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REVIEW

Features of the Mechanism of Proton Transport in ESR, Retinal Protein from *Exiguobacterium sibiricum*

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Abstract—Retinal-containing light-sensitive proteins – rhodopsins – are found in many microorganisms. Interest in them is largely explained by their role in light energy storage and photoregulation in microorganisms, as well as the prospects for their use in optogenetics to control neuronal activity, including treatment of various diseases. One of the representatives of microbial rhodopsins is ESR, the retinal protein of *Exiguobacterium sibiricum*. What distinguishes ESR from homologous proteins is the presence of a lysine residue (Lys96) as a proton donor for the Schiff base. This feature, along with the hydrogen bond of the proton acceptor Asp85 with the His57 residue, determines functional characteristics of ESR as a proton pump. This review examines the results of ESR studies conducted using various methods, including direct electrometry. Comparison of the obtained data with the results of structural studies and with other retinal proteins allows us to draw conclusions about the mechanisms of transport of hydrogen ions in ESR and similar retinal proteins.

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Keywords: retinal protein, proteorhodopsin, Schiff base, proton acceptor, proton donor, photocycle, direct electrometric method

INTRODUCTION

Microbial rhodopsins belong to the family of retinal-containing proteins that perform light-dependent ion transport, sensory and other functions [1-3]. The molecules of these proteins have similar structure, including seven or eight (in the case of enzyme rhodopsins) transmembrane alpha-helical segments and a retinal covalently linked through a Schiff base to a lysine residue [3, 4]. Absorption of a light quantum initiates the isomerization of the retinal from all-*trans* to the 13-*cis* configuration. Relaxation of the retinal to its original state is accompanied by a number of conformational transformations in the protein molecule associated with proton transport (in rhodopsins, which perform the function of a proton pump) [5, 6]. The details of this process have been most extensively studied for the bacteriorhodopsin from *Halobacterium salinarum* (BR), the first member of the microbial rhodopsin family, which was discovered over 50 years ago [7-9]. It has been established that during the photocycle, the Schiff base formed by retinal and a lysine residue is deprotonated and a proton is transferred to the acceptor residue Asp85. At the same time, a proton is released on the extracellular surface of the protein with the participation of residues Glu194 and Glu204 (the so-called PRG – proton-releasing group [10]). The Schiff base is then reprotonated by the donor residue Asp96, which then receives a proton from the cytoplasm. At the end of the photocycle, the proton is transferred from the acceptor to the residues of the PRG [7, 10]. These molecular events correspond to the emergence and decay of spectrally identified intermediate states (intermediates) of the photocycle BR $\xrightarrow{h\nu}$ K → L ↔ M1 ↔ M2 ↔ N1 ↔

Abbreviations: BR, bacteriorhodopsin from *Halobacterium salinarum*; ESR, retinal protein from *Exiguobacterium sibiricum*; PR, proteorhodopsin; PRG, proton-releasing group.

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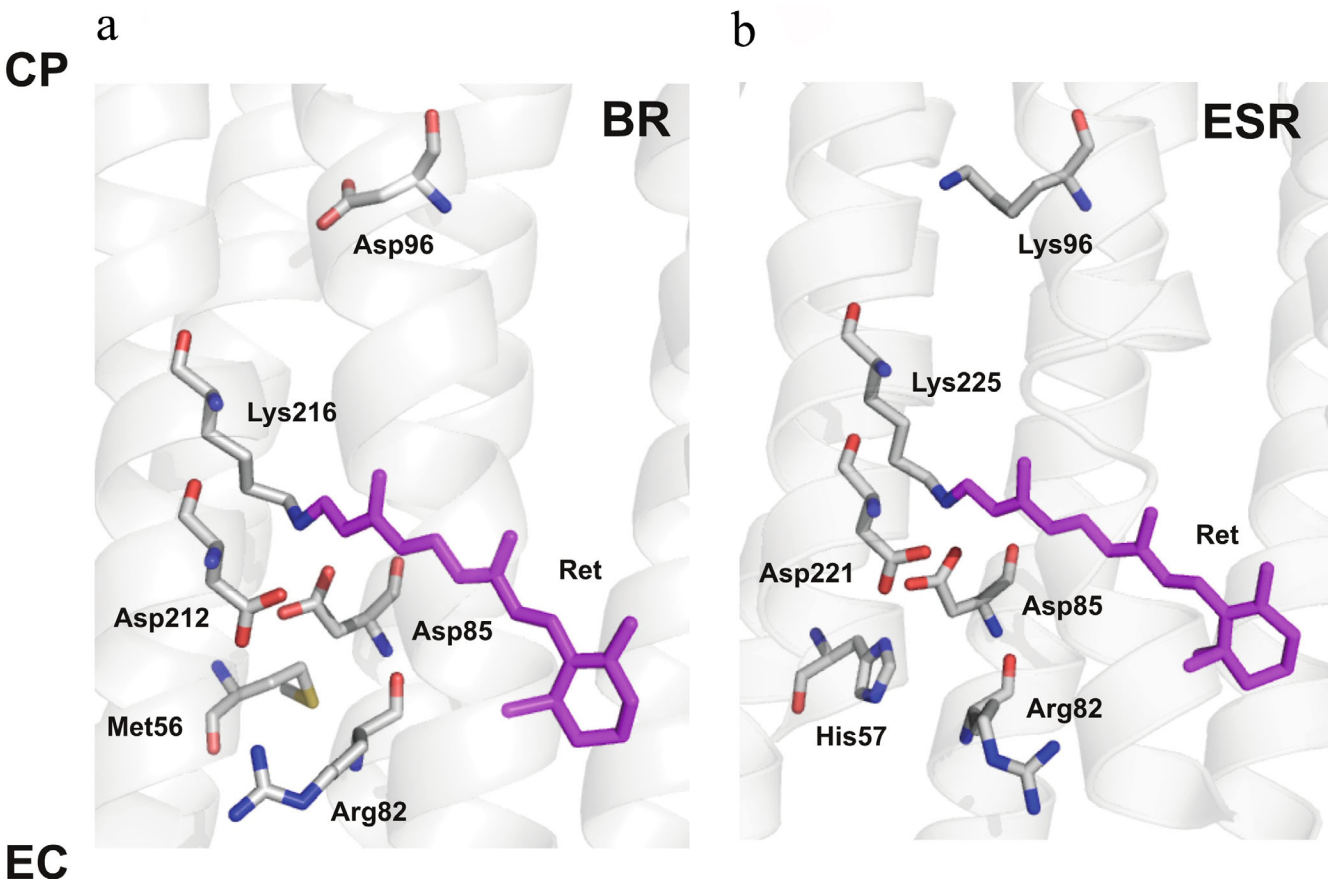

Fig. 1. Comparison of structure of BR (a) and ESR (b). Regions near the Schiff base are shown. The figures were prepared in PyMol using pdb structures 1C3W and 4HYJ, respectively. Ret, all-*trans* retinal; CP, cytoplasmic surface of the protein; EC, extracellular protein surface.

↔ N2 ↔ O → BR [7, 10]. Data from the functional studies of BR are in good agreement with its structural changes in various states [11-13].

Subsequently, in the course of various genomic and metagenomic studies, many new proteins were discovered that were homologous to BR and performed similar functions [3, 14, 15]. In particular, proteorhodopsins (PR) were found in many members of Proteobacteria. They play an important role in the survival of microorganisms under unfavorable conditions. Studies of PRs have revealed that, like BRs, they have acceptor and donor residues (aspartate and glutamate, respectively), but there is no PRG in their molecules [16, 17]. An important feature of PR is the hydrogen bond between the acceptor residue (aspartate) and the side group of the histidine residue; it regulates the degree of protonation and the functional state of the acceptor (depending on pH) and is involved in proton release [18, 19].

ESR, the retinal protein from soil bacterium *Exiguobacterium sibiricum*, also belongs to the proteorhodopsins, but exhibits a significant structural difference from typical representatives of the family. The amino acid sequence of ESR has Lys96 residue in the position corresponding to the carboxyl residues with the function of proton donor for the Schiff base in BR and PR molecules (Asp96 and Glu108, respectively) [20] (Fig. 1). This feature, along with others, sparked interest in research into the structure and mechanism of ESR functioning, which we have been conducting for more than 10 years. In 2015, we published a review of these works [21], but since then a number of important new results have been obtained, largely thanks to the use of the direct electrometry method developed by L. A. Drachev and his colleagues [22, 23].

We employed the version of this method which is based on the use of proteoliposomes containing the protein under study and the macroscopic flat phospholipid membrane reinforced with a collodion film for greater stability. Prior to the measurement, proteoliposomes are adsorbed on the surface of the macroscopic membrane separating the compartments of the measuring cell [23]. In response to the flash, the transmembrane transfer of protons, as well as the movement of charged groups of the protein perpendicular to the membrane plane, generate the electrical potential difference at the proteoliposome membrane ($\Delta\Psi p$) and, proportionally, at the macroscopic membrane ($\Delta\Psi m$) which is recorded using electrometric technology with high time resolution.

BR was one of the first proteins for which direct electrometric measurements were applied [22, 24].

Subsequently, the method was widely used in studies of various retinal proteins [25-27], components of photosystems I and II [28-30], cytochrome oxidase [31-35], and other objects. This review discusses our recent results of the studies of ESR and its mutant variants, including those obtained using direct electrometry, and compares them with those previously published for BR and other retinal proteins.

ESR PHOTOCYCLE: REACTIONS COUPLING WITH PROTON UPTAKE AND RELEASE

The study of light-induced absorption changes of ESR using flash photolysis revealed the presence of main intermediates also characteristic of BR [36] and PR [37] photocycle. Following the decay of intermediates K and L, an increase in absorption is observed at 410 nm on microsecond time scale, which corresponds to the formation of the M-state (deprotonated Schiff base). It is interesting to note that this intermediate is detected only at pH > 8 for ESR in micelles of the detergent DDM (n-dodecyl-β-D-maltopyranoside), while for the protein in micelles of lipid-like detergent LPG (1-palmitoyl-2-hydroxy-sn-glycero-3-phospho-1'-rac-glycerol) or in proteoliposomes, the pK_a of its formation is ~6.5 [38, 39]. Reprotonation of the Schiff base is accompanied by a decrease in absorption at 410 nm (reflecting the decay of the M state) and the appearance of intermediates N1 and N2, which are characterized by the absorption increase at 510 and 550 nm. As a result of retinal reisomerization, intermediate O is formed, which absorbs at 590 nm and decays to the initial ESR state. The N-intermediate is present in the ESR photocycle up to its completion [40], thus, the photocycle diagram includes the following transitions: $ESR \xrightarrow{h\nu} K,L \rightarrow M1 \leftrightarrow \leftrightarrow M2 \leftrightarrow N1 \leftrightarrow N2/O \rightarrow ESR$ [39].

Illumination of *Escherichia coli* cells suspension expressing ESR or ESR-containing proteoliposomes resulted in acidification of the suspension, confirming transmembrane proton transfer by this protein [20, 38]. Addition of protonophore CCCP to the cells eliminates this effect, which confirms the proton transport function of ESR [38]. It was also found that, in contrast to BR, ESR releases protons at the end of the photocycle, which correlates with the absence of residues homologous to PRG in its molecule [20]. Measurements carried out in the presence of the pH-sensitive dye pyranine showed the temporal relationship of proton release with the decay of long-wavelength photocycle intermediates and the return to the initial state [41]. Kinetics comparison of the proton uptake and intermediate M decay in wild-type ESR demonstrated that, unlike in BR, proton uptake in this protein precedes the reprotonation of the Schiff base, which is especially noticeable when measurements are performed in D_2O [41].

ELECTROGENIC STAGES OF THE ESR PHOTOCYCLE

The time-resolved direct electrometry makes it possible to isolate and study individual electrogenic (i.e., directed perpendicularly to the membrane plane) stages of charge transfer within the protein in response to a single flash; simultaneous analysis of the absorption changes kinetics allows us to relate them to the stages of the retinal protein photocycle. Complimentary to the dye-involving methods, (i) such measurements make it possible to obtain directly the information about the proton movement inside the protein, in contrast to just an information on the disappearance or appearance of a proton on its surface, and (ii) they are not limited to pH region close to the pK_a of the dye, which generally opens up the possibilities for reconstructing the complete picture of the functioning of the proton pump at different pH values.

For ESR-containing proteoliposomes measurements were carried out at pH 5.1-9.5 [39]. The total amplitude of the photoelectric response at neutral pH values reached 50 mV, which is comparable to the maximum response amplitude for BR [22]. However, the directions of the response for these proteins were opposite, due to the different orientations of their molecules in proteoliposomes (ESR is incorporated in the same orientation as in cells, with the N-terminus facing out [20, 38], whereas BR is oriented in the opposite direction [42]). Accordingly, ESR transports protons from proteoliposomes to the outside, while transport of protons into proteoliposomes is observed for BR due to the reverse orientation as compared to that in cells.

It was previously demonstrated that the photoelectric response of BR involves three main electrogenic processes, with the fastest (<1 μs) having a negative sign and corresponding to the stages $BR \rightarrow K \rightarrow L$ (photoinduced isomerization of retinal and subsequent relaxation of adjacent residues). The second stage (30-50 μs) reflects the proton movement from the Schiff base to the Asp85 acceptor and the simultaneous proton release into the external medium by the proton-releasing group. The amplitude of the latter phase (5-20 ms) accounts for up to 80% of the total response and corresponds to proton transfer from the internal donor Asp96 to the Schiff base and subsequent reprotonation of the donor, as well as proton transfer from Asp85 to PRG [43] (Fig. 2a).

The kinetics of membrane potential formation for ESR differs significantly from that described above for BR. In particular, the photoelectric response of ESR does not exhibit the fast negative phase characteristic of BR (Fig. 2b). A detailed analysis of the kinetic curves at pH 5.1, however, made it possible to detect its presence, "hidden" by positive signals from electrogenic phases coinciding with it in time. The relative total amplitudes of the microsecond electrogenic phases

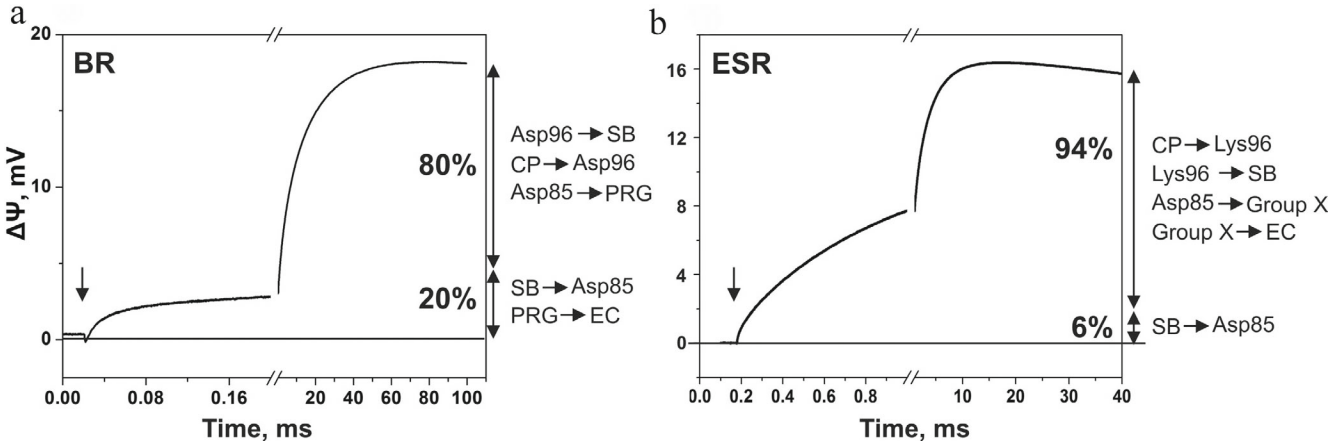

Fig. 2. Kinetics of light-induced formation of membrane electric potential (ΔΨ) by proteoliposomes containing BR (a) and ESR (b), at pH 7.5. The arrows indicate the moment of laser flash. The relative amplitudes of the microsecond and millisecond electrogenic phases are shown, indicating the corresponding stages of proton transfer. SB, Schiff base; PRG, proton releasing group; EC, extracellular surface of the protein; CP, cytoplasmic surface of the protein; Group X is an unidentified residue/group of residues that release protons at the extracellular surface of ESR. For convenience, the photoelectric responses of both proteins are displayed in the same direction.

of ESR (3, 24, and 100 μs at pH 7.5) were approximately three times smaller than those of BR, which may be due to the lack of early proton release in ESR molecule. The decay of intermediate M includes two electrogenic phases, 0.6 and 3.4 ms, with a total contribution of 75%, presumably reflecting the transition M ↔ N1 ↔ N2/O. The last electrogenic phase of ESR (~18.4 ms) is associated with the return to the initial state (N2/O → ESR) and reflects the deprotonation of Asp85 acceptor and proton release by the protein molecule into the external aqueous medium [39].

It should be noted that the photoelectric response of ESR-containing proteoliposomes at pH 8.4 demonstrates a 10-fold (as compared to pH 6.5) slowdown of the electrogenic stages corresponding to the decay of the intermediate M, resulting from redistribution of the relative contributions of kinetic components in favor of the slower ones. In this case, a separate electrogenic phase with $\tau \approx 0.25$ ms emerges, corresponding to the protonation of the initially neutral Lys96 [39]. These data suggest that in ESR, Lys96 and/or the water molecule interacting with it acquires a proton after formation of the state M and transfers it to the Schiff base shortly thereafter. This confirmed the previously made conclusion that the Lys96 residue is not protonated in the initial state at neutral pH and is protonated in the M state with $pK_a \approx 8.5$ [41]. In ESR molecule, Lys96 is surrounded predominantly by hydrophobic residues [44], which presumably contribute to the a reduced pK_a of Lys96 in the initial state. A similar large shift in pK_a (but in opposite direction) is observed for Asp96 residue in BR, which is neutral, has a very high pK_a in the initial state (~11.4) and, accordingly, is protonated due to the highly hydrophobic environment [45].

A decrease of pH to 5.1 is accompanied by an almost complete suppression of ESR photoelectric response and the appearance of noticeable negative phases, presumably as a result of protonation of Asp85 acceptor and/or the associated His57 residue in the initial state.

Lys96 – EFFICIENT PROTON DONOR FOR THE SCHIFF BASE IN ESR

To determine the functional role of the Lys96 residue, we used a classical approach involving generation and study of mutant ESR variants [41, 46]. At first, the K96A mutant was constructed; characteristic features of its photocycle included a significant slowdown (more than 100-fold) of the intermediate M decay (Fig. 3a) and strong dependence of the rate of this process on pH [41]. *E. coli* cells expressing this mutant showed a significant reduction in proton transport as compared to cells containing the wild-type protein. Thus, it was shown that the presence of the Lys96 residue accelerates the reprotonation of the Schiff base in the ESR molecule.

Measurements of the photoelectric response of K96A-containing proteoliposomes demonstrate that this mutation is accompanied by the almost complete disappearance of the millisecond electrogenic phase, which is associated with the reprotonation of the Schiff base (decay of intermediate M) [47] (Fig. 3b). The slowdown of this process, characteristic of the K96A mutant, in itself should not have led to such an effect.

Approximation of the experimental kinetics from K96A with a sum of kinetic components revealed the presence of several electrogenic phases with a negative sign in the millisecond part of the kinetics. The negative amplitude of the millisecond phases, corresponding to the rapid decay of the intermediate M in the mutant protein, indicates that the reprotonation of the Schiff base occurs from an unusual direction – from the extra-

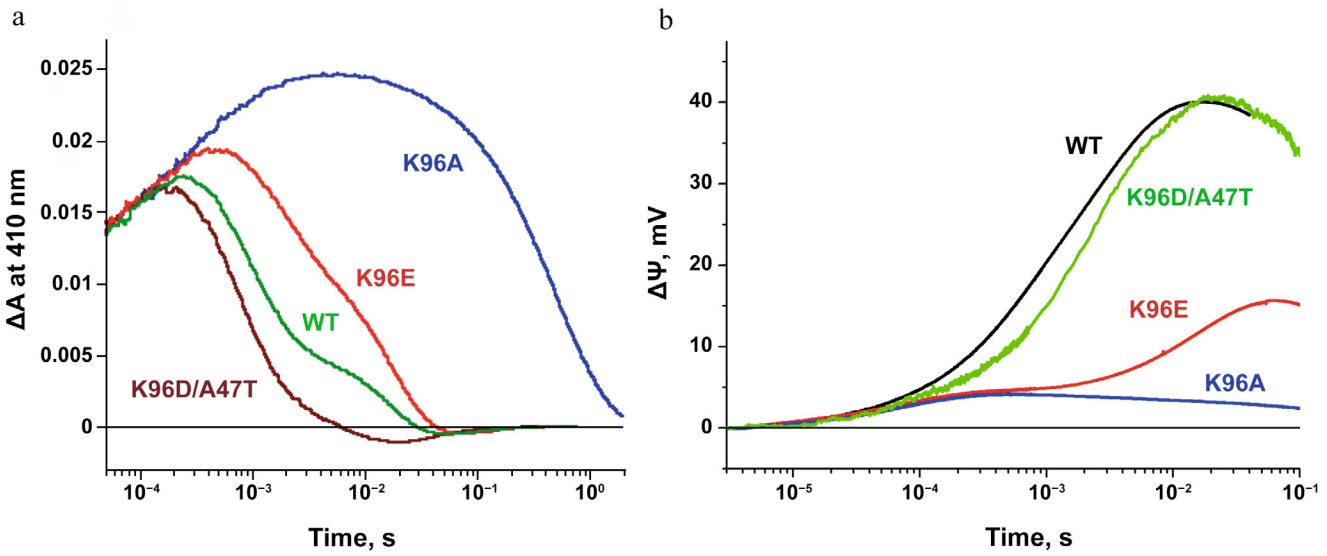

Fig. 3. Properties of mutant ESR variants with substitutions of the Lys96 residue. a) Kinetics of light-induced absorption changes at 410 nm of wild type ESR (WT) and its mutant variants in 0.06% lipid-like detergent LPG, 100 mM NaCl, pH 7.5; b) kinetics of the formation of transmembrane potential difference (ΔΨ) at proteoliposomes containing wild type ESR and its mutant variants at pH 7.5. Adapted from [46], with modifications.

cellular surface of the protein. This is also substantiated by the negligible amplitude of the positive component of the electrogenic response, which apparently includes both the diffusion of the proton from the cytoplasmic surface of the protein and its movement in the opposite direction. At low pH, the first of these mechanisms dominates, while at pH 8.5 the second one is prevailing. The addition of azide to this mutant led to an accelerated delivery of protons from the cytoplasmic side of the protein and to an increase in the amplitude of the positive components of the electrogenic response [47].

Thus, the experiments demonstrated that the reduced efficiency of proton transport in K96A mutant can be explained not only by the slowdown in the photocycle, but also by the decrease in forward transport and the increase of contribution of back (reverse) reactions, presumably from His57-Asp85 acceptor site to the Schiff base. It should be noted that mutations of the donor residue in BR (D96N and D96A) are accompanied by the slowdown in the photoelectric response in the millisecond region, but not by its disappearance, which results from the different configurations of the proton acceptor sites in BR and ESR (Fig. 1) and indicates differences in the mechanism of proton transport in these proteins [48, 49]. Additional arguments in favor of the role of His57 residue in the back reactions were obtained with the mutation study of this residue (see below).

CARBOXYL AMINO ACID RESIDUES IN PLACE OF Lys96 IN ESR

"Can carboxyl residues act as proton donors in ESR molecule?" – was the question we asked in the previous review [21]. Now there is a definite answer: "In general, yes, but there are differences as compared to how they operate in BR." We examined the kinetics of the photocycle, proton transfer, and electrogenicity of ESR mutants K96E and K96D/A47T [46]. In contrast to the K96A mutant, the rates of proton uptake and proton transfer to the Schiff base in these proteins are comparable to those in the wild type protein. In K96E, intermediate M decays somewhat slower than in the wild type – with a larger contribution of the slow component, and in K96D/A47T – even faster than in the wild type (the time constant of the fast component is ~0.7 ms versus 1 ms in the wild type) (Fig. 3a). Reprotonation of the Schiff base and proton uptake from the medium in these mutants occur almost simultaneously with the M to N transition (as in wild-type ESR at neutral pH), whereas in BR these two stages are well separated and coincide in time with the M to N and N to O transitions, respectively [48, 50]. *This reflects the different mechanism of donor involvement in the reprotonation of the Schiff base in BR and ESR*. It is interesting to note that in K96D/A47T and K96E mutants, the formation of the M state is accompanied by partial proton release, presumably at the cytoplasmic surface of the protein (i.e., by movement in the opposite direction). The reasons for this phenomenon will be discussed below.

The photoelectric responses of K96D/A47T and K96E mutants include electrogenic millisecond phases associated with Schiff base reprotonation that are similar to those of the wild type but are absent in the K96A mutant. The main different feature of the electrogenic kinetic of the K96E mutant is the much slower main millisecond component of ΔΨ generation (Fig. 3b). In proteoliposomes containing K96D/A47T, generation

of $\Delta\Psi$ occurs primarily during the decay of the intermediate M, while in the K96E mutant it occurs during the decay of N. This provides independent evidence that carboxyl residues functioning as proton donors in the ESR with the efficiency comparable to that of lysine, in the case of the double mutant, and with a slightly lower efficiency in K96E.

It should be noted that Japanese scientists published a study of mutant PR variants, in which the donor aspartate residue was replaced by lysine and glutamine (E108K and E108Q), as well as ESR mutants with substitutions K96D, K96Q, and K96E [51]. The K96D mutant exhibited a fast decay rate of intermediate M as compared to the wild type, while the K96E mutation exhibited a slower M decay rate. It has also been shown that Lys can replace native Glu in the donor region of PR [51].

The absence of a significant delay between proton uptake and reprotonation of the Schiff base in K96D/A47T and K96E mutants, as well as the early release of the proton from the opposite side of the membrane, indicate that in ESR mutants the carboxyl residues at position 96 are protonated (uncharged) in the initial state, similarly to BR. Presumably, after the formation of the intermediate product M, they are in a more hydrophilic environment as compared to the initial state, resulting in a decrease in their pK_a to 6.5-8.5. This results in partial deprotonation of the donor residues and release of a proton into the medium, followed by reprotonation of the Schiff base and almost simultaneous proton uptake [46]. Therefore, donor residues in the ESR are able to rapidly switch from equilibrium with the medium to equilibrium with the Schiff base during the M to N transition, which is consistent with data from structural studies. The side chain of Lys96 has a certain mobility, and the cavity around this residue is very close to the surface of the protein and is separated from the aqueous medium only by the polar side chain of Thr43, which distinguishes ESR from the more covered position of proton donor groups in BR and xanthorhodopsin (XR) [44]. A similar conclusion was reached by Sasaki et al. [51], who proposed that protein conformational changes associated with Schiff base reprotonation are smaller in ESR than in BR mutants and may involve side chain movements of the donor residue connecting it to the environment or to the Schiff base.

His57 REPLACEMENT ALLOWS PROTON TRANSPORT BY ESR OVER A WIDE pH RANGE

The presence of the histidine residue with the hydrogen bond to the aspartate acceptor residue is a distinctive feature of proteins belonging to proteorhodopsin family, including ESR, as well as XR [38, 52, 53]. The interaction between the histidine side chain and residues of the adjacent subunit plays an important role in the oligomerization of PR and *Gloeobacter* rhodopsin (GR) [54-56]. The close interaction between His57 and Asp85 in the ESR molecule was revealed both in functional studies of mutants of this residue [38] and by determining the spatial structure of the protein [44] (Fig. 1). It has been established that the His57 substitution has a radical effect on various properties of ESR, including the pH dependence of the absorption maximum and the pK_a of the formation of the intermediate M [38]. Unlike wild type ESR, which absorption maximum weakly depends on pH in the range of 3-8, the H57M mutant is characterized by a significant (47 nm) blue shift of the absorption maximum as pH increases from 5 to 8 from deprotonation of Asp85. At pH 5, the absorption maximum of H57M is at 565 nm, which corresponds to the absorption maximum of the D85N mutant. Thus, interaction with His57 determines the degree of protonation of the Asp85 residue, i.e., its ability to function as a proton acceptor from a Schiff base. In order to clarify the functional role of this interaction, we examined the properties of ESR with the H57N substitution and the double mutant H57N/K96A using direct electrometry.

In the studies of the photocycle of proteoliposomes containing H57N mutant, it was established that this substitution leads to an acceleration of the formation of the M intermediate and elimination of slow spectral changes in this process, previously found in the photocycle of ESR and the K96A mutant (Fig. 4a). A corresponding component is also absent in the photoelectric response, which indicates a connection between the slow phase of M generation in ESR and the His57 residue (a possible explanation for this connection is given in the next section). As compared to the wild type ESR, the decay of the intermediate M in the H57N mutant also occurs faster and consists of a single phase, since it is not accompanied by the accumulation of the N1 state.

Interestingly, in the double mutant H57N/K96A, the decay of the M intermediate occurs 1000 times slower than in the wild type and in the H57N mutant, and 10 times slower than in K96A (Fig. 4a). This phenomenon can be explained by the removal of the backward proton movement to the Schiff base as a result of the H57N substitution. Presumably, the replacement of the His57 residue in the environment of the proton acceptor Asp85 leads to changes in the charge or other properties of the environment, resulting in the creation of a kinetic barrier to such movement.

Previously, investigation of the pH dependence of proton transport in *E. coli* cells with ESR expressed revealed that the efficiency of the wild-type protein as a proton pump is significantly reduced at pH below 5 [38]. It turned out that under the same conditions the H57N and H57N/K96A mutants demonstrate high transport efficiency [57]. In accordance with these data, mutants with the H57N substitution retain the amplitude

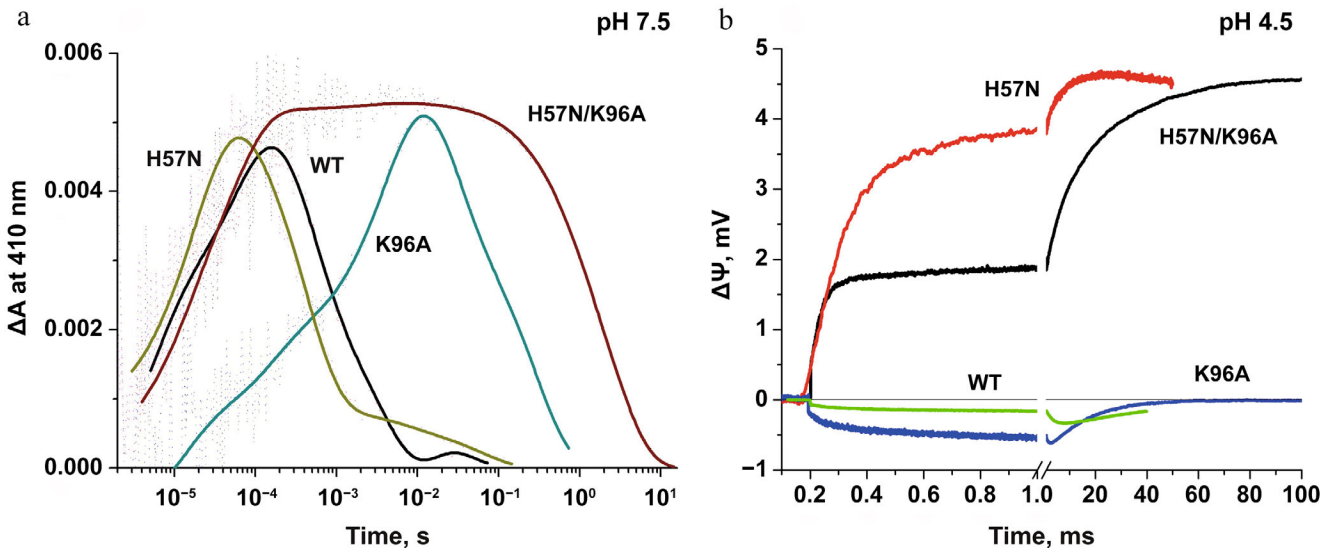

Fig. 4. Properties of mutant ESR variants with substitutions of the His57 residue. a) Kinetics of light-induced absorption changes at 410 nm by proteoliposomes containing wild-type (WT) ESR and its mutant variants at pH 7.5; b) kinetics of the transmembrane potential difference (ΔΨ) formation by proteoliposomes containing wild-type ESR and its mutant variants at pH 4.5. Adapted from [57], with modifications.

and direction of the photoelectric response at pH 4.5, in contrast to the wild type and the mutant with a single K96A substitution (Fig. 4b). Thus, the interaction of His57 and Asp85 limits proton transport at low pH for wild-type ESR. In the H57N mutant at pH 4.5, the Lys96 residue is presumably protonated in the initial state. Due to this, reprotonation of the Schiff base during the decay of the M intermediate occurs at a high rate, after which the donor receives a proton from the environment, similarly to what happens at neutral pH in BR, where the donor Asp96 is initially protonated. The data obtained indicate that Lys96 can effectively function as a donor at low pH values.

As mentioned above, based on the pH dependence of the absorption maximum it was previously concluded that Asp85, the proton acceptor from the Schiff base in the H57N mutant, should be in the protonated state in the detergent DDM at pH < 6 [38]. However, subsequent experiments demonstrated the efficient functioning of this mutant as a proton pump at pH below 5 in liposome membranes [57]. It can be assumed that the pK_a of Asp85 in H57N decreases in more hydrophobic environment of liposomes and after isomerization of retinal to the 13-*cis* configuration, opening the possibility for proton transfer to the Schiff base during the decay of intermediate L to N without significant accumulation of M.

FEATURES OF ESR FUNCTIONING AS A PROTON PUMP

Based on the obtained results, we proposed mechanisms for the functioning of ESR and its mutant variants under various conditions [21, 39, 46, 47, 57]. The previously published sequence of proton transfer reactions for the wild type ESR included: (i) proton transfer from the Schiff base to the Asp85 acceptor during the formation of intermediate M; (ii) protonation of the Lys96 donor from the cytoplasmic surface of the protein; (iii) reprotonation of the Schiff base involving Lys96, and (iv) deprotonation of Asp85 and release of the proton at the extracellular surface of the protein involving an unidentified group of residues [41]. The key steps of this scheme were directly confirmed and characterized by time-resolved measurements of electrogenic proton transport events during the ESR photocycle. In addition, the scheme was updated taking into account the data obtained by the direct electrometric method. In particular, the origin of the slow stages of intermediate M formation, which are observed in ESR at neutral pH values, was clarified. Since in the H57N mutant this process is accelerated and does not include slow components, same as in the wild-type protein under alkaline conditions, it was concluded that they are related to the presence of a positive charge at the histidine residue, which prevents the transfer of a proton from the Schiff base to the Asp85 acceptor.

The presence of the His57 residue and its interaction with Asp85 also explains some other features of proton transport in the ESR, including reduced efficiency of transport at low pH values and an increased contribution of back reactions in the K96A mutant as compared to similar BR mutants. Back reactions, i.e., the return of proton from the Asp85 acceptor to the Schiff base, may be driven by the lower pK_a of the ESR acceptor (Asp85-His57) in the M state as compared to BR or by a lower barrier of reverse transfer from Asp85 to the Schiff base. The high pK value of Asp85 in BR at this stage (~11)

is caused by the separation of the positively charged arginine residue away from Asp85, increased hydrophobicity of Asp85 environment, and early proton release from PRG [10, 58]. In the ESR molecule, proton release occurs at the end of the photocycle [20, 38], as a result of which the extracellular part of the protein can retain a positive charge for quite a long time. This presumably leads to an increase in the probability of reverse proton transfer from the acceptor to the Schiff base [47]. In wild-type ESR, due to the presence of the Lys96 donor and the rapid reprotonation of the Schiff base, backward reactions are insignificant, but in the K96A mutant their effect is dramatic, leading to a significant decrease in the efficiency of proton transport [41, 47].

Thus, an important function of Lys96 in the ESR molecule, in addition to direct reprotonation of the Schiff base, is the prevention of back reactions that reduce the efficiency of the pump. Unlike BR and PR, in which the function of a proton donor is performed by initially protonated carboxyl residues, the lysine residue in the ESR molecule acquires a proton immediately before transferring it to the Schiff base. This sequence of events was previously established in experiments using a pH-sensitive dye [41] and then confirmed using direct electrometry [39]. The discovery of a separate electrogenic stage, corresponding to the protonation of the donor during the M1 ↔ M2 transition as a result of an increase in pH to 8.4 and a corresponding slowdown in the reprotonation of the Schiff base, further demonstrated the capabilities of this method, since this stage did not manifest in any way in optical measurements. The described patterns may be important for understanding the mechanism of functioning of the retinal proteins that were discovered recently and which also contain a lysine residue as a proton donor for the Schiff base [59, 60].

As noted above, the lack of PRG and no early release of protons make an important difference between ESR and BR. However, during the study of mutant protein variants, partial release of the proton at earlier stages of the photocycle (at the time of intermediate M formation) was revealed. Thus, the relative amplitude of the corresponding electrogenic phase in the H57N mutant turned out to be significantly greater than in the wild type ESR (28 and 5%, respectively). Presumably, in the mutant it also involves movement of a proton or a positive charge to the extracellular side of the protein, similar to what occurs in BR [57]. Early proton release was also observed in the mutants containing substitutions of lysine for carboxyl residues, K96D/A47T and K96E. In this case, it apparently occurs at the cytoplasmic surface of the protein as a result of an increase in the hydration of the cavity containing donor residues during the formation of intermediate M [46]. To clarify the mechanisms of these processes, additional studies will be carried out in the future.

CONCLUSION

In recent years, numerous studies in the field of retinal proteins have demonstrated their impressive natural diversity, as well as their wide abundance among microorganisms inhabiting various ecological niches. In particular, it has been established that more than half of the representatives of the microbial communities of the world ocean have rhodopsin genes, which make a significant contribution to the absorption of solar energy by microorganisms [61, 62]. The general structure of the molecule and the basic mechanisms of functioning of these proteins are universal for the entire family, however, evolutionary adaptation to specific living conditions leads to the emergence of fine tuning of these mechanisms [5]. Using the example of ESR, a retinal protein from soil bacterium, we demonstrated how changes in the primary structure are reflected in the features of proton transport in its molecule. An invaluable role in these studies is played by biophysical methods that make it possible to observe the processes occurring in the protein molecule in response to the absorption of a light quantum with high time resolution. In this regard, the method of direct electrometry occupies a special niche, providing unique information about the movement of the proton inside the molecules of proton pumps. Investigation of the details of the structural and functional characteristics of microbial rhodopsins contributes to a more complete understanding of the principles of their function, and opens up the possibility of developing approaches for targeted changes in their properties, for example, with the aim of creating new tools for optogenetics [6, 63-65].

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