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Topical Review

Protein Electric Response Signals from Dielectrically Polarized Systems

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Introduction

Charges move, dipoles rotate inside the proteins during the functioning. A few examples: in myoglobin the Fe^{2+} ion moves in and out the heme plane during binding and release of CO (Phillips, 1978); charged particles or dipoles move when Na^+-K^+ channels are opened and closed (gating current; Armstrong & Gully, 1979); and charged particles move through proteins in case of ion pumps (for example, Na^+,K^+-ATPase translocates Na^+ and K^+ ions; Kyte, 1981).

Moving charges, rotating dipoles in proteins (as in any dielectric medium), induce displacement currents. With suitable methods the kinetics of these currents can be measured, providing important information about the processes related to the activity of proteins. This electrical signal can be named protein electric response signal (PERS) (Keszthelyi & Ormos, 1980).

Two requirements have to be fulfilled in order to observe the time course of PERS: (i) a large number of proteins should be synchronized in their function, and (ii) the systems must be asymmetric with respect to the measuring electrodes.

The first requirement can be easily met in the case of light-activated systems by short light-flash excitation. Most systems can be synchronized by light-flash excitation in an indirect way by the light-induced release of protons (Gutman, 1986) and ATP (Christensen et al., 1988), Ca, etc., from caged substances. Several systems can be excited with electric pulses (as in the case of neurons).

The second requirement needs asymmetric systems located between two electrodes. Membrane-bound proteins are the first candidates if (i) they are in the outer membrane of large enough cells, and the two electrodes can be located inside the cell and on the cell surface, respectively; (ii) the cells or vesicles containing the proteins are small (diameter <20 μm) but they can be attached to a bilayer separating two electrolytes (in this case proteins attached to the bilayer produce more displacement current than the others); (iii) some proteins are in rigid membrane fragments which do not form closed vesicles and thus possess large permanent electric dipole moments. This permits orientation of the fragments in very small electric fields (10–20 V/cm, (Keszthelyi, 1980)) (soluble proteins need $\sim 10^4$ times higher field for orientation).

The best known example is bacteriorhodopsin (bR) in the purple membrane (pm) of *Halobacterium halobium* (Stoeckenius, Lozier & Bogomolni, 1979). Also the open sheets can be attached to bilayers in an oriented way (Drachev, Kaulen & Skulachev, 1978; Fahr, Läuger & Bamberg, 1981; Läuger et al., 1981; Rayfield, 1982).

Vesicles and open sheets adjoined to membranes separating two compartments (capacitive systems) and oriented open sheets (dielectrically polarized systems) yield data on PERS (Trissl et al., 1984). The essential features of capacitive systems were reviewed by Läuger et al. (1981); therefore, in this paper we restrict the discussion to the dielectrically polarized systems and to a special application of the idea.

Even in this relation the main emphasis will be on bR molecules, which translocate protons after photon absorption; bR is the best studied protein in this topic.

Experimental Details of the Measurement of PERS in Di-electrically Polarized Systems

The average diameter of pm's isolated from *Halobacterium halobium* is 0.5 μm and thickness is 5 nm. Their permanent electric dipole moment is $\sim 10^6$ Debye (Barabás et al., 1983). They can be ori-

Key Words bacteriorhodopsin · charge displacement · halorhodopsin · myoglobin · oriented systems · photocurrent

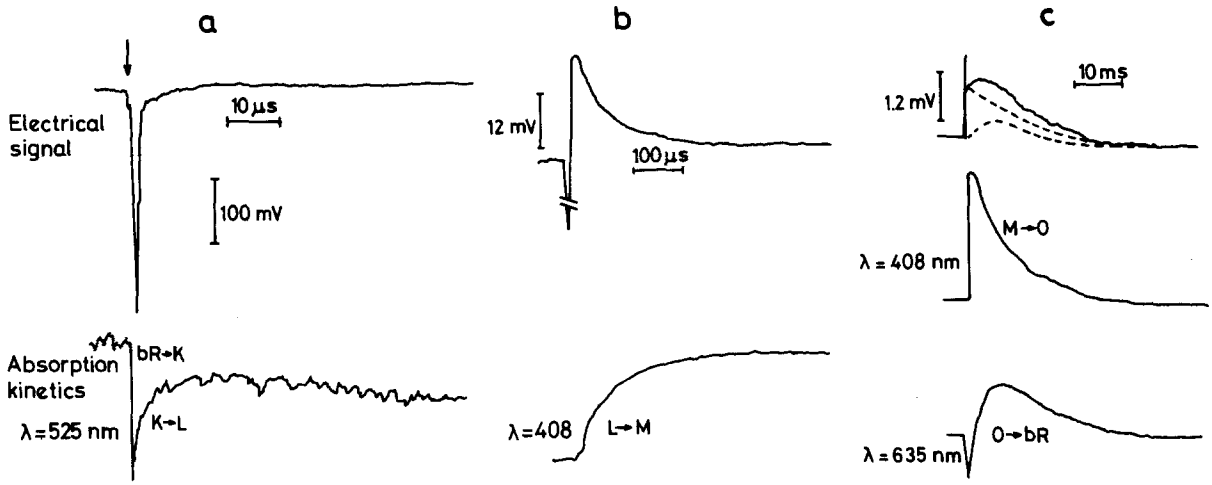

Fig. 1. Characteristic photoelectric (PERS) and absorption kinetic signals measured on water suspension of pm's oriented by DC electric field of 9 V. (*a–c*) Signals measured with different time resolution. The electric signals were measured after the orienting field was switched off, but the orientation was still present. (*a*) $T = 5°C$. (*b,c*) $T = 22°C$

ented practically to saturation by an electric field of 10–15 V/cm; $\sim 10^{14}$ proteins are excited by a laser flash and the protons to be translocated move in one direction. The time and amplitude distribution of the current is easy to measure (for experimental details *see* Keszthelyi & Ormos, 1980, 1983).

The laser is fired either during the presence of the orienting electric field or after it is switched off. In the first case, in addition to PERS currents are also generated due to conductivity changes from transiently emitted ions (protons and others); in the second case, only the PERS appears—which can be obtained because the membranes are still oriented (the relaxation of orientation is 300–500 msec).

The oriented membranes can be immobilized in gel (Dér, Hargittai & Simon, 1985*b*) or deposited on a transparent electrode (Váró, 1981). In these cases PERS is measured without the presence of an external electric field and consequently without the disturbing effect of the conductivity changes.

The main advantage of this method compared with the method using capacitive systems is the large quantity of the proteins involved. Therefore, spectroscopic measurements are easy to perform on the same sample used for PERS measurements. Spectroscopy is extremely difficult with pm attached to bilayers.

In Fig. 1 we reproduce a representative series of electric and light absorption signals. The light absorption signals (life times and wavelength) characterize the different intermediates (*K*, *L*, *M* and *O*) of the photocycle of the bR in accordance with previous results (Stoeckenius et al., 1979). The time constants of the electric signals coincide with the time constants of the intermediates of the photocycle. The coincidence is preserved in a broad temperature (Fig. 2) and pH range (Fig. 3), though it was found to cease at pH > 8 (Ormos, Hristova & Keszthelyi, 1985). Recently, however, coincidence in the whole pH range was demonstrated by Müller et al. (1988).

Based on these data the five kinetic components of the PERS are assigned to the five known transitions of the bR photocycle. The "instantaneous" absorbance decrease which decays with the time constant of the electric circuit corresponds to bR–*K* transitions, the second small negative signal belongs to *K–L* transitions while the consecutive positive signals can be assigned to the transitions *L–M*, *M–O* and *O*–bR, respectively.

The rise of the first negative signal has two components if it is measured in suspension or gel with a time resolution of 10 nsec (Fig. 4). The rate of the first fast change (20 nsec) is limited by the duration of the laser pulse, while the time constant of the second process (τ_2) depends on the resistance of the sample, similar to the time constant of the decay of the signal (τ_3). In a series of measurements $\tau_3/\tau_2 \sim 10$ was found. On the other hand, τ_2 was absent when the electrodes were in immediate contact with the bR layers. In this sample the rise of the first PERS was found to be $\tau_1 < 20$ psec (Groma et al., 1988*a*).

Interpretation of the PERS

The oriented pm's are excited by the laser flash. The displacement current is picked up by the two electrodes (E_1, E_2 at a distance D; Fig. 5*a*) con-

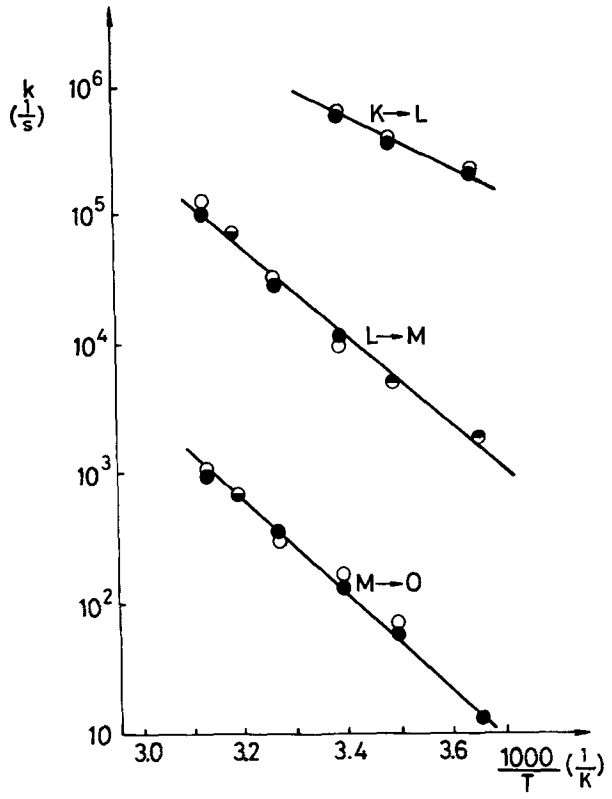

Fig. 2. Arrhenius plot of the temperature dependence of the rate constants of the different transitions as determined from electric (○) and absorption kinetic (●) data, pH = 6.5

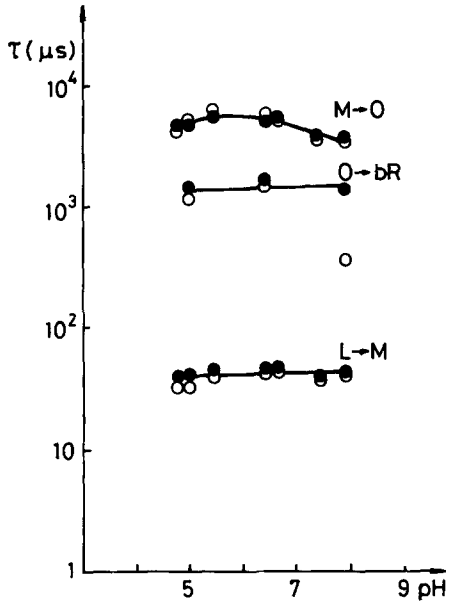

Fig. 3. pH dependence of the time constants of the different transitions as determined from electric (○) and absorption kinetic (●) data. The measurements were made on water suspension of pm's, the pH was set by adding HCl or NaOH to the sample

nected to an *RC* circuit. An approximate theory outlined below can account for the majority of processes.

Let us select a single purple membrane and calculate the current $i(t)$ when a charge Q moves from site 1 to site 2 in a distance d (Fig. 5*b*):

$$i(t) = \frac{Qv(t)}{\varepsilon D'} \tag{1}$$

where $v(t)$ is the time-dependent velocity of the charge, D' the distance of the electrodes, and ε is the dielectric constant of the protein. We assume that $v(t)$ is very large, i.e., the charge jumps from site 1 to site 2 (the time needed is $\sim 10^{-13}$ sec (Läuger et al., 1981). By integrating Eq. (1) with respect to time:

$$Q_{\text{ind}} = \int_o^\infty i(t)dt = \frac{Q}{\varepsilon D'} \int_o^\infty v(t)dt = \frac{Qd}{\varepsilon D'}. \tag{2}$$

Q_{ind} charges the capacitance C in the measuring circuit, which discharges through the resistance R. The voltage generated is

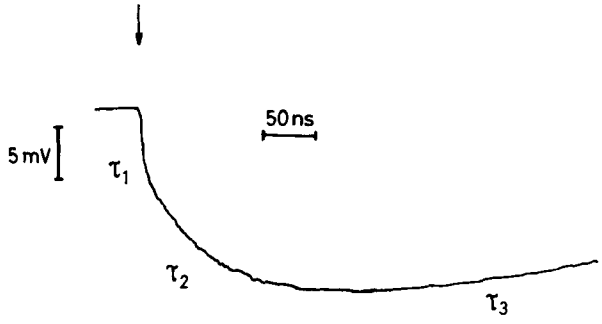

Fig. 4. Submicrosecond kinetics of PERS measured on oriented water suspension of pm's stabilized in gel. pH = 6.5. T = 22°C. The duration of the laser pulse is 20 nsec

$$V_1(t) = \frac{Q_{\text{ind}}}{C} e^{-t/RC} = \frac{Qd}{\varepsilon CD'} e^{-t/RC}. \tag{3}$$

In the real case N charges move with an exponential time distribution:

$$\rho(t) = kNe^{-kt} \tag{4}$$

where k is the rate constant. Every induced charge displacement produces a voltage as in Eq. (3). To obtain $V_N(t)$ we take the electrode distance D and sum the N uncorrelated $V_1(t)$ functions for all times $t' < t$. This means the folding of Eqs. (3) and (4):

Table. The distances that the proton moves perpendicular to the plane of pm during the transitions of the bR photocycle as calculated according to Eq. (8)[a]

Transition	Distance (nm)	Normalized distance (nm)
bR–*K*	−0.5	−0.12
K–*L*	−0.08	−0.02
L–*M*	2	0.5
M–*O*	12.4	3.1
O–bR	6	1.5
Σd_i	19.8	4.96

[a] The normalized distances were obtained by forcing the total displacement to be equal to the membrane thickness (5 nm)

$$V_N(t) = \frac{NQdk}{\varepsilon DC}\int_o^t e^{-kt'} \cdot e^{-(t-t')/RC}dt' = \frac{NQdk}{\varepsilon D}\frac{R}{1-kRC}(e^{-kt} - e^{-t/RC}). \quad (5)$$

Two special limiting cases are important:

$$V_N(t) = \frac{NQd}{\varepsilon DC}e^{-t/RC} \text{ if } k \gg 1/RC \quad (6)$$

and

$$V_N(t) = \frac{NQd}{\varepsilon D}kRe^{-kt} \text{ if } k \ll 1/RC. \quad (7)$$

If there are more components of charge movement with a different time constant in series, as in the case of bR, then more exponential components appear. The population of the component appearing in the series is given by the Bateman functions elaborated for the radioactive decay series (the version specialized for bR has been given by Läuger et al. (1981)). Let us denote it by $f(k_1, k_2, \ldots k_i, t)$ then the voltage for the i-th transition with a displacement d_i

$$V_N^{(i)}(t) = \frac{NQd_i}{\varepsilon_i D}k_i f(k_1, k_2, \ldots k_i) \quad (8)$$

here the possible variation of the dielectric constant is accounted for by ε_i.

Equation (8) contains measurable data $V_N^{(i)}(t)$, N, D, $f(k_1, k_2, \ldots k_i)$ and unknowns d_i and ε_i. Assuming ε to be constant all through the protein,

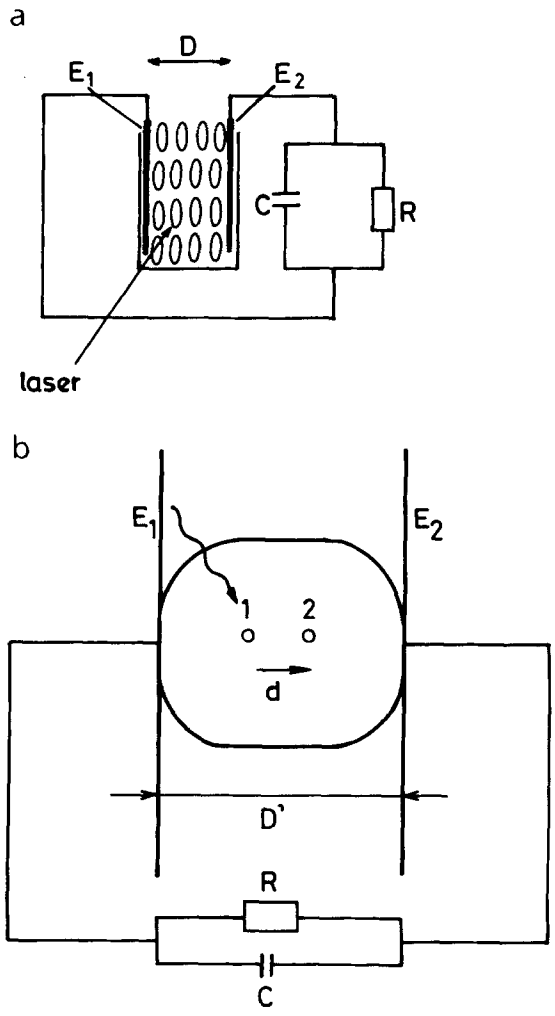

Fig. 5. (*a*) The displacement currents caused by the elementary acts in the bR proteins in purple membranes (small ellipses) are summed by electrodes E_1 and E_2 at a distance D on resistance R. C is the capacitance of the measuring system (i.e., capacitances of electrodes, wirings, input capacitance of the amplifier, etc.). Typical value of $C \sim 10$ pF. (*b*) Assumed elementary act in the measurement of displacement current. bR protein embedded in membrane; charge moves from point *1* to *2*; E_1 and E_2, hypothetical electrodes in a distance D'

$\varepsilon = 2$, and that the moving charge is a single proton, d_i's can be calculated for all the five transitions (Table).

The simplest control of the results is to compare the calculated Σd_i with the membrane thickness. As seen in the Table, the simple theory works surprisingly well, reproducing the membrane thickness within a factor of four. The distances determined after normalization to the membrane thickness of 5 nm also are given in the Table.

This simple derivation does not explain the PERS in the submicrosecond range (Fig. 4). In reality N charges move in a very short time during the

bR–K transition (<20 psec, Groma et al., 1988*a*), but the length of the laser flash (~20 nsec) stretches the time to $\tau_1 = 20$ nsec which is detected (Fig. 4). This is the time of the fast rise; its effect is negligible in τ_2.

A tentative explanation of the time constant τ_2 is the following (Fig. 6): the effect of charge motion should cross the double layer formed between the membrane surface and solution (McLaughlin, 1989). The capacity C_P (the capacitance of the pm) and resistance R_P (the resistance around the pm) form a time constant which delays the build up of the charge at the measuring capacity C. This $C_P R_P$ depends on the conductivity of the solution as does the resistance of the solution R_E which determines τ_3 in our case when $R \gg R_E$. Detailed investigations of this interesting phenomenon are in progress in our laboratory.

An alternative analysis of the same experimental method has been performed by Liu and Ebrey (1988), which is based on the frequency response of their photocurrent measuring system. The two evaluation methods—based on transients as in Eq. (8) or on frequency response—are equivalent and yield the same understanding.

An additional value of the paper by Liu and Ebrey is the consideration of the double layer capacitance between the electrode and solution C_l. If the time constant formed by C_l and the series resistance in the circuit (R) is not much larger than the decay times of the slower components of the photocurrent then these components are distorted. This is easy to avoid in case of platinized Pt electrodes where $C_l \sim 10^{-3}$ F and $R < 3 \times 10^4$–$10^6\ \Omega$. In case of bR the long components have a life time of 10–100 msec (depending on pH, temperature, etc.) as compared to 10–10^3 sec.

The problems in the submicrosecond time range do not disturb the analysis of the components in the longer time range. The bR probably has a 6-nsec component (at room temperature), which has been traced by optical methods (Shichida et al., 1983; Midler & Kliger, 1988). The electric equivalent of this component has been detected only on dried oriented samples (Groma et al., 1988*a*) where τ_2 does not appear because of the immediate contact of pm's with the electrodes. The instrumental time constant in this measurement was $\tau_3 = 450$ psec; therefore, for the component with 6 nsec, Eq. (7) is valid. For the very fast component ($\tau_1 < 20$ psec) τ_3 is long; therefore, Eq. (6) is valid.

Discussion of the Distances

The distances d_i are calculated with important assumptions. Separate data are therefore needed to confirm the values of the distances obtained from PERS experiments.

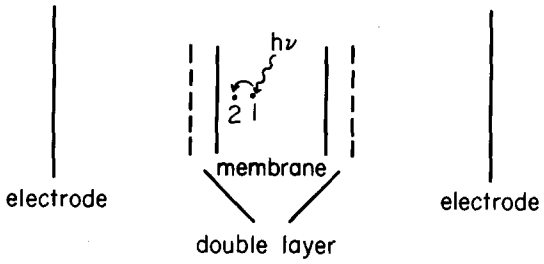

Fig. 6. Schematics of the pm suspension system with the double layer adjacent to the purple membrane. The charge jumps from site *1* to *2* inside the membrane

The sum of distances $d_1 + d_2 = -0.15$ nm (Table) is very near to the projection in the membrane normal direction of the distance $d = 0.16$ nm made by the NH^+ group during all-*trans*–13 *cis* isomerization (Keszthelyi, 1984). Models of bR structure locate the Schiff-base at 1/3 of membrane thickness from the internal surface of the membrane (Stoeckenius et al., 1979; Otomi et al., 1988). This is reproduced by the data $d_4 = 3.1$ nm and $d_5 = 1.5$ nm. These long distances correspond to proton motion from the Schiff-base region to the external surface and from the internal surface to the Schiff-base region of bR.

Charged parts of the amino acid side chains may move in correlation with the photocycle. It has been shown by photoselection measurements in the UV spectral range that the motion of the tryptophane and tyrosine side chains is highly restricted (Czégé et al., 1982). The possible corrections—which are negligible compared to the distances d_i—were discussed by Keszthelyi (1984).

The activation enthalpy ΔH for charge motions and consequently the time constants of the transitions are affected in an external electric field (Zwolinsky, Eyring & Reece, 1949).

$$\tau(V) = A^{-1} \exp\left(\frac{\Delta H + \frac{d}{2D} FV}{RT}\right) = \tau_o \exp\left(\frac{\frac{d}{2D} FV}{RT}\right) \tag{9}$$

where V is the potential on the protein in the membrane of thickness D, d the thickness of the barrier, i.e., the displacement of charge, F the Faraday number, R the Boltzmann constant and T the temperature. Equation (9) has been used to determine d_3, applying electric field externally to dried ori-

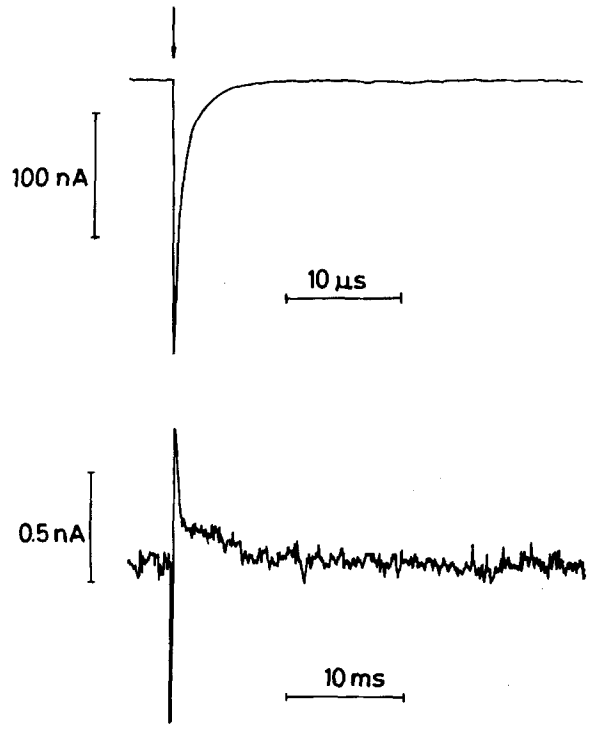

Fig. 7. PERS on halorhodopsin measured on oriented membrane fragments containing hR stabilized in gel in 100 mM NaCl. T = 22°C

ented samples by Váró and Keszthelyi (1985) (Groma et al., 1988*b*), and on pm's bound to bilayer (Braun et al., 1988) to determine the distance of the rate-limiting step in the bR photocycle, which is the *M–O* transition. The values obtained are $d_3 = 0.49 \pm 0.02$ nm and $d_4 = 3.15$ nm. These values are in good agreement with those in the Table, demonstrating that the assumption in determining the values d_i from Eqs. (6)–(7) are reasonable.

Applications

General

The method of PERS measurement on dielectrically polarized samples is applicable only for such protein-containing membrane fragments that are rigid enough not to form closed vesicles. These membrane fragments generally possess large permanent electric dipole moment because the biological membranes are asymmetric in nature.

The easiest system to study is the oriented membrane suspension which is immobilized in gel (Dér et al., 1985*b*). In this case such problems as electrolysis due to the applied electric field, electrophoretic motion of the membranes, conductivity changes due to liberated charges, proper timing of the laser flash measuring a small signal on a much higher one due to the field needed for orientation of the membranes in a suspension are avoided. The bathing solution is easy to change (though a couple of hours is needed for penetration into the gel), and a comparatively higher conductivity is possible. PERS was registered on bR-containing gel in 1 M NaCl solution (Dér et al., 1985*b*), which would be impossible in suspension.

Bacteriorhodopsin

Data on PERS in case of bR provided important knowledge about the mechanism of proton translocation. The studies may be divided into three groups:

1) Environmental effects: pH (Ormos et al., 1985), water content (Váró & Keszthelyi, 1983, 1985; Váró & Eisenstein, 1987), diamines in solution (Tóth-Boconádi, Taneva & Keszthelyi, 1986; Dér, Tóth-Boconádi & Keszthelyi, 1988);

2) Chemical modification of the amino acid side chains: iodination of tyrosines (Packer et al., 1984, 1987), cross linking of carboxyl groups (Packer et al., 1987) and lysine groups (Tóth-Boconádi et al., 1988);

3) Enzymatic digestion of parts of bR (Hristova et al., 1986).

These studies were reviewed by Keszthelyi in 1984, 1986 and 1987 and most recently in 1988. In this last review an attempt has been made to correlate the data obtained by light absorption changes, laser Raman, NMR and infrared spectroscopy with the data obtained from PERS.

Halorhodopsin

Halorhodopsin (hR) also in the plasmamembrane of *Halobacterium halobium* translocates Cl^- ions after light absorption (Lányi & Vodyanoy, 1986). Membrane fragments containing hR could be oriented and immobilized in gel (Dér et al., 1985*a*). Light flashes produced PERS similar to those on oriented pm's. Figure 7 shows the most recent results (Dér et al., 1989). The large, fast negative peak corresponds very probably to the *trans-cis* isomerization of the retinal linked through a protonated Schiff-base to a lysine residue in hR; a second small negative signal with a life time of 2 μsec appears similar

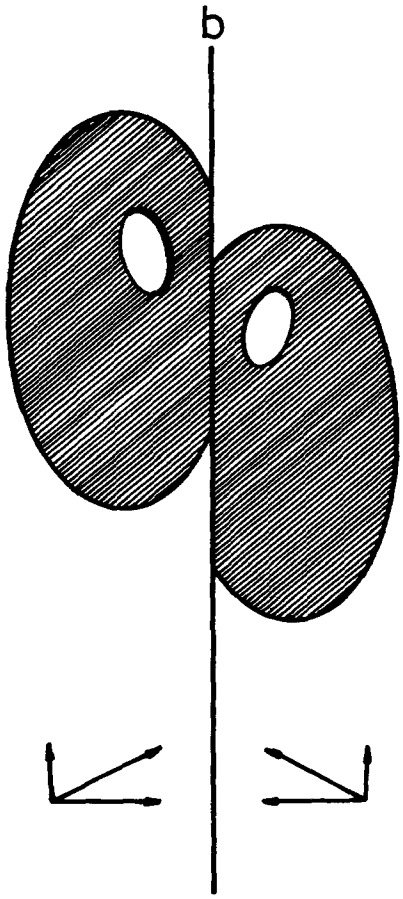

Fig. 8. Schematics of the elementary cell of MbCO crystal. The two MbCO molecules in the unit cell are shown. The arrows indicate the motion of the Fe^{2+} ion during dissociation

to bR, which may correspond to further relaxation of the *cis* isomer. Two positive signals follow with life times of 0.6 and 4 msec. The latter is absent if the solution does not contain Cl^- ions; therefore, it is assigned to the motion of this ion through the protein.

Myoglobin

Protein crystals are naturally oriented systems; therefore, they are candidates to observe PERS due to charge motions correlated with the function of the protein. Myoglobin (Mb) is known to bind CO, which is connected with the motion of the Fe^{2+} ion into the heme plane (Phillips, 1978). The bound CO is deligated from MbCO by light very rapidly and rebinds on a time scale of msec.

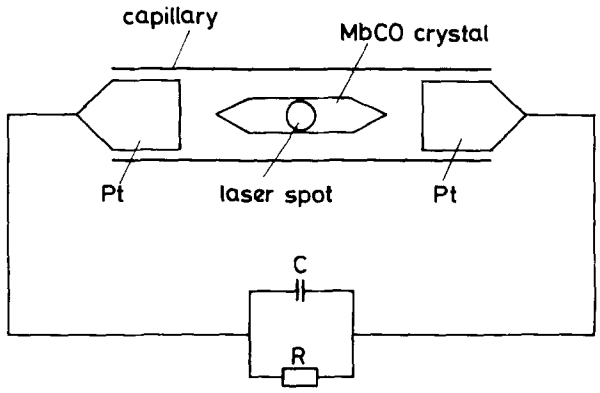

Fig. 9. Experimental cell to measure PERS on MbCO crystal

The crystal structure of Mb is favorable: the two Mb molecules in the elementary cell leave the projection of charge motion in the direction of the *b* axis uncompensated (Fig. 8). A simple experimental setup made the observation of PERS due to Fe^{2+} motion possible (Fig. 9). The results are shown in Fig. 10 (Iben, Váró & Keszthelyi, 1989). One observes a very fast signal, very probably due to the jump of the ion out of the heme plane when the bond is broken by light; the slow, oppositely oriented signal corresponds to the rebinding of CO during which the ion returns to its original position. In this process no net charge is translocated; therefore, the area of PERS should be zero, which is indeed the case.

Perspectives

In this review the method of measuring PERS was demonstrated for three different systems. It is believed that many more membrane-bound proteins or protein crystals can be investigated by this method. It is important to find membrane fragments of biological interest which do not form closed vesicles, therefore are orientable. Sizes of 0.1–0.5 μm diameter usually have large electric dipole moment for easy orientation. There is also a possibility of increasing the rigidity of membranes by chemical methods. PERS can be triggered by direct interaction with light as in examples given above, or by indirect methods such as pH jump, ATP or ADP, Ca^{2+} and Mg^{2+} jump from the "caged" molecules. Since in oriented systems the light-liberated substances and the proteins driven by them are homogeneously distributed, time losses due to diffusion are minimized.

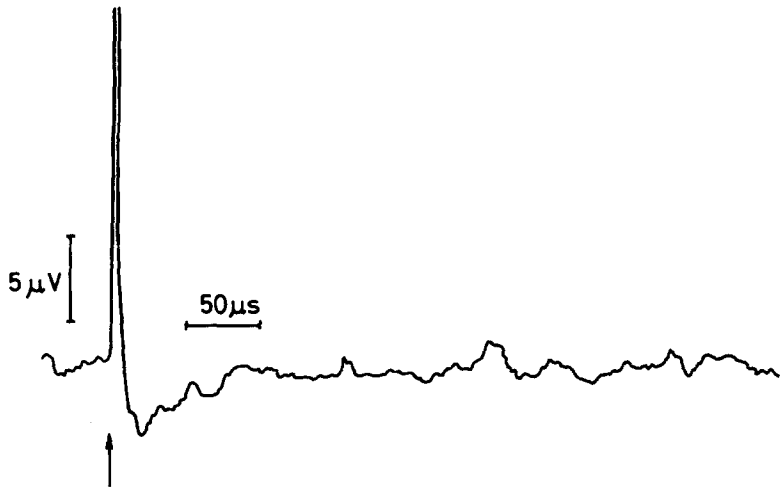

Fig. 10. Electric signals measured on MbCO crystals following photolysis by a laser flash (arrow). This presentation shows the slow component in the right scale; the fast component is much larger

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