Membrane Insertion Kinetics of a Protein Domain In Vivo

THE BACTERIOOPSIN N TERMINUS INSERTS CO-TRANSLATIONALLY*

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The pathway by which segments of a polytopic membrane protein are inserted into the membrane has not been resolved in vivo. We have developed an in vivo kinetic assay to examine the insertion pathway of the polytopic protein bacterioopsin, the apoprotein of Halobacterium salinarum bacteriorhodopsin. Strains were constructed that express the bacteriorhodopsin mutants I4C:H6 and T5C:H6, which carry a unique Cys in the N-terminal extracellular domain and a polyhistidine tag at the C terminus. Translocation of the N-terminal domain was detected using a membrane-impermeant gel shift reagent to derivatize the Cys residue of nascent radiolabeled molecules. Derivatization was assessed by gel electrophoresis of the fully elongated radiolabeled population. The time required to translocate and fully derivatize the Cys residues of I4C:H6 and T5C:H6 is 46 ± 9 and 61 ± 6 s, respectively. This is significantly shorter than the elongation times of the proteins, which are 114 ± 26 and 169 ± 16 s, respectively. These results establish that translocation of the bacterioopsin N terminus and insertion of the first transmembrane segment occur co-translationally and confirm the use of the assay to monitor the kinetics of polytopic membrane protein insertion in vivo.

An essential step in the biogenesis of polytopic or multi-spanning membrane proteins is the insertion of polypeptide segments into the membrane. To elucidate the insertion mechanism in vivo, the cellular factors that participate in this process must be characterized, and the pathway or sequence with which the polypeptide segments are inserted must be determined.

Cellular factors have been identified that mediate protein insertion into the eukaryotic endoplasmic reticulum (1, 2) and bacterial cytoplasmic membranes (3–5). These include the signal recognition particle (SRP), a cytosolic ribonucleoprotein complex, and the secretory translocase, a membrane protein complex. SRP directs ribosome-bound nascent polypeptides to the secretory translocase, which in eukaryotes forms an aqueous channel into which the polypeptides are inserted (6, 7). Sequence conservation of SRP and secretory translocase subunits (8, 9) suggests that aspects of the insertion mechanism are universal.

Less is known about the insertion pathway of polytopic membrane proteins. In eukaryotes, a co-translational, sequential insertion pathway (10, 11) is favored (12). In support of this model, the insertion of transmembrane segments of polytopic membrane proteins has been shown to be mechanistically coupled to translation (13–15) and to occur sequentially (15–17). However, other evidence contradicts this model. At least one eukaryotic polytopic membrane protein appears to insert post-translationally (18), and several others exhibit multiple topologies that may reflect a nonsequential insertion pathway (19–21). In bacteria, it is not known whether insertion is co-translational and sequential or more closely resembles protein secretion, where translocation is independent from elongation (22, 23). There is evidence for both post-translational (24) and co-translational (4, 5, 25) insertion mechanisms, but the issue remains controversial.

Studies of the insertion pathway have been problematic for several reasons. First, methods used to monitor the membrane insertion of protein segments are not time-resolved. Significant time elapses between polypeptide translation and analysis of insertion by methods such as cross-linking (14, 26) or proteolysis (16). During this time, polypeptides may rearrange to yield a topology that does not reflect the state of the polypeptides during insertion. Second, the topology of most polytopic proteins is poorly defined, making it difficult to interpret insertion studies unambiguously. Third, topological reporters commonly used to study insertion, such as fusion domains (27, 28) or glycosylation sequences (21), may alter protein topology or insertion. Finally, the insertion pathway determined in vitro may differ from the in vivo pathway because of differences in the concentrations of SRP or secretory translocase subunits, the membrane potential, or ionic conditions.

To examine the insertion pathway of a polytopic membrane protein in vivo, we have studied bacteriorhodopsin (BR), a light-driven proton pump of known structure (29) expressed in the sole membrane of the archaeon Halobacterium salinarum. BR contains a 248-amino acid polypeptide, bacterioopsin (BO), which forms seven transmembrane α-helices that surround a covalently bound retinal cofactor. The polypeptide is initially synthesized with a 13-amino acid presequence that is cleaved during biogenesis (30).

In this work, we have developed an in vivo assay of BO insertion and used it to characterize the translocation kinetics of the first extracellular domain of the protein. The assay monitors the translocation of engineered Cys residues in BO with a membrane-impermeant gel shift reagent specific for sulfhydryl groups. Comparison of the translocation time of Cys residues near the N terminus and the BO elongation time indicated that the N-terminal domain inserts co-translationally. These results establish the assay as a direct method to monitor the insertion of transmembrane segments in vivo and raise the possibility that the predominant pathway of polytopic...
membrane protein insertion is co-translational in all taxonomic domains.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides were obtained from Life Technologies Inc., *Taq* polymerase and ligase from Promega (Madison, WI), and restriction endonucleases from New England Biolabs (Beverly, MA). Ni²⁺-nitrilotriacetic acid Superflow was obtained from Qiagen (Valencia, CA). Redivue [³⁵S]Met (>1,000 Ci/mmol) was obtained from Amer sham Pharmacia Biotech, and 4-acetamido-4-maleimidylstilbene-2′-2′-disulfonic acid disodium salt (AMS) was from Molecular Probes (Eugene, OR).

**Plasmid and Strain Construction**—Mutations in *bop* were constructed in *Escherichia coli* by polymerase chain reaction. To introduce a sequence encoding six His residues at the BO C terminus, the primers TGGTGGCCAGCTGTAAG and CCGGAGGAGCCGGCGGGCAGC-CCACCACGACGACGTCGAGCCGCGCAG were used (codon changes are underlined). The last three codons of *bop* were removed by these changes. Constructs encoding Cys at amino acids 4 and 5 of mature BO were created with the primers GCGCGCCGATCGCGGAGA and CGCGACGTCGGTACCGTCGCGCAAGA and CCGACGTCGCCAGATCGGCCGCGCAGA. Restriction fragments derived from the polymerase chain reaction products were cloned in pMPK62 (31) to yield plasmids encoding His-tagged BO with a single Cys near the N terminus. Recombinant *H. salinarum* strains expressing the BR variants were created by targeted gene replacement (31). The *bop* sequence of the recombinant strains was confirmed by fluorescent dye terminator cycle sequencing (ABI Prism, Foster City, CA).

**Expression and Characterization of Mutant BR**—To induce BR synthesis, *H. salinarum* was cultured with illumination (32) in 120 ml of peptone medium for 2 days (33). Cell lysates were prepared, and BR levels were determined as described (34) from the change in absorbance at 585 nm between light- and dark-adapted samples. BR levels were expressed as percentages of total cell protein determined by the BCA assay (Pierce). For spectral analysis, mutant proteins were purified (33) and scanned in 30 mM sodium phosphate, 0.025% sodium azide, pH 6.9, with a Perkin-Elmer λ2 spectrophotometer.

**Purification of His-tagged BO**—Full-length His-tagged BO was purified from *H. salinarum* to >90% homogeneity by denaturing Ni²⁺ affinity chromatography at room temperature. Cultures (120 ml) were harvested at 8,000 × *g* for 10 min at room temperature, and the pellet was resuspended in 1.2 ml of medium salts (33). A 25-ml aliquot was mixed with 1.0 ml of 60 units DNase/ml and 100 µg phenylmethylsulfonyl fluoride/ml (lysis solution) and incubated for 40 min. Samples were combined with 40 µl of Ni²⁺-nitrilotriacetic acid beads and adjusted with buffer to a final concentration of 300 mM NaCl, 50 mM Tris-Cl, pH 7.0, 0.1% SDS in 1.4 ml. After incubation for 2 h, beads were sedimented and washed with 1 ml of 0.01% SDS, 300 mM NaCl, 50 mM Tris-Cl, pH 7.0, 5 mM imidazole. Bound protein was eluted by incubating for 0.5 h with 50 mM imidazole, 20 mM EDTA, 50 mM diethothreitol (DTT). Samples (0.5 ml) were added to 100 µl of a specific protein are compared (38). Cells were radiolabeled and lysed by the polymerase chain reaction products were cloned in pMPK62 by polymerase chain reaction. To introduce these changes. Constructs encoding Cys at amino acids 4 and 5 of mature BO were created with the primers GCGCGCCGATCGCGGAGA and CGCGACGTCGGTACCGTCGCGCAAGA and CCGACGTCGCCAGATCGGCCGCGCAGA. Restriction fragments derived from the polymerase chain reaction products were cloned in pMPK62 (31) to yield plasmids encoding His-tagged BO with a single Cys near the N terminus. Recombinant *H. salinarum* strains expressing the BR variants were created by targeted gene replacement (31). The *bop* sequence of the recombinant strains was confirmed by fluorescent dye terminator cycle sequencing (ABI Prism, Foster City, CA).

**Measurement of Elongation Time**—To determine the lag in radiolabeling due to uptake and equilibration of Met during the pulse and chase, cells were radiolabeled or pulse-chase radiolabeled with [³⁵S]Met as above. At various times, aliquots were removed and added to 40 volumes of lysis solution. Total protein in 10 µl was precipitated onto filter paper with trichloroacetic acid (10% w/v). [³⁵S]Met incorporation was quantified by PhosphorImager analysis, plotted as a function of time after the pulse and fit by linear regression. The lag in [³⁵S]Met incorporation after the pulse is given by the x intercept of the fit to data from radiolabeled cells. The lag in incorporation of nonradioactive Met after the chase is given by the time between chase addition and the intersection of the curves fit to data from radiolabeled and pulse-chase radiolabeled cells.

**Measurement of the Translocation Time**—To measure translocation times, cells expressing mutant BR were pulse-chase radiolabeled as above, except 2 µl of 200 mM AMS in dimethyl sulfoxide were added to 400 µl of the cells 1 min before radiolabeling. At various times after the pulse, 50 µl of the cells were combined with 50 µl of 20 mM DTT, incubated for 10 min at 37 °C to complete elongation of the radiolabeled protein, and mixed with 1.0 ml of lysis solution. His-tagged protein was recovered in a nitrilotriacetic acid sepharose (55). Samples were run on a 14% Tris-Cl, pH 7.0, 5 mM imidazole. Bound protein was eluted by incubating for 0.5 h with 50 mM imidazole, 20 mM EDTA, 50 mM diethothreitol (DTT). Samples (0.5 ml) were added to 100 µl of a specific protein are compared (38). Cells were radiolabeled and lysed by the polymerase chain reaction products were cloned in pMPK62 by polymerase chain reaction. To introduce these changes. Constructs encoding Cys at amino acids 4 and 5 of mature BO were created with the primers GCGCGCCGATCGCGGAGA and CGCGACGTCGGTACCGTCGCGCAAGA and CCGACGTCGCCAGATCGGCCGCGCAGA. Restriction fragments derived from the polymerase chain reaction products were cloned in pMPK62 (31) to yield plasmids encoding His-tagged BO with a single Cys near the N terminus. Recombinant *H. salinarum* strains expressing the BR variants were created by targeted gene replacement (31). The *bop* sequence of the recombinant strains was confirmed by fluorescent dye terminator cycle sequencing (ABI Prism, Foster City, CA).

**Expression and Characterization of Mutant Proteins**—To de-
shown immediately after the pulse (an extracellular Cys near the N terminus. The nascent population is quenched ([35S]Met; I4C:H6 and T5C:H6, were expressed in proteins for purification. The His-tagged Cys mutant proteins, reduced at the C terminus of the wild-type and Cys mutant which includes residues 9–31. A hexahistidine tag was intro-

Fig. 1. Assay scheme. Top panel, timeline of the assay. Cells expressing a membrane protein containing a unique extracellular Cys are preincubated with AMS and pulse-chase radiolabeled with [35S]Met. At various times after the pulse, AMS is quenched with DTT. Cells are incubated further to completely elongate the radiolabeled proteins before purification. Bottom panel, assay of a membrane protein containing an extracellular Cys near the N terminus. The nascent population is shown immediately after the pulse (I), after the AMS reaction is quenched (II), and after the radiolabeled population is elongated (III). Met residues are assumed to be evenly spaced. *, incorporation of [35S]Met; C, Cys; circled C, AMS-derivatized Cys.

devlop the assay, Cys was substituted for Ile or Thr, located in the 8-amino acid extracellular domain at the N terminus of mature BO (41). Translocation of this domain reports on the insertion of the adjacent transmembrane α-helix of mature BO, which includes residues 9–31. A hexahistidine tag was introduced at the C terminus of the wild-type and Cys mutant proteins for purification. The His-tagged Cys mutant proteins, I4C:H6 and T5C:H6, were expressed in *H. salinarum* at significantly lower levels than wild-type BR but only slightly lower than BR:H6 (Table I). The proteins could be purified in a form similar to the wild-type purple membrane (33) and had normal spectral properties (Table I), indicating that they assemble normally and bind the retinalchromophore.

The reactivity of the Cys residues, the mutant proteins were derivatized with AMS. A shift was observed with T5C:H6 (Fig. 2A) and I4C:H6 (data not shown) but not BO:H6, which lacks Cys (Fig. 2A). The reaction of AMS with T5C:H6 in intact cells was complete in ~10 s and was effectively quenched by DTT (Fig. 2B). Thus, AMS is sulfhydryl-specific and reacts rapidly.

Translocation Time of the BO N Terminus—As the first step in establishing the translocation time of the N-terminal domain, AMS derivatization of T5C:H6 and I4C:H6 was measured at various times after radiolabeling according to the scheme in Fig. 1. Results are shown for T5C:H6 (Fig. 3); similar results were obtained for I4C:H6 (data not shown). A substantial fraction of radiolabeled T5C:H6 was derivatized at the earliest time point, indicating that many of the nascent polypeptides are inserted at the start of the assay (Fig. 3A). Derivatization increased to a plateau of ~98% (Fig. 3B), consistent with maturation of the nascent radiolabeled population.

Several observations suggest that the appearance of derivatized protein at early times is not due to derivatization of nascent BO inside the cell. First, AMS is unlikely to cross the membrane because of its two negatively charged sulfonate groups. It has been used in other systems as a membrane impermeant reagent (42–44). Second, translocation time courses similar to that in Fig. 3B were obtained when the preincubation with AMS was extended by several minutes or when the reagent was added after pulse-chase radiolabeling (data not shown). Finally, different translocation time courses are observed with Cys located in other extracellular regions of the protein, arguing that AMS derivatization reflects Cys translocation.2

Measurement of Elongation Time—To determine whether translocation of Cys residues near the N terminus occurs co- or post-translationally, we measured the BO elongation time by labeling cells with a short pulse of [35S]Met followed by a nonradioactive Met chase. The point at which incorporation of [35S]Met into full-length BO reaches a plateau, corrected for the lag in Met equilibration, reflects the elongation time of the protein. [35S]Met incorporation into T5C:H6 is shown in Fig. 4A; similar results were obtained with I4C:H6 and BO:H6 (data not shown). A plot of the data, normalized for the total number of Met residues in the protein (Fig. 4B, open circles), was fit with a curve that accounts for the distribution of Met in BO

2 H. Dale and M. Krebs, unpublished results.

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**Table I**

| Expression levels and spectral characteristics of wild-type and mutant BR | Absorption maximum |
|---|---|
| | Total cell protein (W T) | BR:H6 | Dark-adapted | Light-adapted |
| Wild type | 3.6 | 569 | 569 |
| BR:H6 | 1.9 (53) | 558 | 569 |
| I4C:H6 | 1.0 (28) | 558 | 568 |
| T5C:H6 | 1.5 (42) | 558 | 568 |

*BR expression levels from 2-day-old cultures were determined by light-dark spectroscopy of crude cell lysates as described (34). Values reported are the average of two experiments.*
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**DISCUSSION**

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We have developed a kinetic assay to monitor the membrane insertion of polytopic proteins and used it to demonstrate that the first transmembrane segment of BO inserts into the *H. salinarum* membrane co-translationally. To our knowledge, the
insertion kinetics of a polytopic membrane protein have not been established in vivo in any other organism. The assay was feasible because the H. salinarum membrane is accessible to the external environment, AMS reacts with translocated Cys very rapidly, and BO translation is sufficiently slow to resolve translocation events. Although H. salinarum is ideal for this approach, it may be possible to apply the method to other prokaryotes in which the cytoplasmic membrane is accessible and translation is slow. Examination of other Cys substitutions in prokaryotes in which the cytoplasmic membrane is accessible may nucleate folding of the remaining transmembrane segments and must be considered in studies of BO folding. This is reasonable, given that H. salinarum SP is implicated in BO biogenesis and that homologs of secretory translocase subunits exist in several archaea, including H. salinarum. Direct studies are required to test whether these cellular factors mediate BO insertion.

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TABLE II

| Translocation time | Elongation time | Elongation rate | Translocated chain length |
|-------------------|----------------|----------------|--------------------------|
|                   | s              | s              | aa/s                     | aa                        |
| BR:H₆             | 46 ± 9         | 114 ± 26       | 2.0 ± 0.1                | 102 ± 4                   |
| I4C:H₆            | 129 ± 5        | 169 ± 16       | 2.3 ± 0.6                | 92 ± 12                   |
| T5C:H₆            | 61 ± 6         | 167 ± 31       | 1.5 ± 0.1                |                           |

* Elongation time and rate were determined as described in the legend to Fig. 4.

Co-translational translocation of the BO N terminus is consistent with both a translocase-dependent mechanism, in which protein factors within the lipid bilayer mediate insertion (10, 11), and a spontaneous mechanism, in which no such factors are involved (51, 52). Although our results do not distinguish between these possibilities, we propose that BO insertion is mediated by SRP and the secretory translocase, as has been demonstrated for eukaryotic and bacterial membrane proteins. This is reasonable, given that H. salinarum SP is implicated in BO biogenesis and that homologs of secretory translocase subunits exist in several archaea (9), including H. salinarum. Direct studies are required to test whether these cellular factors mediate BO insertion.

3 C. M. Angevine and M. P. Krebs, unpublished results.
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