

ESR – A Retinal Protein with Unusual Properties from *Exiguobacterium sibiricum*

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Abstract—This review covers the properties of a retinal protein (ESR) from the psychrotrophic bacterium *Exiguobacterium sibiricum* that functions as a light-driven proton pump. The presence of a lysine residue at the position corresponding to intramolecular proton donor for the Schiff base represents a unique structural feature of ESR. We have shown that Lys96 successfully facilitates delivery of protons from the cytoplasmic surface to the Schiff base, thus acting as a proton donor in ESR. Since proton uptake during the photocycle precedes Schiff base reprotonation, we conclude that this residue is initially in the uncharged state and acquires a proton for a short time after Schiff base deprotonation and M intermediate formation. Involvement of Lys as a proton donor distinguishes ESR from the related retinal proteins – bacteriorhodopsin (BR), proteorhodopsin (PR), and xanthorhodopsin (XR), in which the donor function is performed by residues with a carboxyl side chain. Like other eubacterial proton pumps (PR and XR), ESR contains a histidine residue interacting with the proton acceptor Asp85. In contrast to PR, this interaction leads to shift of the acceptor's pK_a to more acidic pH, thus providing its ability to function over a wide pH range. The presence of a strong H-bond between Asp85 and His57, the structure of the proton-conducting pathways from cytoplasmic surface to the Schiff base and to extracellular surface, and other properties of ESR were demonstrated by solving its three-dimensional structure, which revealed several differences from known structures of BR and XR. The structure of ESR, its photocycle, and proton transfer reactions are discussed in comparison with homologous retinal proteins.

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In 2008, an article was published in *BMC Genomics* devoted to deciphering the genome of *Exiguobacterium sibiricum* – a microorganism isolated from permafrost aged about three million years. Based on the data of cod-

ing sequences annotation, it was suggested that this psychrotrophic bacterium is able to produce rhodopsin [1]. Expression of the corresponding gene in *Escherichia coli* revealed functional similarity of the recombinant with

Abbreviations: BR, bacteriorhodopsin from *Halobacterium salinarum*; DDM, *n*-dodecyl- β -D-maltopyranoside; ESR, retinal protein from *Exiguobacterium sibiricum*; GPCR, G-protein-coupled receptors; GR, xanthorhodopsin from *Gloeobacter violaceus*; LPG, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-(12-*rac*-glycerol); PR, proteorhodopsin; XR, xanthorhodopsin from *Salinibacter ruber*.

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other retinal proteins – proton transporters [2]. By that time, it has already been 40 years since the first proton pump of this kind was discovered – bacteriorhodopsin (BR) of purple membranes of the haloarchaea *Halobacterium salinarum* [3]. During these years, hundreds of reports focused on determination of the mechanism that allows this protein to transform energy of absorbed light into electrochemical proton gradient across the membrane.

The most important landmarks on this way were identification of the amino acid sequence of BR [4, 5], demonstration of electrogenicity of the proton transfer [6, 7], determination of the three-dimensional structure of the protein [8, 9], developing a mutagenesis system in *H. salinarum* [10], and obtaining of high resolution X-ray crystallography data [11]. It was established that BR molecules consist of two components: opsin (protein) and a chromophore (*all-trans* retinal linked to Lys216 residue through a Schiff base [4]). After light absorption, retinal isomerization into 13-*cis* configuration occurs [12, 13] with formation of the short-lived intermediate K [14, 15]. As a result of subsequent conformational changes in the chromophore returning it into *all-trans* conformation and accompanying structural rearrangements in the protein that proceeds through formation of intermediates L, M, N, and O, a proton is released at the outer surface of the protein and taken up from the cytoplasmic surface [16].

A key event in the BR photocycle is proton transfer from the Schiff base to the acceptor – the Asp85 [17] – that leads to formation of intermediate M (deprotonated Schiff base). It was shown that the system of hydrogen bonds connecting proton acceptor Asp85 with adjacent residues (Arg82, Asp212, etc.) is crucial for the functioning of the protein [11]. The Schiff base is reprotonated with participation of an intraprotein donor – the Asp96 residue [18]. It has been demonstrated that proton transport occurs due to sequential changes in pK_a of the Schiff base and key amino acids due to conformational rearrangements [19]. Three-dimensional structures of different BR intermediates have been studied by X-ray crystallography at low temperatures [20–22]. On the whole, at present BR is one of the best-studied membrane proteins.

In 2000, the family of the retinal-containing proteins was supplemented by proteorhodopsin (PR), whose gene was discovered during sequencing of a metagenomic library obtained from marine microplankton [23]. It turned out that “green” (called this way due to location of its absorption maximum) proteorhodopsin (GPR) goes through the same key photocycle stages that are typical for BR. However, the pK_a of the primary acceptor Asp97 in its molecule is significantly higher (7.5 against 2.5 in BR). Furthermore, proton release by the PR molecule occurs during the second part of photocycle, after its uptake from the cytoplasm.

Then, a vast number of BR structural analogs were discovered in Proteobacteria, Actinomycetales, Cyano-

bacteria, Fungi, and other microorganisms [24]. The amino acid sequences of these proteins are homologous to some extent. Their key positions are occupied by the same or homologous amino acids with analogous functional groups as in BR. Thus, aspartate is a proton acceptor in all the transport rhodopsin molecules, while the role of proton donor may be played by either aspartate or glutamate. Hence, finding a new retinal protein gene in 2008 was not a sensation anymore; so, there must have been substantial reasons to start isolating and studying this protein.

There were two reasons. First, the presence of the coding sequence of a retinal protein (we called it ESR after *Exiguobacterium sibiricum* rhodopsin) in the genome of a psychrotrophic soil bacterium was of interest [2]. All the known microbial rhodopsins belonged to halophilic Archaea, or marine microorganisms, while *E. sibiricum* was isolated from soil of freshwater origin. Second, the amino acid sequence alignment of ESR, BR, and PR revealed conservatism of all the key amino acid residues that are presumably involved in the proton transfer, excluding one. At the position that corresponds to the proton donor for the Schiff base, uniquely in ESR, instead of the typical carboxyl residue, there was a lysine (Lys96; Fig. 1). Later, the same residue at the corresponding position was found in proteorhodopsins belonging to related species, for example, *Exiguobacterium* AT1b living in hot springs of Yellowstone National Park [25], and in putative retinal proteins of oceanic proteobacteria [26]. The presence of such a unique structural feature raised a number of questions that we tried to answer during our studies of this interesting retinal protein.

FEATURES OF ESR PHOTOCYCLE IN DIFFERENT MEDIA AND ITS RELATION TO PROTON RELEASE

We expressed recombinant ESR having a C-terminal hexa-histidine tag in *E. coli*, extracted it from the membrane fraction in the presence of the detergent *n*-dodecyl- β -D-maltopyranoside (DDM), and purified it using immobilized-metal-affinity chromatography [2]. At pH 7.0, the absorption spectrum of the preparation had a maximum at 532 nm (Fig. 2a), shorter than that of BR and XR and closer to the absorption maximum of GPR. To prove the proton transfer activity of ESR, we tested light-induced pH changes in suspension of proteoliposomes with the incorporated protein or in *E. coli* cells expressing this protein. In response to illumination, the solution was acidified, which corresponds to transfer of protons from the cells or proteoliposomes containing ESR (Fig. 2b). The proton pump functioning was observed over a wide pH range – between 4.5 and 8.5. Based on these data, we concluded that the new retinal protein is a representative of the transport rhodopsin family that functions as proton pumps.

ESR/1-252	1	-----MEEVNLVLA-----QYMFVWGFVGMAGTLYFLVERNSLA-PE	39
PR/1-249	1	MKLLLILGSVIALPTFAAGGGDLASDYTGVSFWLVTAALLASTVFFVVERDRVS-AK	57
XR/1-273	1	-----MLQELPTLTPG-----QYSLVFNMFSTVATMTASFVFFVLARNNVA-PK	44
BR/1-249	1	-----QAQITGRPE-----WIWVALGTALMGLGTLVFLVKGMGVSDPD	38
ESR/1-252	40	YRSATVAALVTFVAAIHYFVKDAVGTSGLLSEIDGFP-----EIRYIDWLVITTP	91
PR/1-249	58	WKTSLTVSGLVTGIAFWHYMYMR-----GVWIE TGDSPT-----VFRYIDWLLTVP	103
XR/1-273	45	YRISMMVSAVVFIAGYHYFRITSSWEAAYALQNGMYQP TGELFNDAAYRYVDWLLTVP	102
BR/1-249	39	AKKFYAITTLVPAIAFTMYLSMLLYGLTMVPPFGGEQNP IY-----WARYADWLFITTP	91
ESR/1-252	92	LLLVKFP LLLGLKGR LGRPLLTKLV IADVIMIVGGYIGESSINIAGGF TQLGLWSYL I	149
PR/1-249	104	LLICEFYLI LAAATNVAGSLFKKLLVGSLVMLVFGYMG EAGIMAA-----WPAFII	154
XR/1-273	103	LLTVELVLMGLPKNERGPLAAKLGFLAALMIVLGYPG EVSENAALFGTR-GLWGFLS	159
BR/1-249	92	LLLLDLALLVDADQG-----TILALVGADGIMIGTGLV GALT KVYSYR-----FVWVAI	140
ESR/1-252	150	GCFAWIYI IYLLFTNVTKAAENKPAP-IRDALLKMRLFILIGWAIYPIGYAVTLFAPG	206
PR/1-249	155	GCLAWVYMIYELWAGEGKSACNTASPAVQSAYNTMMYI IIFGWAIYPVGYFTGYLMGD	212
XR/1-273	160	-TIPFVWILYILFTQLGDTIQRQSSR-VS TLLGNARLLLLLATWGFYPIAYMIPMAFPE	215
BR/1-249	141	STAAML YILYVLF FGFT-SKAESMRPEVASTFKVL RNVTVVLSAYPVVWLIGSEGAG	197
ESR/1-252	207	V-----EIQLVREL IYNFADLTKVGFGLIAFFAVKTMSLSSSKGKTLTS-----	252
PR/1-249	213	G-----GSALN LNIYNLADFVNKILFGLI IWNVAVKES SNA-----	249
XR/1-273	216	AFPSNTPGTIVALQVGYTIADVLAKAGYGVLIYNI AKAKSEEEGFNVSEMV EPATASA	273
BR/1-249	198	IVP-----LNIETLLFMVLDVSAKVGFGILLRSRAIFGEAEAPEPSAGDGAAATSD	249

Fig. 1. Alignment of amino acid sequences of ESR and homologous retinal proteins using CLUSTAL 2.1 software. Positions corresponding to amino acid residues Asp85, Lys96, and Lys225 in the ESR molecule are indicated with frames.

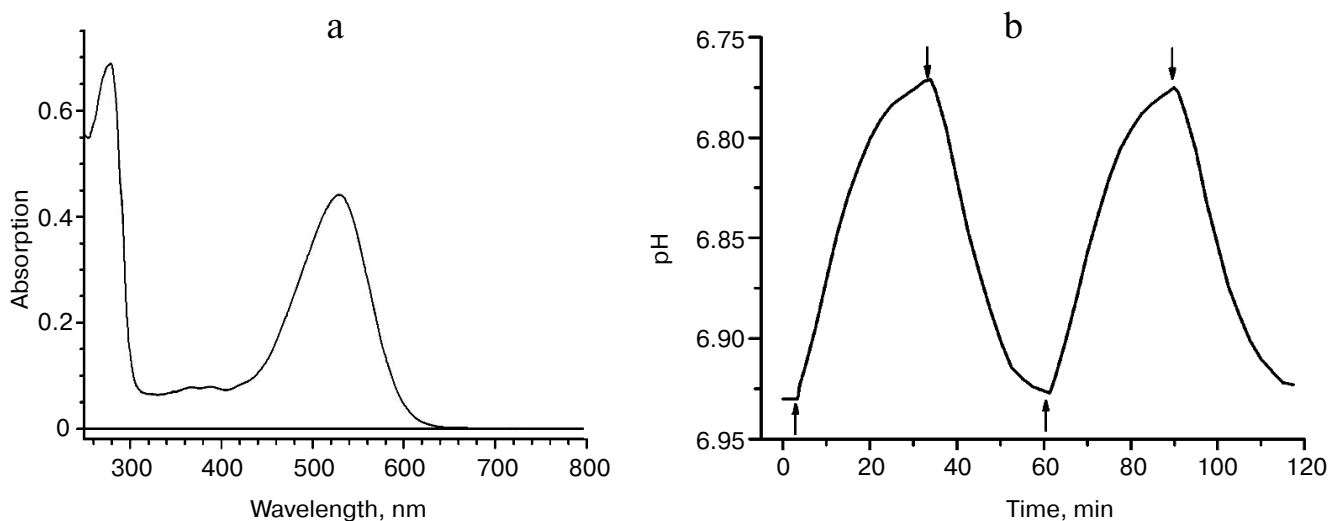


Fig. 2. Properties of recombinant ESR. a) Absorption spectrum for the sample in buffer: 50 mM sodium phosphate, 10 mM NaCl, 0.2% DDM, pH 8.0; b) light-induced proton release by ESR-containing proteoliposomes. The time points of turning the light on and off are indicated with arrows.

The photocycle of ESR was studied using laser flash spectroscopy (flash photolysis). It turned out that at neutral pH values in DDM micelles, in response to a flash ESR forms predominantly intermediates (K-, L-, and

N/O-like) absorbing in the long wavelength region, while the quantity of an M-like intermediate, that corresponds to the deprotonated Schiff base having an absorption maximum at 410 nm, is very small (Fig. 3a). Increase of

pH to above 9.0 results in fast emergence of an M intermediate that reaches its maximum 4 ms after the flash (Fig. 3b). The pK_a of M formation in DDM micelles is $\sim 8.8-9.0$ [27].

After M decay via Schiff base protonation, the state analogous to N- or O-intermediate of the BR photocycle is formed. As shown with Fourier transform infrared spectroscopy (FTIR), retinal re-isomerization is one of the slowest stages and occurs at the very end of the ESR photocycle, which leads to the accumulation of mostly an N-type intermediate with retinal in the 13-*cis* configuration [28].

Studies on the photocycle of ESR in proteoliposomes or lipid-like detergent LPG (16:0 Lyso PG) [29] revealed the same pattern as in the protein solubilized in DDM micelles, but the M-intermediate in these media

appeared already at neutral pH (the pK_a of its formation was ~ 6.5) and decayed much faster (Fig. 3, c and d). As in DDM, the quantity of M-intermediate in the lipid environment increased along with pH. Thus, significant dependence of photocycle parameters on the environment is an interesting feature of ESR.

Experiments using a pH-sensitive dye, pyranine ($pK_a \sim 7.2$), were carried out to study temporal events in the process of proton transfer by ESR. Increase in pyranine absorption at 460 nm in response to a flash corresponds to proton uptake from the environment, while decrease corresponds to proton release. Despite the small quantity of M-intermediate in the ESR photocycle in the DDM micelles, at pH 7.2 a substantial signal from pyranine was registered. In contrast to BR, in the ESR protein proton uptake occurs first ($\tau \sim 12$ ms) and then its release

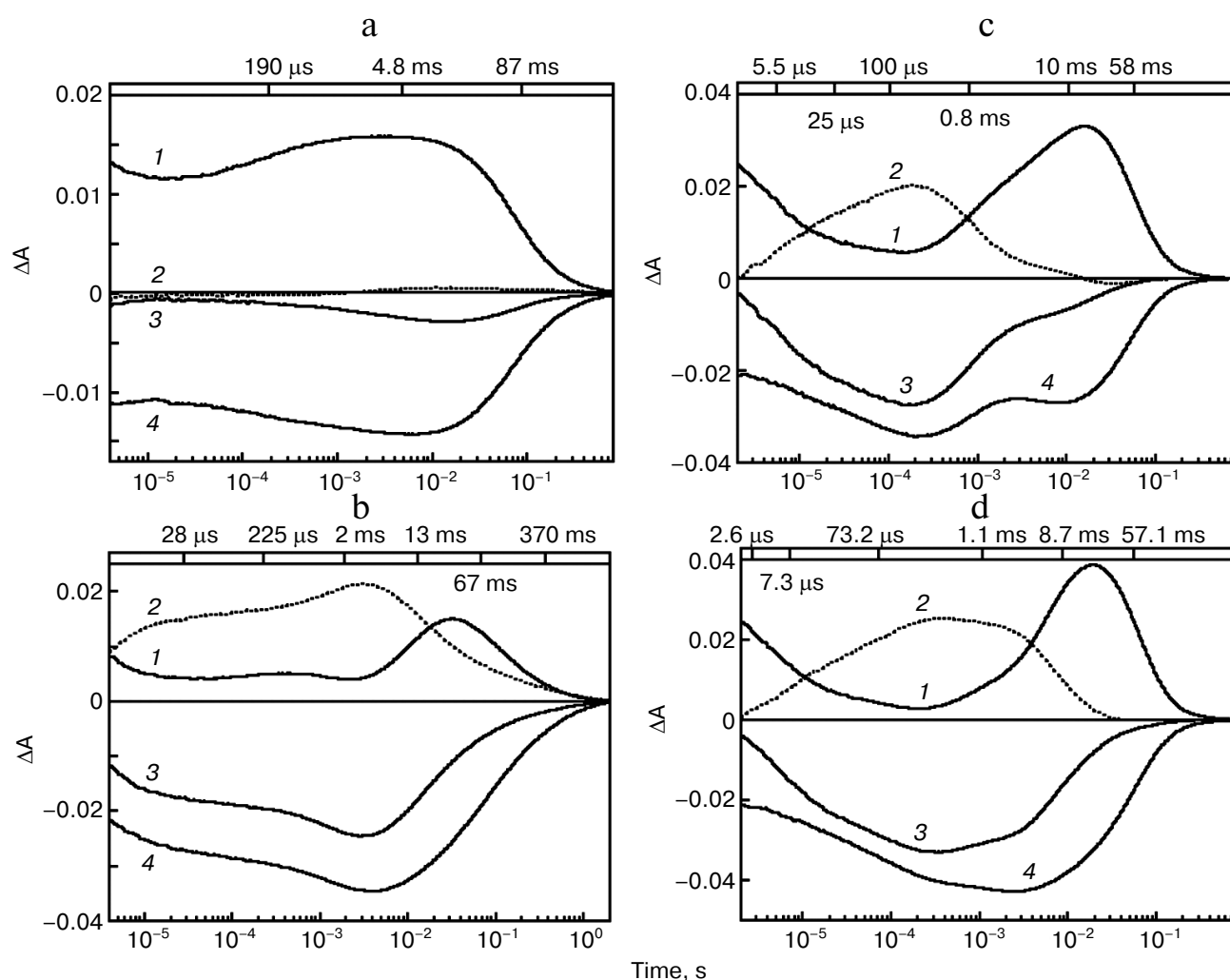


Fig. 3. Photocycle of ESR in DDM micelles (a, b) and LPG (c, d) at pH 7.6 (a), 9.1 (b), 7.1 (c), 8.7 (d). 1) Absorption changes at 590 nm due to formation and decay of the K-, N-, and O-intermediates; 2) absorption changes at 410 nm due to M-intermediate formation and decay; 3) absorption changes at 550 nm due to return to the initial state and formation and decay of the K-, M-, and N-intermediates; 4) absorption changes at 510 nm due to formation of the L-intermediate and decay of the K- and O-intermediates. Numbers on the upper panel of the figure are time constants determined from kinetic analyses of the absorption changes. Adapted from [27] and [29] with permission from the American Chemical Society and American Society for Biochemistry and Molecular Biology.

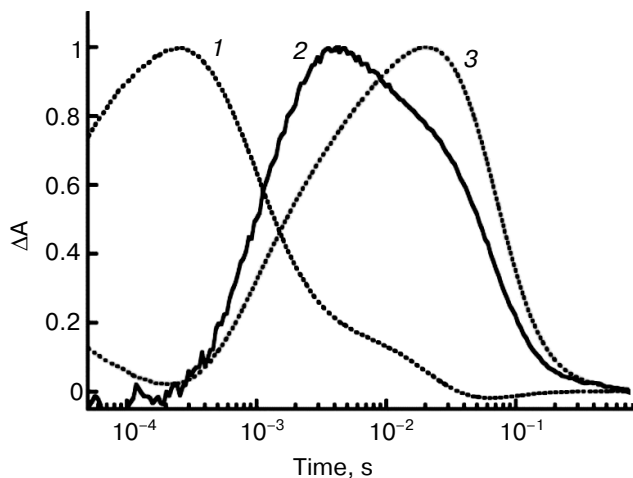


Fig. 4. Kinetics of light-induced proton uptake and release by ESR as compared to kinetics of M- and N-photocycle intermediates at pH 7.2: 1) absorption changes at 410 nm due to M-intermediate formation and its transition to N; 2) pyranine absorption changes at 455 nm due to its proton uptake and release; 3) absorption changes at 590 nm due to formation of intermediates N and O. Adapted from [29].

outward occurs ($\tau \sim 120$ ms). A similar order of events is typical for the majority of eubacterial proton pumps. It is explained by the fact that residues homologous to Glu194 and Glu204 that form a proton release group in BR are absent in these molecules (for reviews see [30, 31]).

Study of the ESR preparation solubilized in LPG micelles has allowed comparison of the kinetics of proton transfer and of the protein photocycle in detail owing to presence of high levels of the M-intermediate at pH 7.2 already. Under these conditions, the proton uptake occurred at $\tau \sim 0.9$ ms that was equal to the time of the M-intermediate decay and formation of the N-intermediate, i.e. Schiff base reprotonation. The proton release is featured by $\tau \sim 64$ ms and correlates with decay of the long-wavelength intermediate and returning into the initial state (Fig. 4).

To determine if an intermediate donor–acceptor group is involved in H^+ transfer, it was required to establish whether the proton uptake precedes reprotonation of the Schiff base or *vice versa*. For this, replacement of H_2O with D_2O was helpful [29]. It was known from BR studies that different photocycle stages are not slowed equally in heavy water. This kinetic effect appeared in our case as well: M-intermediate decay was 3-fold decelerated ($\tau \sim 3$ ms), while the proton uptake rate was only 2.3-fold decelerated ($\tau \sim 2$ ms). Hence, using heavy water distinguished in time the processes of proton uptake and Schiff base reprotonation more clearly and demonstrated that the first precedes the second. At first, the proton uptake occurs by a distinct group of the protein that does not function in the initial state. Then, after a short delay, the proton is transferred to the Schiff base. Thus, this group serves as the proton donor for the Schiff base in the ESR

molecule. The nature of this group was studied in experiments with ESR mutants.

Is the M-intermediate present in the ESR photocycle at $pH < pK_a$ of its formation? The data that clarified the nature and the order of intermediates in the ESR photocycle under different conditions were obtained using Fourier transform infrared spectroscopy (FTIR) [28]. The band corresponding to the C=O stretch of protonated counter ion, i.e. deprotonated Schiff base, was found in these spectra already at $pH \sim 5.0$. Furthermore, in the K96A mutant photocycle in DDM, owing to low rate of the M-intermediate decay, even at $pH 7.6$ its slow formation ($\tau \sim 12$ ms) is observed. One can hypothesize that the absence of M-intermediate accumulation in the wild-type protein photocycle in DDM at neutral pH values is for kinetic reasons, i.e. decay rate for this intermediate overrides its formation rate.

ROLE OF Lys96 RESIDUE IN SCHIFF BASE REPROTONATION

As mentioned above, an intriguing feature of the ESR amino acid sequence is the presence of a lysine residue at the position that corresponds to the intra-protein proton donor for the Schiff base. To characterize its functional role in the reprotonation process, we prepared and studied different mutant variants of this protein that have substitutions of this residue [29]. Mutant K96A, whose properties are considered in this section, was studied to the highest degree.

At pH 7.4, the rate of M formation is similar for K96A and the wild-type protein in LPG micelles. However, the rate of its decay, i.e. reprotonation of the Schiff base, is as much as 500-fold slower for the mutant (Fig. 5a). This leads to the apparent absence of the long-wavelength intermediates that follow M in the ESR photocycle and to a general deceleration of the photocycle of this mutant, for which the Schiff base reprotonation is a rate-limiting step. Since in the K96A mutant the Schiff base reprotonation happens directly from the environment, increasing pH from 7.0 to 9.0 is accompanied by linear deceleration of the M-intermediate decay, by more than one order of magnitude. Moreover, the mutant ESR in proteoliposomes or expressed in the *E. coli* cell membrane demonstrates decreased ability to transfer protons as compared to the wild-type protein (Fig. 5b).

Thus, we can state that this residue in the ESR molecule facilitates reprotonation, i.e. it functions as a proton donor for the Schiff base. Hence, ESR represents the first example of a proton-transfer rhodopsin in which this role is played by a residue having no carboxyl group. It was considered before that the presence of such group in a position corresponding to the proton donor as well as the carboxyl residue in the acceptor position of the retinal proteins is an essential condition for carrying out the proton

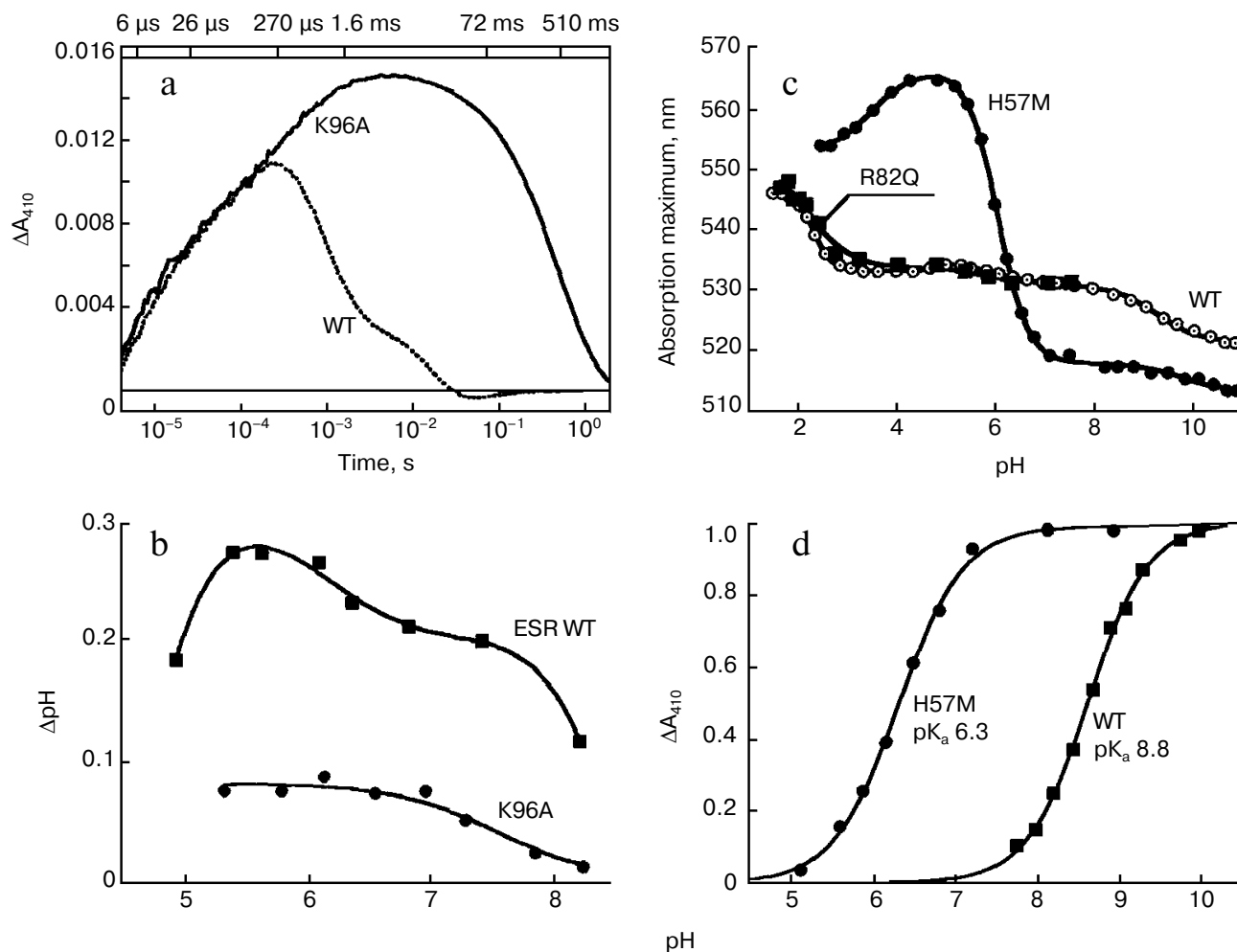


Fig. 5. Properties of the ESR mutants. a) Light-induced absorption change at 410 nm in wild-type ESR as compared to K96A demonstrating deceleration of the Schiff base reprotonation in the mutant; b) light-induced pH changes in a suspension of *E. coli* cells expressing wild-type ESR and K96A mutant; c) pH-dependence of absorption maximum of H57M in comparison with wild type and R82Q mutant; d) pH-dependence of light-induced absorption changes at 410 nm (M-intermediate accumulation) of H57M as compared to wild-type protein. Adapted from [27] and [29].

transfer [32]. From the discovery and study of ESR, this rule was broken for the first time [29].

One can imagine two ways for the participation of Lys96 in reprotonation of the Schiff base in the ESR molecule. The donor function could be carried out directly by the ε-amino group of the lysine residue that temporarily acquires the proton after M-intermediate formation. Then, after transfer of the proton to the Schiff base, it returns to the initial non-protonated state. It is known that the pK_a of lysine is very much dependent on the environment [33]. In the initial state of ESR, Lys96 is predominantly surrounded by hydrophobic residues that may decrease its pK_a . On the formation of the M-intermediate, enlargement of the hydrophilic cavity in the cytoplasmic part of the molecule may occur, thus increasing the pK_a of the lysine and causing capture of the proton.

At present, a second variant cannot be ruled out, the participation of the lysine side chain in a system of hydro-

gen bonds that involves water molecules which can serve as the proton donor. In this case, the protonation status of Lys96 would be unchanged.

Do carboxyl residues function as proton donors in the ESR molecule? To address this question, we made ESR mutants containing replacements of Lys96 with carboxylic residues (Asp or Glu) and planned to determine whether the Schiff base reprotonation in these mutants would occur as fast as in the wild-type protein.

ROLE OF His57 RESIDUE IN MODULATING pK_a OF PROTON ACCEPTOR Asp85

It is known that the function and the location of the absorption maxima for retinal proteins depend on protonation of an aspartic acid residue (Asp85 in BR), which is a proton acceptor from the Schiff base. Upon increase of

the pH to a certain value, loss of a proton by the side chain of this residue occurs. As a result, it acquires the ability to carry out an acceptor function, i.e. to accept the proton from the Schiff base and to serve as a counter-ion for the Schiff base in the initial state. Deprotonation is accompanied by a shift in the absorption spectrum toward shorter wavelengths. Therefore, studying the pH-dependence of the absorption maxima of retinal proteins provides important information regarding the mechanism of their functioning under different conditions and the factors that affect the mechanism. Thus, the titration curve of the BR absorption maximum shows complex dependence with two pK_a – 2.6 and 9.7 [34] – reflecting interaction of the proton acceptor Asp85 with residues that form a proton release group, Arg82, Glu204, and Glu194 [30]. For PR, the shift of the maximum in the 543–520 nm range is typical, which happens with pK_a 7.1 and corresponds to deprotonation of the Asp97 residue [35]. The absorption maximum of both these proteins at low pH values coincided with the absorption maximum of mutant variants having replacements of aspartic acid residues to asparagine, which eliminates the negative charge of the counter-ion.

Dependence of the ESR absorption maximum on the pH is complex and includes three transitions with pK_a 2.3, 6.0, and 9.1. The total shift of the maximum is 24 nm (from 545 to 521 nm) in the pH range 2.0–10.5 (Fig. 5c). Mutant variant D85N having Asp85 replaced with asparagine has an absorption maximum at 563 nm. Thus, at $pH > 3.0$ the majority of the ESR molecules contain the acceptor in the deprotonated state, which allows the protein to transport protons over a wide pH range. We studied the properties of some mutant variants of the protein to find the reason for the complex dependence of the spectral characteristics of ESR during titration.

It turned out that the pH-dependence of the absorption maximum for the R82Q mutant is identical to the curve obtained for the wild-type ESR (Fig. 5c). This suggests that in the ESR molecule, in contrast to BR [36, 37], the Arg82 residue is weakly bound to the proton acceptor from the Schiff base. A similar situation was observed for PR [38]. In contrast, replacement of His57 with a methionine residue (H57M) leads to substantial change in properties of the acceptor complex. At pH 5.0, the absorption maximum for this mutant is at 565 nm, which almost coincides with the absorption maximum for the D85N mutant. This means that at pH 5.0 the proton acceptor Asp85 in the H57M protein is fully protonated. Along with pH increase to 8.5, significant shift (47 nm) of the absorption maximum of the mutant to 517 nm was observed, with pK_a 6.3 corresponding to pK_a of Asp85 in H57M (Fig. 5c).

Such substantial increase in the Asp85 proton acceptor pK_a in the H57M mutant as compared to the wild-type protein indicates that in the latter the His57 residue tightly interacts with the Asp85 residue, decreasing its pK_a . As a result, acceptor Asp85 is deprotonated over a wide pH

range. This interaction apparently causes the complex character of the pH-dependence of the wild-type ESR absorption maximum.

The presence of the histidine residue at the position that corresponds to His57 of ESR is a feature of eubacterial transport rhodopsins, including PR [39], XR [40], and GR [41]. At the same time, XR, similarly to ESR, shows only marginal absorption maximum shift (3–5 nm) with pH increase from 4.0 to 10.0. Examination of the three-dimensional structure of XR revealed that the His62 and Asp96 residues are at a distance of ~ 2.5 Å from each other and form a strong hydrogen bond [40]. Unfortunately, data regarding properties of XR mutants having His62 replacements are not currently available due to the absence of an efficient system for expression of this protein in *E. coli*. Titration of PR in the pH range 6.0–10.0 leads to a more significant shift (23 nm) of its absorption maximum. In contrast to ESR, His75 replacements in this protein lead to shift of pH-dependence of the absorption maximum of the mutants toward the acidic region [39, 42]. Thus, we can state that structure of the acceptor complex in this protein apparently significantly differs from that of ESR.

Studying the H57M mutant photocycle at different pH values revealed that the appearance of the M-intermediate (deprotonated Schiff base) occurs at lower pH values as compared to the wild-type protein, the mutant having pK_a 6.3 (Fig. 5d). This value corresponds to the pK_a of the absorption maximum shift for H57M (see above), i.e. the pK_a of the proton acceptor deprotonation in the initial state. In H57M, Schiff base deprotonation happens much faster than in the wild-type protein, which corresponds to higher pK_a of the acceptor (Asp85). In general, the photocycle of this mutant features higher rate than the photocycle of the wild-type ESR, which is also typical for His75 mutants of PR [42].

Hence, the results obtained provide ample grounds to assume that at neutral and acidic pH values in the wild type protein His57 residue is protonated, interacts tightly with Asp85, and decreases pK_a of the latter. In the process of photocycle, retinal isomerization, apparently, leads to disruption of a hydrogen bond between these residues and to deprotonation of His57 or to change of orientation of His57 relatively to Asp85 similar to what happens with Arg82 residue in BR [36, 43]. As a result of these events, pK_a of the acceptor temporarily increases and it acquires an ability to accept the proton from the Schiff base.

THREE-DIMENSIONAL STRUCTURE OF ESR

The tertiary structure of ESR was studied by X-ray crystallography at 2.3 Å resolution [44]. According to the crystallographic data, ESR, like BR, belongs to the structural family of membrane proteins having seven α -helix segments that comprises all the currently known retinal

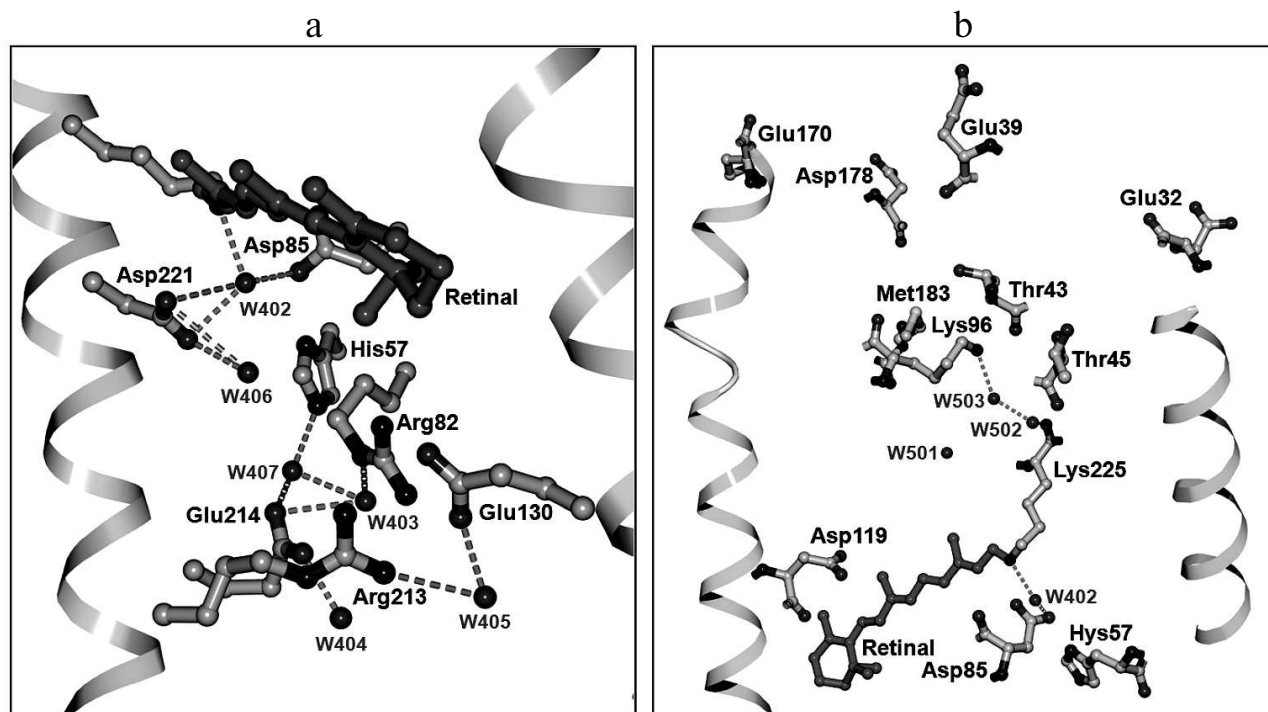


Fig. 6. Three-dimensional structure of ESR: a) outer protein moiety; b) cytoplasmic protein moiety.

proteins, including visual rhodopsin and G protein-coupled receptors (GPCR) [46–48].

Similarly, like the other representatives of microbial transport rhodopsins, ESR contains a retinal residue in *all-trans* configuration covalently linked to the Lys225 residue. The protonated Schiff base is turned toward the extracellular part of the protein and is bound by hydrogen bonds through water molecule W402 to carboxylic residues that form the counter-ion – Asp85 and Asp221 (Fig. 6a). This configuration stabilizing positive charge of the Schiff base was found in the majority of known transport rhodopsins functioning as proton pumps. The side chain of the Asp85 residue in the ESR molecule is oriented just as in BR, in contrast to XR, where it is turned differently [40]. Similarly to BR, in the close vicinity of retinal there is another water molecule, W406, that forms a hydrogen bond with the Asp221 residue. While for BR in the initial state Arg82 is typically coupled to the proton acceptor Asp85, in the ESR molecule the side chain of this residue is directed outward from the Schiff base, similarly to its position in the M-intermediate of BR, and it is bound by a hydrogen bond to the Glu130 residue. This configuration explains the above-discussed absence of an influence of Arg82 on the functional state (protonation status) of the proton acceptor Asp85 in ESR [27]. ESR differs from the earlier transport rhodopsins by having a bend in α -helix F in the region of residue 185. The corresponding region of the helix is stabilized due to hydrogen bonding with side chains of Trp154 and Asn224 residues

that are present at homologous positions in molecules of all the proteorhodopsins. The reason for such universality remains elusive.

Explanation for the influence of the His57 residue on properties of the acceptor could be given based on the tertiary structure of ESR. The distance between these residues is very short, $\sim 2.4 \text{ \AA}$, which allows formation of a strong hydrogen bond. A similar bond is present in the XR structure [40], but its configuration differs significantly. In the ESR molecule, the side chain of the histidine is directed to Arg82 and is placed in a hydrophilic cavity containing several water molecules. In XR, the His62 side chain is turned to the opposite direction.

As stated above, the ESR molecule contains no homologs of Glu194 and Glu204 that in BR form, together with water molecules, the proton release group [49]. It is presumed that this is the reason for reverse (as compared to BR) order of proton transfer reactions: first, proton uptake occurs at the cytoplasmic side of the protein, then its release at the opposite side of the membrane by an unknown residue or a group of residues. The presence of the hydrophilic cavity that connects the extracellular part of ESR with the acceptor complex His57–Asp85 suggests their direct role as the proton source. Moreover, there are a large number of polar and charged residues associated by hydrogen bonds in this part of the protein that might also be the proton source. In particular, it is worth paying attention to the chain of hydrogen bonds connecting His57 with Asp214 through water molecule W407.

As discussed above, unlike the other representatives of the proton transport rhodopsin family, in ESR at the position that corresponds to the proton donor for the Schiff base there is a lysine residue rather than a carboxylic residue. Lys96 is surrounded primarily by hydrophobic residues; however, it is only separated from the hydrophilic cavity by the polar side chain of Thr43 residue (Fig. 6b). Such proximity of the proton donor to the environment distinguishes ESR from other proton pumps with known structures (BR, XR, and HmBR [50]) and may provide fast access of the protons from the cytoplasm in the reprotonation process. The side chain of the Leu93 residue as well as at least two water molecules are located between the Schiff base and Lys96.

Thus, studies on the three-dimensional structure of ESR have revealed the presence of structural elements common for the other representatives of the family of eubacterial proton pumps, XR [40] and BPR [51], but also distinct features of this protein related to the presence of the lysine residue as the proton donor and the complex system of hydrogen bonds between the Schiff base and the outer protein surface.

SUGGESTED SCHEME FOR THE FUNCTIONING OF ESR AS A PROTON PUMP IN COMPARISON WITH OTHER TRANSPORT RHODOPSINS

Based on spectroscopic and structural studies, the order of events accompanying light-dependent proton transfer by ESR can be described as follows. In the initial state of the protein, retinal is in *all-trans* configuration, the Schiff base is protonated (positively charged), proton acceptor Asp85 is deprotonated (negatively charged), and the proton donor is in the neutral state. The ion pair formed by the Schiff base and Asp85 is stabilized by water molecule W402 (Fig. 6a). Absorption of a light quantum leads to retinal isomerization from *all-trans* to 13-*cis* configuration and destabilization of this pair. As a result, the proton is transferred to the acceptor Asp85 that is bound to His57 by a hydrogen bond (L-M1 stage). The mechanism of the fast (femtosecond-microsecond range) primary processes in ESR has not been studied in detail; however, we expect that it is similar to those described for BR, PR, and XR [52-55]. During the M1–M2 transition, proton uptake from the environment takes place by the donor Lys96 residue, which acquires positive charge for a short while. The proximity of this residue to the hydrophilic cavity in the cytoplasmic moiety of the protein accelerates opening of the “channel” that leads to the increase in pK_a of the lysine.

M2 decay is accompanied by proton transfer from donor to the Schiff base at the N1 stage. In the final stage of the photocycle, deprotonation of the acceptor group, proton release into the medium by an unidentified group X, and retinal re-isomerization into *all-trans*

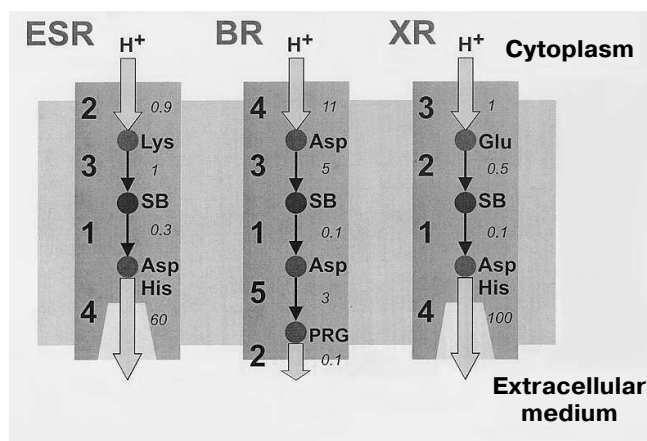


Fig. 7. Proton transfer scheme in ESR, BR, and XR. The proton transfer stages are indicated with numbers, and typical proton transfer times in milliseconds are italicized. For ESR: 1) Schiff base deprotonation (formation of M-intermediate); 2) proton uptake from the cytoplasm; 3) Schiff base reprotonation (M-intermediate decay); 4) acceptor deprotonation and proton release. For BR: 1) Schiff base deprotonation (formation of M-intermediate); 2) proton release by PRG (proton release group); 3) Schiff base reprotonation (M-intermediate decay); 4) proton uptake from the cytoplasm; 5) acceptor deprotonation and proton release. For XR: 1) Schiff base deprotonation (formation of M-intermediate); 2) Schiff base reprotonation (M-intermediate decay); 3) proton uptake from cytoplasm; 4) acceptor deprotonation and proton release.

configuration occur. Thus, the protein returns to its initial state.

The main differences of the photocycle of ESR from the other proton-transport rhodopsins (BR, XR, and PR) are related to the presence of a lysine residue at the ESR position that corresponds to the position of the proton donor Asp96 in BR and also the absence of residues that form the proton release group in BR (Glu194 and Glu204). Both Lys96 of ESR and Asp96 of BR in the initial state are surrounded by hydrophobic residues, which facilitate maintaining their electroneutrality. However, while for the Asp96 residue (having initial pK_a greater than 11) this means the presence of a proton on it, in the ESR molecule the Lys96 donor is not protonated. At this stage, its pK_a is apparently about 6.0, and only later it increases to ~ 8.5 . According to these differences, in the photocycle of BR the Schiff base reprotonation (M-intermediate decay) occurs during the M to N1 transition due to H^+ transfer from Asp96 until the moment of proton capture by this residue. During the process of proton capture, the pK_a of Asp96 drops to 7.5 [56, 57]. The Schiff base reprotonation occurs similarly in the XR and PR molecules, where the proton donor is a glutamic acid residue. On the contrary, Lys96 of ESR first obtains a proton from cytoplasm and only then transfers it to the Schiff base (Fig. 7).

The proton release at one of the last photocycle stages is an important feature that distinguishes ESR from

BR. In the BR photocycle, it happens during the M1–M2 transition, i.e. at an earlier stage. However, mutant variants of BR having no Glu194 and Glu204 residues demonstrate so-called “late” proton release, like ESR [58, 59]. This order of events is also typical for XR and PR due to the absence of the proton release group in their molecules.

During last 15 years, the discovery of many new retinal proteins [60] – proteorhodopsins [61], xanthorhodopsins [62], channel rhodopsins [63, 64], chloride and sodium pumps [65, 66] – along with substantial progress in determining their tertiary structures [40, 50, 51, 67–69] has restored interest in studying the light-dependent ion transport started over 40 years ago by D. Oesterhelt and W. Stoeckenius [3]. Studying the new representatives of the family supplements our knowledge of the structural basis of such transport [66] and promotes their application in bio-optoelectronics as a basis for molecular memory elements and photoactive media in dynamic holography [70–72], and in a new field – optogenetics [73] – for regulation of neuronal activity [74–76]. The sensitivity of photocycle reaction kinetics and fluorescence of the proton pumps to electrochemical potentials [77, 78] revealed the possibility of using them as sensors for detection of intraprotein [79] and transmembrane changes of potentials [80].

Our studies on ESR – a new retinal protein from *E. sibiricum* – have broaden our knowledge of the mechanism of Schiff base reprotonation and of the nature of the amino acid residues that play the role of the intraprotein proton donor. The example of ESR is the first demonstration that a lysine residue can serve as the donor facilitating proton delivery from the cytoplasmic protein surface to the Schiff base. The interaction between Asp85 and His57 residues that leads to ESR proton acceptor pK_a shift toward acidic pH that we discovered provides its ability to function over a broad pH range. The presence of the hydrogen bond between these residues and other features typical for proteorhodopsin and xanthorhodopsin families was demonstrated from the three-dimensional structure of ESR. Studies devoted to ESR are continuing, and they will undoubtedly lead to discovery of interesting new properties of this protein. It remains to be determined, which amino acid residues are responsible for the proton release under different conditions, how the ESR photocycle changes depending on lipid environment, and whether the proton transfer is accompanied by intramolecular charge transfer. It is possible that these studies will assist in addressing the question of evolutionary mechanisms by which light-sensitive retinal protein was encoded in the psychrotrophic bacterium genome from the Siberian tundra permafrost.

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