognized by the enzyme (31, 32). The addition of a single Leu residue after the L12A10V stretch (construct L11A10LV) almost completely abolishes signal peptide cleavage, and the addition of six Leu residues appears to make the C-terminal end of the stretch sufficiently hydrophobic to be pulled back into the membrane as seen by a shift in the minimal glycosylation distance (Fig. 4). To minimize the sequence complexity, we have used model transmembrane poly-Leu segments in this study; however, given that helical hairpins can also be induced in natural transmembrane segments (12), we assume that our results should be at least qualitatively correct for transmembrane segments in general.

Taken together, our results show that Ala has a slight preference for the lipid-water interface region over the central acyl chain part of the ER membrane and that this preference can have important consequences for membrane protein topology and signal peptidase cleavage. Thermodynamic analysis of the binding of pentaepitides to model lipid bilayers and the partition of tripeptides between water and octanol also indicates that Ala has a slight preference for the lipid-water interface region over the acyl chain region (17). Solid-state NMR studies of peptide-bilayer interactions point in the same direction (33). It thus appears that the interactions between a nascent polypeptide chain and the ER translocon that determine membrane protein topology in vitro are remarkably similar to peptide-lipid interactions in highly simplified in vitro systems.

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