===== REVIEW =====

Lel A. Drachev and the Direct Electrometric Method

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Abstract—In the bioenergetics studies, the direct electrometric method played an important role. This method is based on measuring the electrical potential difference ($\Delta\psi$) between two compartments of the experimental cell generated by some membrane proteins. These proteins are incorporated into closed lipid–protein membrane vesicles associated with an artificial lipid membrane that separates the compartments. The very existence of such proteins able to generate $\Delta\psi$ was one of the consequences of Peter Mitchell's chemiosmotic concept. The discovery and investigation of their functioning contributed to the recognition of this concept and, eventually the well-deserved awarding of the Nobel Prize to P. Mitchell. Lel A. Drachev (1926-2022) was one of the main authors of the direct electrometrical method. With his participation, key studies were carried out on the electrogenesis of photosynthetic and respiratory membrane proteins, including bacteriorhodopsin, visual rhodopsin, photosynthetic bacterial reaction centers, cytochrome oxidase and others.

INTRODUCTION

Lel Aleksandrovich Drachev started his work at the Interfaculty Research Laboratory of Molecular Biology and Bioorganic Chemistry (hereinafter, referred to as Interfaculty Laboratory; currently, Belozersky Institute of Physical-Chemical Biology of the Moscow State University) in 1973 as a Chair of the newly established Department of New Physical Methods. He was offered this position by Rem Viktorovich Khokhlov, Rector of the Moscow State University, who had been appointed shortly before that and who was Drachev's course mate at the Physical Faculty of Moscow State University, through the intermediary of another course mate, well-known biophysicist Efim Arsentyevich Liberman. At that time, Liberman, who several years before had developed the method of membrane potential measurements with synthetic penetrating ions, closely collaborated with the newly appointed Director of the Interfaculty Laboratory, Vladimir Petrovich Skulachev and his co-workers.

MITCHELL'S HYPOTHESIS AND SEARCH FOR APPROPRIATE RESEARCH METHODS

Direct electrometric method is based on the concept of transmembrane electric potential difference ($\Delta\psi$) formed on the intracellular, the so-called coupling, membranes of mitochondria and chloroplasts and on the inner bacterial membranes. This concept has stemmed from the chemiosmotic theory of coupling between the respiratory and photosynthetic electron transfer and phosphorylation suggested by the future Nobel laureate P. Mitchell. The chemiosmotic theory considered the transmembrane difference of electrochemical potentials of hydrogen ions ($\Delta\mu_{H^+}$) as an intermediate form of energy in the processes of oxidative and photosynthetic phosphorylation. Existence of $\Delta\mu_{H^+}$ on the coupling membranes proved to be one of the experimentally verified statements of the Mitchell's hypothesis, and the efforts of research teams headed by Skulachev and Liberman, who were among the first to accept the chemiosmotic theory, had been focused on proving it. Note that as it

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has become clear much later, $\Delta\psi$ is not always the main component of $\Delta\mu_{H^+}$. In chloroplasts, $F\Delta\psi$ (where F is the Faraday constant) is much lower than the concentration component of $\Delta\mu_{H^+}$ ($-RT\Delta pH$) [1, 2]. In the sodium bioenergetics, which was discovered later, the transmembrane electrochemical potential of sodium ions plays the role of the membrane form of energy, which allowed to generalize the Mitchell's concepts [3]. Here, we will omit the discussion between Mitchell and R. Williams about the role of protons in membrane energization, as it has been already described in detail in the literature (e.g., see the article by Weber and Prebble [4]).

Selection of an appropriate research method was essential for $\Delta\psi$ evaluation, so development of quite different methods for measuring $\Delta\psi$ had started at that time almost simultaneously. Apparently, it was the discussion of the Mitchell's hypothesis that has initiated the outburst of research activity in the field. In 1968, Junge and Witt [5] used electrochromic shift in the absorption spectrum of chlorophyll *b* (though they did not refer to it as such) to study photoinduced generation of the transmembrane potential and ion transport in photosynthesis in isolated spinach chloroplasts. A little later, Bulychev et al. [6-8] developed a microelectrode method for measuring $\Delta\psi$ that was used to study giant (up to 15-25 μm) chloroplasts of the higher plant *Peperomia metallica*, both isolated and *in vivo*. The method for measuring electric potential in a light gradient was proposed shortly afterwards [9, 10]. This method used macroscopic changes in $\Delta\psi$ (and consequently, macroelectrode techniques to record them) that originate due to the summation of dipole moments of individual vesicles in an optically dense suspension of chloroplast thylakoids. Here, we will not discuss these methods, which have been widely used later and provided a lot of valuable data on the light-dependent reactions of photosynthesis. Instead, let us get straight to their limitations, as this will be important in the context of further article. The method for recording transmembrane potential based on the measurement of electrochromic shifts of photosynthetic pigments (mainly carotenoids), is widely used in the studies of plant chloroplasts and chromatophores of purple bacteria; however, it proved to be inefficient for investigating cyanobacteria and isolated pigment−protein complexes in model systems. Also, this method provided a relatively low signal to noise ratio, a problem that was largely solved only 1.5 decades later in the works of Joliot and Joliot [11].

The methods based on the microelectrode technique have provided an important information on the light-dependent currents generated on the chloroplast membranes but could not be extended to smaller objects (including proteoliposomes and chromatophores of purple bacteria). Unavoidably high active resistance of microelectrodes also limited the time resolution of this method (about 1 ms). The light gradient method has
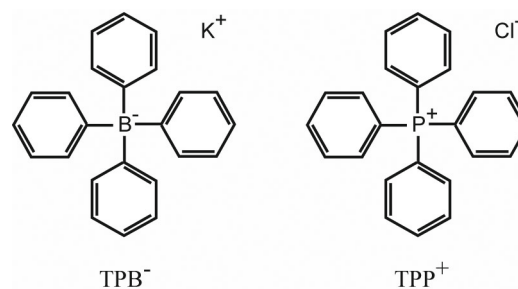

Fig. 1. Lipophilic tetraphenylboron (TPB⁻) and tetraphenylphosphonium (TPP⁺) ions.

allowed to record the kinetics of membrane potential generation within a time interval from 20 ps to 50 ns; however, in the case of slower processes, recording was limited by the dissipation of the formed electric potential gradient due to the ionic conductivity of the buffer solution in the suspension of membrane vesicles.

Further development of methods for measuring in a light gradient using multilayered electrically oriented fragments of protein−lipid membranes has followed a decade and a half later [12]. Switching to gels and decreasing ionic conductivity has allowed to raise the upper limit of the time window [13]. We should mention that the initial variants of the method described below, which was developed by Liberman and Skulachev, as well as of the direct electrometric method that has evolved from it, also has limitation with respect to many of the above-mentioned parameters.

Liberman and Skulachev have solved this problem by coming up with an ingenious idea of synthetic penetrating ions. "Penetrating (through the membrane) ions" seemed as an oxymoron at that time, because the charge of an ion obviously makes it hydrophilic and creates an energy barrier to its immersion in the hydrophobic environment of the membrane. However, Liberman assumed that the barrier could be considerably reduced by distributing the charge over a large volume of the particle or surrounding it by relatively hydrophobic molecular groups. The search for such "lipophilic ions" in the collections of colleagues working in chemistry had been successful and helped to confirm the original idea: some of the found compounds, in particular, phenyl dicarbaundecaborane (PCB⁻) and tetraphenylboron (TPB⁻) anions and tetraphenylphosphonium (TPP⁺) cation turned out to be able to easily penetrate through the membrane (Fig. 1). At the same time, their distribution on the two sides of the membrane was determined, in accordance with the Nernst equation, by the electrochemical gradient $\Delta\mu_{H^+}$: positively charged ions accumulated inside the mitochondria that generated $\Delta\psi$ on the inner membrane (with a negative sign inside in the case of mitochondria and positive sign in the case of submitochondrial particles) due to the process of oxidative phosphorylation.

As a result, the concentration of these ions in the suspension decreased, which could be recorded potentiometrically [14]. This approach has demonstrated for the first time that the process of cellular respiration is accompanied by the $\Delta\psi$ generation on the membranes of mitochondria and submitochondrial particles, which could be suppressed by respiratory inhibitors or uncouplers of oxidation and phosphorylation [15].

SYNTHESIS OF TWO APPROACHES

In 1971, Oesterhelt and Stoeckenius [16] published an article in *Nature New Biology*, in which they described a rhodopsin-like protein from the purple membranes of *Halobacterium halobium*. At the initiative of Skulachev, a strain of *H. halobium* was brought to Moscow and the protein bacteriorhodopsin (bR) capable of light-dependent proton translocation across the membrane was isolated from the purple membranes. The studies of this membrane protein at the Department of Bioenergetics and then at the Department of New Physical Methods of the Interfaculty Laboratory were the first step on the path to the development of direct electrometric method.

In the summer of 1973, Drachev moved from the All-Union Institute of Scientific and Technical Information (VINITI) to the Interfaculty Laboratory. Together with the graduate student A. D. Kaulen, he performed direct measurements of the electric potential generated by bR embedded in a flat artificial lipid membrane (ALM) separating two sections of a Teflon cell. bR was incorporated in the membrane directly from a phospholipid solution in n-decane, to which it had been added in a solubilized form in the presence of detergent [17]. In contrast to the indirect measurements with the involvement of penetrating ions, this approach allowed direct measurements of $\Delta\psi$ generation by the protein. However, it was characterized by considerable variations in the values and even directions of photoinduced signal from one experiment to another. Later, it has become clear that these variations were associated with random orientation of bR in the membrane: approximately the same amounts of protein were incorporated in the membrane in the "plus" and "minus" orientations, and even minor variations in their ratio led to significant changes in the determined difference between the signals and fluctuations in the sign of $\Delta\psi$.

In the autumn of 1973, one of the authors of this article (A.Yu.S.), who had just graduated from the Department of Molecular Biology, Faculty of Biology, Moscow State University, became involved in this work to study bR-containing proteoliposomes, which had been introduced in the research practice shortly before by Kagawa and Racker, specialists in bioenergetics [18]. bR incorporated in proteoliposomes generated transmembrane potential in response to illumination [19].
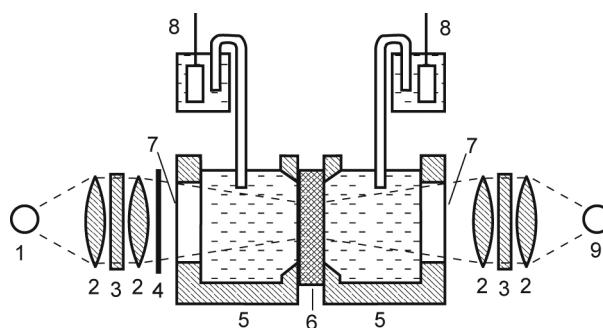

Fig. 2. Scheme of the experimental system: 1) continuous light source (halogen lamp); 2) lens; 3) optical filter; 4) shutter; 5) Teflon cuvette; 6) ALM formed on membrane porous filter; 7) glass window; 8) AgCl electrode; 9) pulse laser.

The experimental system was a cell consisting of two sections filled with an electrolyte solution and separated with a flat ALM applied onto a hole in the partition between the sections. Silver chloride electrodes connected to an electrometric voltmeter were submerged into both sections of the cell to measure changes in the potential difference between the sections throughout the experiment (Fig. 2). Penetrating anions (PCB⁻) were added to both cells, and bR proteoliposomes were added to one of them. When the cell was illuminated, PCB⁻ ions were absorbed by proteoliposomes due to the generation of transmembrane potential, resulting in the generation of potential difference between the sections. Although the amplitude of this potential difference was small (~0.01 V), it was sufficient for reliable measurement.

As we know from the history of science, discovery of a new phenomenon is often a matter of chance. Becquerel stored uranium salts together with photographic plates; Pasteur kept tartaric acid crystals for too long; Fleming failed to prevent Petri dishes from fungal contamination. In our case, ALMs were not stable enough during long-term experiments and periodically lost their resistance because of electrical or mechanical fluctuations. As a result, the photoelectric signal disappeared and a drop of phospholipid solution had to be injected into the hole in the Teflon cell partition to form the ALM again. Another factor contributing to the discovery of this effect, which had become the key element in the direct electrometric method development, was high concentration of ammonium ions in the medium. Since the electrochemical potential on the liposome membrane could contain both electrical and concentration (pH-dependent) components, to maximally improve the measured electric signal, it was decided to reduce the "competing" transmembrane pH gradient. For this purpose, ammonium salts were added to the medium at a significant concentration (0.1 M) as a pH buffer penetrating into proteoliposomes. Under these conditions, repeated formation of ALM was accompanied by the increase in the photoelectric signal with time, which was difficult to explain within the framework of existing ideas

on the action mechanism of penetrating ions. The fact that this effect was observed at high salt concentrations suggested that it could be related to changes in the surface charges. When the singly-charged ions in the background electrolyte were replaced with lower concentrations (10-20 mM) of doubly-charged ions (Ca^{2+} and Mg^{2+}), the signal increased much more rapidly. This suggested the shielding of fixed negative charges of phospholipids by cations of the background electrolyte, in which doubly-charged cations were much more efficient than singly-charged ones. The shielding of surface charges on the proteoliposome membranes and ALM should have accelerated proteoliposome aggregation and adsorption on the ALM separating the cell sections. This implied that the increase in the light-induced $\Delta\psi$ occurred due to the activity of bR in the membranes of proteoliposomes attached to the ALM. To verify this suggestion, Liberman proposed to perform the same experiment in the absence of penetrating ions. Indeed, even without the addition of PCB⁻ to the medium, incubation of proteoliposome suspension for 30 min in the presence of 20 mM $CaCl_2$ was accompanied by a gradual increase in the amplitude of photoinduced $\Delta\psi$ up to ~250 mV.

Unlike the method of ALM formation by mixing a bR suspension in detergent with a solution of phospholipids in n-decane, which had been originally used by Drachev and Kaulen, experiments with bR incorporated into liposome membranes showed a high degree of orientation asymmetry: more than 95% protein molecules were oriented with the "plus" directed inward (this was proven later using lanthanum ions inhibiting the activity of bR upon its binding in the "entrance" proton channel on the proton-donor side). As a result, light-induced $\Delta\psi$ always had the same sign and a relatively small dispersion of values that was mostly determined by the accuracy with which the total internal volume of proteoliposomes was controlled. Apparently, such asymmetry of protein incorporation was due to the asymmetry of the membrane that had a nonzero curvature in the case of closed spherical vesicles, while flat ALMs lacked the curvature that could provide the asymmetry of protein incorporation.

Therefore, both methods (based on synthetic penetrating ions and mixing bR with a solution of phospholipids) had their pros and cons. The discovery of proteoliposome adsorption on the ALM surface allowed to create a "hybrid" of these two methods by combining their advantages: a high degree of asymmetry of protein incorporation into proteoliposome membrane (and, therefore, higher signal value) typical of the indirect method of synthetic penetrating ions and direct measurement of protein-generated potential in the direct electrometric method. Further development of this hybrid method, that has received the name of direct electrometric method, or Drachev's method, has been associated with the works of Drachev and his coworkers A. D. Kaulen and A. Yu. Semenov.

PROOF OF EQUIVALENT CIRCUIT AND SELECTION OF ARTIFICIAL MEMBRANE

"Hybridization" of the two methodological approaches has provided new experimental opportunities but simultaneously posed new problems. Above, we mentioned association of proteoliposomes with flat ALM surface. But was this protein−lipid system equivalent to the one formed by direct protein incorporation in the ALM in the presence of detergent (except for the differences in the asymmetry of incorporation)? This is what correct quantitative interpretation of measurements depended on. The systems would be equivalent if after adsorption on a flat ALM, the membrane of a proteoliposome ruptured, proteoliposome phospholipids "spread" over the ALM, and membrane proteins were incorporated in the ALM. However, an alternative scenario is possible, when proteoliposome membranes do not undergo topological changes and proteoliposomes remain associated with the ALM surface as closed vesicles. In this case, proteoliposomes retain aqueous phase inside (Fig. 3a). At the same time, hypothetically, there might be also different variants of protein distribution, as proteins could be transferred from the proteoliposome membrane into the ALM or remain in the proteoliposome membrane. Later, a similar question was raised for the distribution of a pool of ubiquinone-10 molecules in the chromatophore membranes in the studies using chromatophores of photosynthetic bacteria (natural closed vesicles). However, if hydrophobic ubiquinone freely redistributed between the chromatophore membranes and ALM, no such exchange was observed for protein molecules. The proof that the aqueous phase (which is referred to as the "third water" in the literature) was retained inside the vesicles and all proteins were located on the vesicle external surface was based on the measurements of potentials generated in the presence of protonophore uncouplers of different types. For example, gramicidin A, which forms channels in the membrane, substantially reduced the potential generated by bR upon illumination, but had almost no effect on the total electrical conductivity of the membrane. At the same time, the "shuttle" protonophores (e.g., carbonyl cyanide-m-chlorophenylhydrazone) decreased both the light-dependent potential and the membrane conductivity [20]. Such difference could result only from the presence of two types of membranes in the system. Gramicidin formed channels in the bilayer membrane containing $\Delta\mu_H^+$ generators (bR), but not in a relatively thick flat ALM that separated the compartments of the experimental system and determined conductivity of this system; although ALM remained permeable for the "shuttle" protonophores (Fig. 3b).
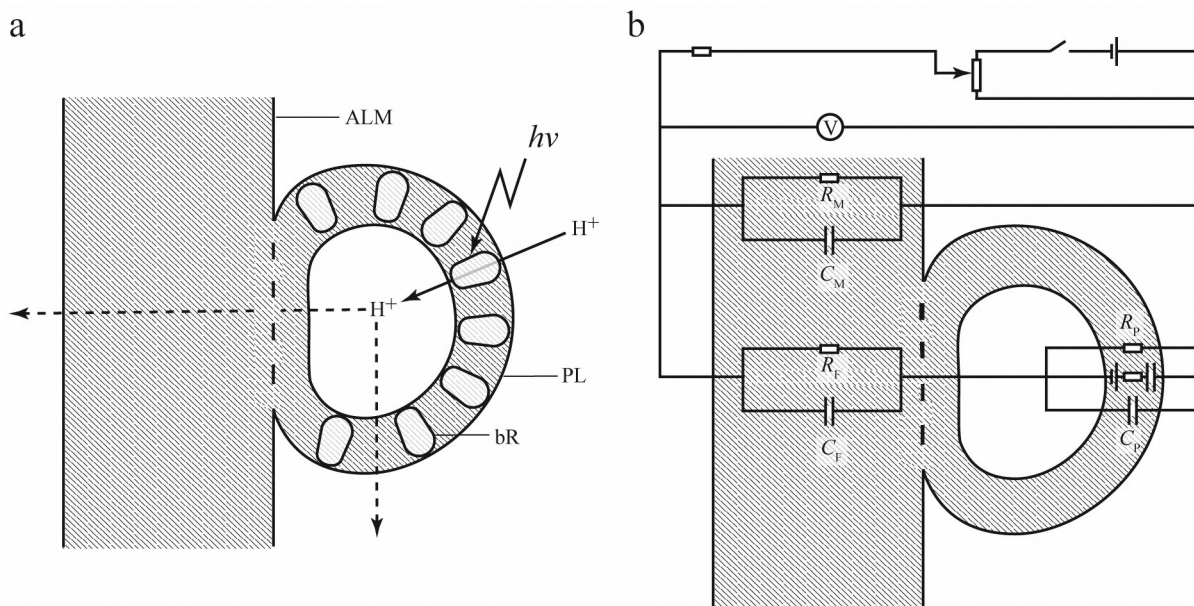
Fig. 3. Interaction between bR-containing proteoliposome (PL) and flat phospholipid ALM (a) and equivalent circuit diagram (b). Orientation of bR molecules in the PL membrane is asymmetric (~95% molecules transferred protons inside PL). According to the experimental data, bR was almost absent in the area of membrane fusion. V, electrometric voltmeter with high input resistance; C_P, R_P, C_M, R_M, C_F, R_F, electrical conductivity (C) and resistance (R) of PL membrane, ALM, and area of membrane fusion, respectively. The image is out of scale, as the ALM thickness exceeds the PL membrane thickness by several orders of magnitude.

Experiments with direct incorporation of protein molecules into a flat membrane conducted by Drachev and Kaulen, as well as experiments of Semenov with proteoliposomes, were performed using thick (about 100-µm) phospholipid membranes separating two compartments of a Teflon cell. Although proteins cannot not penetrate such membrane or transfer a proton across it, their incorporation into the membrane, nevertheless, transformed it in an analog of H$^+$-selective electrode, which made possible the recording of an electric signal. However, such membrane had one significant drawback: because of a significant volume of lipids, it had low electrical capacity, resulting in a considerable increase in the electrical response time. The minimum characteristic time of $\Delta\psi$ generation that could be obtained in this system was about 10 ms, which was significantly higher than the characteristic times of main processes of charge transfer in bR, so experiments on the kinetics of this transfer required much thinner membrane. Unfortunately, a phospholipid bilayer (analog of natural membrane) could not be used in this case, because despite numerous attempts, the bilayer membrane (referred to as "black membrane" due to reduced optical transmission resulting from the interference of light reflected from the two surfaces) remained extremely unstable. The attempts to incorporate proteins into the "black membrane" failed as well, since addition of detergent-containing proteins to the electrolyte surrounding the membrane resulted in membrane rupture. The attempts to use flat bilayer membranes in the electrometric method had been abandoned, demanding the search for new approaches to the problem.

This search for other variants of flat ALM has been very long and difficult. It became obvious that such membrane had to be somehow "reinforced" to increase its strength, and various porous membrane filters soaked in a phospholipid solution have been tested as rigid base for the ALM. Although the filters themselves were rather thick (about 0.1 mm, which is similar to the thickness of thick phospholipid membrane), there was hope that the phospholipid films formed over the filter pores would be thinner. These studies have also shown that not only the filter material and pore size, but also the nature of the vesicle was important for the development of the experimental system. Thus, almost all tested types of filters could be used for the measurements with bR-containing proteoliposomes, while the results of studies of chromatophores were affected by the filter properties. Filters made of chemically inert materials (Fluoropore, Teflon) provided the maximal electric responses that were comparable with the potentials obtained with the phospholipid membrane (up to 0.25 V; [21, 22]) irrespectively of the pore diameter. Electrical responses recorded for chromatophores (but not proteoliposomes) with cellulose-based filters were lower (and even less for cellulose acetate−nitrate filters compared to pure cellulose nitrate filters). In the case of cellulose acetate-containing filters, the results also depended on the pore size, as larger pores ensured higher values of measured potentials [21]. However, irrespectively of the filter used, the same insurmountable time resolution limit remained − about 10 ms.

In addition to the use of available commercially filters, there were attempts to create suitable "frames"

using contemporary "high technologies". This brings to mind the visit of Drachev and his young colleagues to the Laboratory of Nuclear Reactions headed by G. N. Flerov at the Joint Institute for Nuclear Research in Dubna, where the scientists synthesized their own filters for different technical tasks by placing polymer films under a particle accelerator beam which turned them into "sieves". The diameter and distribution density of pores could be varied by varying the beam parameters. However, none of such filters turned to be suitable for the membrane experiments, as a phospholipid membrane failed to settle on long narrow cylindrical pores and did not impregnate the filter.

Finally, the filters that were found by Drachev and his colleagues and proved to be the best for their studies, were nitrocellulose collodion films. These films were actively used as supports in electron microscopy and, therefore, were available. Nitrocellulose collodion films consisted of long cellulose filaments that form a small number of layers and had a comparatively small thickness (~200-300 nm). When impregnated with the phospholipid solution, they ensured sufficient membrane stability and, simultaneously, a fast electrical response (about 200 ns). Most of the following studies at the Interfaculty Laboratory were performed using collodion ALMs. The search for this key component of the measurement system took almost five years.

MAIN RESULTS OBTAINED BY DIRECT ELECTROMETRIC METHOD

From 1974 to 1980, experiments using a combination of thick phospholipid membrane and closed vesicles (proteoliposomes containing various membrane proteins or chromatophores) have demonstrated generation of potential difference by bR, visual rhodopsin, mitochondrial cytochrome c oxidase, ATPase, and transhydrogenase, as well as by chromatophores of photosynthetic bacteria [23-27]. In 1979-1980, analogous measurements were performed using different membrane pore filters impregnated with a phospholipid solution in n-decane. A typical photoelectric response of the membrane filter/bR-containing proteoliposome system to light switching on and off is shown in Fig. 4. In this case, illumination caused rapid generation of $\Delta\psi$ with a positive sign inside the proteoliposomes and amplitude up to ~200 mV. The photoelectric signal remained at approximately the same level for at least 10 min of light exposure and completely decayed when light was switched off. When the system was kept in the dark, the light-dependent potential with an amplitude of >150 mV was maintained for 72 h and then decayed simultaneously with the decrease in the ALM resistance.

In the previous section of this article, we mentioned that when using "thick" phospholipid ALMs and
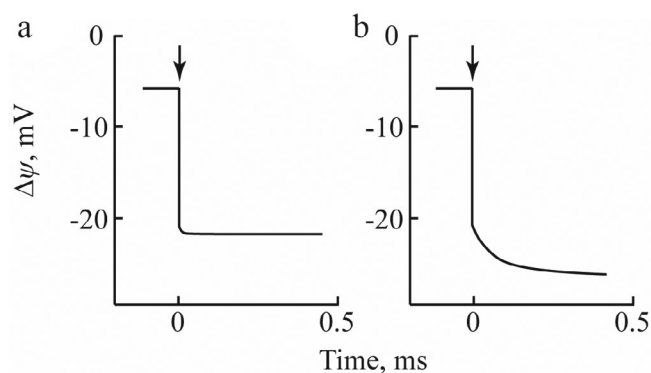

Fig. 4. Photoelectric response of proteoliposomes containing bacterial photosynthetic RC without additions (a) and in the presence of 2 mM ascorbate and 2 µM natural electron donor cytochrome c (b).

ALMs based on a membrane filter, low-capacity C_M comparable with the input capacity of the operational amplifier (~5 pF) distorted the kinetics of $\Delta\psi$ generation in response to a laser flash. Therefore, rapid kinetics was studied using collodion phospholipid ALMs (~200 nm thick) with the C_M value of ~5000 pF. This system characterized by the time resolution of 0.2 µs, was used to investigate the kinetics of laser-induced $\Delta\psi$ generation in proteoliposomes containing bR and bacterial RCs and chromatophores from photosynthetic bacteria [28-30].

As shown in Fig. 4a, in the system of collodion ALM/proteoliposomes with incorporated photosynthetic RC from *Rhodobacter sphaeroides*, laser flash in the absence of electron donor caused generation of $\Delta\psi$ with a negative sign inside with an amplitude of ~15-20 mV due to the formation of the ion–radical pair $P_{870}^+Q_A^-$ during electron transfer from the primary donor of *R. sphaeroides* RCs (bacteriochlorophyll dimer P_{870}) to the acceptor (ubiquinone Q_A incorporated in the RC) [31]. Upon addition of 2 mM ascorbate and 2 µM cytochrome c_2 (native electron donor), kinetics of the enhanced photoelectric response, apart from the quick phase, showed an additional slower component with the characteristic time $\tau = 250$ µs (Fig. 4b). The observed increase in the $\Delta\psi$ generation was due to the electrogenic reduction of P_{870}^+ by cytochrome c_2. Beside electrogenic reactions resulting from the reduction of oxidized primary donor P^+ by different c-type cytochromes, the use of chromatophores from *R. sphaeroides*, *Rhodospirillum rubrum*, *Ectothiorhodospira shaposhnikovii*, *Chromatium minutissimum*, and *Blastochloris viridis* demonstrated the presence of one more electrogenic component associated with protonation of the secondary quinone acceptor Q_B from the external aqueous phase in response to even-numbered light flashes [32]. Chromatophores from *R. sphaeroides* were also used to study laser-induced electrogenic responses caused by electron and proton transfer in the cytochrome bc_1 complex [33].
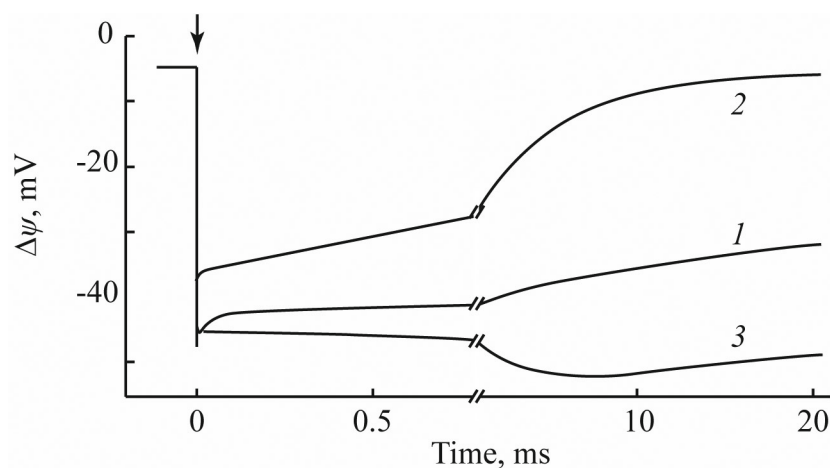
Fig. 5. Photoelectric response of PSI-containing proteoliposomes: *1*) without additions; *2*) in the presence of sodium dithionite; *3*) in the presence of 10 mM ascorbate and 0.4 mM plastocyanin (native PSI external donor).

A similar approach was used to study electrogenic responses in proteoliposomes containing photosystem (PS) I and II complexes from cyanobacteria and higher plants [34, 35]. Figure 5 shows typical photoelectric responses of proteoliposomes containing PSI complexes from the cyanobacterium *Synechocystis* sp. PCC 6803.

In the absence of additions (curve *1*), these preparations demonstrated a photoelectric response with the negative sign inside and amplitude of ~50 mV that decayed with $\tau = 100$ ms. Addition of a strong reducer (100 mM sodium dithionate) slightly decreased the amplitude of $\Delta\psi$ and accelerated its decay to several milliseconds (curve *2*). This effect was due to the reduction by dithionite of the terminal 4Fe4S clusters F_A/F_B; as a result, the decay of $\Delta\psi$ occurred from the preceding 4Fe4S cluster F_X. At the same time, addition of 0.4 M plastocyanin caused an appearance of an additional electrogenic component with $\tau = 3$ ms (curve *3*) resulting from the electrogenic reduction of the photooxidized PSI primary electron donor (chlorophyll dimer P_{700}^+) by plastocyanin.

One of the important features of direct electrometric method is that can be used to evaluate the distribution of dielectric permittivity (ε) in hydrophobic membrane proteins containing redox cofactors. The transfer of a charge (electron or proton) from one group to another inside the protein causes a change in the potential difference between the two sides of the membrane that depends both on the distance over which the charge is transferred in the membrane (or more precisely a transfer component, which is normal to the membrane plain) and the ε value of the protein domain where this transfer takes place. The potential difference caused by the charge transfer is lower in a domain with a greater number of polar amino acid residues and, consequently, higher ε value, but is greater in the domains with hydrophobic residues (and low ε value). Evaluations of

the distances between cofactors (based on X-ray structure) have allowed to employ the data of direct electrometric method to estimate the average ε value in protein regions between two cofactors. The use of this method made it possible to determine ε distribution in the bacterial photosynthetic RC from *R. sphaeroides*, PSI from cyanobacteria, and PSII from higher plants [36, 37]. Of course, these data were merely evaluative because the polarity of adjacent protein domains also affects the value of the measured $\Delta\psi$. However, estimated ε values were in good agreement with the data on the distribution of polar and nonpolar residues in proteins. Figure 6 shows the distribution of PSI dielectric permittivity along the normal to the membrane plain estimated by direct electrometric method [38]. One can see that it is in agreement with the distribution of polar groups in the structure of protein complex. The proportion of atoms carrying a noticeable partial charge (side-chains of Asp and Glu marked in blue and those of Lys, Arg, and His marked in red) and causing the dielectric response of the protein atomic subsystem determined based on the X-ray structure analysis of the PSI three-dimensional structure is presented in the same graph. In the figure, redox cofactors (primary electron donor P_{700}, primary and secondary acceptors A_0 and A_1 in the two branches of redox cofactors *A* and *B*, and 4Fe4S acceptors F_X, F_A, and F_B) and the relative contributions of each electron transfer stage to $\Delta\psi$ are also shown.

The data on distribution of ε in PS1 served as a basis for creating an algorithm for calculation of electrostatic interactions in protein complexes and, eventually, for determination of redox characteristics of electron carrier cofactor. The other contribution to the proposed algorithm was the ideas of L. I. Krishtalik on how to overcome the apparent contradiction between the paradigm of macroscopic electrostatics and calculations using X-ray analysis data on the 3D protein structure, in particular, partial atom charges. Krishtalik has shown
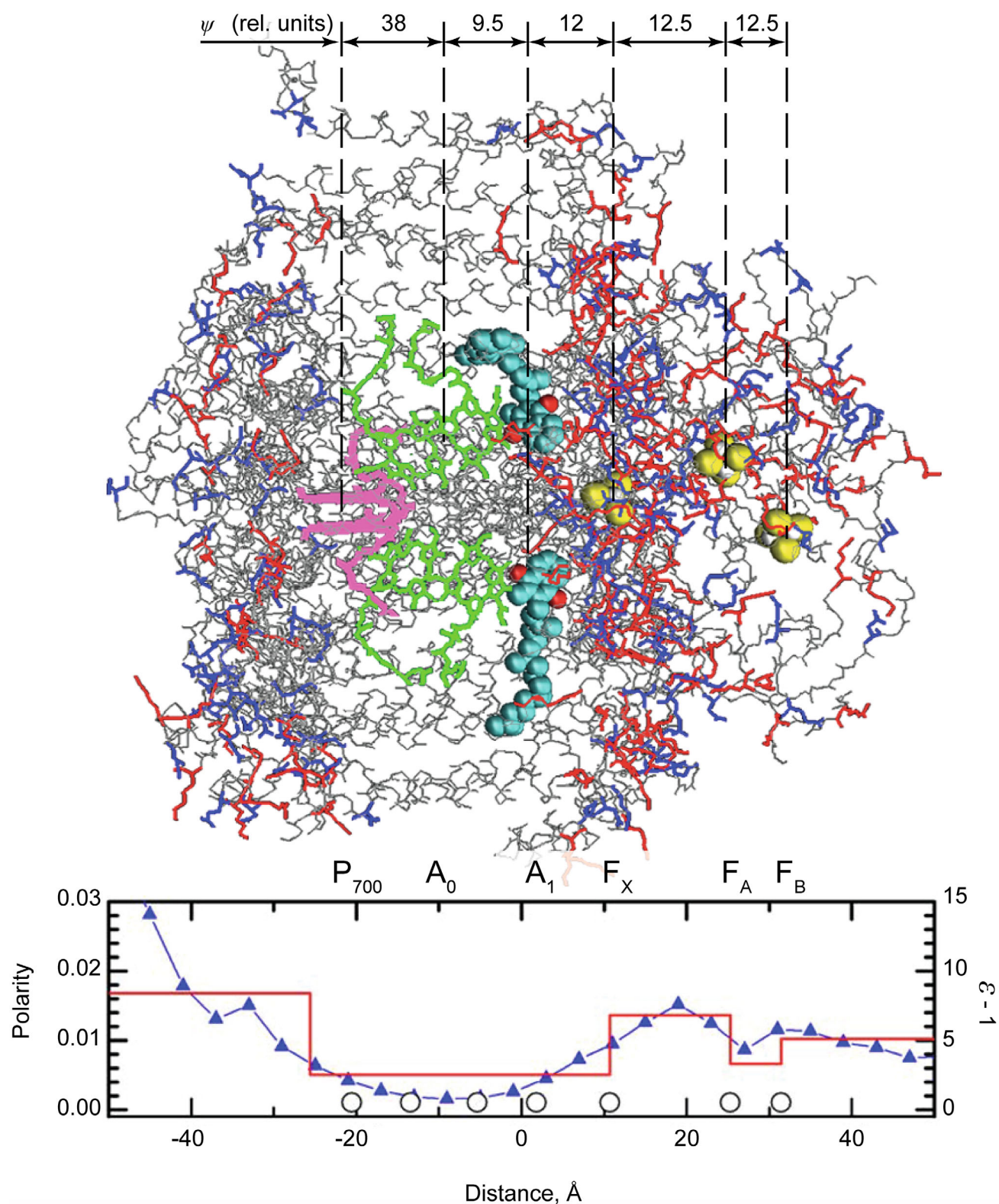
Fig. 6. Three-dimensional structure of PSI (the donor side exposed to the lumen is on the left; the acceptor side exposed to the right) and distribution of the polarity and dielectric permittivity of protein complexes along the normal to the membrane plane. Polarity was calculated as a proportion of atoms carrying a partial charge (N, O, and S atoms of amino acid side chains). Circles on the x-axis indicate coordinates of redox cofactors. Electron transfer between the primary donor P_{700} (pink) and quinone acceptors A_1 (turquoise and red spheres, carbon and oxygen atoms, respectively) can occur via two different branches: A and B (upper and lower, respectively, in the figure). Each branch contains a pair of chlorophyll molecules (green) between P_{700} and A_1 that function as a single cofactor A_0 due to the electronic coupling between two parallel porphyrin rings. In the structure of PSI proteins, only peptide backbone (gray) and side chains of aniono- and cationogenic amino acid residues (blue and red, respectively) are shown.

that these data carry different kinds of information about various components of changes in the free energy of transfer of cofactor into the protein. Therefore, these components should be calculated with different approaches and by taking into account the properties of protein via using various dielectric constants [39, 40]. As a result, for the first time, redox potentials of all PSI cofactors, from the primary donor P_{700}/P_{700}^+ to the iron–sulfur clusters F_X, F_A, and F_B, were calculated within the framework of the same approach [38].

CONCLUSIONS

One of the consequences of the Mitchell's chemiosmotic concept was discovery of $\Delta\psi$ generators as a class of membrane proteins. Existence of these proteins was demonstrated by the direct electrometric method, which served as one of the proofs of the chemiosmosis theory and contributed to the decision to award Mitchell with a well-deserved Nobel Prize in Chemistry.

Direct electrometric method and its variants were (and are) used not only in the Drachev's laboratory and, later, in the Kaulen and Semenov's laboratories, but also in some groups in other countries. In particular, this method was used in the studies of electrogenic reactions in bacterial RCs at the laboratory of G. Feher (La Jolla, USA) [41], electrogenic reactions in PSI at the laboratory of P. Brzezinski (Göteborg, Sweden) [42], kinetics of $\Delta\psi$ generation during transitions between the S states in PSII complexes at the laboratory of W. Junge (Osnabrück, Germany) [43], and electrogenic reactions during the functioning of terminal oxidases of mitochondria and bacteria at the laboratories of M. Wikström and M. Verkhovsky (Helsinki, Finland) (see review by Wikström and Verkhovsky [44]).

The development of direct electrometric method has been recognized as one of the main achievements (among approximately 40) of experimental studies in the field of oxidative and photosynthetic phosphorylation in the 20th century, along with the works by G. Embden and O. Meyerhof, V. Engelhardt, O. Warburg, V. Belitzer and E. Tsybakova, A. Lehninger, D. Arnon, B. Chance, P. Boyer, etc. [45].

We would like to emphasize the role of Drachev in the development and modification of direct electrometric method. Drachev was a radiophysicist, a highest-level specialist in electronics and laser technology. When he started to work at the Interfaculty Laboratory of the Moscow State University, he had a rather superficial understanding of proteins, as well as biochemistry and molecular biology in general. Liberman and Skulachev had played an essential role in attracting his interest to membrane proteins.

From 1973 to 1993, he had worked with younger biochemists Kaulen and Semenov, who carried out biochemical experiments. They isolated proteins, obtained proteoliposomes and chromatophores, varied the composition of proteoliposome phospholipids and ALM, and tested membrane filters as a support for ALM. They were also directly engaged in the measurement of $\Delta\psi$ produced by various membrane proteins in response to light or addition of respective substrates, performed pH and redox titration of photoelectric signals, and studied kinetics of $\Delta\psi$ generation in response to laser flashes. However, detailed elaboration of the method, including development of a system for recording membrane potential generation and corroboration of equivalent electric circuit would be impossible without painstaking and highly professional work of Drachev. Among other things, he was a top-level engineer, who identified and very effectively eliminated instrumentation problems.

Once his co-workers told him in some context: "...You are our scientific supervisor!" Drachev answered half-jokingly: "I am not a scientific supervisor, but a technical director". Of course, Lel Aleksandrovich has significantly understated his role. In fact, all scientific findings were discussed by Drachev and his co-workers, and his opinion played an extremely important role in the data interpretation and planning of future experiments.

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