Review–Hypothesis

Extensions of the signal hypothesis – Sequential insertion model versus amphipathic tunnel hypothesis

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Signal hypothesis *Protein translocation* *Membrane insertion* *Amphipathic tunnel hypothesis*

1. INTRODUCTION

It is well established that hydrophobic signal peptides trigger the translocation of secretory proteins across membranes [1,2] (for review see [3]). They are mostly located at the N-terminus of a precursor protein and cleaved off after membrane transfer of the polypeptide. Studies in vitro have shown that the signal peptide is recognized by a signal recognition particle (SRP) as soon as it emerges from the ribosome. Thereby translation is stopped until binding to the SRP receptor in the rough endoplasmic reticulum membrane (docking protein) has taken place and translocation is initiated [4–6]. A similar mechanism may hold for *E. coli*, although the details are less clear (see [7]).

The actual translocation process remains mysterious. According to the signal hypothesis [2] an aqueous tunnel is transiently formed by transmembrane proteins through which the growing nascent polypeptide chain is transported. Direct evidence for a tunnel is lacking as yet. However, recently temperature-sensitive pleiotropic mutants have been found in yeast which have been interpreted in favour of a protein channel [8].

Signal peptides, functionally identical with those of secretory proteins, also trigger the incorporation of many integral membrane proteins [9–11]. Of course, the translocation process has to stop at some point in order to retain a protein in the membrane. Moreover, membrane proteins have very different structures and orientations, so that additional factors besides signal peptide-initiated translocation have to be postulated to explain their biosynthesis.

Here two models are discussed: one, the 'sequential insertion model', is a summary of hypotheses proposed by Blobel [12,12a] and Sabatini et al. [13], and a new one, the 'amphipathic tunnel hypothesis', which is based mainly on thermodynamic considerations. Both models are extensions of the signal hypothesis and are suggested in order to explain in particular the biosynthesis of complex membrane proteins. We shall first explain the models and later discuss discriminating features, some of which are amenable to experimental testing.

2. DESCRIPTION OF THE MODELS

2.1. *The sequential insertion model*

This model extends the signal hypothesis by 2 assumptions:

(i) A second type of topogenic sequences is postulated: stop-transfer peptides [12,12a] (or halt-transfer sequences [13]). These sequences are assumed to trigger a translocation stop, i.e. reverse

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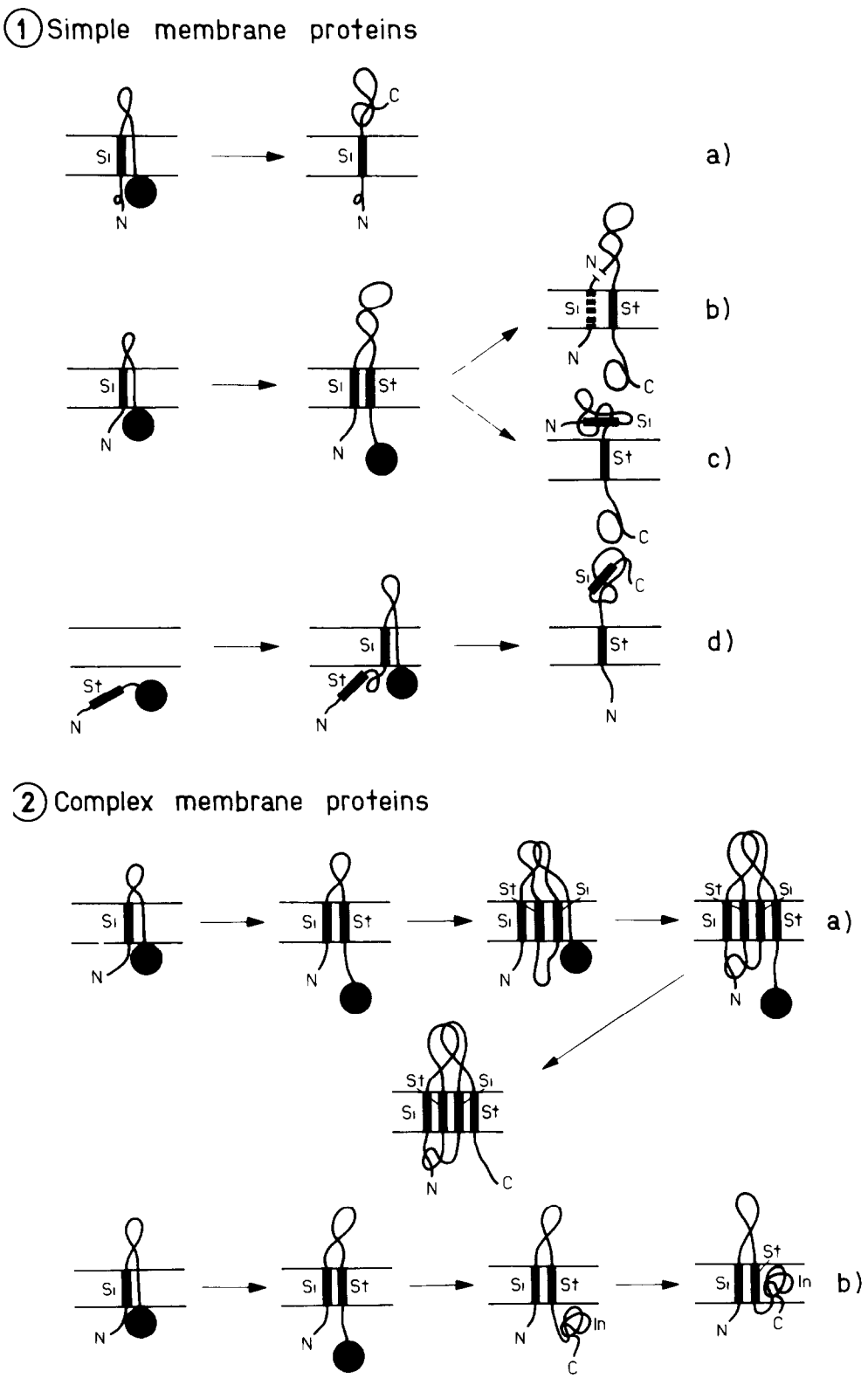
1 Simple membrane proteins
Si
St
N
C
a)
b)
c)
d)
2 Complex membrane proteins
In
a)
b)

the previous function of a signal peptide. A special type of receptor has to be assumed that recognizes this type of sequence.

(ii) It is postulated that more than one signal peptide can be present in a single polypeptide chain [12,13]. Assuming the presence of alternating signal and stop-transfer sequences, the polypeptide would be incorporated in a step-wise manner by repeated initiation and termination of translocation.

In its simplest formulation, the model assumes that each topogenic sequence determines the location of the following, not the preceding part of the nascent polypeptide chain (fig.1). The amino acid sequence C-terminal of a signal peptide would therefore be synthesized by a membrane-bound ribosome and translocated until a stop-transfer peptide emerges from the ribosome. Thereafter the synthesis of the polypeptide chain would continue in the cytoplasm until a second signal peptide redirects the ribosome to the membrane. The membrane-spanning segments would be signal and stop-transfer peptides in an alternating manner [13]. Taking into account that the N-terminal signal peptide need not be cleaved off [14], it could also serve as membrane-spanning segment (see table 1).

In a more sophisticated version of the model, it is assumed that a signal peptide may also transport a polypeptide segment previously synthesized [12,12a]. In this case, a stop-transfer peptide may precede a signal peptide and would identify the 'upstream' stop of translocation (fig.1, case 1d).

Finally, the model may be supplemented by assuming the existence of 'insertion' [12] or 'imbedding' [3] sequences. These allow portions of the polypeptide chain to be spontaneously incorporated into the phospholipid bilayer without mediation of a membrane protein. Such a sequence is found, for example, at the C-terminus of cytochrome b_5 [15]. The original assumption that insertion sequences only trigger the 'unilateral' integration of protein segments into the lipid bilayer [12,12a] can be extended to permit them spanning the membrane. Combining the concept of the signal hypothesis with the possible existence of imbedding domains, one may assume that parts of the polypeptide are incorporated into the membrane by signal and stop-transfer peptides and that the rest is drawn into the bilayer by imbedding independent of a translocation apparatus (fig.1, case 2b).

2.2. *The amphipathic tunnel hypothesis*

Our hypothesis is based on the assumption that translocated proteins fold to the thermodynamically most stable state, as is also assumed for soluble proteins (see [16]). Obviously, the membrane constitutes a great energetic barrier which must be lowered considerably to allow efficient folding. The translocation apparatus may provide the necessary condition by disrupting the membrane structure temporarily and creating a suitable environment. The hypothesis detailed below offers *one* possibility of how the energetic barrier of protein folding could be lowered; other mechanisms of 'catalysis' of protein folding are conceivable.

Our hypothesis differs from the sequential insertion model in that the presence of only one signal peptide is assumed in a translocated polypeptide, regardless of its complexity. No other topogenic sequences, triggering the translocation process, are

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Fig.1. Hypothetical cases of sequential membrane insertion of proteins. The schemes show the cotranslational incorporation of hypothetical polypeptides into membranes. The lower side corresponds to the cytoplasmic side. The filled circles indicate the ribosomes synthesizing membrane proteins. They can be either bound to the membrane or 'dangling' at the nascent polypeptide chain. Si, St and In denote signal, stop-transfer and insertion (imbedding) sequences, respectively. Case 1a: the signal peptide is uncleaved and serves as membrane-spanning segment. In the final state the N-terminus remains in the cytoplasm. Case 1b and 1c: in the final state the protein spans the membrane once with a stop-transfer sequence. The N-terminal signal peptide may be cleaved off (case 1b) or translocated across (case 1c). Case 1d: the stop-transfer peptide precedes the signal peptide. Case 2a: a polytopic membrane protein is inserted into the membrane by alternating signal and stop-transfer peptides. Case 2b: the N-terminal part of a complex membrane protein is inserted by a signal and stop-transfer peptide, the C-terminal part by an insertion (imbedding) sequence (or domain).

postulated. The model is an extension of the signal hypothesis by making more specific assumptions for the properties of the transient tunnel in the membrane. It is assumed that it contains an aqueous environment but that its inner surface has both hydrophilic and hydrophobic areas. Hydrophilic surfaces could be formed by transmembrane proteins or by induced inverted micelles. Hydrophobic surface areas could be provided either by transmembrane proteins or by the hydrocarbon chains of the lipids.

Formation of a tunnel would require energy. This step is assumed to be essentially irreversible. Disruption of the tunnel is assumed to be coupled with termination of protein synthesis and may be triggered by dissociation of the ribosome into subunits when the termination codon on the mRNA has been reached.

The tunnel would provide binding sites for parts of the polypeptide chain transported through it. Hydrophobic parts of sufficient length would have a tendency to be retained at the hydrophobic surface area. Hydrophilic portions would normally not be retained unless a special charge distribution or H-bonding pattern were complementary to the polar parts of the channel. If several parts of a polypeptide chain within the tunnel can interact with each other so as to form a hydrophilic surface, they are able to pass through it without further hindrance. When the protein is completed, any parts of the polypeptide retained within the tunnel during its biosynthesis are tested for compatibility with a hydrophobic environment. If charges cannot be compensated or several H-bonds cannot be formed between H-bond donors and acceptors, the polypeptide segment(s) are expelled

Table 1

Sequences presumed to be membrane-spanning or translocated signal peptides

Protein	Sequence	Reference
Membrane-spanning signal peptides		
Influenza (Lee) neuraminidase	H_2N-MLPST VQTLTLLLTSGGVLLSLYVSASLSYLLYS...	38
Influenza (Victoria) neuraminidase	H_2N-MNPNQKIITIGSVSLTIATICFLMQIAILVTTVT...	39
Influenza (WSN) neuraminidase	H_2N-MNPNQKIITIGSICMVVGIISLILQIGNIISIWI...	39
Cytochrome P450 LM_2	H_2N-MEFSLLLLLAFLAGLLLLLF...	40
Cytochrome P450 PB	H_2N-MEPSILLLLALLVGFLLLLV...	41
Cytochrome P450 b	H_2N-EPTILLLLALLVGFLLLLVRG ...	42
Cytochrome P450 a	H_2N-MLDTGLLLVVILATLTVMLLLTL...	42
Epoxide hydratase	H_2N-MWLELVLASLLGFVIYWFVS .	43
Translocated signal peptides		
Semliki Forest virus p62	H_2N-SAPLITAMCVLANATFPCFQPPCVPCCYENAEA...	44
Sindbis virus p62	H_2N-SAAPLVTAMCLLGNVSFPCDRPPTCYTREPSRA...	45
Ovalbumin	CH_3-CONH-GSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMV YLGAKDSTR...	46
Bovine opsin	H_2N-MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEP...	47

Amino acid residues are given in the one-letter code. Hydrophobic residues are underlined. Asterisks indicate sites of attachment of carbohydrate. Proline residues are emphasized by thick dots. Charged residues are also indicated

from the membrane. Expulsion would occur so as to minimize the energy demand, i.e. the segment would generally be liberated into the aqueous phase where most of the polypeptide chain is already located. If, on the other hand, charge bridges and H-bonds can be formed so that a structure stable in the hydrophobic environment is achieved, the protein will be retained in the mem-

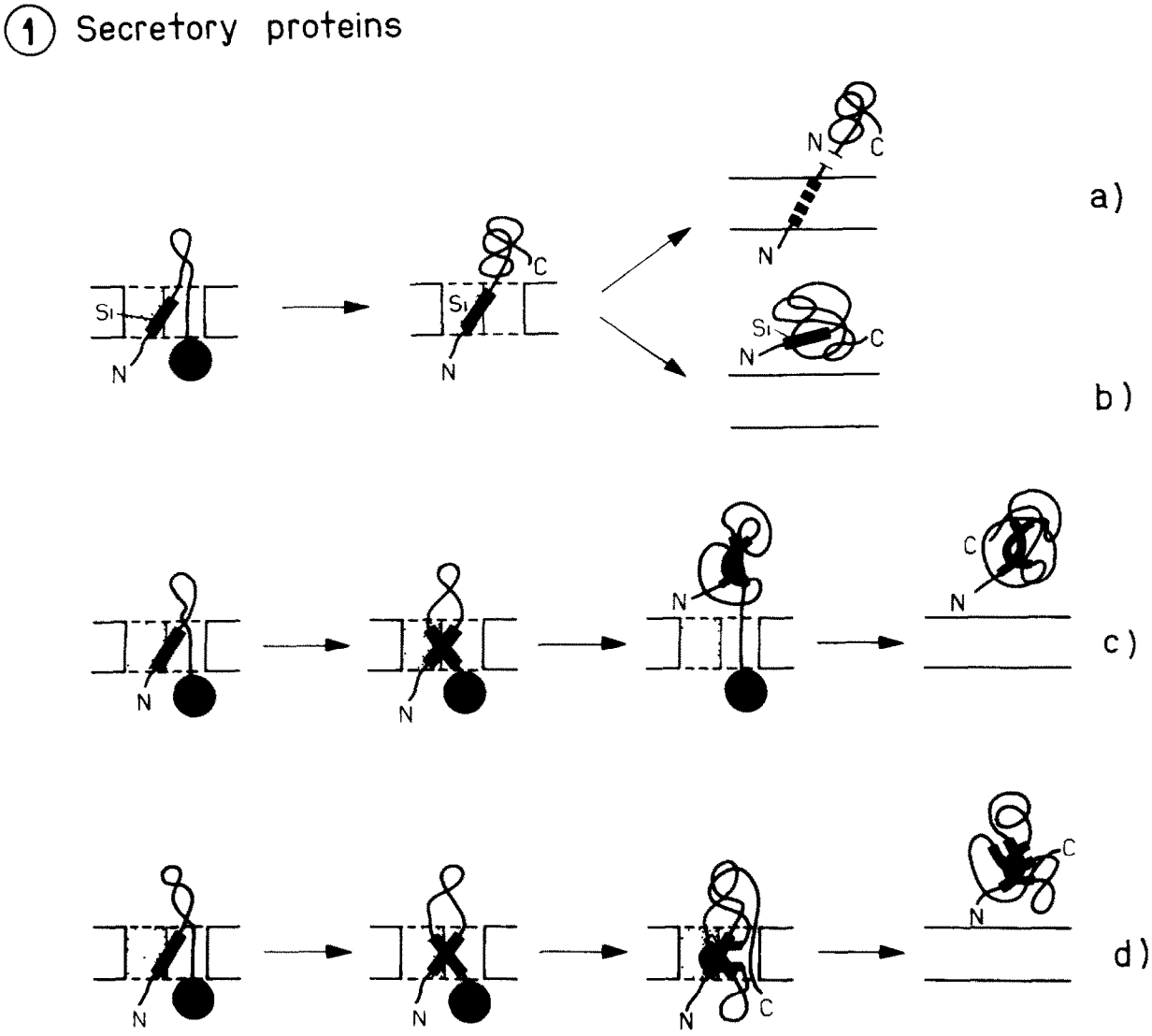

Fig.2. Translocation of proteins across membranes according to the amphipathic tunnel hypothesis: secretory proteins. The schemes show the cotranslational translocation of hypothetical polypeptides. The hydrophobic and hydrophilic parts of the presumed tunnel in the membrane are indicated by dotted and blank areas, respectively. Cylinders indicate portions of the polypeptide chain retained in the tunnel. Hydrophobic and hydrophilic parts of the retained segments are indicated in grey and black, respectively. Si denotes the signal peptide, N and C the NH_2- and COOH-termini, respectively. The filled circles are the ribosomes which remain membrane-bound until completion of the polypeptide chain (cf. fig.1). Case 1a and 1b: the signal peptide is either cleaved off (case 1a) or translocated across the membrane (case 1b) since it is not compatible with membrane insertion (the hydrophobic portion is too short). Case 1c and 1d: the protein contains several segments transiently retained in the tunnel. These parts fold into a hydrophilic globular structure capable of traversing the membrane either before (case 1c) or after (case 1d) completion of the protein.

brane. It should be noted that the constraints on membrane compatibility are greater than for retention in the amphipathic tunnel: a short hydrophobic sequence may be bound to the tunnel but could be expelled from the phospholipid bilayer. The application of the hypothesis to several cases is illustrated in figs 2 and 3 in a schematic way.

Different secretory proteins may differ in their mode of membrane transfer (fig.2). They could be translocated linearly through the membrane (case 1a or b) or even after completion of the protein if

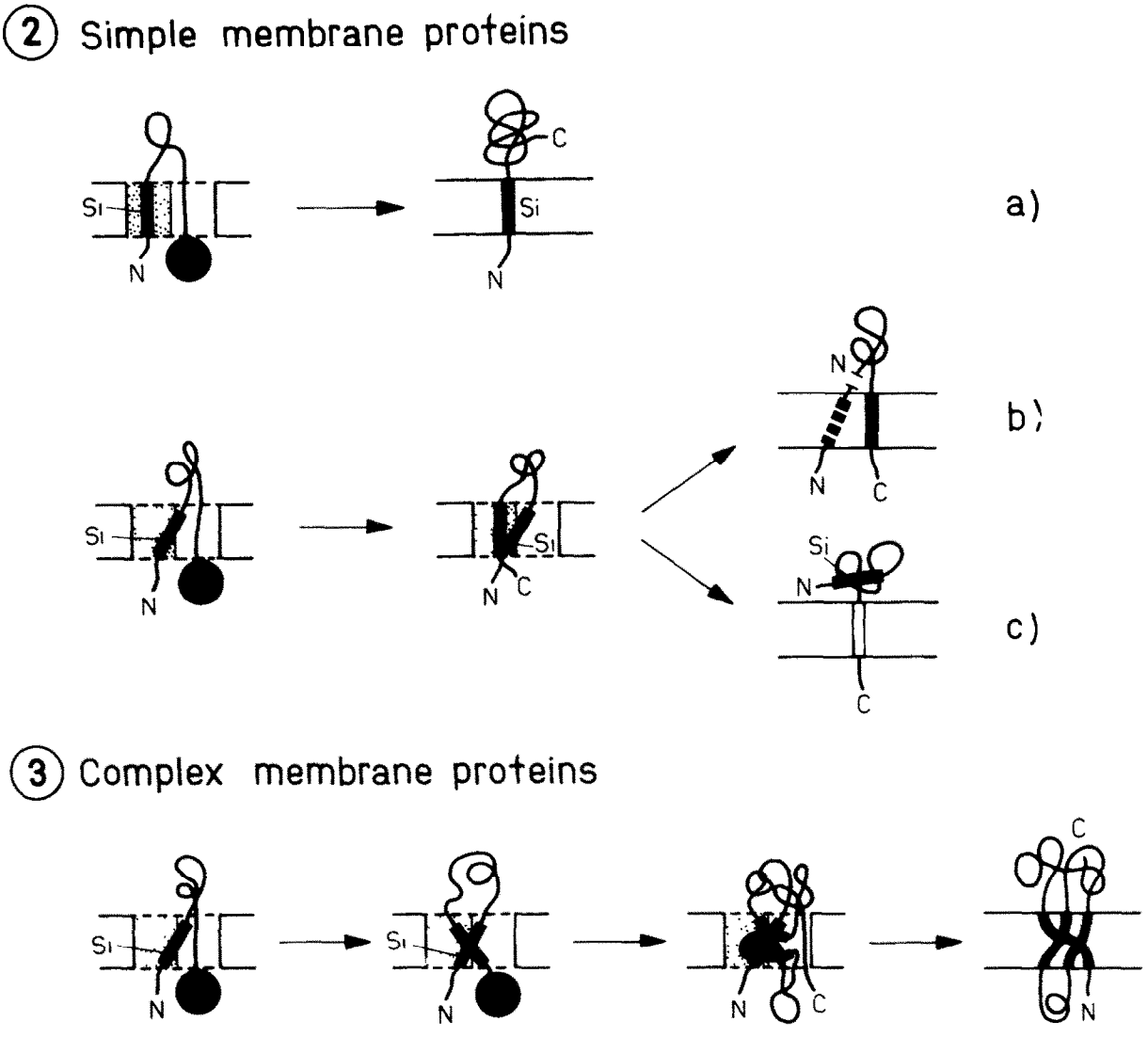

Fig.3. Translocation of proteins across membranes according to the amphipathic tunnel hypothesis: membrane proteins. All symbols are explained in the legend to fig.2. Case 2a: the signal peptide is compatible with membrane insertion (the hydrophobic portion is long enough, cf. fig.2, case 1b) and serves as membrane-spanning segment. Case 2b and 2c: the signal peptide is followed by a second hydrophobic segment retained in the tunnel. The signal peptide can be either cleaved off or translocated across the membrane. In both cases the C-terminus would remain in the cytoplasm. Case 3: one example of a complex membrane protein is considered with several segments retained in the amphipathic tunnel. These parts of the protein could fold to yield a hydrophobic surface compatible with the hydrophobic membrane environment. Note that the incorporated polypeptide segments need not traverse the membrane perpendicularly.

the parts transiently retained in the tunnel are unstable in the phospholipid bilayer (case 1d). It is also evident that the segments of the polypeptide retained in the tunnel could associate in a very complex manner typical for tertiary structure formation, forming either a hydrophilic (fig.2, case 1d) or a hydrophobic (fig.3, case 3) surface.

It should be noted that the amphipathic tunnel hypothesis does not exclude the existence of insertion (imbedding) sequences or domains which allow the direct integration of protein segments into a membrane without involvement of a translocation apparatus. In contrast to the 'membrane-triggered folding hypothesis' [16a] it is, however, assumed that such a mechanism is not responsible for the transfer of large folded polypeptide domains across a membrane or the complex folding of membrane-incorporated protein segments in general.

3. COMPARISON OF THE TWO MODELS

3.1. *Folding pathway similar to soluble proteins or determined by sequential membrane insertion?*

The most discriminating feature of the two models is the way by which a complex membrane protein would achieve its final conformation. According to the sequential insertion model, the folding pathway would be determined by the order of the topogenic sequences. Each membrane-spanning segment of a polypeptide chain or at most two consecutive ones would be incorporated separately into the membrane. This follows from the assumption that the translocation apparatus disassembles after each stop-transfer sequence (see [12]). These intermediates in the biosynthesis and folding of the protein should be stable in the bilayer. On the other hand, according to the amphipathic tunnel hypothesis, folding would only yield a membrane-compatible structure when all sequence parts required for the final conformation have been synthesized. Such a folding pathway is more similar to that generally assumed for globular, soluble proteins (see [16]). Indeed, there is no a priori reason to assume that sequential insertion will result in a structure corresponding to a global energetic minimum. However, from the structural information for bacteriorhodopsin [17] and for the major envelope proteins of proteins of *E. coli* [18] it may be concluded that the final conformation of membrane proteins is thermodynamically determined. All charged residues appear to be oriented away from contact with the lipids whereas the hydrophobic amino acid residues face the hydrophobic environment. Denaturation-renaturation studies, carried out for bacteriorhodopsin, support the conclusion by showing that an entirely different folding pathway leads to the same native structure [19]. Given the fact that shielding of charged residues and saturation of H-bonds are only expected after synthesis of large portions of a polypeptide chain, it appears unlikely that intermediates in the sequential insertion could exhibit a similar energetic compensation. These arguments favour the amphipathic tunnel hypothesis.

According to the sequential insertion model, stop-transfer sequences would be recognized by a specific receptor, whereas according to the alternative hypothesis, thermodynamic circumstances would determine the membrane location of the peptide. A particularly interesting test for the models is provided by translocated proteins carrying uncleaved presumed signal sequences at their N-terminus, which can be either peptides spanning the membrane or translocated across the phospholipid bilayer to the ectoplasmic aqueous phase. As shown in table 1, membrane-spanning segments assumed to be signal peptides consist of at least 18 consecutive, uncharged and mostly hydrophobic amino acid residues. By contrast, translocated signal peptides appear to have shorter uncharged regions or proline residues within them. Furthermore, in some examples of translocated signal peptides, there is a carbohydrate chain attached to a neighbouring amino acid residue. Such features may indeed prevent a stable membrane anchoring of these peptides. One may conclude that a specific receptor need not be invoked. Rather, mere thermodynamic considerations fully explain the behaviour of peptides in translocation.

The amphipathic tunnel hypothesis also provides a clue as to how the hydrophobic fusion peptides of some viral envelope proteins cross the membrane during their biosynthesis (see, e.g., [20,21]). Since these peptides appear to be buried in the interior of the final molecule we suggest that they are transferred when folding of the polypep-

tide chain in the tunnel permits their shielding by hydrophilic residues.

It should be noted that according to our hypothesis only the folding of the polypeptide is thermodynamically determined whereas the orientation (i.e. sidedness) depends on the 'history' of the system. Fixation of the signal peptide in a hairpin structure, as postulated in the loop model [22], would prevent a flip-flop of the nascent polypeptide chain across the membrane (see figs 1–3).

3.2. *Domain transfer or linear extrusion?*

In the sequential insertion model a linear extrusion of the polypeptide chain is assumed which is strictly coupled to chain elongation, whereas according to the amphipathic tunnel hypothesis, folding *could* occur during translocation or even be required for it. Whereas translocation across the rough endoplasmic reticulum membrane is believed to proceed indeed linearly (for discussion see [23]), for *E. coli* evidence exists that entire polypeptide domains are translocated at once across the membrane [24]. For a number of exported proteins a post-translational mode of transfer has been demonstrated (see [3]). These data are difficult to reconcile with the sequential insertion model.

It should be noted that our model suggests the cotranslational opening of a tunnel, even if the protein is transferred only after its completion. This assumption could explain the apparently contradictory observations that translocation and synthesis of exported proteins appear to be coupled in *E. coli* [25,26] (see also [7]) whereas translocation can occur post-translationally. In some cases translocation would have to occur by necessity *after* synthesis of the 'stop-transfer' sequence postulated by the sequential insertion model. For example, membrane-bound immunoglobulin M has only 3 cytoplasmically located amino acid residues at its C-terminus following the membrane anchor [11]. Since about 20–40 amino acid residues are buried within the ribosome [27,28], the membrane anchor must be inserted into the membrane after termination of protein chain synthesis. Such a post-translational positioning of the polypeptide chain would be fully compatible with the amphipathic tunnel hypothesis.

It has been shown that amino acid residues in the mature parts of translocated proteins modulate the efficiency of export (see [7]). For example, such an 'enhancer' sequence has been identified between residues 89 and 189 of the mature maltose-binding protein [7]. Such findings are, of course, in agreement with the expectations of the amphipathic tunnel hypothesis.

3.3. *More than one topogenic sequence in a polypeptide chain?*

The existence of only one signal peptide in even complex membrane protein, as postulated in the amphipathic tunnel hypothesis, fits with the occurrence of cleaved-off peptides in such cases. The major envelope proteins of *E. coli*, ompA, ompF and lamB possess cleaved signal peptides with typical structures (for compilation see [29]). Bacteriorhodopsin also has a cleaved-off peptide which, although not particularly hydrophobic, may be the signal peptide in the halobacterium [30]. There is no reason to believe that further signal peptides are required for membrane insertion. Indeed, for the lamB protein it is clear that mutation of the cleaved-off peptide converts the protein into a cytoplasmic one [31]. If there were additional signal peptides, one would expect a protein partially incorporated into the membrane.

According to the sequential insertion model, at least some of the 7 helices of bacteriorhodopsin which traverse the membrane should be alternating signal and stop-transfer peptides. Functional differentiation between the helices is, however, difficult to justify by their primary structures.

Another example is the acetylcholine receptor consisting of homologous subunits which are incorporated with their C-termini in the membrane in a complex manner [31a]. Among the 5 peptides presumably traversing the membrane, one appears to form an amphipathic helix which apparently is only stabilized by interaction with other parts of the protein. Again, differentiation between signal and stop-transfer sequences appears unjustified.

Some major envelope proteins of *E. coli* do not show any long hydrophobic or uncharged segment which could span the membrane and serve as internal signal sequence. For example, both the ompF and lamB proteins contain many charged residues and the length of uncharged segments is less than 13 residues (see [32]). It may also be doubted that the polypeptide traverses the membrane perpendicularly; structures involving complex folding

may be expected. Indeed, both proteins contain a high percentage of β-structure, indicating H-bonding between distant polypeptide segments.

How could one distinguish experimentally between the sequential insertion and the amphipathic tunnel hypothesis? According to the latter hypothesis, SRP would be needed only once whereas according to the alternative model, it would be required as many times as there are signal peptides in a polypeptide chain. The dependence on SRP may be testable in synchronized translation experiments, where SRP is added or the action of SRP is blocked after varying time periods. Read-out translation with polysomes isolated from intact cells may also be tried to see if SRP is needed *after* initiation of translocation for incorporation of complex membrane proteins. The cycling of ribosomes between a free and a membrane-bound state during the synthesis of a membrane protein, postulated in the sequential insertion model, could also be tested experimentally. A protein factor, apparently required for ribosome detachment from the rough endoplasmic reticulum membrane [33], may be a valuable tool for the analysis of the possible cycling.

3.4. *Generalization of protein translocation across membranes*

How do the two models apply to other cases of protein transport across membranes, such as across mitochondrial [34], chloroplast [35] or peroxisomal [36] membranes, or the transfer of toxins into cells [37]? In all these cases, the transport of the polypeptides occurs post-translationally. Generally the proteins appear to carry signal peptides or perhaps signal domains which are recognized by receptors in the target membranes. It is obvious that the sequential insertion model meets with difficulties in dealing with post-translational translocation, whereas the amphipathic tunnel hypothesis would be easily applicable. The signal peptide (or domain) triggered transient formation of an amphipathic tunnel could be the basis for protein translocation across and insertion into membranes in general.

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