Biophysical Chemistry 29 (1988) 79–84
Elsevier

BPC 01215

Ion transport across biological membranes

Diffusion in water solution or conduction in the solid state?

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Accepted 15 October 1987

Action potential; Axonal membrane; Ion channel; Na^+ flux; Solid-state conductance

The mechanism of the highly efficient and precisely regulated ion flux across axonal membranes has usually been considered as free diffusion in aqueous channels. The evidence for single-file mechanisms has not been convincing. In this report, solid-state ionic conductance is proposed as an alternative mechanism. The sequence of the Na^+'channel' protein is scrutinized for elements that may form an ionic core that is well insulated by a hydrophobic mantle from other segments of the protein. A specific structure is proposed that may correspond to ionic lattices as they are known to exist in crystals of small, inorganic electrolytes that exhibit considerable ionic conduction.

1. Introduction

The transport of ions across membranes is usually regarded as diffusion as it occurs in aqueous bulk solutions. This concept may appropriately describe specific channel systems, such as the porins, a class of proteins that have been shown to form voltage-dependent, water-filled channels across outer membranes of bacterial cells [1–3]. Whether this same concept may be applicable as a general mechanism has been a long-standing debate that considered pore models [4,5] and single-file diffusion [6,7] as alternatives. The latter hypothesis was first formulated in 1955, when Hodgkin and Keynes [8] used it to explain observations that revealed deviations from predictions derived from the quantitative description of the electrical events occurring upon action potentials, as introduced in 1952 by Hodgkin and Huxley [9]. Since ion fluxes are of crucial importance in nerve excitability, the underlying mechanism has attracted an unusual amount of interest (for reviews, see refs. 10–12). Although, as a consequence of the studies of Hodgkin and Keynes [8], the single-file mechanism was followed up closely [13–15], it was eventually abandoned as a model mechanism for Na^+ flux, mostly on the basis of energy considerations [14,15]. This may also have been due to the lack of an adequate concept for the structural correlate of a single-file mechanism. But, mostly, it may be ascribed to the extensive studies of gramicidin A at high resolution, with respect to both function [14,16,17] and structure [18–20], which unequivocally confirmed its channel structure. This ionophoretic antibiotic also provided a seemingly easy explanation for the rates of Na^+ flux across axonal membranes. Yet, the voltage control, originally described as the movement of the equivalent of six gating particles (or charges) across the membrane boundary [9], and the selectivity were difficult to explain with an intrinsically uncharged molecule such as gramici-

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din. Gating was also puzzling once the notions on the structure of membrane proteins developed, based primarily on studies with bacteriorhodopsin [21,22] and a reaction center of photosynthetic bacteria [23]. They seemed to indicate that membrane proteins were characterized by hydrophobic domains, with a maximal exclusion of charged residues from within an environment with a low dielectric constant.

When the primary structure of the Na^+ channel protein was unraveled by Numa and co-workers [24], most of the structure predictions springing from that achievement [24–28] attempted to identify channel-lining elements. Although gating charges were considered, they were relegated to domains either beyond the membrane boundary [24], or within it, though not immediately contiguous to the channel [26–28], as suggested originally [10,15]. Some potential clarification of this paradox between functional and structural concepts appeared to come from the comparison of the highly specific Na^+ channel with porin, mentioned previously. This is a transmembrane protein containing highly unspecific voltage-dependent pores. We have postulated that it contains, within the membrane domain [29,30], many polar and ionizable residues that are also inaccessible from the channels, a proposal that in view of its structure appeared all but inevitable. Current efforts concentrate on verifying this by high-resolution X-ray analysis [31–33].

For Na^+ transport, the fundamental question thus arose again as to whether ionic conduction could be explained by a mechanism other than diffusion in an aqueous environment. Ever since solid-state physics became established, it was clear that specific crystalline electrolytes, though insulators by themselves, exhibit ionic conductivity either by doping, or through crystal defects (see, e.g., refs. 34 and 35). Such defects (the most relevant within this context being Schottky defects) appear to migrate randomly across crystal lattices by ions constantly hopping into randomly vacant positions, thereby creating new defects. Such a notion of a seemingly unordered movement appeared of little relevance to the structure of biological molecules. Yet, specific solid electrolytes, consisting of alkali-metal-iodide complexes [36], for instance, have long been known to exhibit high conductances ($\Gamma = 0.2$ S cm^{-1} as compared to 0.5 S cm^{-1} in aqueous solution). An X-ray structure analysis to high resolution [37] of one representative example of such an electrolyte, $RbAg_4I_5$, revealed that the coordinates of its Rb^+ and I^- occupied uniquely defined positions within the crystal lattice, whereas for Ag^+, of which there are 16 in the cubic unit cell, 56 sites (belonging to three different classes) are available. Startlingly, these potential binding sites were found to occur within pathways traversing the crystal [37].

With this in mind, the sequence of the Na^+ channel protein [24] was scrutinized in an attempt to identify segments that may represent the constituents of local ionic lattices, formed by charged residue contributed by side chains of the protein. Thus, the criteria based on hydrophobicity and related parameters were at first disregarded as primary determinants. Rather, maintaining local as well as global electroneutrality, as well as maximal saturation of the entire hydrogen bonding potential [38], were considered as being of prime significance. The structural model that was obtained with this procedure indeed exhibited a series of intercommunicating vacancies that appear devoid of protein charge and mass, although their coordinates seem also uniquely defined by the structure of the protein [39]. In analogy to the metal-iodide complex, such vacancies would be filled by Na^+. But is such a structure applicable to ion pathways, and might it be extended to other ion-translocating proteins?

2. A local membrane-spanning ion lattice

The potential 'folding' of the Na^+-transducing protein, proposed here, may be described in a series of purely conceptual steps which, of course, do not imply any correlate to the in vivo folding. The center of the sequence of this protein from the electroplax of the electric eel contains an uninterrupted stretch of nine glutamyl residues. If neutralized with Na^+ forming salt pairs, it might be arranged as an α-helix, just as it was shown to occur with protonated polyglutamyl segments [40]. It may be worthy of note that this α-helix is one

of the strongest predictions from the power spectra of Greenblatt and co-workers [26]. It could thus span a distance of 13.5 Å, but may be complemented by neighboring or distant acidic segments occurring in the sequence, extending the total length up to about 30 Å [39]. Incidentally, in the present model, the central sodium wire being surrounded by other protein segments, the thickness of a typical hydrophobic membrane core may not be critical in such considerations. Around this central α-helix, the four polycationic S4 segments (the nomenclature used [24] is that of Noda and co-workers) would twist in 3_{10}-helical configurations, with their cationic groups (mostly Arg) matching the carboxylate groups on the anionic central α-helix. 3_{10}-helices had indeed been proposed previously [24], but this configuration was withdrawn subsequently [28], because such structures have been observed neither in soluble proteins, nor in the only high-resolution structure of a membrane protein currently existing [23]. In the structure under consideration here, it seems conspicuous that every fourth residue on the α-helix is 6 Å apart (4 × 1.5 Å as projected on the helix axis), and that all the negatively charged residues could be matched by every third cationic residue of the four 3_{10}-helices with distances of 6 Å (3 × 2.0 Å) between them. (For distances, as projected onto helix axes, see ref. 41.) Indeed, four 3_{10}-helices match all residues on the central α-helix, irrespective of its length. In the structure proposed here, very strong electrostatic interactions would occur, comparable to those in ionic lattices of small electrolytes. It should be noted that α-helices instead of the 3_{10}-array would not satisfy the geometry as considered here. Extensive 3_{10}-helical configurations have also been suggested, based on an entirely different and independent rationale [25]. The open question in that model is whether such assignments are justified in view of the lack of local and global electroneutrality. Also, the assignment of 3_{10}-helical configurations to anionic segments does not appear obvious at all. The debate on 3_{10}-structures remains open in any event, as synthetic peptides that are membrane-spanning have recently been proposed to exist in that configuration [42].

Since the four S4 segments in the Na^+-transducer protein exhibit four to six Arg (few Lys) groups, all of the proposed 3_{10}-helices would be strictly amphipathic and exhibit lengths of about 25 Å. At the outset of this description, it was assumed that carboxyl residues in the central α-helix were paired with Na^+. Thus, the excess of positive charge due to the guanidinium groups from the 3_{10}-helices would have to be neutralized by additional carboxylate groups, contributed by the protein. The corresponding segments would be expected to contain anionic groups in a conformation such that these would form amphipathic anionic bands constituting fully hydrogen-bonded segments [38]. Negative charges would be located periodically at distances of about 6 Å, as described above. With 2.2_7 helices, 2_7 ribbons, or β-strands, every other residue would be a carboxylate group. Indeed, there are four segments containing four to six acidic residues in the sequence of the protein [24] in alternating positions. They would span distances of 20–30 Å, depending on their precise conformation. A periodic array of anions at fixed 6 Å repeats could also be fulfilled by amphipathic α-helices, for which one segment (residues 748–760 according to ref. 24) could be a candidate. This segment has been predicted as being potentially membrane-spanning based on the criteria of weak evolutionary conservation (s_g in ref. 26; S6 in ref. 27).

3. Insulation of the ion wire

The set of ionic groups as described may now be envisaged to be arranged within a local ion lattice and thus act as the active core of the protein. Three of the four positions in any 'elementary unit' of the ionic lattice would be contributed by the protein, whilst the fourth, a 'vacancy', with coordinates also determined uniquely by the protein structure, would be filled by Na^+. Local as well as global electroneutrality would be conserved, and the hydrogen-bonding potential of the backbone peptide bonds would be satisfied [38,43]. This hypothetical structure would thus represent a sodium wire within an ion-exchange system [8] which would be provided by the protein.

Insulation of this hypothetical lattice from the

surrounding membrane domain could be provided by the highly hydrophobic, and presumably α-helical segments S5 and S6 of the four evolutionarily most conserved domains [24]. They may be complemented by segments among S1–S3 that contain few charged residues. If they were also α-helical, as seems to be suggested [44] by the highly conserved Gly-X-Y-Z-Lys positions at the ends of all segments S2, the charges present could be oriented towards the protein center, as they would all be confined [39] to angles within 120°, if projected onto a helical wheel. If one envisages 16 α-helical segments exposing no electrical charge on the face in contact with the membrane lipid phase, there would still remain enough polypeptide chain to form approximately nine additional potentially membrane-spanning fragments. These could, as it were, link the central ionic lattice with the hydrophobic insulating sheath. The sequences of these segments by themselves do not contain obvious information concerning their secondary structure. If potentially neighboring segments are considered, however, such as the periodic anionic bands as contraints, it seems apparent that *intra*segmental hydrogen bonding within such segments (in 2.2_7 or α-helices, or 2_7 ribbons) would not, a priori, put constraints on the configuration of other segments in the intermediary zone. Alternatively, if the anionic amphipathic structures existed in the β-configuration, several adjacent segments would be expected to exist in the same configuration, so as to allow the backbone hydrogen-bonding potential to be fully saturated by the formation of a β-barrel, allowing *inter*segmental bonds to be formed [39,43]. Clearly, prediction bearing on the intermediary zone is not possible, nor would it appear useful at this time. There seems, in any event, little cogent reason why the finding of a uniformly α-helical arrangement in the reaction center [23] and in bacteriorhodopsin [21], or the appararently exclusive occurrence of the β-structure in porin [45], should be taken to represent generally applicable rules.

4. Weighting prediction criteria

The uncertainty of the prediction in the intermediary zone may indeed deserve special emphasis. It is proposed in the present model that the core of the protein is an anionic α-helix, as suggested by the sequence of the protein from *Electrophorus electricus*. This sequence is not conserved, however, in other Na^+- or Ca^{2+}-transducing proteins [28,46]. The considerations on the possible polymorphism of the intermediary zone may therefore be worthy of a more detailed examination in the present context. It seems that the central α-helix could be replaced by a polyanionic bundle, drafted, among other segments, from acidic segments in the insert between homologous domains I and II in the protein from rat brain [28]. Alternatively, instead of a central sodium wire, there may be individual sodium wires in each of the four domains, all involving the S4 segment. From electrophysiological studies, it appears difficult to distinguish between a single pathway and four individual, though potentially interacting, pathways. This alternative might be tested rather readily by single-site deletion or site-directed mutagenesis [47], as proposed previously [39]. In any of these models, the highly conserved S4 fragments would constitute a framework within which evolution has adapted to the particular challenges of the function and environment of a particular protein. Needless to emphasize, it is not pretended that the proposal advanced here is correct. Yet, it may evoke the possibility that ionic conduction across a protein in the solid state may be considered as an alternative mechanism to the diffusion in an aqueous environment for the transport of ions across axonal membranes.

In principle, the mode of ion conduction suggested here should be testable by studying the effect of temperature on ionic flux. In the case of diffusion in solution, a linear dependence would be expected, for solid-state ionic conduction, an exponential relation would apply [35]. Measurements of Q_{10} have been reported [9,48] but, due to the limited temperature range available for biological experimentation, these results do not allow an unequivocal answer. The activation energies involved may be calculated to be within the range

of those of other electrolytes [49]. Molecular approaches, such as site-directed mutagenesis that has already been applied to the acetylcholine receptor protein [47], should prove revealing. An eventually detailed understanding of the mechanism at the molecular level will nonetheless have to await the results that only high-resolution structural methods can give.

5. Conclusion

(1) The model presented is a speculation that is based on the functional properties of the Na^+-transducing protein rather than on conventional structural criteria such as hydrophobicity and α-helical configuration. As a result of the various considerations, *an ionic lattice serving as an ion-selective sodium wire and as a gate is proposed.* It would be surrounded by a highly insulating mantle. The concepts used rely, in part, on the rather strong evidence that another transmembrane protein, porin, contains many polar as well as ionizable residues buried in its interior, and hence within the membrane domain [29,30].

(2) Although the general *physical principles applying to protein folding are the same* as those in soluble proteins, it may be misleading to assume that the vast body of determined structures may be transferred to a membrane protein such as the Na^+-translocating protein. *No precedent is known of a protein catalyzing vectorial charge flux at very high rates that is subject to fast and subtle regulation.* The knowledge of the high-resolution structure of a single membrane protein that provides an electron pathway presumably exhibits a fundamentally different structure [23].

(3) The highly specific predictions provided here and previously [39] should be considered as a test to determine whether *solid-state conductance* deserves consideration *as an alternative to diffusion across aqueous channels.* Of course, the details are certain to be incorrect. Yet, it seems nonetheless justified to consider the fundamentally different pathway as a possible mechanism for ion translocation across the membrane barrier.

(4) Disregarding any of the details proposed, the model is implicitly governed by two criteria. Firstly, electroneutrality must apply at both the local as well as global level. Secondly, it stipulates that the entire hydrogen-bonding potential, and this includes the backbone as well as side chains, must be saturated within the membrane domain. Specifically, fully saturating all possible hydrogen bonds of ion pairs (or their uncharged correlates) may actually greatly stabilize the structure of membrane proteins which exist in a hydrophobic environment. The unusual stability of bacteriorhodopsin, the reaction centers, and porin may highlight this point.

(5) The secondary structure of the zone presumed to insulate the ion wire from the transmembrane segments of the insulating has deliberately not been predicted. Conventional algorithms consider only *intra*segmental hydrogen bonds, whilst interactions among transmembrane segments cannot be excluded a priori.

Acknowledgements

The investigation reported here was funded by the Swiss National Science Foundation (grants 3.656.80 and 3.294.85) and the Roche Research Foundation. I thank my colleagues, notably R. Bookman, H.-J. Güntherodt and J. Nicholls, for most stimulating discussions. The secretarial help of E. Müller is gratefully acknowledged.

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