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Thermodynamics, the Structure of Integral Membrane Proteins, and Transport

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Membranes are structures whose lipid and protein components are at, or close to, equilibrium in the plane of the membrane, but are not at equilibrium across the membrane. The thermodynamic tendency of ionic and highly polar molecules to be in contact with water rather than with nonpolar media (hydrophilic interactions) is important in determining these equilibrium and nonequilibrium states. In this paper, we speculate about the structures and orientations of integral proteins in a membrane, and about how the equilibrium and nonequilibrium features of such structures and orientations might be influenced by the special mechanisms of biosynthesis, processing, and membrane insertion of these proteins. The relevance of these speculations to the mechanisms of the translocation event in membrane transport is discussed, and specific protein models of transport that have been proposed are analyzed.

Key words: peripheral and integral proteins, membrane biosynthesis, hydrophobic and hydrophilic interactions

It has become clear in recent years that over long distances in the plane of the membrane each membrane surface is close to equilibrium with the aqueous phase bathing it, whereas over the much shorter distance across the membrane the 2 surfaces are normally far from equilibrium. The near equilibrium state in the plane of the membrane is reflected in part by the rapid intermixing of components that can occur over the entire membrane surface (8, 34). By contrast, the nonequilibrium state across the membrane is reflected in the normally very slow or negligible rates of mixing of membrane components from one surface to the other (see below). This nonequilibrium state is not simply due to the fact that the 2 aqueous compartments that are separated by the membrane of a living cell are themselves not in equilibrium with one another, but is rather an intrinsic property of the membrane-water system itself, because the asymmetry of the membrane persists when the cell is lyzed and the membranes are isolated.

Some years ago, we discussed semiquantitatively some of the thermodynamic factors influencing membrane structure (29). In particular, the important roles played by hydrophobic and hydrophilic interactions were stressed. Hydrophobic interactions have become

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well-appreciated in molecular biology since the classic paper by Kauzmann (15), and reflect, crudely speaking, the thermodynamic tendency of nonpolar structures to sequester themselves from contact with water. Hydrophilic interactions have received less attention. As a class of interactions, they are responsible for the strong thermodynamic tendency of highly polar and ionic structures to remain in contact with water if given the choice between an aqueous and a nonpolar environment.

The near equilibrium and nonequilibrium features of membrane structure discussed above follow from these hydrophobic and hydrophilic interactions. The well-known bilayer structure of phospholipids in membranes, for example, is one result of these interactions. At equilibrium the nonpolar fatty acyl chains are sequestered from contact with water, thereby maximizing hydrophobic interactions, while the polar head groups are exposed to the aqueous phase, maximizing hydrophilic interactions. On the other hand the very slow rates of trans-bilayer rotations (flip rates) of phospholipids [with an estimated minimum half-time of 80 days in synthetic bilayer vesicles (24)], very likely reflect hydrophilic interactions, namely, the large free energy of activation required to transfer the polar headgroup of a phospholipid molecule through the nonpolar interior of the membrane (29–31).

The structures of membrane proteins must also result from the interplay of these thermodynamic factors. Lenard and I (18) and Wallach and Zahler (37) suggested independently that membrane proteins [or, rather, those that we now call integral proteins (29)] would generally exhibit amphipathic molecular structures, with predominantly hydrophilic segments (containing all the ionic and highly polar residues, such as saccharides) exposed to the aqueous phase, and predominantly hydrophobic segments embedded in the membrane interior. At the time that this proposal was made, no integral membrane proteins had been studied in sufficient structural detail to test it, but since then the existence of a large number of such amphipathic structures has been demonstrated.

We further suggested (31) that integral membrane proteins would exhibit negligibly slow trans-membrane rotations, again because of the very large free energies of activation involved in moving their hydrophilic segments across the nonpolar membrane interior. This accords with the facts: so far as is known, each integral protein is present in a unique orientation in its membrane. This orientation is most likely not an equilibrium condition, but rather must reflect the specific mechanisms by which the integral proteins are inserted into the membrane during or after biosynthesis, as discussed below.

It seems appropriate, now that the crude first approximation suggestions about the structure of integral proteins have been borne out, and a considerable number of such proteins is currently under investigation, to examine in somewhat greater detail than previously what factors might influence the structures of integral proteins, in particular what might be the equilibrium and nonequilibrium effects on such structures, and how these structures bear on the problem of membrane transport. Some of the ideas about membrane protein biosynthesis discussed in this paper have been recently put forward by others as well (25, 27).

THE STRUCTURES OF INTEGRAL PROTEINS

At this early stage in our knowledge of the structures of integral proteins, at least 4 classes of such structures have to be considered. These are depicted in Fig. 1.

In what follows, we will emphasize the biosynthetic sites of these proteins with respect to the membrane. Reference to the 2 faces of the generalized membrane in Fig. 1 as cis and trans reflects this emphasis. Thus, in single membrane procaryotes, the cyto-

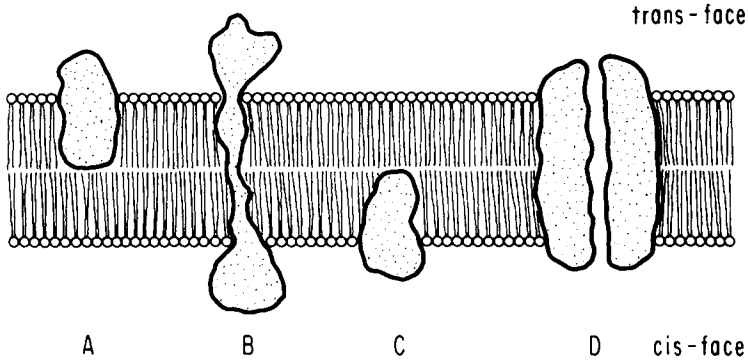

Fig. 1. At least 4 classes of membrane integral proteins need to be considered, depicted schematically in this figure. The cis and trans faces are defined in the text, the cis side being related to that surface to which membrane-bound ribosomes are attached. A and C represent proteins that are only part-way embedded in the bilayer, from the trans and cis sides, respectively. Whether proteins of type A actually exist, however, is not clear. Proteins of type B are trans-membrane proteins with hydrophilic segments protruding from both membrane faces, and a hydrophobic segment in between which is embedded in the hydrophobic interior of the bilayer. Transport proteins, it is suggested, are all type D, subunit aggregates with aqueous channels running down the axis of the aggregate. See text for further details.

plasmic face of the plasma membrane is the cis face, the one to which membrane-bound ribosomes become attached. For the plasma membranes of eucaryotic cells, however, the corresponding cis face is that facing outside, the cytoplasmic surface being the trans face. This is because protein biosynthesis on membrane-bound ribosomes occurs in the endoplasmic reticulum of eucaryotic cells; new plasma membrane most likely arises by a process of vesiculation of the reticulum and fusion of the vesicles with already existing plasma membrane. This process results in an inversion of the surfaces of the vesicle and plasma membranes (22, 12). It is therefore more useful to define the membrane face in terms of biosynthetic origins rather than final membrane orientation.

Returning to the protein structures of Fig. 1, cytochrome b_5 of endoplasmic reticulum is a well-known example of type 1C (32). It has one hydrophilic domain containing the NH_2 terminus, protruding from the cis face of the membrane, and a hydrophobic domain, containing the COOH terminus, embedded in the membrane. A distinctive feature of such a type 1C protein is its spontaneous binding to lipid bilayers and membranes (33) suggesting the absence of a significant second hydrophilic domain attached to the hydrophobic one, as in type 1B. On the other hand, it is not really known how deeply the hydrophobic domain of cytochrome b_5 is embedded in the membrane. It is certainly large enough to span the thickness of the membrane, but probably does not.

Glycophorin is the current paradigm for proteins of type 1B, where a single linear hydrophobic portion of the polypeptide chain is embedded within the membrane, connecting 2 hydrophilic domains exposed on either side (35). It is of interest that the NH_2 terminus of glycophorin is exposed at the trans face of the membrane, and the COOH terminus at the cis face. This feature is discussed in the following section.

The integral proteins depicted in Fig. 1A and C appear to be closely similar in structure. They are considered to be distinct for our purposes, however, since the former

protrudes from the membrane surface opposite to the side where protein biosynthesis occurs (the trans face), whereas the latter is located on the cis face. Bretscher (6) suggested that integral proteins of the type shown in Fig. 1A do not exist, i.e., that integral proteins which project a hydrophilic segment from the trans face of a membrane must all be proteins that span the membrane (type 1B). This suggestion was based on the dispositions of only the 2 major polypeptides of the human erythrocyte membrane. I think, however, that it is premature at this time to exclude the existence of membrane proteins of type 1A. It is difficult to obtain positive evidence for their existence; one can only infer this from a relatively loose hydrophobic association of the protein with the membrane (a criterion which does not discriminate it clearly from a peripheral protein) and from the inability to label the protein from the cis-face side. On such grounds, the IgM-like receptor on B lymphocytes is a good candidate for a type IA protein (36).[1]

Integral proteins of type 1D have not been widely discussed as yet. On thermodynamic grounds, we proposed (29, 30) that all proteins involved in specific transport through membranes are of this type, unaware that for other reasons Jardetzky (14) had made a similar proposal earlier. Proteins of type 1D are molecular aggregates of some small number (2, 3, or 4) of identical or similar subunits which span the membrane. Such aggregates would have (two-, three-, or fourfold) rotation axes perpendicular to the plane of the membrane, i.e., each chain of the aggregate would have the same orientation in the membrane so that the aggregate was asymmetrically positioned in the membrane.

An important feature of such aggregates is that they may generate a narrow water-filled channel down the central axis of the aggregate. The surfaces of the polypeptide chains lining the channel could contain ionic and polar groups, because they could interact with the water in the channel and thereby satisfy their hydrophilic interactions.

Structures similar to type 1D are very common among soluble proteins (20). Of those soluble aggregates with only a single rotation axis, dimers are the most prevalent kind. Cytoplasmic malate dehydrogenase is an example of such an homotropic dimer; its x-ray crystallographic structure is known to a resolution of 3.0 Å (11). A water-filled channel traverses the entire length of the molecule down the twofold axis. In principle, the only structural change required to convert cytoplasmic malate dehydrogenase into a type 1D integral membrane protein is to produce an hydrophobic outer surface on the aggregate where it would come in contact with the hydrophobic interior of the membrane.

At the time this proposal was first made, no integral membrane proteins with such a structure were known. Since then evidence has been obtained with 2 transport proteins that is at least consistent with their having type ID structures: the Na^+, K^+-ATPase (17), and the erythrocyte band 3 anion transport protein (38). In each case, chemical cross-linking and other studies have shown that these 2 proteins are both noncovalently bound homotropic dimers within the membrane; and each monomer chain spans the membrane since it can be labeled from both sides of the membrane (26, 5). What is not yet known is whether these dimers have a continuous aqueous channel down their twofold rotation axes, and whether their respective specific ion binding sites are located within the channel.

In addition to these 2 proteins bacteriorhodopsin has been shown to exist as a trimeric aggregate in the purple membrane (10), and functions as a H^+ ion transport protein (19). However, such proteins may be structurally different from other transport

[1] Parenthetically, one reason that it is very important to know whether type IA proteins do in fact exist is that if they do not, then all integral proteins exposed at the trans face, being trans-membrane, are at least potentially capable of direct linkage to cytoskeletal proteins (3).

proteins, because H^+ ion transport need not occur by way of a specific binding site within an aqueous channel; it could conceivably occur by transfer of the proton along a chain of different proton-accepting groups within a relatively hydrophobic matrix.

Another important structural feature of integral proteins is the number of trans-membrane folds made by a polypeptide chain that is of type IB or ID. A single bacteriorhodopsin molecule, for example, has about 80% of its chain folded into 7 helical segments spanning the membrane. It is not known how these segments are interconnected, nor what is the disposition of all of the remaining 20% of the chain, but if the helical segments are connected by hydrophilic bends in the chain, then the insertion of such molecules into the membrane raises thermodynamic problems.

THE MECHANISM OF TRANSLOCATION IN TRANSPORT

A few years ago, the "rotating carrier" mechanism for the translocation event in transport was popular. In this scheme, a transport protein translocated its ligand-bound active site from one membrane surface to the other by rotating about an axis parallel to the plane of the membrane. For thermodynamic reasons, however, we considered that such protein rotations or trans-membrane diffusions were unlikely. Furthermore, several investigators in recent years have shown (16, 13, 7) that the attachment of large proteins (antibodies or lectins) to transport proteins in intact membranes does not alter their transport or transport-coupled enzyme activities, a result that is very difficult to reconcile with a rotating carrier mechanism.

Jardetzky (14) and I (29, 30) have suggested an alternative mechanism. Specific transport proteins are all proposed to be type ID proteins, capable of existing in at least 2 conformationally distinct states each of which retains the same basic orientation in the membrane. In one state, however, the ligand-binding site is accessible to the aqueous phase on one side of the membrane; in the other state, the site is accessible to the other side (Fig. 2). The affinity of the site for the ligand is different in the 2 states. Conversion from one state to the other involves an energy-requiring rearrangement of the subunits, driven by the concentration gradient of the ligand in the case of facilitated diffusion, or by some other energy source (ATP hydrolysis, membrane potential, etc.) in the case of active transport.

With a homotropic dimer of type ID, for example, 2 ligand binding sites would be present, related by the twofold rotation axis. These sites might exhibit cooperativity in binding and transport of either the positive or negative type (28).

With minor modifications, the same basic mechanism could be extended to account for exchange diffusion and for group translocation types of transport.

An important feature of a subunit aggregate is that it is a structure which allows large changes in the spatial arrangement of the aggregate to occur with a relatively small input of energy. This allows an active site within the channel to be exposed alternatively to 2 different aqueous compartments bathing the membrane without much change in the disposition of the site in the direction perpendicular to the membrane (Fig. 2).

The role of the periplasmic binding proteins in a large number of cases of bacterial transport has been mystifying. On the one hand, the evidence is very extensive that they are critically involved in their respective transport processes. On the other hand, their localization in the periplasmic space and their solubility properties have been difficult to reconcile with a membrane-mediated role in transport. The possibility that they shuttled back and forth across the inner bacterial membrane was often considered in the past. In

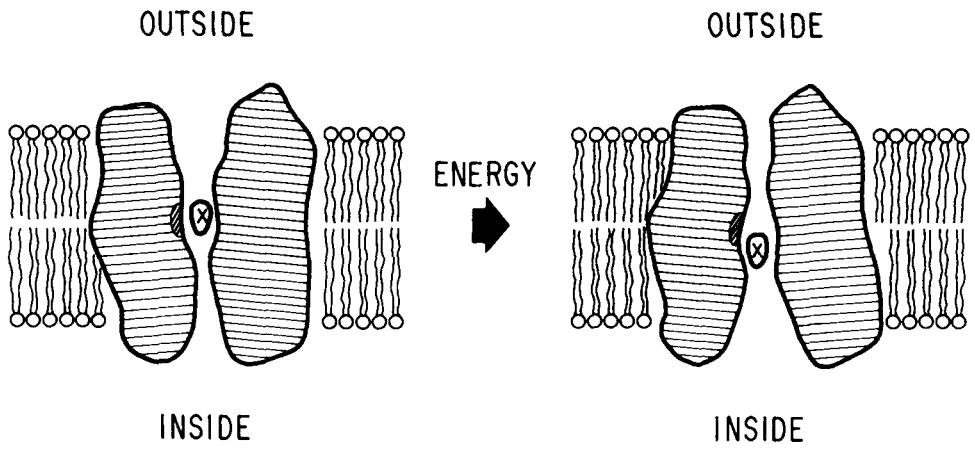

Fig. 2. The aggregate rearrangement mechanism for the translocation event in active transport. See text for details. [Reprinted from (30), with permission.]

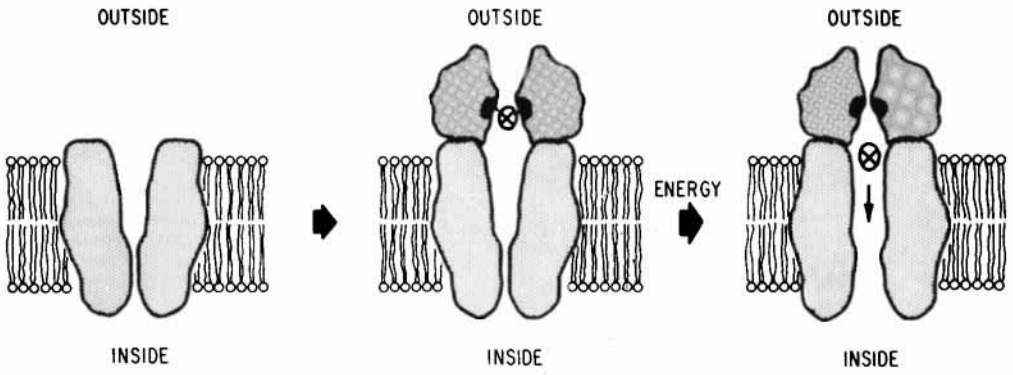

Fig. 3. A schematic diagram of a proposed mechanism for the involvement of periplasmic binding proteins in transport. The binding protein (shaded), with an active site for the ligand X, is considered to be a peripheral protein attached to a so-called portal protein (stippled) which is an integral protein of type D, Fig. 1. The mechanism of translocation of X is then projected to be similar to that depicted in Fig. 2. [Reprinted from (30), with permission.]

view of the thermodynamic considerations mentioned earlier, and the model of transport represented in Fig. 2, we suggested (30) a general mechanism for their action, depicted in Fig. 3. The significant features of this mechanism are: a) the periplasmic binding proteins are proposed to be peripheral proteins (29) which, when functional in transport, are attached to the trans face of the inner membrane; b) their attachment is (noncovalently) to exposed sites on specific integral proteins (which might be called portal proteins) spanning the membrane; c) these putative portal proteins exist as subunit aggregates in the membrane, forming channels much like those in Fig. 2, except that they do not possess the active sites to bind the transported ligand. These active sites are on the periplasmic binding

protein; and d) the mechanism of translocation of the ligand across the membrane during transport involves a coordinated subunit rearrangement of the periplasmic and portal proteins much like that depicted in Fig. 2.

At the time we first considered this proposal, we sought for evidence in the literature to support it. We came upon the elegant studies of Ames and Lever (1) which showed that the high-affinity histidine transport system in S. typhimurium required the products of 2 genes in order to function: one gene, his J, coded for a periplasmic binding protein; the other, his P, coded for an unknown product. We suggested that the his P gene product was the portal protein. Although the his P gene product has not yet been identified, and it is not known whether it is an integral protein of the membrane, the suggestion that the his J-his P protein interaction is a peripheral-intergral protein interaction has been adopted by Ames and Spudich (2), and important evidence consistent with this suggestion has been adduced by these authors. There is also preliminary evidence that such a system of 2 components may function in other cases involving periplasmic binding proteins (Hogg, this volume, p 411 [MAMT, p 273]).

While the basic scheme of translocation is the same for the 2 types of transport systems depicted in Figs. 2 and 3, the advantage of the latter is that, in principle, several different periplasmic binding proteins could use the same portal protein channel, as required; quite possibly, even other types of periplasmic proteins, such as those involved in chemotaxis (9), can use the same portal proteins.

It should be obvious that the schemes depicted in Figs. 2 and 3 were originally meant to convey general features of the translocation mechanisms proposed. When they were put forward, it was hoped that they would provide a point of view about how these systems work, rather than precise structural prescriptions of the mechanisms. The x-ray crystallographic studies of Hogg and Quiocho (this volume) have shown, for example, that the single chain of the arabinose binding protein is itself a pseudo-dimer: it has 2 similar but not identical domains related by a pseudo twofold axis of rotation, with an aqueous channel down the twofold axis. Only one of the domains has a sugar-binding site, which is located on the face of the channel. Clearly, such a single pseudo-dimer molecule is structurally closely analogous to the true dimer of periplasmic binding protein depicted in Fig. 3. The aqueous channel of a single arabinose binding protein, with its single active site, might then occupy the position of the aqueous channel of the dimer of periplasmic protein shown in that figure. The important features of the mechanism proposed in Fig. 3 are essentially unaltered by such a substitution. Many more specific changes can be accommodated into the mechanism as new information is acquired, assuming that the mechanism is basically correct.

THE BIOSYNTHESIS OF INTEGRAL PROTEINS

The biosynthesis of membrane proteins is a subject of intense speculation and little experimental information at present. Our purpose in discussing it is to stress our opinion that the insertion of at least certain types of integral proteins in membranes cannot occur spontaneously, and must therefore occur by suitable mechanisms, and that, therefore, the biosyntheses and the structures of integral proteins are intimately connected to one another.

The propositions of this section are that: i) membranes are not made de novo, but grow by the synthesis of lipids, and the insertion of integral proteins, within preexisting membranes acting as templates; ii) all integral proteins are originally synthesized on

membrane-bound ribosomes; iii) various forms of posttranslational processing of integral proteins may occur; and iv) the final structures of integral proteins in the membrane, in particular whether they are equilibrium or nonequilibrium structures, reflect these biosynthetic and processing events.

It is not our object here to try to document these propositions fully. The first 2 are discussed in the interesting review article by Sabatini and Kreibich (1976). The second is at present a matter of controversy, some of which arises from a failure to discriminate between peripheral and integral proteins of membranes. Peripheral proteins are indeed very likely made on free ribosomes, as are cytoplasmic proteins. Our own indirect evidence regarding the site of biosynthesis of cytochrome b_5 is briefly discussed below.

It is our conviction on thermodynamic grounds that proteins of type IA and IB, with hydrophilic segments protruding from the trans face of the membrane, cannot find their proper orientation in the membrane by a spontaneous process (i.e., they are not equilibrium structures). It has been proposed (4, 27) that type IB proteins are synthesized and inserted into membranes by a mechanism analogous to the synthesis and disposition of secretory proteins. It is proposed that at the NH_2 terminus of the polypeptide chain, as it is translated off the messenger RNA of the membrane-bound ribosome, there is a short segment of a hydrophobic peptide; this so-called signal peptide (4) directs the nascent polypeptide chain into the lipid interior of the attached endoplasmic reticulum membrane. After passage of the signal peptide through the lipid, a hydrophilic segment of the chain sequence is translated and passes through the membrane via a newly-generated hydrophilic protein channel in the membrane, and the signal peptide is removed by proteolysis. If the protein is a secretory protein, the entire sequence is ultimately transferred across the membrane in this fashion, to fold up in its native soluble conformation in the cisternal space on the trans side of the membrane. If the protein is an integral membrane protein of type IB, however, the first hydrophilic part of the sequence, after traversing the membrane, is followed by a hydrophobic sequence which, it is presumed, remains embedded in the lipid interior of the membrane. The next hydrophilic sequence, after synthesis, is then retained on the cis side of the membrane. The first and second hydrophilic segments, after release of the nascent chain, then fold up independently on their respective sides of the membrane.

Proteins of type IA (if they exist) might be synthesized similarly and either i) have as their COOH termini the hydrophobic segment that followed, in sequence, the signal peptide and the hydrophilic segment of the chain; or ii) be secreted entirely into the cisternal space, to become attached at a later stage by some suitable hydrophobic processing (see below). If the former occurred, the NH_2 terminus would be exposed at the trans face, but the COOH terminus would be embedded in the membrane interior; if the latter occurred, the position along the chain where the hydrophobic processing happened would determine the orientation of the chain termini.

Cytochrome b_5, as mentioned above, is considered to be an integral protein of type IC. It has been suggested that such proteins are synthesized on free ribosomes and are then detached into the cytoplasm, to find their way to the cis-face side of their appropriate membrane (6). If this were so, however, what would be the explanation of the finding that cytochrome b_5 is present on the cis side of the endoplasmic reticulum, and not, for example, on the cis side of the plasma membrane to which it would also have access (23)? José Remacle and I, in unpublished studies, have shown by ferritin-antibody labeling experiments that, in vitro, purified cytochrome b_5 can indeed attach spontaneously to the cis side of plasma membrane fragments of liver cells. This argues that the absence of cytochrome b_5 from the plasma membrane in vivo is not due to some thermodynamic barrier

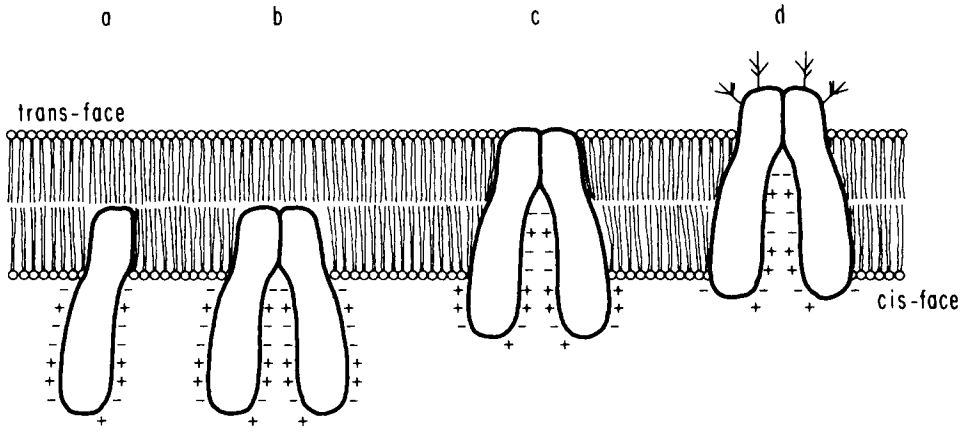

Fig. 4. A hypothetical process for the generation of a protein of type D, Fig. 1, starting from a protein of type C, Fig. 1 (a), which dimerizes to form an extended aqueous channel exterior to the membrane (b), and which after hydrophobic processing of its exposed cis-face hydrophilic surfaces, is embedded more deeply into the membrane (c), and finally, after hydrophilic processing on the trans-face side, becomes a trans-membrane aggregate (d). See text for further details.

to binding, but rather suggests that cytochrome b_5 is directed to the endoplasmic reticulum membrane because it is synthesized on ribosomes attached to that membrane.

It is of interest that the NH_2 terminus of the cytochrome b_2 molecule is part of the hydrophilic segment exposed on the cis side of the membrane. This suggests that if the molecule is made on membrane-bound ribosomes, the hydrophilic NH_2-terminal segment never enters the membrane but remains in the aqueous phase on the cis side of the membrane during translation of the nascent chain. Only after the hydrophobic COOH-terminal segment is synthesized and released, does that portion of the molecule insert spontaneously into the lipid bilayer. It is of great importance to know whether these chain termini positions are generally found with all type IC proteins. If so, then the different positions in the membrane that are taken up by type IB and IA proteins, on the one hand, and type IC proteins, on the other, may simply depend upon whether they are, or are not, initiated by a signal peptide on the NH_2 terminus of their nascent chains.

Of primary interest in connection with transport is the biosynthesis of proteins of type ID, for reasons given above. The monomer subunits of such proteins are presumed to have hydrophilic residues lining the aqueous channels that are formed by their oligomers. It is thermodynamically unreasonable for such trans-membrane monomers to be inserted individually into the membrane because the channel hydrophilic residues would then be in contact with the lipid interior of the membrane. It seems likely, therefore, that type ID and IB proteins, although both trans membrane, are synthesized and inserted into membranes by entirely different mechanisms.

One can simplify matters somewhat by proposing that the synthesis of type ID and IC proteins is related. Two monomers of a type IC protein, after they were individually synthesized and were bound to the cis side of the membrane (Fig. 4a), might spontaneously dimerize (Fig. 4b). The dimer might form an extended aqueous channel that was initially entirely exterior to the membrane. Specific processing or modification of the dimer might then occur that displaced the dimer more deeply into the membrane (Fig. 4c), and even-

tually caused it to protrude from the other side (Fig. 4d). As a consequence, the aqueous channel in the dimer might finally extend entirely across the membrane.

We are already familiar with certain specific posttranslational processing of membrane proteins, such as glycosylation by membrane-bound glycosyl transferases. As the highly polar oligosaccharides of membrane glycoproteins are found exclusively on the trans face of eukaryotic cell membranes (21), these residues must be attached only after the protein has spanned the membrane and become exposed at the trans face. Glycosylation of suitable residues of a protein close to the trans-side water-membrane interface may serve to "pull" a larger volume fraction of the protein through the membrane into the aqueous phase on the trans side. Other processing reactions of the protein on the cis face, such as the acylation of polar and ionic residues situated close to the water-membrane interface, or the formation of nonionic amide linkages between a glutaminyl-residue and an ionic ϵ-NH_3^+ of a lysyl residue (transpeptidation), to mention just a few possibilities, may likewise serve to "push" deeper into the membrane those regions of an integral protein that were exposed on the cis side of the membrane. The sequential operation of such cis side and then trans side processing reactions could therefore result in the proper positioning and conformation of a type ID protein in the membrane (Fig. 4).

It is not intended that these fanciful speculations about biosynthesis be taken very seriously in their details. They are presented primarily to illustrate the problems that arise when thermodynamic principles are applied to integral membrane proteins and to emphasize the likely connection between the biosynthesis and structures of these proteins. It is certain that there are still many surprises ahead of us as the experimental analysis of these proteins continues to develop.

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