Kinetics of the fast electric signal from oriented purple membrane

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ABSTRACT The photoinduced electric response of oriented purple membranes associated with processes before the K-intermediate decay of bacteriorhodopsin was measured in the 180–300 K temperature range. These response signals consist of two kinetically distinct components (both temperature dependent). The experimental data show a correlation between the time constants of the rise of the signal and solution resistance. A model is proposed to assign these components to two diffusion-limited processes of charge displacement in the solution. The displacement is caused by the electric field of the photoinduced transient dipole which is formed in the primary act of the bacteriorhodopsin photocycle. The two processes are assigned as: (*a*) the conduction of electrical current through H-bonds (time resolved only in the temperature range 180–200 K) and (*b*) the diffusion of charges through the interfacial layer.

INTRODUCTION

Bacteriorhodopsin (bR) from *Halobacterium halobium* is known to translocate protons under light excitation (Stoeckenius et al., 1979). The mechanism of this process has been studied by many different experimental approaches. The way used here is to observe the displacement current caused by the charges moving inside the protein.

Bacteriorhodopsin can be isolated from *Halobacterium halobium* embedded in the so-called purple membrane (PM). PMs are membrane fragments (average diameter, 500 nm; thickness, 5 nm) that contain bR and lipids only. PMs have a large permanent electric dipole moment in the direction of the membrane normal (Keszthelyi, 1980), which allows them to be oriented in a moderate electric field. In a typical cuvette a large number of PMs and therefore a large number of bRs may be oriented. It is relatively easy to record the time and amplitude behavior of the displacement current evoked by charges moving in the protein (Keszthelyi and Ormos, 1980). Because of the large number of bR molecules, the changes in the light absorption can be recorded simultaneously on the same sample, thus charge motion and absorption changes may be correlated (Keszthelyi and Ormos, 1980).

Various experimental methods were worked out for the study of the above mentioned electrogenic activity of bR: PMs oriented in suspensions (Keszthelyi and Ormos, 1980); oriented and dried in films (Váró, 1981); and oriented and immobilized in gels (Dér et al., 1985). Quantitative evaluation of the electric signals requires a knowledge of the equivalent circuits of these systems. Keszthelyi and Ormos (1983) and, later, Trissl et al. (1984) discussed only electrical distortions produced by the resistances and capacitances of the samples, electrodes, and amplifiers. The delay between the photoinduced motion of the charges in the PM and the signal was assumed to be negligible. We will show that with proper time resolution of the recording system this delay is measurable.

As a result of the studies of the photoinduced electric signals, five components characterized by decay rates and amplitudes could be separated (Keszthelyi and Ormos, 1983). These were assigned to the five transitions in the bR photocycle: bR-K, K-L, L-M, M-O, and O-bR on the bases of the rate constants (for the characterization of the bR intermediates; see, e.g., Stoeckenius et al., 1979). The very first component (bR-K) is negative and its rise is faster than 5 ps (Simmeth and Rayfield, 1990). At very low temperatures (80–200 K), however, two components have been found (Ormos et al., 1983).

The aim of this work is to investigate the rise of the electric signal in suspension and gel over a broad temperature range. The main question was whether the two components observed at very low temperatures may be attributed to intermediate processes within the protein before the formation of the L state (Ormos et al. [1983] and Shichida et al. [1983] suggested states called K and KL from electric and optical data, respectively) or whether they are experimental artifacts.

The results show that the two components in the rise of the negative signal are due to the propagation of the signal between its source (the bR molecule) and the electrodes, i.e., through the medium surrounding the

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PMs in the suspensions and gels. If there was an electric signal corresponding to the hypothetical K-KL transition it should either be faster than 20 ns at room temperature and 1 μs at 180 K (the time resolutions of the system) or its amplitude should be less than ~5% of the total amplitude of the negative signal.

MATERIALS AND METHODS

The experimental procedure was based on methods described by Keszthelyi (1980), Dér et al. (1985), and Ormos et al. (1983) with only slight alterations.

Purple membrane from *Halobacterium halobium* (strain NRL R1M1) was used in the experiments with a bR concentration of 160 μM.

(*a*) For measurements above 273 K, the temperature of the water suspension and gel samples were regulated by attaching the 1 × 0.1 cm cuvette to a circular Peltier element built for Carl Zeiss Jena microscopes (30 G 516 B; Thornwood, NY). The conductance of the sample was adjusted by adding small amounts of NaCl.

The samples were excited by a homemade Nd-YAG laser with frequency doubling (10 ns duration, 530 nm wavelength, 6–7 mJ/pulse). The signals were collected by a homemade FET input follower of 10^{10} ohms/20 pF input resistance and capacitance, respectively, and transferred to a 100 MHz TEKTRONIX, Inc., 464 storage oscilloscope (Beaverton, OR). Traces were photographed and evaluated graphically. Some data were taken by an IWATSU TS 8123 digital oscilloscope. The rise time of both instruments is ~3 ns.

(*b*) For the low temperature experiments the PMs were suspended in 60% glycerol-water mixture. The sample was in a 1 × 0.1 cm cuvette with two Pt wire electrodes 1 cm apart (not platinized). After light adaptation, the sample was oriented with 15 V (DC) for ~1 min. With the field still present the sample was rapidly cooled in an optical cryostat to 80 K (the change of temperature 300–80 K took ~1 min). At 80 K the orienting field was removed and the sample was heated up to the required temperature. The cycle was initiated with flashes from a flash lamp pumped dye laser (Carl Zeiss, FRG; 580 nm wavelength, 1 μs pulse length, 10 mJ energy). To complete the photocycle (which stops at low temperatures) the sample was warmed up to 260 K and kept at this temperature for ~10 min. The orientation did not change appreciably during this time as shown by the constancy of the full amplitude of the electric signal. The sample was recooled to the next temperature.

Signals were fed into a homemade amplifier (based on a Burr-Brown Corp. 3554BM chip, $R_{\text{input}} = 10^{10}$ ohms; Tucson, AZ) through a coaxial cable (120 pF capacitance), and digitized with an eight-bit, 10 MHz transient recorder (NEO-200-B; KFKI, Hungary). All time constants were estimated by graphical analysis. The method is estimated to be accurate to within 10%. Sample resistance (DC) was measured by an electrometer (model 610CR; Keithley Instruments, Inc., Cleveland, OH). Readings were taken after waiting a few minutes for the stabilization of the current. The temperature was monitored by a thermocouple.

RESULTS

Gel and water suspension (T = 270–300 K)

Signals from single shots were recorded from gel and water suspension samples at different temperatures between 270–300 K. Fig. 1 reproduces a typical data set at T = 283 K. The signal is a superposition of five exponentials and starts with a fast rise of τ_1 = 20 ns (10–90%) which ends abruptly (Fig. 1 *a*). The rise is completed by an exponential of τ_2 = 170 ns. After the maximum the signal decays with τ_3 = 800 ns (Fig. 1 *b*), crosses the zero line with τ_4 = 1.4 μs, and turns back toward the zero line with τ_5 = 72 μs (Fig. 1 *c*). The sign of the signals can be changed by rotating the gel or by an oppositely oriented electric field in the case of the oriented suspension.

To obtain more information about the processes characterized by the five τ values, their dependence on temperature (T) and on the conductivity (G) of the solution was measured. The results are given below.

(*a*) τ_1 does not depend either on temperature or conductance of the solution. The value τ_2 = 20 ns equals the full duration of the laser pulse, i.e., twice the measured half-width of 10 ns. The rise time of the signal is thus compatible with the results of Simmeth and Rayfield (1990), which show that the primary charge motion in bR occurs with 5 ps after the absorption of a photon. Therefore, charges move and generate displacement current during the duration of the laser pulse.

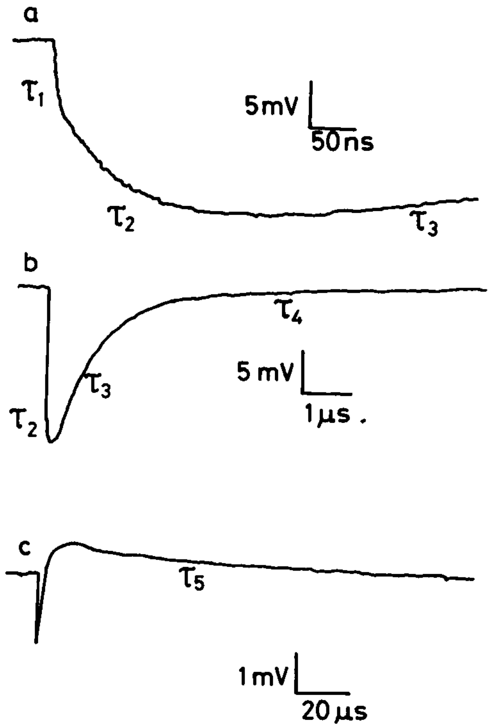

FIGURE 1 Electric response signal from PMs oriented and immobilized in gel. Distilled water solution, T = 295 K. Single signals recorded by IWATSU oscilloscope. (*a–c*) Signals with different amplitude and time scales.

(*b*) Both τ_2 and τ_3 are temperature dependent as shown in Fig. 2 for gel samples with three different conductivities. Similar data were obtained for water suspension samples (not shown). Moreover, it was found that τ_2 and τ_3 have a fixed ratio τ_3/τ_2 = 10–12 in all cases.

τ_3 is determined by the *RC* time constant of the electric circuit. The capacitance, *C*, of the measuring set-up discharges through the sample (the input conductance of the amplifier is negligible). The sample conductance *G* depends on the salt concentration and on the temperature. In Fig. 2 one of the measured set of *G* values is shown normalized to τ_3. (*G* was measured by AC current of 10^3 Hz. The capacitance, *C*, calculated from these *G* values and associated τ_3 values was used to determine the *G* values of other solutions from τ_3.) The data yield a temperature dependence of the conductivity

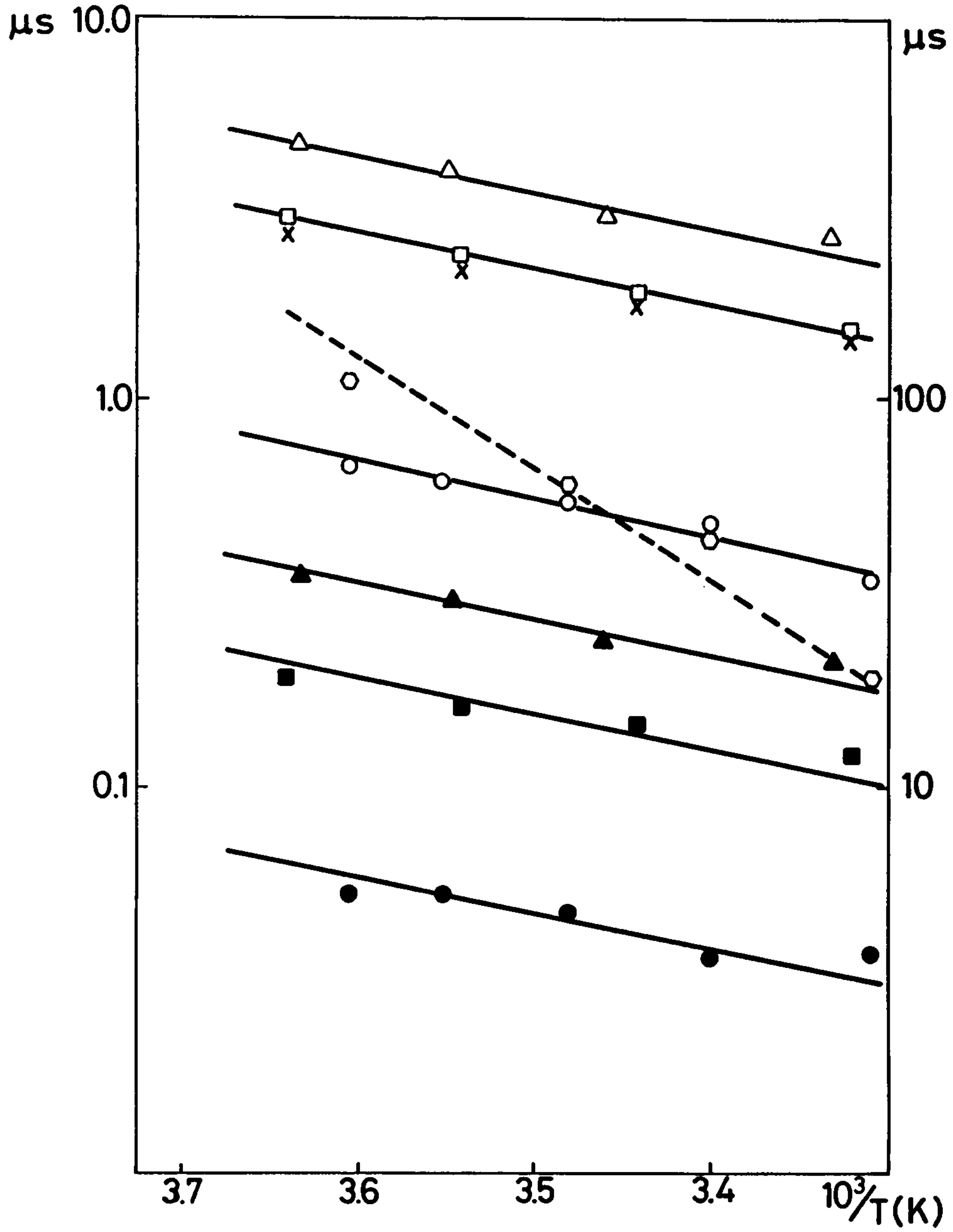

FIGURE 2 Conductance and temperature dependence of the time constants τ_2 and τ_3. The filled symbols relate to τ_2 and the empty to τ_3 (*left scale*). Three series (○, □, △) measured on gel samples with different conductivity (NaCl concentrations: △, 0.26 mM; □, 1 mM; ○, 1.9 mM). (x) Values of G measured separately and normalized to τ_3 at 300 K. The actual value of $G = 6.5 \times 10^{-6}$ ohm^{-1}. (○) Values of τ_5 (L-M transition; *right scale*).

of $(\Delta G)/(G\Delta T) = 0.022/°C$. This value coincides with that of monovalent ions in water solution (Handbook of Chemistry, 1971).

The signal with time constant τ_2 is part of the rise of the electric signal. It appears after the light induced primary charge motion characterized by τ_1 is finished. It is intimately connected with the conductivity of the sample as shown in Fig. 2. Therefore, it may not be associated with a transition in bR.

We note that because $\tau_1, \tau_2 < \tau_3$ the data on these components are photovoltages, while τ_4 and τ_5 are measured as photocurrent because their lifetime is greater than $RC = \tau_3$. (For this point see Ormos et al. [1983] and Trissl et al. [1984].)

The ratio of the amplitudes of the slow (A_s) (τ_2) and fast A_f (τ_1) components of the rise was 1 in water suspension and 2–4 in gel samples. The ratio depends only slightly on the temperature (less than 20% in the range of 275–300 K).

(*c*) The temperature dependence of τ_4 and τ_5 follows the temperature dependence of the K-L and L-M transitions and is more pronounced than that of τ_2 and τ_3 (in Fig. 2 the temperature dependence of τ_5 is also given). τ_4 and τ_5 do not depend on conductivity. Light absorption measurements (not shown) confirmed the assignment of τ_4 to the K-L and τ_5 to the L-M transition, respectively.

The pH of the bathing solution was varied between 5.5–7.0 with 1 mM phosphate buffer at room temperature. The value of τ_2 and the amplitude ratio A_s/A_f were uneffected.

Water-glycerol suspension (180–280 K)

The rise of the electric signals between 180–280 K temperature range consists of two kinetically distinct components τ_1 and τ_2 as at higher temperatures. An example is given in Fig. 3. The results can be summarized as follows.

(*a*) The slow component, which can be approximated with one exponent of life time τ_2, exhibits a marked temperature dependence. In the 280–200 K range the

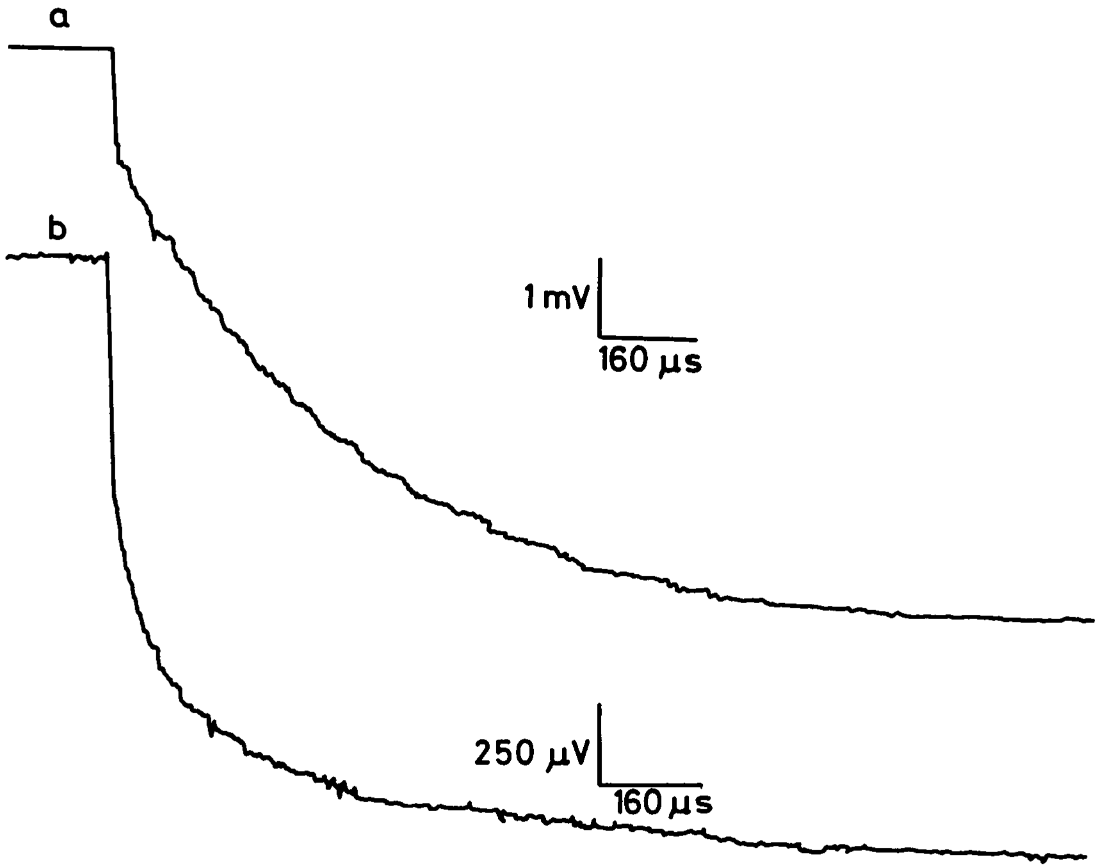

FIGURE 3 The time course of the electric signal associated with K state formation. PMs oriented and frozen in glycerol-water suspension. (*a*) The fast component (~ 20% of the whole amplitude) is followed by the slow component (~ 80% of the whole amplitude), at 233 K, $\tau_2 = 390$ µs. (*b*) The fast component recorded on the same timescale but at 188 K, $\tau_1 = 130$ µs. Note the different amplitude scale.

fast component is unresolved (its time constant is less than 3 μs the time resolution of the system), but in the 200–180 K range a temperature dependence is measurable. Although this process is nonexponential, it was approximated with one time constant.

τ_1 and τ_2 can be fitted to a quasi-Arrhenius dependence with similar activation enthalpies, ΔH_1 = 66 kJ/mol, ΔH_2 = 63 kJ/mol. Their preexponential factors, however, differ by more than three orders of magnitude (Fig. 4).

(*b*) The electrical resistance of the sample was measured between 280–180 K. The resistance increases from $\sim 5 \times 10^5$ ohms at 280 K to more than 10^{12} ohms at 180 K. The resistance values can also be fitted to an Arrhenius relation. An activation enthalpy, ΔH_R = 64 kJ/mol coinciding with H for the rapid and slow components of the electric signals was found.

(*c*) The ratio of the amplitudes of the slow (A_s, τ_2) and fast (A_f, τ_1) components differed from 1 to 20 for different samples or on refreezing the sample, but it remained constant for each particular sample after freezing.

(*d*) Absolute values for the amplitude of the electric signal associated with K formation (the sum of the rapid and the slow components) were measured between 200–280 K. The amplitude was found to be constant within 6% (note that the amplitude depends only on the capacity of the circuit).

(*e*) An additional capacitance was connected in parallel to the sample (and hence to its capacitance). The result is a decrease of the signal amplitude (the measured signal amplitudes are inversely proportional to the total capacitance) without any change of the time constant (data not shown). This is considered as evidence that the source of the two kinetic components in the electric signal associated with K-formation is the sample itself and not the measuring circuit.

DISCUSSION

The rise of the electric signal between 180–300 K is characterized by two components: a fast component (τ_1) (determined by the length of the exciting laser pulse and

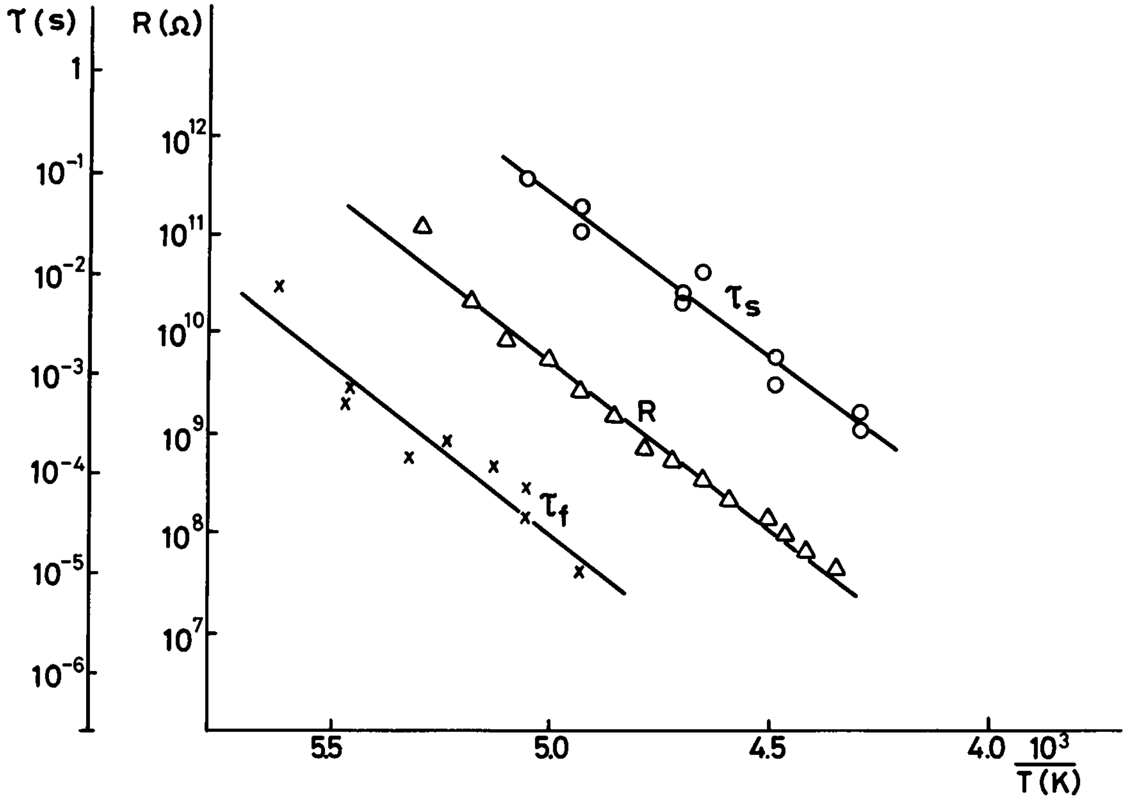

FIGURE 4 Temperature dependence of the time constants of the slow (O) and the fast (x) components of the electric signal, associated with K state formation. The difference in their activation enthalpies ($\Delta H = 63$ kJ/mol, $\Delta H = 66$ kJ/mol) is within experimental error. In the same temperature range (190–230 *K*) the electrical resistance can also be fitted to an Arrhenius dependence with an activation enthalpy $\Delta H = 64$ kJ/mol (all ΔH are least square estimates).

longer than this only at temperatures below 200 K), and a slow component (τ_2). Our main assumption in explaining these time constants is that the charge motion inside the bR during the bR-K transition is very fast (in the ps range, Groma et al., 1988; Simmeth and Rayfield, 1990). The values deviating from the time of the laser flash are characterized by other effects.

The temperature dependence of τ_1 between 180–200 K is described by the same activation enthalpy as for τ_2 and that of the resistance of the sample. We tentatively assign this component to the conduction of protons through H-bonds, where the rate limiting stage is the rotational diffusion during H-bond formation (Eigen and de Maeyer, 1958). Measured activation enthalpy values are in the range of 60–80 kJ/mol (for pure ice). Our value ΔH = 65 kJ/mol for the glycerol-water mixture corresponds to this value.

Data on τ_2 show that it is proportional to the resistance or inversely proportional to the conductance of the sample. This proportionality is valid in the whole temperature range. The specific conductance G^* ($\mathrm{ohm^{-1}m^{-1}}$) can easily be calculated from the resistance taking into account the geometry of the samples. The quantity $1/(\tau_2 \times G^*)$ was found remarkably constant for the different experimental systems throughout the whole temperature range (Table 1).

We may write

$$\frac{1}{\tau_2} = k_2 = \gamma_{\mathrm{exp}} G^*, \tag{1}$$

where $\gamma_{\mathrm{exp}} = (2\text{–}5) \times 10^8$ ohm m s^{-1} for all samples, temperatures and conductivities studied. This simple relationship between k_2 and G^* is explained (at least to an order of magnitude) as follows.

The fast charge separation occurs inside the protein. Its effect immediately appears at the surface of the membrane and should be compensated by ions from the solution through the interfaces, or, in other words, the double layer at the membrane surfaces. The thickness of a double layer according to the Gouy-Chapman theory (McLaughlin, 1977) for single charges can be approximated as:

$$d = \left(\frac{\epsilon\epsilon_0 k_B T}{2e^2 n}\right)^{1/2}, \tag{2}$$

where ϵ, ϵ_0 are the dielectric constant and the vaccum permittivity, respectively, k_B the Boltzman constant, T the temperature, n the ion concentration, and e the elementary charge (using SI units). On the other hand, the specific conductivity may be expressed with the diffusion constant of the ions (D):

$$G^* = \frac{e^2 D n}{k_B T} \qquad D = \frac{G^* k_B T}{e^2 n}. \tag{3}$$

The ions diffuse through the two double layers on the opposite sides of the membranes to compensate the effect of internal charge motion with a rate k:

$$k = \frac{D}{2d^2} = \frac{G^*}{\epsilon\epsilon_0} = \gamma_{\mathrm{theor}} G^*. \tag{4}$$

Using $\epsilon = 80$, $\epsilon_0 = 8.85 \times 10^{-12}$ ohm^{-1} s m^{-1} we obtain a value for $\gamma_{\mathrm{theor}} = 1.4 \times 10^9$ ohm m s^{-1} which deviates roughly with a factor of four from γ exp. In the present stage of our understanding and experimentation, no explanation can be offered for the ratio of the amplitudes of the components of τ_1 and τ_2.

The problem of the division of the electric signal into bR-K and KL-L components (Ormos et al., 1983) can not yet be answered definitively. This study shows that the kinetics of the electric signal associated with primary charge separation is masked in the submicrosecond range by diffusion-limited transport processes in solution. From our data, it may be concluded that the K-KL transition must be either faster than 20 ns at ~275 K at 1 μs at 180K (the time resolution), or if it has longer lifetime at these temperatures its amplitude should be below the limit of our sensitivity (5% of the total amplitude).

Recent data measured by optical (Milder and Kliger, 1988) and electric methods (Groma et al., 1988) point to the former possibility, i.e., to a K-KL transition faster than 10 ns at room temperature.

The proposed mechanism of the appearance of diffusion-limited components on the fast rise of electrical signal from the oriented biological system seems to be of universal nature. It should be measurable (when tested with sufficient time resolution) in any system in which the source of the signal and the measuring electrodes are separated by any medium other than an ideal conductor or ideal isolator. The presence in the medium of any movable charge with finite (no matter whether very high or very low) mobility leads to the appearance of a "false" kinetic component. The measured kinetics

TABLE 1 $\gamma_{\mathrm{exp}} = 1/(\tau_2 \times G^*)$ values for different samples

Sample	$\gamma_{\mathrm{exp}} = 1/(\tau_2 \times G^*)$ ohm s^{-1}m
Water Solution	$(3\text{–}5) \times 10^8$
Gel	$(3\text{–}5) \times 10^8$
Gel in frozen state (270 K)	2.8×10^8
Water-glycerol suspension	$(2\text{–}3) \times 10^8$

τ_2, lifetime of the second component of the rise of the electric signal in s; and G^*, the specific conductance of the sample in ohm^{-1}m^{-1}.

would be determined by rate-limiting stage of charge transport in that medium. The absolute rates would depend on charge mobilities, distances, etc. This mechanism not only masks the real kinetics but allows us to study the transport properties of charges in the medium.

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