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Author's Review

Structural Bases of Membrane Protein Functioning*

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Nowadays membrane proteins arouse a keen interest as direct participants of numerous vitally important processes in the living cell. Of special significance here are the proteins involved in bioenergetic and transport functions as well as in transformation and transfer of various internal stimuli. These are various ATPases, fast sodium channels of excitable membranes and retinally-contained pigments, bacteriorhodopsin and animal rhodopsin. The present report is concerned with current advances in the study of the chemical nature and spatial organization and functioning of these systems.

ATPases, molecular ion pumps, occupy the key position among active transmembrane ion transporting systems. All these enzymes have much in common: disposition in the membrane, vectorial translocation of ions and utilization of ATP as an energy source. On the other hand, two types of ATPases — $F_1 \cdot F_0 \cdot$ ATPase (proton-translocating ATPase) and $E_1 \cdot E_2 \cdot$ ATPase (Na^+, K^+-, Ca^{2+}-, H^+-ATPases) considerably differ as far as the structural organization and enzymatic properties are concerned.

In order to elucidate the functioning of these ion pumps, the structure and spatial organization of Na^+, K^+-ATPase from pig kidney and H^+-ATPase from mitochondria are under close study.

Two types of subunits in equimolar ratio form Na^+, K^+-ATPase molecule: α- (96 kDa) and β-glycoprotein (40 kDa and 7 kDa for protein and carbohydrate moieties, respectively).[1] The ATP-hydrolyzing catalytic site is located on the α-subunit. The functional role of the β-glycoprotein remains obscure. Its carbohydrate moiety consists of two chains linked to the protein by N-glycosidic bonds. One of these chains is attached to the AsN_2 residue.[1]

Today the subunit primary structure is studied according to the conventional scheme involving parallel application of genetic engineering and protein chemistry techniques.

Membrane-bound Na^+, K^+-ATPase can be purified from the pig kidney outer medulla without breaking the native lipoprotein associations. We found that a short trypsin treatment of this preparation provided intensive cleavage of the catalytic α-subunit and preserved the integrity of β-glycoprotein. Wa-

* Dedicated to Professor Mihailo Lj. Mihailović on the occasion of his 60th birthday.

ter-soluble peptides of the hydrolyzate contained about 50% of the α polypeptide chain and were separated by HPLC. We obtained about 40 individual peptides and determined their complete or partial structures. These sequence data gave us the necessary information on the synthesis of specific oligonucleotide probes.[2] Information on clones containing cDNA of the corresponding mRNA of the α-subunit of Na^+, K^+-ATPase is available. Analysis of their structure and the amino acid sequence of peptides allows us to establish the complete structure of the α-subunit of Na^+, K^+-ATPase.

The available data on the subunit composition of the »functional unit« of the enzyme are rather contradictory. We determined the minimal molecular weight of the »functional unit« of membrane bound ATPase as the molecular weight of the protein unit completely inactivated upon site affinity modification with the alkylating ATP analog (ATP-γ-4(N-2-chloroethyl-N-methylamino) benzylamidate. At the point of complete inactivation specific incorporation of the reagent is equal to 2 nmol/mg of the protein, which corresponds to the binding of 1 reagent molecule to the protein structure (molecular mass 480—530 kDa). Thus the membrane-bound Na^+, K^+--ATPase is oligomeric complex $\alpha_4\beta_4$.[3]

With the aid of different immunochemical methods[4] antigenic determinants of both α and β were found on the outer membrane site, while on the inner site only antigenic determinants of α were revealed. So, only α penetrates the lipid bilayer, exposing large hydrophilic segments on both membrane sides. It means that the α-subunit has to participate in the construction of a cation-conducting pathway. Immunochemical data and fluorescamine labeling indicate that β projects only on the outer side of the membrane where its N-terminus with a carbohydrate chain is located.

This agrees with the results of studying the three-dimensional enzyme structure by electron microscopy of two-dimensional crystals.

The cell unit of these crystals has the following parameters: $a = 72$ Å, $b = 123$ Å and $\gamma = 77°$, and the thickness is 120 Å. The resolution is about 20 Å; the crystals belong to two-sided plane group *P*21. The cell unit is formed by two identical protomers αβ. Each protomer protrudes the membrane, and the major part of the protein is exposed on its surface (Figure 1).[5]

The H^+-ATPase from beef heart mitochondria is more complex than the most studied enzyme from *E. coli*. It contains a number of extra subunits and, like all mitochondrial H^+-ATPases, it is sensitive to the antibiotic oligomycin, which inhibits the ATPsynthase activity. Two proteins are responsible for the sensitivity of mitochondrial ATPase to oligomycin. They are the so-called »oligomycin sensitivity-conferring protein« (OSCP) and the coupling factor F_6. Both of them are required for the interaction of the catalytic (F_1) and proton translocating (F_0) moieties of the mitochondrial ATPase to yield a membrane-bound oligomycin and DCCD-sensitive ATPsynthase.

The complete amino acid sequences of these proteins were established.[6,7] The polypeptide chains of OSCP and F_6 consist of 190 and 76 residues, respectively, and both of them contain internal repeats. Thus, both proteins appear to have been evolved by a process of gene duplication. In addition, OSCP and F_6 have homologous structural elements, mostly manifested when comparing the following regions. The N-terminal part of F_6 is homologous with the C-terminal region of the *E. coli* subunit *c* including the DCCD-

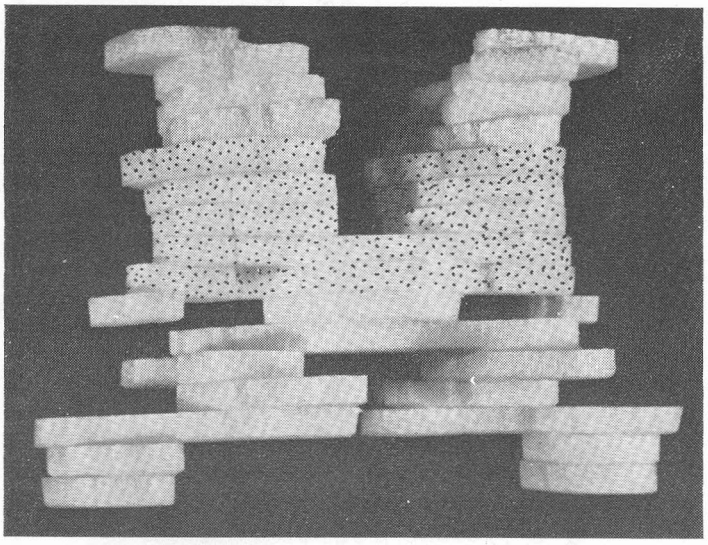

Figure 1. Three-dimensional model of Na^+, K^+-ATPase : ⁚ ⁚ intramembrane region.

-binding site, but the whole molecule F_6 has no counterpart in the bacterial enzyme.

Earlier OSCP was supposed to have no counterpart in chloroplasts and bacteria. Comparison of the amino acid sequences of OSCP and the δ-subunit of *E. coli* F_1 revealed a considerable structural homology between these proteins.

Comparison of the amino acid sequences of OSCP and the β-subunit of *E. coli* F_0, F_1-ATPase also shows homology between the repeating portion of OSCP and the central part of the β-subunit (Figure 2).[8] It was postulated that protein b occupies a central position in the *E. coli* ATPsynthase complex, providing a structural link between F_1 and F_0, and it is as important for H^+-translocation as for the biosynthetic assembly. Apparently, the H^+-ATPase complex of mitochondria lacks this protein since all the attempts to find it failed. This would suggest that OSCP in H^+-ATPase from beef heart mitochondria may play the same role as δ- and b-subunits in *E. coli* H^+-ATPase. Functional role of structural homologies is a point of further studies.

Halophilic microorganisms of the Halobacterium family can utilize solar radiation energy because of the presence of bacteriorhodopsin, a light-driven primary proton translocase. Bacteriorhodpsin is a relatively small protein (about 250 amino acid residues) containing protonated aldimine of the retina as a prosthetic group. In the cell membrane bacteriorhodopsin is concentrated in patches of about 0.5 μm, called purple membranes. Purple membranes are two-dimensional quasi-crystals formed by hexagonally packed protein trimers, the space between them being filled with lipid molecules. Each working cycle of bacteriorhodopsin induced by absorption of light quanta is accompanied by the transfer of at least one proton across the membrane.[10]

Our interest was focused on chemical and biochemical aspects of this unique membrane protein. No doubt such studies are necessary not only

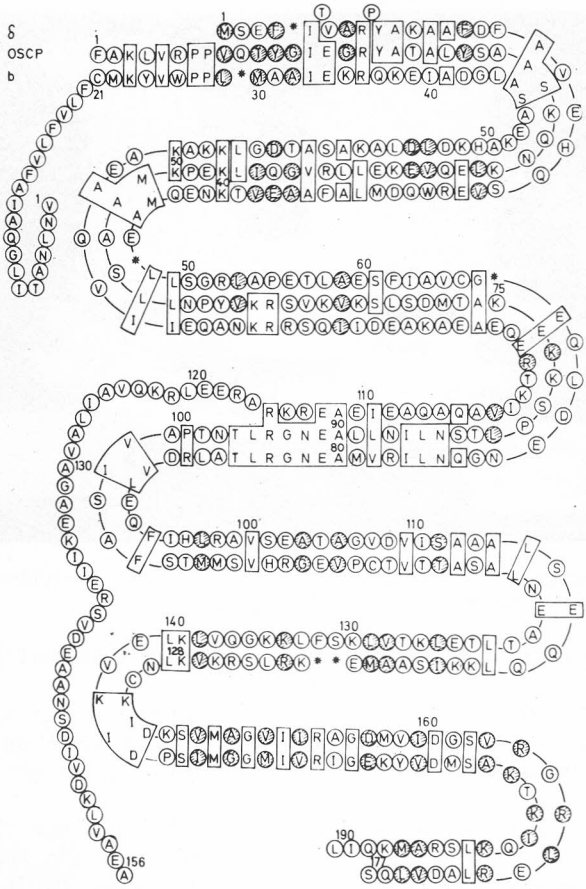

Figure 2. Comparison of amino acid sequences of OSCP and δ- and b-subunits of H^+-ATPase from *E. Coli*. Sequences of *E. coli* proteins are from lit.[9]

for elucidation of the mechanism of proton translocation by bacteriorhodopsin, but also for understanding the dynamics of functioning of even more complex light-transducing membrane proteins — visual pigments; halorhodopsin, etc. The amino acid sequence of the protein is known from both protein and gene sequencing.[11–13]

We started a detailed immunological investigation of bacteriorhodopsin, which proved to be useful in studying the orientation and surface topology of the protein. Khorana *et al.* used this approach to identify distinct antibody--binding sites on the cytoplasmic surface of bacteriorhodopsin that allowed identification of a peptide loop between α-helical segment 3 and 4, and conformation of the cytoplasmic location of the exposed C-terminal tail.[14]

In the course of immunological studies, we obtained five hybridomas producing monoclonal antibodies against different membrane-exposed parts of the polypeptide chain. Specificity of these antibodies was established using modified derivatives of bacteriorhodopsin and a number of overlapping peptides, derived from enzymatic or chemical cleavages of the protein.

The antigenic determinants are situated on the following exposed parts of bacteriorhodopsin: Glu^{1}-Glu^{9} with three N-terminal amino acids: Gly^{35}-Met^{56}, including Asp^{36} and/or Asp^{38} and Phe^{42}; Phe^{156}-Met^{163} with Phe^{256}; Glu^{194}-Leu^{207}, including residue Glu^{194}; and Pro^{200}-Leu^{207}. Thus, bacteriorhodopsin fragments 4—65 and 156—231 have membrane-exposed peptide regions. All experimentally obtained data and those concerning the membrane location of fragments 66—72 and 231—248 evidence that each of the sequences 4—65 and 156—231 traverses the membrane at least twice.[15]

Even more important is the accessibility of Glu^{194} to a monoclonal antibody. In the study of the chromophore orientation in bacteriorhodopsin by crosslinking using the photosensitive *n*-diaziridinophenyl, analogs of retinal Ser^{193} and Glu^{194} were shown to be the sites of crosslinking with the diaziridine group located at the phenyl ring. Based on this finding, a structural model with Glu^{194} well within the membrane was put forward.[14] Our result, however, proves that this residue, being a part of an antigenic determinant, should be located on the membrane surface.[15] Obviously, only further studies will establish the real topography of bacteriorhodopsin and its chromophore within the membrane.

Chemical or enzymatic modification of bacteriorhodopsin can give important structure-function information. We found that removal of the C-terminal 17 amino acid sequence did not affect the efficiency of the proton transport in bacteriorhodopsin.[16] However, the latest results show the light-induced proton release decreases by 50—70% in such preparations without affecting a photocycle.[17] The data now available confirm our finding that removal of the C-terminal tail influences neither the rate nor the efficiency of the proton transport. An apparent decrease in efficiency is due to aggregation of the protease-treated membrane sheets, the effect being completely prevented by a detergent.[18]

Along with these studies, we started an investigation into the genetics of halobacteria, searching for new ways of approaching the mechanism of proton translocation.

Bacterioopsin genes were cloned in *E. coli* using pBr 322 as a vector plasmid. This enabled the following:

1) investigation of the gene structure and the nature of gene-inactivating mutations;

2) creation of a basis for producing mutant bacteriorhodopsin by site-directed mutagenesis of the cloned gene;

3) investigation of the possibility of replication and expression of *H. halobium* genetic material in *E. coli*, connected with the fundamental problems of genetic engineering, since halobacterial DNA is far more alien to *E. coli* enzymatic machinery than, say, human DNA;

4) investigation of the prospects for production of a hydrophobic and unusual membrane protein such as bacterioopsin by *E. coli* cells.

Our data on the primary structure of the wild-type bacterioopsin gene are consistent with those published earlier by the Khorana group.[13]

We found out that genes isolated from bacterioopsin-deficient strains contain inserts within their coding sequences. In the case of *H. halobium* strain R1mR, the bacterioopsin gene contains an insert of 500 base pairs length. An insert in the S1 strain is about 1700 base pairs long. The inserts are called ISH2 and ISH S1 respectively.

Primary structures of inserts ISH S1 and ISH2 were determined together with the structures of the surrounding opsin gene reigons. In both cases there is a duplication of short stretches of the opsin gene DNA at the element-insertion site. These regions are represented only once in the wild-type gene. Moreover, there are inverted nucleotide repeats at the ISH2 and ISH S1 termini. Both mentioned features are characteristics of the majority of known transposable elements in both prokaryotes and eukaryotes.[10,11,19,20]

To detect the level of expression, we decided to exploit the well-known fact that β-galactosidase of *E. coli* enzymatically forms functional hybrids if its several N-terminal amino acids are exchanged for some other polypeptide. It is easy to test the expression of any foreign protein in *E. coli* if its C-terminal part is fused to the gene coding β-galactosidase with several N-terminal codons cut off. Such a hybrid gene placed under promoter control programs a synthesis of the hybrid protein possessing galactosidase activity and containing a polypeptide chain of the foreign protein.

Thus, we fused the bacterioopsin gene to the *lac-Z* gene (a gene of β-galactosidase) and placed this hybrid gene downstream the *E. coli* tryptophan promoter also containing the ribosome-binding site. This plasmid POG contains the full bacterioopsin gene. It means that this gene encodes an opsin precursor 13 amino acid longer than the mature opsin. Judging by the β-galactosidase activity, the level of expression is rather low.

We removed the signal peptide and changed the system of expression regulation. The POG 1 plasmid was reconstructed in the following way. The precursor region of the opsin gene was removed, and a strong promoter of phage λ-179, containing its own ribosome-binding site and initiating ATP--codon, was placed upstream the gene. High galactosidase activity implies a high level of hybrid protein expression (up to 1% of the total cellular protein). The investigation into site-directed mutagenesis of the bacterioopsin gene, aimed at the production of mutated bacterioopsin and their functional studies, was started.

Sodium channels of electrically excitable membranes are the main functional components of the system of nerve impulse generation. Among other components of sodium chanels, the glycoprotein of molecular mass about 260 kDa is identified; in case of mammalian brain membranes it is linked to other proteins.

Sodium channels of rat brain membranes form an equimolar complex of three glycoproteins of molecular masses 260 (γ), 39 (β_1) and 37 (β_2) kDa; α- and β_2-subunits are bound by disulfide bonds.[21,22] The channel subunits contain no free α-amino groups and are characterized by a high content of hydrocarbon components (Table I).[22] The functional role of the subunits is still unclear. However, the voltage clamp technique reveals that polyclonal antibodies produced against the α- and β_2-subunits are capable of blocking the sodium conductivity of the membranes of the isolated neuron from rat brain as well as affecting the process of sodium channel inactivation.

Electron microscopy of the sodium channel shows that its molecule has a form of frustum of 45 · 55 · 110 Å in size. By the method of digital image processing of single protein molecules we succeeded in obtaining a projection of the channel structure on the membrane plane. Noteworthy is the clearly expressed similarity of this projection to the analogous structural characte-

TABLE I

Amino Acid and Carbohydrate Composition of Sodium Channel Subunits

	α (260 kDa)	β_1 (39 kDa)	β_2 (37 kDa)
Asx	10.65	9.47	10.82
Thr	5.1	6.24	6.18
Ser	7.82	7.09	8.31
Glx	11.73	14.04	13.66
Pro	4.45	3.06	4.11
Gly	6.50	7.34	9.55
Ala	6.49	7.92	5.37
Cys	0.91	0.92	0.98
Val	5.9	7.66	6.99
Met	2.87	2.30	1.89
Ile	5.12	4.67	3.52
Leu	9.61	8.25	8.34
Tyr	2.57	3.59	3.0
Phe	5.51	4.61	3.91
His	2.34	1.79	1.87
Lys	6.13	5.79	6.43
Arg	4.97	4.45	4.7
Trp	1.32	0.8	0.38
Fuc	4.33	3.29	5.32
Man	13.49	22.29	19.73
Gal	13.75	13.78	19.58
Glc NAc	17.6	19.41	22.17
Gal NAc	4.7	4.01	4.51
Sialic acid	46.14	37.22	28.68
Protein, %	79.5	72.1	68.12
Carbohydrate, %	20.5	28.9	31.88

ristic of the acetylcholine receptor (Figure 3). Such coincidence allows the general principles of the spatial structural organization of membrane proteins, forming ionic channels, to be proposed.

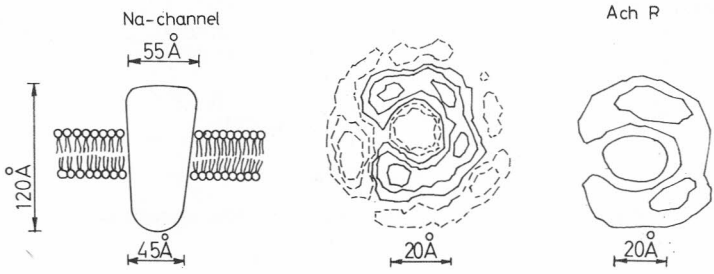

Figure 3. Model of the sodium channel into membrane and projections of the channel and AchR structures on the membrane plane.[23]

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SAŽETAK

Strukturne osnove djelovanja proteina membrane

Yuri A. Ovchinnikov

Zbog neposrednog sudjelovanja u brojnima za život važnim procesima koji se odvijaju u stanicama živih bića membranski proteini pobuđuju široki interes. Od posebnog su značenja proteini koji sudjeluju u bioenergetskim i prijenosnim funkcijama te u pretvorbama i prijenosu raznih unutarnjih podražaja. To su razne ATPaze, pigmenti rožnice, bakteriodopsin, životinjski rodopsin i sl. Dan je pregled novijih dostignuća istraživanja kemijske prirode, prostorne organizacije i djelovanja tih sistema.