Hypothesis

On the physical basis for the *cis*-positive rule describing protein orientation in biological membranes

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Abstract The topology of hydrophobic intramembrane proteins is characterized by a statistical asymmetry in the distribution of positively-charged residues on the two sides of the membrane, the 'inside- or *cis*-positive rule'. A mechanism is proposed involving only neutral residue transfer. For a tightly bound polypeptide adsorbed on the membrane and not at equilibrium, the p*K* values of the ionic residues related to dissociation of the proton into the aqueous phase bulk are increased because of interaction with the negative charges at the membrane surface. The p*K* shift would selectively neutralize aspartate and glutamate residues, favoring their translocation across the membrane, while stabilizing the impermeant positively charged state of lysine and arginine residues.

Key words: Membrane protein; Topology; Translocation; Surface potential; Gouy-Chapman

1. Introduction

The orientation of hydrophobic intramembrane proteins is characterized by a pronounced statistical bias in the distribution of positively-charged amino acids on the two sides of the membrane [1–10]. This bias expresses the relative difficulty of translocating positively charged amino acids, mainly lysine and arginine, across the membrane after they are synthesized on the inside of the cytoplasmic membrane or imported from the *cis* side of an organelle membrane (Fig. 1). A bias in the distribution of side-chains contributed by the negatively-charged residues, aspartate and glutamate, is much less pronounced [1,3,7]. This positive charge bias phenomenon has been described as the 'inside' or '*cis*-positive rule' [1,2], where the 'cis' nomenclature includes protein import into organelles such as chloroplasts and mitochondria. One exception to the rule is that it is not necessarily obeyed by integral membrane proteins with terminal segments or connecting extrinsic loops greater than 60 residues in length [5,7]. This is presumably related to a greater role of the protein translocation machinery in the secretion or import of longer terminal segments and interhelix loops.

The rule also applies to eukaryotic proteins [7], although the magnitude of the positive charge bias is smaller than in prokaryotic membrane proteins [2,7], and decreases with the distance of the *trans*-membrane helix from the N-terminus [7]. Additional parameters [11–14] including the net charge difference across the N-terminal *trans*-membrane segment [13] are topological determinants for eukaryotic membrane proteins. In addition, the overall amino acid composition of extrinsic segments is different on the cytoplasmic and extracellular sides [14].

2. Membrane bilayer-based hypothesis

In seeking an explanation for the *cis*-positive rule, it was assumed that because of its ubiquity it would reside in the general electrostatic parameters of the membrane lipid bilayer, and not in details of structures of specific protein translocation machineries. The hypothesis offered to explain the rule therefore does not provide any details about the mechanism of protein penetration into, or translocation across, the membrane. The latter processes must involve intricacies of the protein translocation machinery [9,15,16]. The hypothesis requires that at some point in the pathway of insertion into the membrane, irrespective of the nature of protein components involved in translocation, the imported polypeptide interacts with the negative membrane surface.

3. The energy barrier

The major energy barrier for insertion of protein into membranes arises from the insertion of charges (i.e. charged amino acids) from water, dielectric constant $\varepsilon = 78$, into the low dielectric, $\varepsilon = 2$–3, core of the membrane.

This energy barrier or 'Born charging energy' [17], E_B, in kcal/mol:

$$= 166 \frac{(z)^2}{r} [1/\varepsilon_m - 1/\varepsilon_w] \quad (1)$$

where z is the valence of an ion of radius r, in Å, and $\varepsilon_m$and ε_w are the dielectric constants, respectively, of the membrane bilayer and the bulk aqueous phase. If $z = 1$, and $r = 2$ Å (terminal ionic group of Lys), or 2.5 Å (terminal ionic group of Arg, Asp, or Glu), then $E_B \approx 40$ and 32 kcal/mol, respectively.

These values should be somewhat lower because of charge

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\psi$, *trans*-membrane potential; ψ_s, membrane surface potential; pK_{sv}, p*K* at membrane surface with the proton release into bulk aqueous volume.

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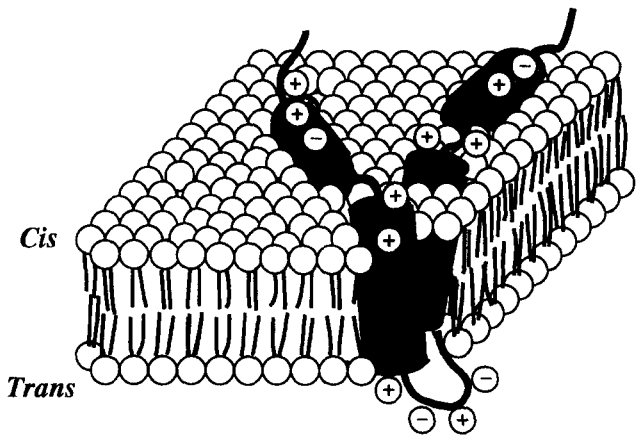

Fig. 1. Folding on the surface of a simple lipid bilayer membrane of a single membrane protein anchored by a hydrophobic hairpin inserted into the membrane bilayer according to the '*cis*-' or 'inside-positive' statistical rule. This rule describes the tendency of: (i) the positively charged (+) basic residues, lysine and arginine, to be found in statistical excess on the *cis* side, the side from which the polypeptide is imported; and (ii) for the negatively charged (−) amino acids, aspartate and glutamate, to be distributed approximately equally on the two sides of the membrane. The net negative charge of the membrane lipid head groups is implicit.

interaction with the polar surroundings of the membrane [18], particularly the polar (ε = 10–20) interfacial lipid head-group layer [19]. Using a three-dielectric model of the membrane [19], the magnitude of the decrease was calculated to be approximately 2 kcal/mol.

3.1. *Bias for anionic residues caused by carbonyl ester dipole potential*

The energy for insertion of anionic residues is reduced by the surface dipole potential, positive inside, suggested to arise from carbonyl ester groups that link the lipid fatty acid chains to the glycerol backbone of the phospholipids. This potential favors the insertion of anionic residues, by approximately 5 kcal/mol, and disfavors cationic residues by the same amount [20]. Therefore, although there is a small bias for the insertion of anionic residues, the energy barrier for insertion of charged residues remains large, approximately 25 kcal/mol for (Asp, Glu), 35 kcal/mol for Arg, and 43 kcal/mol for Lys.

3.2. *Other possibilities for a decreased energy barrier*

(1) Some fraction of the favorable energy, ca. 60 kcal/mol, for insertion into the membrane bilayer of a hydrophobic helical hairpin [21], could offset the Born energy for translocation of charges through the middle of the bilayer. For a charge that will be fully translocated across the membrane, approximately half of the hairpin will be inserted into the membrane when this charge confronts the barrier at the center of the bilayer. Therefore, approximately 30 kcal/mol could be utilized to offset the translocation. This is sufficient for the translocation of only 1–2 charges per hairpin. This compensation is not sufficient in most cases and, moreover, does not explain the bias for selective translocation of anionic residues.

(2) The negative-inside *trans*-membrane potential ($\Delta\psi$) could favor the export of negative and retard that of positive charges. It has been shown that mutants of the M13 coat or leader peptidase proteins can be constructed whose translocation and orientation are sensitive to the presence of high concentrations (50 μM) of the proton ionophore, CCCP, which will collapse the $\Delta\psi$ [22,23]. In spite of these effects on specific mutants, it seems difficult to understand how $\Delta\psi$-dependent electrophoretic effects could provide the general explanation and mechanism for the *cis*-positive rule under physiological conditions, for the following reasons (i–iii): (i) A $\Delta\psi$-dependent electrophoresis would cause an accumulation of positive and negative residues, respectively, on the inside and outside of the membrane; however, such a segregation of Asp and Glu residues generally does not occur [1], although there may be a segregation of glutamic acid, but not aspartic acid, in nuclear-encoded mitochondrial inner membrane proteins [24]. (ii) The *cis*-positive rule with the 60 residue loop cut-off applies to all, or almost all, chloroplast thylakoid integral membrane proteins of the photosystem II, photosystem I, cytochrome *b6f*, light-harvesting, and ATPase complexes whose topologies are reasonably well-established [25–27]. However, the steady-state *trans*-membrane $\Delta\psi$ is very small, ca. 10 mV, across thylakoid membranes (e.g. [28] and references therein). (iii) The magnitude of the energetic contribution of the *trans*-membrane $\Delta\psi$ at the top of the barrier for ions translocation (in the middle of membrane) is small, approximately 0.05–0.08 eV or 1.2–1.8 kcal/mol, for $\Delta\psi$ = 100–160 mV. This is evidently not enough to drive ions over a barrier of at least 25 kcal (vide supra).

4. Neutral residue mechanism

From the preceding energetic considerations, it seems likely that amino acid residues that are translocated across the membrane are electrically neutral. The transport of neutral forms of low-molecular wieght electrolytes is a well-known phenomenon (e.g. [17]). This idea was applied to proteins in [29], and then invoked to explain the preference for Lys over Arg in a β-lactamase signal peptide used for hybrid protein export [30]. However, this mechanism in its simplest form does not discriminate between insertion of basic vs. acidic residues because the amount of the neutral form is determined by the difference of the residue pK_a and the solution pH. At pH 7, the magnitude of this difference is similar for (Glu, Asp) and Lys, and the fraction of their neutral forms is low. Furthermore, the partition coefficient from water into organic solvents of *n*-butylamine, which is a model for the Lys side chain, is approximately 10 times larger than the coefficients of the undissociated propionic propyl and acetic acetyl acids that are models for Glu and Asp [31]. This factor, although not large, would influence the distribution in a direction opposite to that described by the *cis*-positive rule. Therefore, a mechanism is needed at the membrane surface to cause selective neutralization of carboxylate residues.

5. Hypothesis

It is proposed that the explanation of the rule can be based: (a) on the inference that neutral residue side-chains are predominantly translocated; (b) the selectivity arises from the pK increase of ionic residues in polypeptides tightly bound at the membrane surface, caused by interaction with the negative charged lipid head groups and resulting negative surface potential, ψ_s, always present in biological membranes. For low-

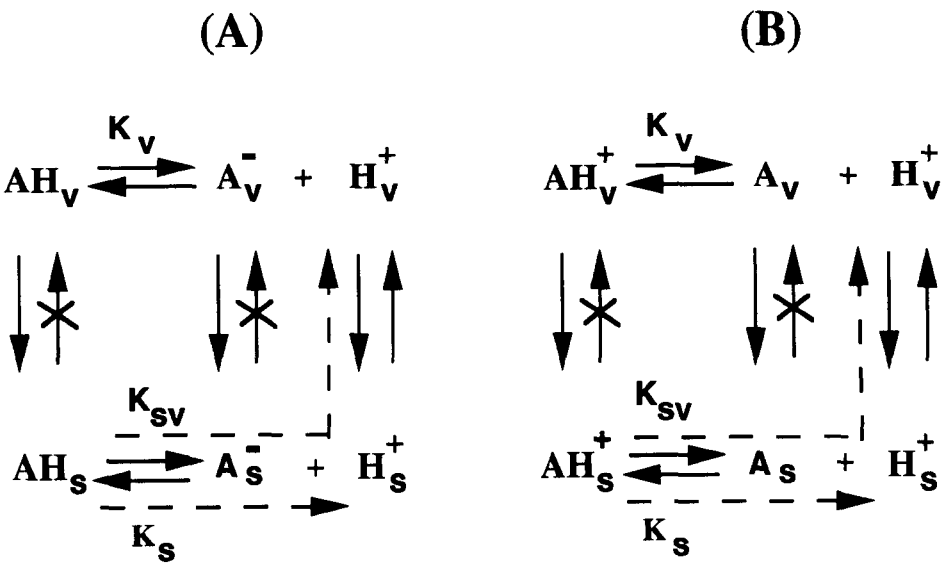

Fig. 2. Schemes of equilibria between species (A) in the bulk volume (subscript 'v') and on the surface ('s'). The surface dissociation constant, K_s, refers to an equilibrium with all reactants at the surface. The 'mixed' dissociation constant, K_{sv}, corresponds to the sum of equilibria of surface dissociation and of proton exchange between surface and bulk. Schemes (A) and (B) refer to neutral and cationic acids, for which neutral forms binding to the membrane are acidic or basic, respectively. Equilibria of surface-bulk exchange that are operative for small molecules but not for surface-adsorbed proteins are crossed.

molecular weight electrolytes, the surface potential cannot influence their membrane penetration because it does not affect the equilibrium between neutral molecules at the surface and in the bulk solution [17]. The essential difference for the case of proteins is that their tight, non-equilibrium binding to membranes prevents exchange of the residues at the surface with the bulk solution. The pK increase of these bound residues will have an oppositely directed effect on neutralization of basic (Lys, Arg) and acidic (Asp, Glu) residues.

5.1. Qualitative effect of negative membrane surface

Carboxylates and basic residues can be tightly bound to the membrane surface when they belong to a polypeptide that: (i) has basic domains [32–34] that contribute cooperatively to a large and favorable electrostatic binding component to the negatively charged surface; and/or (ii) one or more hydrophobic segments that bind in the interfacial region or the hydrophobic membrane core. The desorption rate of cooperatively bound proteins is slow compared to the electrolytic dissociation of acids that occurs in μs–s for pK values from 4 to 10. Therefore, the ionic residues of adsorbed proteins are in equilibrium with the bulk solution only through their electrolytic dissociation, but not through exchange of the residues.

Surface ('s') or mixed surface-bulk dissociation ('sv') is depicted in Fig. 2. The corresponding reactions are: $AH_s \rightleftarrows A_s^- + H_s^+ \rightleftarrows A_s^- + H_v^+$, and $AH_s^+ \rightleftarrows A_s + H_s^+ \rightleftarrows A_s + H_v^+$, respectively. The negative membrane surface causes the energy of A_s^- to increase, and of AH_s^+ to decrease, resulting in both cases in a more ready protonation, i.e. an increase, ΔpK_{sv}, of the mixed surface-volume pK_{sv} for all components relative to that in the bulk volume (pK_v). This shifts the equilibrium towards an undissociated acid or protonated base. Alternatively, we can consider the equilibrium with both A_s and H_s^+ at the surface at the same potential. In this case, $pK_s = pK_v$, but the surface concentration of H^+ increases, resulting quantitatively in the same increase of AH_s concentration. This effect can qualitatively explain the *cis*-positive rule, because the electrolytic dissociation equilibria of carboxylates and basic residues will, respectively, be shifted closer to, and farther from, neutrality.

6. Discussion

*6.1. Contributions to the interfacial Δp*K

Electrostatic effects arising from the negative membrane surface charge will cause a positive ΔpK shift for both acidic and basic groups, and therefore an asymmetric effect relative to a pH 7 reference. A surface charge density arising from a 30% anionic lipid content gives rise to a Gouy-Chapman surface potential, $\psi_s = -30$ mV, and a $\Delta pK_{sv} = +0.5$ for all groups [35]. The Gouy-Chapman potential is an averaged value corresponding to the charge evenly distributed on the surface. A discrete nature of the membrane surface charges would effectively increase their interaction at short distances with the discrete charges of peptides. This could explain the magnitude of the binding energies of basic poly-Lys or poly-Arg peptides [32,33] that would translate to a ΔpK_{sv} as large as +1.0–+1.4.

An approach close to, or a partial insertion into, the low-dielectric membrane would shift all pK's, making the neutral forms more favorable [36,37]. Such effects were observed for the fatty acid myristate ($\Delta pK = +2.1$) and for basic tertiary amine anaesthetics ($\Delta pK = -1.0$–1.5) in neutral DMPC liposomes [38].

Thus, charge and dielectric effects, both of which cause a positive ΔpK of the carboxylate amino acids, Asp and Glu, at the surface of the membrane and in the interfacial layer, can shift their pK values within one unit or less of neutrality, so that a significant fraction of the carboxylates can be in the neutral state at any one time. For the basic amino acids, Lys and Arg, the two effects exert oppositely directed influences. The pK values of these residues will tend to be similar to their values

of approximately 10 and 12 in bulk solution, which corresponds to only 10^{-3}–10^{-5} of these residues being neutral at pH 7. The substantially higher fraction of the neutral state for acidic compared to basic residues is proposed to underly the preference for translocation of Asp and Glu residues over Lys and Arg.

6.2. *Statistical nature of the cis-positive rule*

Differences in the charge and charge distribution of the bound protein, local anionic lipid surface density, closeness of approach of amino acid residues to lipid head-groups and the low dielectric phase, and the contribution of partly or fully inserted hydrophobic segments, will result in a spectrum of possible pK shifts.

Translocation of a basic group can be facilitated by penetration into the membrane of an ion (e.g. carboxylate-Lys) pair, as proposed for the *E. coli* leader peptidase [39], bacteriorhodopsin [40] and the lactose permease [41]. Such ion pairs very likely exist in a mutually neutralized form because of the large Born charging energy (Eqn. 1) associated with insertion of the individual ions into the bilayer. Together with the natural variation in surface binding affinity and resulting ΔpK_{sv}, this could explain why the translocation of basic residues is not strictly prohibited, and why the *cis*-positive rule has a statistical character.

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