Photochemically induced charge separation occurring in bacteriorhodopsin

Detection by time-resolved dielectric loss

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ABSTRACT Time-resolved dielectric loss (TRDL) measurements are reported for the photochemical excitation of bacteriorhodopsin (bR) in solid films of *Halobacterium halobium* purple membranes. These measurements provide an independent confirmation for the existence of an important component of charge separation in these membranes after photochemical excitation. The separation of charge is detected by the absorption of microwave energy by the multilayer films of purple membranes in a microwave cavity during flash photolysis experiments. The TRDL method has the advantage of being sensitive to charge separation occurring in both oriented and unoriented films of purple membranes. One disadvantage is that the water content of the samples must be minimized, however, there is some absorbed water present in our electrodeposited solid film samples. To the best of our knowledge, TRDL measurements have not been reported previously for photochemical charge separation in biological membranes. It is significant that an early decay component of TRDL in the 20-μs time domain corresponds to the relaxation of the negative charge displacement photocurrent in oriented samples of purple membranes. In addition, a component of charge separation persists during the first several hundred microseconds of the bR photocycle.

INTRODUCTION

The primary photochemistry of bacteriorhodopsin (bR) is a 13-*trans* to 13-*cis* photoisomerization of the retinylidene pigment which causes the protonated Schiff base to be separated from its negative counterion (1–3). This primary event is followed by a series of thermal reactions which are considered to be part of the photocycle spanning the picosecond to millisecond time domain, and proton pumping across the membrane is the final result. While it is clear that the primary event involves a component of energy storage in the form of electrostatic energy, there is also a significant component of energy storage in the form of strain energy in the retinylidene protein (4, 5). It has also been proposed that these two kinds of energy storage undergo interconversion (5) during microsecond relaxation processes after light absorption by retinylidene pigment proteins.

There are several important physical properties associated with these two kinds of energy storage in the bR photocycle. Three recent review articles provide a detailed coverage of various aspects of the problematics of energy storage in the photocycle: Trissl (6) has reviewed the charge displacement current measurements in purple membranes, Birge (7) has reviewed the spectroscopic and energetic aspects of the photocycle, and Birge (8) also has reviewed the photophysical and mechanistic aspects of the photocycle. These reviews show that much progress has been made to elucidate possible models for the energy storage act. However, the mechanistic and dynamical details of the conversion of the stored energy to promote charge displacements are not fully understood at this time (8). The photoelectric measurements (6) provide a means of following the charge displacements in the purple membranes, however, these measurements do not permit a direct measure of the charge separation or dielectric components which can occur before and during the charge displacements.

Models of the electrostatic environment of the retinal cavity pocket must include the participation of several counterions in close proximity to the retinal chromophore. Consequently, the photochemistry must give rise to the splitting of existing electric dipoles and the creation of new dipoles: these changes are directly related to the question of energy storage in the form of charge separation. Furthermore, it is clear that the significant changes in atomic charge distribution (9) occurring upon photochemical excitation of the retinal chromophore will perturb the nearby charged and polar amino acid residues so as to maximize the electrostatic stabilization (8). Consequently, there could be changes in pK_a values arising from the changes in the electrostatic environment (10). A further major complication in the analysis of a model for the retinal cavity pocket is the realization that the cavity may not be very rigid: the presence or absence of contrary motions by the polar protein residues after the primary photoact is still an open question (8).

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A complete understanding of the charge displacement and the charge separation or dielectric changes occurring during the photocycle will only be achieved when we have a clear picture of the protein cavity or binding site for the retinal pigment. In this regard, Birge (8) has concluded that the nature of the binding site has been a subject of intense study but only of modest agreement; and therefore we need to do more work to achieve a better picture of the cavity pocket. Studies which focus on the question of electric dipole splitting and dipole creation seem to be particularly pertinent.

One method which should shed some light upon the bioenergetic mechanism of energy storage is the technique of time-resolved dielectric loss (TRDL) or microwave conductivity which measures the charge separation component of energy storage by photochemical transients and the kinetics of ionic processes (11, 12). In these time-resolved dielectric measurements, the absorption of microwave energy is usually associated with the electric mobilities of one type of charge carrier at high microwave frequencies. For instance, in semiconductors and photographic films (12), the most mobile charge carriers are usually electrons; and the microwave mobilities of these carriers are directly responsible for the absorption of microwave energy. For TRDL measurements in general, even though the mobile carriers are detected by microwave absorption, the physical quantity which is being measured is charge separation in the dielectric medium under study. The TRDL technique has several advantages including high sensitivity and linearity at low microwave power levels as well as the absence of electrode contacts (12, 13).

An application of TRDL measurements to the bR photocycle appears to be pertinent as it can provide dynamical information about dielectric changes during the events of the photocycle (14). We note that the dielectric loss of partially hydrated purple membrane films has been measured by an a.c. impedance method (15) which was sensitive to bound water, but these measurements of the capacitance and dielectric loss of the same samples (for frequencies between 10 kHz and 10 MHz) were not directly related to the photocycle. However, microwave absorption in the gigahertz frequency domain has the advantage of being particularly sensitive to the charge separation phenomena occurring during the photocycle where various dynamical intermolecular interactions play a pivotal role. It is important to emphasize that TRDL measurements only detect the changes in charge separation and are not sensitive to the static dielectric dispersions which represent the dipolar interactions between membrane proteins and absorbed water molecules or interfacial phenomena (16).

In addition to the TRDL measurements of photochemical molecular transients (11, 13), microwave conductivity techniques have been widely used for many years to detect hole-electron pairs and to measure their recombination rates in solid and dispersed semiconductor interfacial systems (17). A successful TRDL measurement depends upon a frequency-dependent polarizability of the photochemically produced dipoles in the sample which gives rise to a time-dependent dissipation of energy or dielectric loss in the sample under study. A parallel requirement is that the electric mobility of the photochemically created dipoles in the sample must be nonzero. Microwave Hall mobilities have been measured for the static electric dipoles in a few biological membranes (18, 19), but TRDL measurements have apparently not been reported previously for photochemical charge separation in biological membranes. The previous Hall mobility measurements indicate that the charge carriers of the static electric dipoles in biological membranes have electric mobilities of ~ 1–5 $cm^2s^{-1}V^{-1}$ which are intermediate between those of semiconductors and organic pigments. It should be possible to follow the kinetics of the bR photochemical cycle with measurable TRDL signals provided that the Hall mobilities of the photochemically generated charge carriers are at least as large as those of the static carriers, that is, ~ 1–5 $cm^2s^{-1}V^{-1}$. We note here that Hall mobility measurements have not been carried out for the static charge carriers in purple membranes, but we expect that they should have mobilities similar to those of other biological membranes (19). We also make the assumption that the photochemically produced charge carriers in purple membranes are directly related to the preexisting charge carriers which are probably free and ionizable protons and the carboxylate groups of amino acid residues (8). While the photochemically induced charge separation in bR is a picosecond phenomenon, the subsequent steps occur on a microsecond time scale; and the photocycle shows charge recombination from several intermediates, particularly from the K intermediate (20). The purpose of this study is to report our initial findings concerning the transient TRDL signals, measured at X-band with microsecond time resolution, associated with the bR photocycle in oriented and unoriented purple membrane films.

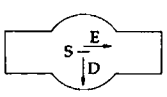
MATERIALS AND METHODS

Thin oriented films of purple membrane patches, isolated from the *Halobacterium halobium* S9 strain, were electrodeposited onto SnO_2 electrodes on thin (cover slip) glass supports as previously reported (21). In some cases, blue deionized membranes were prepared by passing milliampere/centimeter^2 currents through the samples for ~ 1 min after electrodeposition. Some time-resolved optical absorption measurements were performed on thin film samples by 532 nm laser excitation and measurement at 45° incidence as previously reported (21). To maximize the photochemical activity (22), all samples were

dried at 50% room humidity and were introduced into a Varian Associates (Mountain View, CA) model E-238 microwave cavity which operated in the TM_{110} mode with an unloaded Q factor of 12,000. On introducing the solid membrane multilayers into the cavity of the Varian E-12 EPR spectrometer, the cavity Q dropped to ~1,000–2,000 as measured by the width at half maximum of the cavity absorption, close to 9.4 Ghz. We note that cavity filling factors and carrier charge densities are important parameters in these measurements (12), but we found that excellent sensitivity was obtained with very small volumes of these membrane samples, typically only several micrometers thick with superficial areas of ~0.2 cm^2. The sample (S) was placed in a vertical orientation (top view of the cavity shown in the scheme) close to the center of the cavity in a region of weak microwave electric field (E) to permit the TRDL measurements. Also in the scheme, the arrow (D) shows the direction of charge displacements in the purple membrane stacks. We used relatively low microwave power levels to maintain linearity (12) and also to avoid the noise present at high power levels. A Photonics Ltd. (Southampton, England) model 2-500N-4J pulsed argon flash lamp was used to provide ~1 μs light flashes at a repetition rate of 0.2 Hz, and the light was filtered by a Corning 3-69 cut off filter (Swift Glass, Elmira, NY) which transmitted light pulses with $\lambda > 490$ nm, with an energy of ~1 mJ per pulse at the sample position. Under these conditions, heating artifacts in the microwave cavity were negligible; and there was no light absorption by the thin SnO_2 film. The microwave absorption signal at the output of the microwave bridge was amplified by a CLC-100 wideband (DC to 500 MHz) amplifier (Comlinear Corp., Loveland, CO) and was digitized and signal averaged (17). The microwave absorption signal was AC coupled in the bridge at 20 Hz which limited our measurements to <2 ms, but the instrument limited risetime was 0.5 μs. All TRDL signals were measured after a light-minus-dark protocol (17) to eliminate a small amplitude of radio frequency noise associated with the discharge of the flash lamp. Usually 500–1,000 light flash acquisitions were sufficient to obtain acceptable signal-to-noise ratios for the signals from light-adapted purple membrane samples with absorbances between 0.5 and 2.0 at 568 nm.

RESULTS AND DISCUSSION

The time-resolved optical absorption detection of the bR deprotonated Schiff base or M intermediate, measured at 412 nm, is shown in Fig. 1 for solid oriented films of purple membranes prepared by electrodeposition onto SnO_2 electrodes. We note that the decay of the M intermediate for these samples lasts as long as several seconds, however significant decay has occurred 100 ms to 1 s after flash excitation which is similar to the behavior of previous samples (21), prepared by a slightly different electrodeposition protocol. We found that all membrane samples had a comparable photochemical yield and were fully reversible during the 5-s interval between successive light flashes in these experiments. In addition, Fig. 1 shows the formation of the M intermediate with a characteristic log(time) rise function which



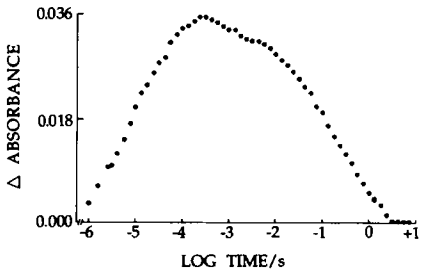


FIGURE 1 Typical time-resolved optical absorption measured at 412 nm at 298 K and 50% relative humidity of electrodeposited purple membrane patches on a SnO_2 electrode on a glass support. The optical density of the bacteriorhodopsin film was $A_{568} = 0.35$ with Nd/YAG laser excitation at 532 nm (1 mJ per laser flash) and 16 laser flashes were signal averaged.

indicates the presence of a distribution of rise kinetics for the M intermediate in these samples. This behavior for the solid oriented films is not anomalous as several kinetically distinct M intermediates have been proposed to explain the rise and decay kinetics of M, even in aqueous suspensions of purple membrane fragments (23, 24).

Oriented purple membranes in solid SnO_2/bR/Ag electrode sandwich cells displayed the characteristic biphasic (negative and positive) charge displacement currents as shown in Fig. 2. Here, we measured integrated charge displacement currents (21), and these measurements were in agreement with those of other oriented samples in solid films (21, 22), where the negative phase persists typically for ~20 μs.

A typical TRDL measurement of oriented purple membranes is shown in Fig. 3, where we observed a rise time of ≤0.5 μs, the characteristic response time of the microwave bridge detection system in the absorption mode. In addition, a significant decay of microwave absorption was usually observed in the first ~20 μs, which corresponded to the early negative phase of the charge displacement photocurrents (as in Fig. 2). The

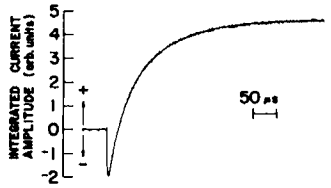


FIGURE 2 Time-resolved electric displacement currents measured for a SnO_2/bR/Ag electrode sandwich cell with $\lambda > 490$ nm excitation. The kinetic profile was signal averaged with 64 flashes of the pulsed argon light source with 100 μJ per flash incident on the sample with $A_{568} = 0.3$.

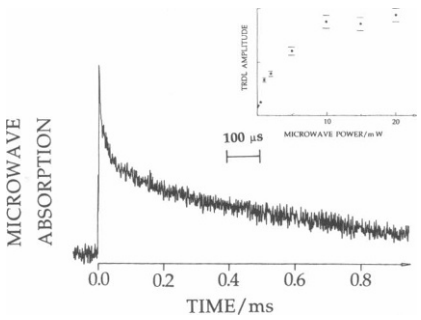


FIGURE 3 Flash induced time-resolved dielectric loss changes (5 mW microwave power in the microwave cavity tuned at 9.4 GHz) measured for a sample of purple membrane patches oriented by electrodeposition on a SnO_2/glass support and measured at 298 K and 50% relative humidity. The optical density of the bacteriorhodopsin film was A_{568} = 2.0, and signal averaging was used with excitation by 500 flashes with $\lambda > 490$ nm from the pulsed argon flash lamp (with 0.5 mJ per flash). (*Inset*) Microwave power dependence of the flash-induced TRDL amplitudes.

microwave power dependence of the TRDL signal is shown in the inset to Fig. 3 which indicates a microwave saturation phenomenon for these hydrated samples which has been observed previously for various samples (12, 17), and is representative of a nonlinear response at high power levels associated with a saturation of the charge carrier mobilities at 9.4 GHz. The TRDL signals exhibited a complex decay function over a time period of ~1 ms for the oriented purple membrane samples. For these samples, we observed that most of the TRDL decay occurred during the time period of formation of the M intermediate, as shown in Fig. 1. However, on comparing Figs. 1 and 3, it was clear that part of the TRDL decay was slower than the optical absorption rise for the oriented bR samples. For instance, 100 μs after flash excitation, the TRDL signal has decayed to ~0.4 of its original value whereas the M absorbance signal has attained ~0.95 of its maximum value. Besides being slower, the TRDL decay was complex, containing at least multiexponentials, but it could not be fitted to linear log(time) kinetics as in the case of the M absorption rise. We emphasize that the observed TRDL decay was not subject to instrumental limitations attributable either to the 20 Hz AC coupling of the microwave signal or to the overall bandwidth of the detection system which permitted 0.5 μs time resolution. Also the TRDL response should have been proportional to the number density of charge carriers, although it is difficult to give an absolute estimate of the density of charge carriers (12). The observation of a slower TRDL decay component apparently represents some charge separation persisting during the early part of the decay profile of the M intermediate. We will return to this question in Conclusions.

Unoriented samples of purple membranes were also prepared by evaporating purple membrane aqueous suspensions on the same thin glass cover slips. A typical measurement of largely unoriented purple membranes is shown in Fig. 4 which also shows a complex decay profile in the ~1 ms time domain. The TRDL decay in the first ~20 μs was not as pronounced as for the oriented samples, however, these samples also showed slower rise kinetics for the M intermediate in optical absorption (data not shown). Finally, we attempted to measure TRDL signals from oriented blue or deionized membranes, but we were unable to detect a significant signal from these samples. In many cases, any signals which were detected seemed to be correlated with a small fraction of purple membranes present in these deionized films. In addition, the solid films of blue deionized membranes appeared to be ~50% dehydrated relative to the water content of oriented purple membranes, based on the relative microwave cavity Q factors observed when the two kinds of samples were compared.

CONCLUSIONS

It is clear that we have measured the changes in dielectric loss occurring in partially hydrated samples of purple membranes during the bR photocycle. We recall that there are only ~100 water molecules per pigment protein complex (22) and that most of these water molecules are relatively far removed from the retinal cavity. In addition, neutron diffraction studies (25) of the primary photoisomerization, leading to the M intermediate, indicate that the protein structural changes are not dominated by a redistribution of water or exchange-

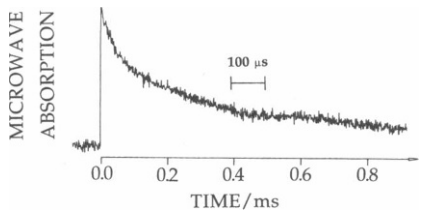


FIGURE 4 Flash induced time-resolved dielectric loss changes (5 mW microwave power in the microwave cavity tuned at 9.4 GHz) measured for a sample of unoriented purple membrane patches on a glass support and measured at 298 K and 50% relative humidity. The optical density of the bacteriorhodopsin film was A_{568} = 1.0, and signal averaging was used with excitation by 1,000 flashes with $\lambda > 490$ nm from the pulsed argon flash lamp (with 0.5 mJ per flash).

able hydrogens but by alterations in the protein conformation close to the chromophore. Therefore, most or nearly all of the initial dielectric loss changes could be directly related to the electrostatic changes occurring in the retinal cavity pocket.

We will now consider three possible sources for the observed TRDL signals: (*a*) molecular dipole changes associated with photochemical excitation (13), (*b*) charge separation associated with the primary photochemical event of photoisomerization and concomitant charge redistribution, and (*c*) proton pumping across the membrane which may give rise to a measurable TRDL signal involving some charge separation but dependent upon the microwave electric mobilities of the pumped protons. First of all, it is known (9) that significant changes occur in the molecular dipole moment in the pigment's excited singlet state, but it is very unlikely that any part of these picosecond transients could be detected by the 0.5 μs response system employed in the current study. Secondly, the primary photochemistry occurs on a picosecond time scale but should give rise to a charge separation phenomenon in the retinal pigment cavity which could persist for microseconds depending upon the nature of the cavity: these changes should give rise to a detectable TRDL signal. Thirdly, the transduction of stored energy resulting in a deprotonated Schiff base and mobile proton(s) could also give rise to a charge separation phenomenon; but it should give rise to a smaller TRDL signal than that of the primary photochemistry where a significant fraction of the energy is stored in the form of charge separation (4, 7).

It is clear that the rise of the TRDL signal was always instrument limited for all oriented and unoriented purple membrane samples. This observation leads us to propose that the electrostatic changes occurring in the retinal cavity pocket, during and immediately after the primary photochemistry, were the major source of the rapidly rising, <0.5 μs TRDL signals. For instance, there was no correlation with the distributed rise kinetics of the M intermediate in Fig. 1 and the beginning of proton pumping in these samples (26–28). Therefore, the observation of a rapidly rising TRDL signal is strong independent evidence for there being significant net charge separation associated with and immediately after the primary photochemistry. This information is useful because the current models for the primary photoisomerization appear to be equally divided between two possibilities (7, 8). In one model, there is net movement of the positively charged imine proton and retinal C_{15} away from negatively charged counterions (probably at least Asp-212 and Tyr-185). In another model, the positively charged part of the chromophore moves toward Asp-212: for this model, there would be no dielectric loss because the net dipole moment would decrease. Therefore, within the observed microsecond time resolution, the TRDL result appears to agree with the first model involving some charge separation.

A second important question (7, 8) concerns the possible presence of contrary motions by polar protein residues after the primary event. The observation of the strong TRDL signals of this work is good evidence for the retinal cavity pocket being relatively rigid during and subsequent to the primary photoisomerization. Indeed, if this were not the case, motions by certain polar amino acid residues contrary to that of the displaced charges would tend to minimize the observed charge separation during and after the primary photoisomerization.

With regard to energy transduction leading to proton pumping, we offer the following evidence that proton pumping cannot be a major source of the early TRDL signals. It is clear that proton pumping starts during the rise and decay of the M intermediate (26–28) which is very slow in these samples (see Fig. 1). Therefore, the key observation is that the temporal profile of the absorption signals for the M intermediate is different from that of the TRDL signals: we particularly point to the slow distributed rise kinetics of M for the samples of this study. Therefore, the rapidly rising TRDL signals cannot be correlated with proton pumping.

Significantly, we have observed some temporal similarity between the kinetic profiles of the photocurrents and of the TRDL signals, both obtained from oriented purple membranes in solid films. Indeed, the negative phase of the photoelectric signal persists for ~ 20 μs for these samples (see Fig. 2). During the same time period, we observed a significant change in the charge separation component of energy storage as seen from the TRDL decay observed for oriented purple membrane samples (see Fig. 3). The negative phase of the photoelectric effect has previously been assigned to the K to L transition of the bR photocycle (26, 27). We suggest that the 20-μs TRDL decay for these oriented samples could be related to energy transduction events occurring during the K to L transition, leading eventually to the formation of M. An alternative explanation would be to assign at least part of the TRDL decay to charge recombination events. On the basis of the TRDL decay alone, we cannot distinguish between these two possibilities, however, the observation of the 20-μs photoelectric signal is probably a signature of competent bR energy transduction. To reinforce this interpretation, we point to the significant decay of the TRDL signal during the first several hundred microseconds after flash excitation which is the time domain for the formation of M according to its absorption rise kinetics in Fig. 1. However, there also could be some charge recombina-

tion and some very limited proton pumping occurring during the 20 μs < time < 1 ms period. As previously discussed, proton pumping cannot be a major component in these samples, but given some microwave mobility of the mobile protons, we could expect a measurable TRDL signal from these mobile charge carriers in the slower portion of the TRDL decay.

We believe that the 20-μs decay phase of the TRDL signal is not as well resolved in unoriented purple membrane samples due to structural changes in the purple membranes associated with a different sample preparation protocol involving prolonged evaporation in these latter samples. Of course, it was not possible to measure the corresponding photoelectric signals from the unoriented film samples. In this regard, TRDL measurements have the advantage of generality over the photoelectric measurements, but it is clearly advantageous to be able to perform both measurements on the same samples. From the point of view of possible artifacts in the TRDL measurements, we reject the possibility that there was any fundamental difference between oriented and unoriented samples involving the physical nature of microwave absorption in the two kinds of samples. In this regard, we deem electrostriction of the protein or of the stacked membranes to be an unlikely occurrence in either oriented or unoriented membranes.

The lack of a detectable TRDL signal for blue deionized membranes may be related to the observation of an inverted phase and a slower decay for the photoelectric charge displacement time profile (21). As the TRDL measurements are sensitive to changes in charge separation, one possible explanation for the lack of a detectable TRDL signal in deionized membranes would be a much greater energy storage in the protein's conformational changes rather than in charge separation for the initial phase of the deionized membranes' photocycle. It is clear that the primary photochemistry cannot give rise to a significant component of charge separation for the deionized blue membranes.

We propose that the TRDL method could eventually provide a means of discriminating between the conformational and charge separation components for energy storage in the time-resolved bR photocycle. However, it is difficult to give precise estimates of transient microwave absorption or microwave cavity Q attenuation at present: this avenue could be pursued in future work possibly with much more sophisticated microwave detection systems. Another important factor in quantitative studies will be measurements of the microwave electric mobilities of the various photochemically produced charge carriers: they are presently unknown as discussed earlier.

On comparing the TRDL signals of this work with those measured for inorganic semiconductors (17), we estimate that the former signals are ~100 times weaker, probably largely due to the lower microwave mobilities for the charge carriers in biological samples. Considering these lower microwave mobilities, the TRDL signals of this work are actually reasonably strong, taking into account the small sample volumes and small cavity-filling factor. The measurement of the strong TRDL signals of this study provides support for charge separation being a major component of the 0–20 μs energy transduction events in the partially hydrated purple membranes of this work. It is also significant that charge separation persists for several hundred microseconds in these partially hydrated samples. From the point of view of distributed kinetics in these samples, this latter observation points to those bR reaction centers which have slower energy transduction steps including the following elements: the relatively slow formation of the M intermediate, the presence of some limited proton pumping, and slow charge recombination rates. We cannot estimate the relative size of the TRDL signal due to proton pumping because we do not have information on the relative magnitudes of the microwave mobilities of the early charge carriers relative to those of mobile protons. In addition, the presence of some charge recombination or back reactions from the K, L, and M intermediates (20) would tend to result in decreased microwave absorption and faster TRDL decay at various times in the bR photocycle.

With regard to future perspectives, the measurement of TRDL for the photochemical reaction centers of bR in purple membranes appear to be a technique of moderate sensitivity. For instance, on the basis of the observed signal-to-noise ratios, it is not as sensitive as the measurements of charge displacement currents in oriented purple membrane systems as shown in Fig. 2 and elsewhere (21, 22, 26, 27). Nonetheless, given sufficiently large microwave mobilities, one could reasonably anticipate the measurement of TRDL associated with charge separation in other biological membranes, perhaps eventually even including aqueous interfaces by working with samples in aqueous EPR flat cells (17).

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