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Charge Clusters and the Orientation of Membrane Proteins

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Summary. Although hydrophobic forces probably dominate in determining whether or not a protein will insert into a membrane, recent studies in our laboratory suggest that electrostatic forces may influence the final orientation of the inserted protein. A negatively charged hepatic receptor protein was found to respond to *trans*-positive membrane potentials as though "electrophoresing" into the bilayer. In the presence of ligand, the protein appeared to cross the membrane and expose binding sites on the opposite side. Similarly, a positively charged portion of the peptide melittin crosses a lipid membrane reversibly in response to a *trans*-negative potential. These findings, and others by Date and co-workers, have led us to postulate that transmembrane proteins would have hydrophobic transmembrane segments bracketed by positively charged residues on the cytoplasmic side and negatively charged residues on the extra-cytoplasmic side. In the thermodynamic sense, these asymmetrically placed charge clusters would create a compelling preference for correct orientation of the protein, given the inside-negative potential of most or all cells. This prediction is borne out by examination of the few transmembrane proteins (glycophorin, M13 coat protein, H-$2K^b$, HLA-A2, HLA-B7, and mouse Ig μ heavy chain) for which we have sufficient information on both sequence and orientation.

In addition to the usual diffusion and pump potentials measurable with electrodes, the "microscopic" membrane potential reflects surface charge effects. Asymmetries in surface charge arising from either ionic or lipid asymmetries would be expected to enhance the bias for correct protein orientation, at least with respect to plasma membranes. We introduce a generalized form of Stern equation to assess surface charge and binding effects quantitatively. In the kinetic sense, dipole potentials within the membrane would tend to prevent positively charged residues from crossing the membrane to leave the cytoplasm. These considerations are consistent with the observed protein orientations. Finally, the electrostatic and hydrophobic factors noted here are combined in two hypothetical models of translocation, the first involving initial interaction of the presumptive transmembrane segment with the membrane; the second assuming initial interaction of a leader sequence.

Key words membrane proteins · membrane biosynthesis · membrane potential · surface potential · surface charge · dipole potential · amino acid sequence

The Asymmetry of Membrane Proteins

All membrane proteins studied thus far are oriented asymmetrically with respect to the plane of the membrane. Hydrophobic segments reside within the lipid bilayer, while hydrophilic segments extend into the cytoplasm or into an extra-cytoplasmic space. A central problem in the study of biological membranes is to identify how the asymmetry is established and maintained.

A possible answer has been provided by the "signal hypothesis" (Blobel & Dobberstein, 1974*a*, *b*), an idea initially formulated to explain translocation of secretory proteins from ribosomes into saccules of the endoplasmic reticulum. According to this hypothesis, an amino-terminal leader on the growing peptide chain is recognized by a receptor protein in the membrane. The peptide is driven through a protein-lined pore, and the leader sequence is then clipped off by a "signal peptidase." For membrane proteins the signal hypothesis is modified to include a stop translocation signal to arrest passage of the peptide part way through the membrane (Rothman & Lenard, 1977). Orientation of the protein is thus established almost trivially by the location of various elements of the synthetic machinery: ribosomes and leader sequence receptor on the cytoplasmic side of the endoplasmic reticulum membrane, signal peptidase inside the saccule.

"Self-assembly" models for protein translocation (Wickner, 1980), on the other hand, invoke more general sorts of physical interaction between hydrophobic portions of the protein and the lipid bilayer. Given these models, the problem of orientation becomes more substantive, or at least more accessible to explanation in terms of broad physical and chemical principles. In the next several pages we will examine how electrostatic potentials could provide the basis for orientation of membrane proteins. The ideas grow out of our recent studies on hepatic asialoglycoprotein receptor and melittin.

The Transmembrane Potential As a Modulator of Protein Disposition in Membranes

Recently, we studied a hepatic asialoglycoprotein receptor (Ashwell & Morell, 1974) inserted from the

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aqueous medium into artificial lipid bilayers (Blumenthal, Klausner & Weinstein, 1980; Klausner et al., 1980). When ligand was added, the receptor changed its conformation or disposition in the membrane. If a transmembrane potential of more than 20 mV (opposite side positive) was then established, the receptor, a negatively charged protein, appeared to "electrophorese" across the membrane, exposing binding sites on the other side. These studies provided the first demonstration that an intrinsic membrane protein could cross, or partially cross, a lipid bilayer in response to an electrical potential. We have also shown that melittin will assume a transbilayer orientation under the influence of an applied potential (Kempf et al., 1982). The complete sequence of this 26-amino acid peptide allows explicit modeling of the influence of voltage in terms of the folding of the peptide and its distribution of charges. In a similar vein, correct assembly of the M13 viral procoat protein into *Escherichia coli* membranes appears to be prevented if the membrane potential is collapsed, suggesting a role for the potential in physiological insertion of M13 procoat protein (Date, Goodman & Wickner, 1980; Date, Zwizinski, Ludmerer & Wickner, 1980).

There are a number of other precedents to the notion that the electrical potential can modulate the position, and perhaps the orientation, of molecules in a membrane. Asymmetrical conductances have been attributed to such effects on antibiotic ionophores (Ehrenstein & Lecar, 1977), toxins (Finkelstein, Rubin & Tzeng, 1976; Schein, Kagan & Finkelstein, 1978; Blumenthal & Klausner, 1982), lipids (McLaughlin & Harary, 1974), cellular transport molecules (Schein, Colombini & Finkelstein, 1976; Blumenthal & Shamoo, 1979), immune cytotoxic factors (Henkart & Blumenthal, 1975; Michaels, Abramovitz, Hammer & Mayer, 1976), and "gating" components of the sodium and potassium channels of nerve (Hodgkin & Huxley, 1952; Miller & Rosenberg, 1979).

Charge Clusters

The possible role of a transmembrane potential in protein orientation leads us to specific predictions about charged amino acids in the protein: we would expect an asymmetric clustering of charges on the two sides of a hydrophobic membrane-spanning segment; if the cytoplasmic compartment is assumed to be electrically negative, then there should be a cluster of positive charges (lysine and arginine) on that side of the hydrophobic segment and a cluster of negative charges (glutamic and aspartic residues) on the extra-cytoplasmic side. If we examine the few transmembrane proteins for which the requisite information on orientation and amino acid sequence (Dayhoff et al., 1981) is available, the prediction is largely borne out (*see* Fig. 1).

Glycophorin, the major glycoprotein of red blood cells, is a clear example: There is a cluster of six negative residues (with one positive) on the electrically positive outer surface and five positive residues (with one negative) on the electrically negative cytoplasmic surface (Marchesi, Furthmayr & Tomita, 1976). When assembled into the membrane of *E. coli*, the M13 coat protein has five negative charges and two positive external to the bilayer, with four positive and one negative on the cytoplasmic side (Nakashima & Konigsberg, 1974), if the N- and C-termini are included. The heavy chain of mouse surface immunoglobulin μ has six negative charges on one side and two positive plus the C-terminus on the other (Rogers et al., 1980). The human histocompatibility antigens HLA-A2 (Fig. 1) and HLA-B7 (not shown) each have the predicted positive clusters on the cytoplasmic side (Robb, Terhorst & Strominger, 1978), but the sequence just to the other side of the hydrophobic segment is still uncertain. The H-2K^b antigen has the positive cluster (Coligan et al., 1981) but only two negative with one positive on the opposite side. Bacteriorhodopsin appears not to fit. Its peptide chain is thought to loop seven times through the membrane, and no consistent pattern of charge asymmetry is evident in the structural model proposed by Engelman, Henderson, McLachlin and Wallace (1980). We might hazard the guess that proteins functioning as channels or having charged, hydrophilic interiors will show more complex patterns than proteins, such as those shown in Fig. 1, which are simply anchored in the membrane by hydrophobic segments.

Distribution of Charge Clusters in a Transmembrane Electric Field

How powerful a force for orientation of the protein is the electrical potential difference? The following points indicate our emphasis in approaching that question: (i) We are not trying to explain how hydrophilic segments of the protein can overcome energetic barriers to their passage through the membrane. Though that issue will return later, we assume for now that the translocation takes place by an unknown mechanism and ask only about the bias for one final orientation or another. (ii) For simplicity, the peptide chain is assumed to pass only once through the membrane. The possibility of generalizing to more complex proteins will be obvious.

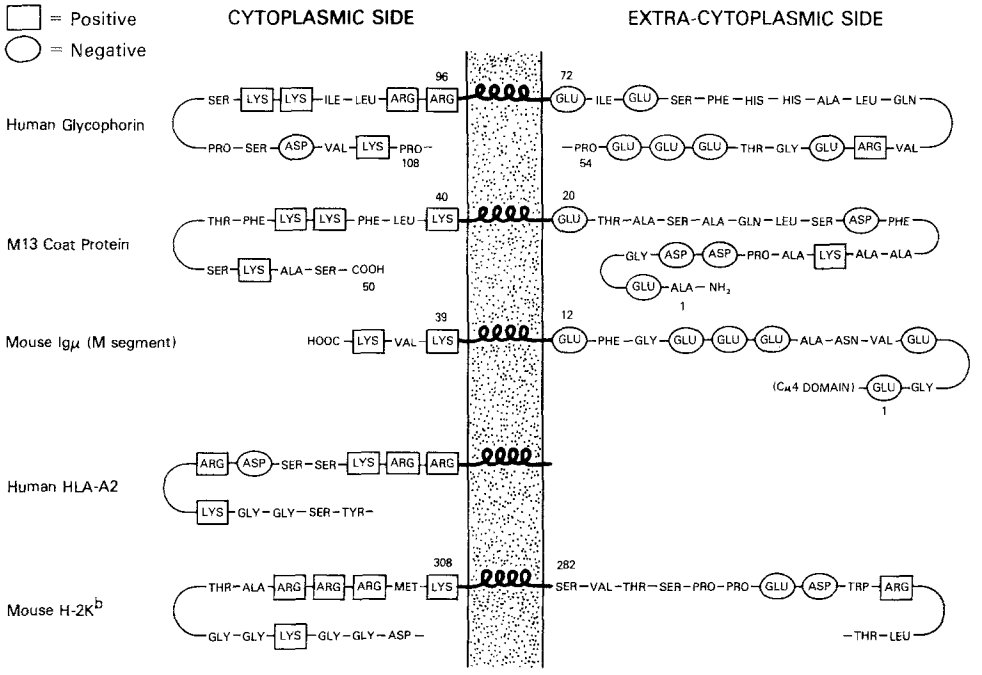

Fig. 1. Clusters of charged residues in transmembrane proteins. Each protein has a predominantly positive cluster on the cytoplasmic side and a predominantly negative cluster on the extra-cytoplasmic side, consistent with expectation given the membrane potential. Placement of the membrane boundary in each case is, to a degree, arbitrary

(iii) Only charged residues clustered near the hydrophobic segment will be considered. We are thus postulating that the immediate region of the hydrophobic segment becomes oriented, that the orientation becomes irreversible, and that the more distant parts of the peptide chain take up their final position by a different mechanism. (iv) We will begin the analysis with thermodynamic considerations. It seems unlikely that the process of insertion is entirely under thermodynamic, as opposed to kinetic, control (though there is no evidence either way); however, the free energies calculated will at the very least suggest what might be available to bias free energies of activation. We will then briefly consider kinetic factors. (v) Orientation in the endoplasmic reticulum probably determines eventual orientation in the plasma membranes of eukaryotes; hydrophilic segments extending into the saccule are destined for the extracellular space and cytoplasmic segments remain cytoplasmic. However, these relationships are not firmly established, and we will consider both plasma membrane and endoplasmic reticulum in the following discussion. In the case of bacteria, the inner or outer membrane, or both, must be penetrated. (vi) We will assume that the nascent protein has not yet been glycosylated or phosphorylated when its orientation is established.

Figure 2 contains a schematic view of the problem. The charge clusters can become oriented in any

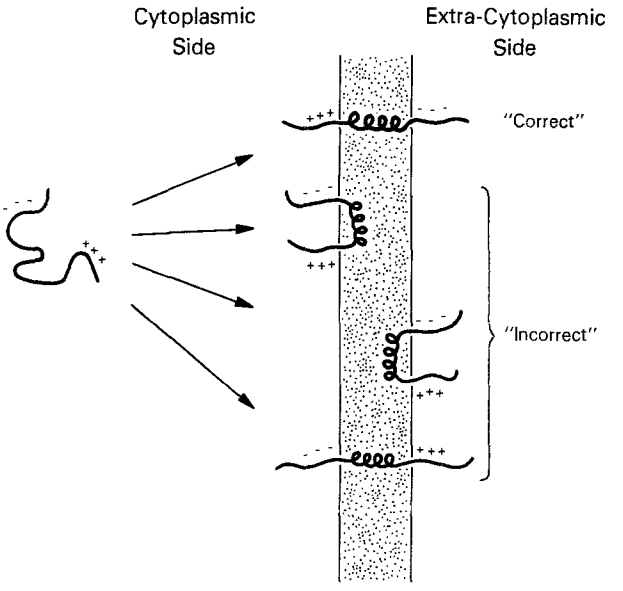

Fig. 2. Four possible orientations of a membrane protein. The first is "correct" with respect to prediction from the membrane potential

of four ways with respect to the membrane. The transmembrane hydrophobic segment is shown as a helix to indicate that it almost certainly must assume a high degree of secondary structure (probably helical) to satisfy the hydrogen bonding requirements of its peptide backbone. It might be helical in

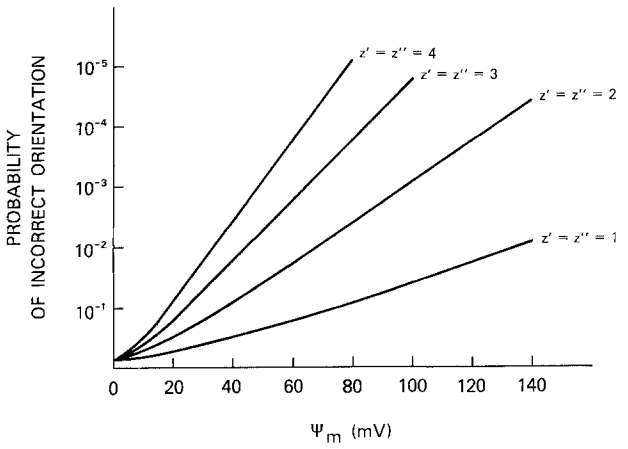

Fig. 3. Probability of "incorrect" orientation of a transmembrane protein in the electrical field according to the 4-state model. Each charge cluster has a valence of $z'=z''$

the aqueous phase as well. If we assume orientation to be determined solely by the transmembrane electrostatic potential operating on the charge clusters, and if the concentration of peptides is not so high as to perturb the potential significantly, then the ratio of electrically "correct" (α) to electrically "incorrect" (β) cluster locations is given by a Boltzmann distribution $\alpha/\beta=\exp(-zqV_m/kT)$ where z is the valence of the charge cluster (assumed to move as a unit), q is the electronic charge, k is the Boltzmann constant, T is the temperature (°K), and V_m is the transmembrane potential in mV (defined as cytoplasmic minus extra-cytoplasmic). At 20 °C, $kT/q=25.7$ mV. The probability of correct cluster location is just $P=\alpha/(\alpha+\beta)$. Then the probability that both charge clusters of the protein are oriented correctly is the product $P'\times P''$, where $'$ and $''$ indicate parameters of the two clusters. Figure 3 shows the probability of incorrect protein orientation (i.e., $1-P'\times P''$) for various cluster sizes, where $z'=z''$. At reasonable membrane potentials, the thermodynamic bias is immense for even relatively small clusters. For example, if $z'=z''=3$ and $V_m=-60$ mV, only 0.17% of the protein molecules, on average, would be incorrectly oriented. If, instead of allowing the four configurations, we require that the protein be transmembrane in one of the two possible orientations, then all six charges must be oriented cooperatively and the frequency of incorrect orientations with $z=6$ is only 0.000076%. The free energy difference (ΔG) between correct and incorrect orientations is given by the expression $\Delta G=zFV_m$, where F is the Faraday constant. For $z=6$ and $V_m=-60$ mV, $\Delta G=34.7$ kJ/mole $=8.3$ kcal/mole, or about 14 times kT. This considerable energy is available to establish orientation. It is probably not sufficient by itself to account for passage of a hydrophilic segment across an unperturbed lipid bilayer.

The Boltzmann expression for the bias in orientation implies a reversible process, and our studies with melittin suggest that voltage dependent orientation of a hydrophilic peptide in the bilayer can indeed be reversible (Kempf et al., 1982). However, melittin is only a model for that part of membrane proteins that directly interacts with or neighbors the bilayer. Once other forces are present that stabilize the orientation of entire membrane proteins, we do not expect this orientation to be reversible with changes in membrane potential.

Components of the Transmembrane Potential

We will now consider several components (Bockris & Reddy, 1970; McLaughlin, 1977) of the membrane potential and their possible roles in orienting membrane proteins. The relationships are shown schematically in Fig. 4.

The Microscopic (V_m) and Macroscopic (V) Transmembrane Potential

If the charge clusters are assumed to lie essentially at the membrane surface, then the potential difference effective in orienting a protein is the "microscopic" transmembrane potential, V_m. This is the quantity that would be measured if electrodes could be placed at the surface. V_m is thought to be reflected experimentally in the potential-dependence of membrane enzyme function (Wojtczak & Nalecz, 1979), in the gating components of axons (Gilbert & Ehrenstein, 1969), and in the fluorescence signals of "fast" potential-sensitive dyes (Waggoner, 1979). In general, V_m differs in value from the "macroscopic" potential V, the quantity actually determined by electrodes. In biological systems V is usually associated with electrogenic pumps and with restrictions to the passive diffusion of ions through leakage pathways. Cells for which measurements have been made have inside-negative values of V and V_m, correlating well with the charge cluster orientations in Fig. 1.

The situation is not so clear for endoplasmic reticulum. By the time it has been isolated as a microsomal preparation, both the geometry and the ionic composition have changed. Some studies suggest a cytoplasm-negative polarity due to an inwardly directed electrogenic Ca^{++} pump (Zimniak & Racker, 1978), but there is dissent (Beeler, Russell & Martonosi, 1979).

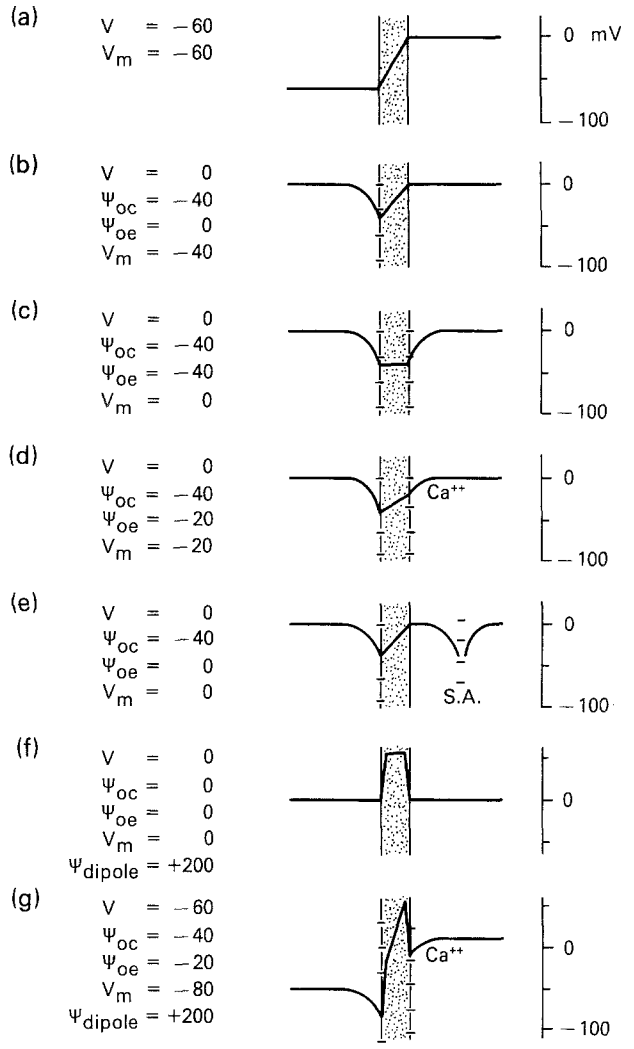

Fig. 4. Components of membrane potential. *See* text for explanation and for definitions of symbols. *S.A.*, sialic acid. Dipole potentials are not to scale. For simplicity, potential profiles across the membrane are depicted as linear

The Surface Potential ψ_0

Figure 4*b* illustrates the effect of placing a negative surface charge on the cytoplasmic side of the membrane. Free cations from the medium accumulate in the interfacial region and "screen" the charges. The distribution of these cations can be described approximately by the classical Gouy-Chapman formulation for the diffuse double layer. A balance is struck between electrostatic attraction of the cations to the negative charges at the membrane surface and the entropic tendency of the cations to spread uniformly throughout the medium. At physiological ionic strength, the surface potential falls off nearly exponentially from the membrane surface with a characteristic distance of about 9 Å. It is negligible beyond about 30 Å from the membrane and therefore cannot be detected by electrodes. ψ_o can be related to the effective surface charge density (σ) by the expression (Grahame, 1947)

$$\sigma = \left\{ \frac{\varepsilon kT}{2\pi} \sum_i C_i [\exp(-z_i q \psi_o / kT) - 1] \right\}^{1/2} \quad (1)$$

where ε is the dielectric constant of water and C_i (in moles/liter) is the activity of the i-th cation in the bulk medium. At 25 °C, $2\pi/\varepsilon kT = 7.40 \times 10^4$ mole·Å^2/liter. The surface potentials on biological membranes have been estimated to range from −15 to about −60 mV (McLaughlin, 1977). The following sample calculation will show how values in that range can be obtained from charge on the membrane lipids.

Approximately 15% of the phospholipids of both plasma membranes and endoplasmic reticulum are negatively charged, principally phosphatidyl serine and phosphatidyl inositol (Robinson, 1975). Assuming 60 Å^2 per phospholipid molecule (for the endoplasmic reticulum, which has little cholesterol) and assuming that the proteins contribute just enough charge to account for their fraction of membrane area, then the surface charge density would be $0.15 \times (1/60) = 1$ charge per 400 Å^2. For a monovalent cation activity of 130 mM in both cytoplasm and extra-cytoplasmic space, Eq. (1) predicts a potential of −39 mV on each surface of the membrane. As illustrated in Fig. 4*c*, symmetrical surface potentials would not affect V_m, and would therefore not bias the equilibrium orientation of charge clusters. However, as will now be discussed at some length, the surface potential is likely to be asymmetrical both because of the intrinsic transmembrane distribution of charged lipids and because of asymmetries in the ionic milieux.

In erythrocytes almost all of the charged lipid appears to reside in the cytoplasmic leaflet of the membrane. For other plasma membranes qualitatively the same polarity has been found in most studies, but the evidence is not so clear (Op den Kamp, 1979). The cytoplasmic surface of endoplasmic reticulum has been claimed to contain most of the phosphatidyl serine (DePierre & Dallner, 1975; Nilsson & Dallner, 1977*a*, *b*), but there is disagreement (Higgins & Dawson, 1977; Sundler, Sarcione, Alberts & Vagelos, 1977; Bollen & Higgins, 1980). Clearly, the question is not yet resolved. If all of the charged lipid were indeed on the cytoplasmic side (Fig. 4*b*), then the surface potential calculated from Eq. (1) would be −70 mV. This potential would add to any macroscopic potential V in biasing charge orientation.

Ions decrease the surface potential by electro-

static screening, as reflected in Eq. (1). Divalent cations are dramatically more effective at screening than are monovalent cations, but calculations according to Eq. (1) indicate that screening is unlikely to be a major factor under physiological conditions. However, cations also bind to bilayers, more strongly to negatively charged lipids than to zwitterionic ones. The effects of this binding on ψ_o can be calculated from a Stern equation (*see* McLaughlin, 1977). In brief, Eq. (1) is combined with the Langmuir isotherm for surface adsorption of each ion, with a Boltzmann expression for each ion, and with an expression for the conservation of each ionic and lipid species. For a membrane considered to have one type of negative and one type of zwitterionic lipid, we find that

$$\sigma = -\frac{\{P^-\}^{\text{tot}}\left[1-\sum_i [K_i^- C_i \beta_i (z_i-1)]\right]}{1+\sum_i [K_i^- C_i \beta_i]} + \frac{\{P^\pm\}^{\text{tot}} \sum_i [K_i^\pm C_i \beta_i z_i]}{1+\sum_i [K_i^\pm C_i \beta_i]} \quad (2)$$

where P^- and P^- are the surface concentrations of zwitterionic and negative lipid, respectively, in molecules/Å^2; each K_i is an instrinsic 1:1 association constant; and $\beta_i = \exp(-z_i q \psi_0 / kT)$. Equations (1) and (2) can be solved iteratively for ψ_o as a function of any of the other parameters. *See* McLaughlin (1977) and McLaughlin et al. (1981) for discussion of the limitations of the Stern treatment and for explanation of the assumption that binding of divalent ions to phosphatidyl serine is 1:1.

To analyze the sensitivity of ψ_o to various changes, we assume a membrane composed of phosphatidyl serine (negative) and phosphatidyl choline (zwitterionic). Figure 5 gives the results as a function of phosphatidyl serine content of the membrane for reasonable estimates of the ionic activities in mammalian cytoplasm and extracellular space. Even if the membrane is assumed symmetrical with respect to lipid composition, binding appears to cause a greater reduction of surface potential on the external surface of the membrane, as illustrated in Fig. 4*d*. With 15% phosphatidyl serine in each leaflet, ψ_{oc} would be reduced in magnitude from -39 to -26 mV, whereas ψ_{oce} would be reduced from -39 to -15 mV (*c* and *e* indicate cytoplasmic and extracytoplasmic sides, respectively). The data are not available for a meaningful calculation for endoplasmic reticulum, but it is worth noting that Ca^{++} is pumped to high concentration in its saccules (Moore & Pastan, 1978). Most of this Ca^{++} is probably

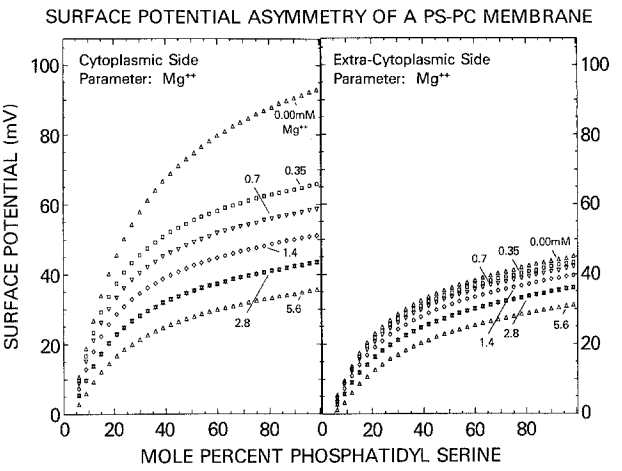

Fig. 5. Surface potential calculated as a function of phosphatidyl serine content of the membrane. Intrinsic 1:1 binding constants (McLaughlin, Grathwohl & McLaughlin, 1978; Eisenberg, Gresalfi, Riccio & McLaughlin, 1979; McLaughlin et al., 1981) for Ca^{++}, Mg^{++}, Na^+, and K^+ binding to phosphatidyl serine are taken as 12, 8, 0.6, and 0.15 liter/mole, respectively. For phosphatidyl choline the Ca^{++} and Mg^{++} binding constants are taken as 3 and 2 liter/mole, respectively, whereas Na^+ and K^+ are assumed not to bind significantly. As estimates of the Ca^{++}, Na^+, and K^+ activities in extracellular fluid, we use 1.5, 130, and 5 mM. Cytoplasmic activities are less certain but are estimated as 10^{-4}, 10 and 130 mM, respectively. Mg^{++} values in this sample calculation are allowed to vary around the reasonable physiological values of 1.0 and 0.7 mM for extracellular and cytoplasmic compartments, respectively. These calculations suggest that a modest asymmetry in surface potential such as shown in Fig. 4*d* would arise even if lipid compositions of the two leaflets were identical. Mg^{++} appears to be an important determinant of cytoplasmic, but not extra-cytoplasmic, surface potential

bound to molecules other than the membrane lipids, but any residual high activity would tend to promote surface potential asymmetry of the polarity in Fig. 4*d*. It has been reported that *Streptococcus faecalis* can grow, and presumably insert membrane proteins, with V short curcuited (Harold & Van Brunt, 1977). Surface potential might be important under that experimental condition.

Negative charge associated with sialic acid plays a major role in the electrophoresis of whole cells, but Fig. 4*e* illustrates why it probably does not influence V_m. Polysaccharide chains are quite rigid compared with peptide chains, and most of the sialic acid residues have been estimated to reside well outside the electrostatic interfacial region. The 9 Å characteristic length for electrostatic screening has two further implications deserving mention. First, without knowing the tertiary structure of the protein it is not possible to say how many of the charges in each cluster will effectively be in the interfacial region. Second, we cannot exclude the possibility that loci of protein translocation or the peptides themselves have "discrete charge" (*see* McLaughlin, 1977)

such that the surface potential there differs from the average for the membrane surface as a whole.

The Dipole Potential

Zwitterionic phospholipid membranes are generally orders of magnitude more permeable to hydrophobic anions than to equivalent cations. This finding can be attributed to dipole potentials such as those shown in Fig. 4*f*. It is unclear whether these potentials are a function of the ester linkages of the lipid or whether they arise from interaction of the headgroups with water (Hladky & Haydon, 1973), but they can amount to several hundred millivolts. Since dipole potentials, if symmetrical, would not affect V_m, they would not be expected to alter the thermodynamics of charge cluster orientation. But in the kinetic sense, they could lower the potential energy barrier for translocation of negative charges across the membrane, while increasing the barrier for positively charged residues. This is precisely the bias required to achieve the orientations shown in Fig. 1. Fig. 4*g* summarizes how the various components of potential might concatenate in a biological membrane to produce high V_m and ψ_{dipole}.

Discussion

We have now identified a number of electrostatic principles which, singly or in combination, could influence the orientation of proteins in biological membranes. There is no reason to suppose that they all operate, but it may be instructive to assemble them into two speculative collages.

The first is shown on the left side of Fig. 6. Either post-translationally or as a hydrophobic segment leaves the ribosome, the peptide chain approaches the cytoplasmic membrane surface (Fig. 6*a*). Its positively charged cluster is drawn into the potential energy well represented by the negative surface potential. If the hydrophobic segment is extended at this point, the negative and positive charge clusters could be as much as 70 Å apart. Thus, the negative charges could reside outside of the electrically negative interfacial region during initial interaction of the hydrophobic segment with the membrane. Ignoring the configurational free energy required to keep the negative cluster away from the interface, we can use a Stern equation to approximate the concentration ratio of positive clusters between interface and bulk solution in the absence of binding. If the quantity of positive charge on the peptide at the interface is assumed small relative to the quantity of fixed negative charge and if discrete charge effects are ignored, the Stern equation reduces to a Boltzmann relation between bulk and interfacial activities. A -39-mV surface potential would result in a 4.6-fold concentration of single charges at the interface, and a 95-fold concentration of 3-charge clusters. Any nonelectrostatic binding of the positive residues to the membrane surface would amplify the effect.

The hydrophobic segment enters the membrane and takes on a helical conformation (Fig. 6*b*) to satisfy the hydrogen-bonding requirements of its peptide backbone. The free energy of this interaction might aid in pulling negatively charged residues through the interfacial region (Fig. 6*c*). The positive dipole potential would tend to decrease the energetic barrier to passage of the negative cluster through the interior of the membrane.

Because H^+ is concentrated in the interfacial region (again according to the Boltzmann distribution), the pH is calculated to be 0.66 units lower than that in the bulk cytoplasm for a -39 mV surface potential. This lowering of pH would facilitate entry of glutamic (pK 4.3) and aspartic (pK 3.8) residues into the membrane (against the countervailing tendency of the negative surface charge to exclude anionic residues from the interfacial region). Because the free energies for titration of the basic amino acids are considerably greater than those for titration of acidic ones (*see*, e.g., Engelman & Steitz, 1981), and because of the dipole potential, the basic residues should preferentially remain on their side of origination, i.e., in the cytoplasm. Indeed, as one looks at the proteins shown in Fig. 1 (or, correlatively, at leader sequences of secreted and membrane proteins (Steiner et al., 1980)), the positive cluster is more striking, and perhaps more influential, than the negative cluster.

The electrostatic principles described in this paper do not indicate how the rest of the peptide chain would get through the membrane. Perhaps the problem is not energetically very difficult once the stage of Fig. 6*d* has been reached. Von Heijne and Blomberg (1979) have calculated the activation energy for moving a peptide chain through lipid by requiring that one residue leave on the opposite side for each one that enters. Engelman and Steitz (1981) have proposed models similar in spirit. If charged residues are assumed largely neutralized, the chain is expected to slide without too great an energetic barrier except as the hydrophobic region is reached. However, it seems more likely to us that the chain would pass through some "defect" in the structure of the lipid bilayer (Fig. 6*d*). The defect might be a protein pore, as in the signal hypothesis, a fluctuation in lipid packing at a phase boundary (Klausner, Kleinfeld, Hoover & Karnovsky, 1980), or a nonbilayer lipid structure.

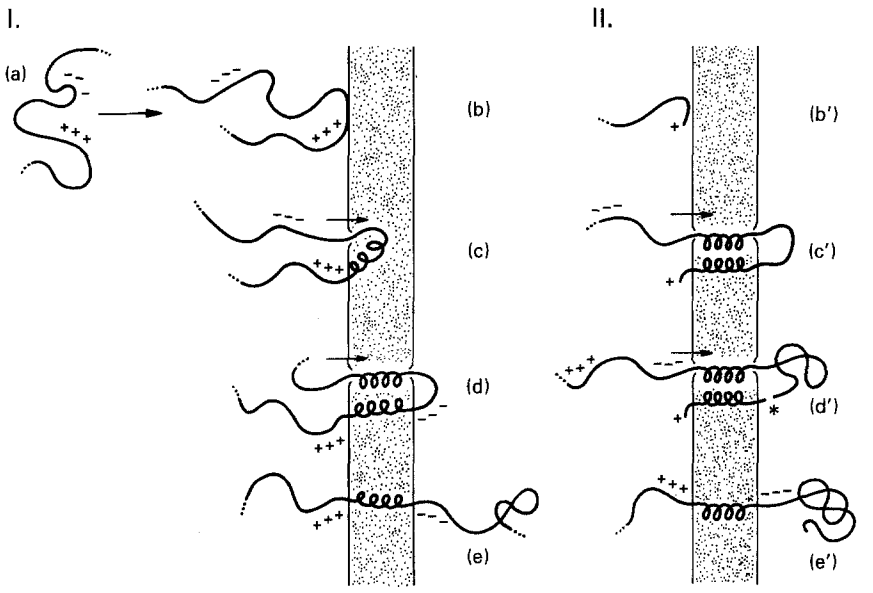

Fig. 6. Hypothetical views of the insertion of a transmembrane protein. (*I*): Insertion is initiated at the transmembrane hydrophobic segment and/or the adjacent positive charge cluster. (*II*): Insertion is initiated at a leader sequence, and protein is threaded through membrane until hydrophobic segment is in place. Small arrows indicate direction of threading. * indicates cleavage by peptidase, which could take place at any time in the process after (*b'*). Mechanisms *I* and *II* could be either co- or post-translational. *See* text for discussion

The model on the right-hand side of Fig. 6 brings similar electrostatic principles into play but with a different topology. For membrane proteins with a leader sequence (e.g., M13 coat protein), the first interaction is at the leader. The protein then threads through the membrane (perhaps at a "defect") until the hydrophobic sequence has reached a transmembrane position. The positive charge cluster would constitute a firm "stop translocation" signal, perhaps with involvement of multivalent binding to negatively charged membrane surface components, and the negative cluster might serve to anchor the protein in the appropriate position. These two models are topologically similar to ones suggested by a number of workers (Von Heijne & Blomberg, 1979; Date et al., 1980; Inouye & Halegoua, 1980; Engelman & Steitz, 1981). Our own emphasis is not on the topology or on the hydrophobic interactions but on an analysis of the electrostatics.

We are not proposing that the transmembrane potential and asymmetric distribution of charged residues around a hydrophobic region can by themselves explain the energetics of protein translocation. Rather, we stress that both thermodynamic and kinetic considerations suggest the following: if a protein must be oriented in one way or another in the membrane, it might more easily locate itself with a positive cluster on the cytoplasmic surface and a negative cluster on the extra-cytoplasmic side. This hypothesis led to our initial expectation of finding charge cluster asymmetries, and that expectation has been borne out so far by the amino acid sequences shown in Fig. 1. Our second aim in this paper was to begin an analysis of the electrostatic effects which could influence orientation. More detailed examination (including discrete charge effects, dipole moment of alpha helix, boundary potentials, nonindependence of charged groups, partially titrated residues, and pH changes near the membrane surface) will be justified as data become available for analysis. The ideas presented here suggest a clear line of further experiment: With artificial lipid membrane systems (liposomes and planar bilayers) one can manipulate lipid composition, lipid charge, transmembrane potential, and ionic environment independently. Using melittin and the hepatic asialoglycoprotein receptor (as well as other peptides and proteins), we are now examining in more detail the various components of electrostatic influence on orientation.

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