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Absolute quantum yields and proof of proton and nonproton transient release and uptake in photoexcited bacteriorhodopsin

(conductivity/pH change/free ion)

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ABSTRACT **Using a sensitive differential ac conductance apparatus, we have measured transient ion movements in and the heating of bacteriorhodopsin suspensions after a light flash. The signal from the heating serves as an internal calibration of the absorbed photons and therefore the method gives the absolute quantum yield (ϕ) from a single measurement. At pH 4, H^+ uptake precedes release, with $\phi = 0.4$. By varying the buffer composition, we can prove that this signal is due to protons. At pH 8, however, the transient conductance increase is virtually independent of the buffer composition, showing that ions other than H^+ are first released and then taken up by the purple membrane. If these ions are typical monovalent cations such as Na^+ ($\Lambda = 50$ ohm^{-1}·cm^2·equiv^{-1}), this process has a quantum yield of 2 or more at high salt concentrations.**

There are three biological systems in which a light-driven reaction stores chemical free energy. One is photosynthesis. The other two involve the retinal-containing proteins found in the membranes of halophilic bacteria, halorhodopsin and bacteriorhodopsin (which function as light-driven ion and proton pumps, respectively) (for review, see refs. 1–3). Proton release and uptake by bacteriorhodopsin has been measured by using pH indicators (4–6) and volume changes (7) after light flashes or by steady-state pH changes under continuous illumination (8–10) in membrane suspensions, vesicles, cell envelopes, and whole cells. Quantum yields obtained with bacteriorhodopsin suspensions ranged from 0.25 to 0.7, depending on ionic strength (6, 7); in whole cells, the reported values range from 0.3 to 0.7 (8, 10). The results for suspensions may not be directly comparable with those for closed systems because, in the former, one cannot distinguish protons pumped across the purple membrane from those released and taken up on the same side.

We present here measurements of transient conductances observed in bacteriorhodopsin suspensions after a flash of light. Changes in $[H^+]$ can be measured even in buffered solution—in fact, the behavior of the signal amplitude at different buffer compositions directly proves whether or not protons are involved. The method has general application to any system involving release or uptake of ions. It is particularly convenient for photoinitiated reactions and gives the absolute quantum yield without extra light measurements.

MATERIALS AND METHODS

The core of the apparatus is a Wheatstone bridge containing two identical cells for sample and reference. The cells are 1-cm-square Pyrex with Pt wires protruding about 1 cm from diagonal corners of the bottom. At the low polarizing voltages used (250 mV rms), the cells are well described from 10 Hz to 200 kHz as a capacitance (≈ 0.1 μF) in series with a resistance inversely proportional to the ionic strength. Above 10 kHz, the impedance is frequency independent even at the highest salt concentration (0.3 M) used. Slight differences in the cells are compensated for by a variable resistance/capacitance network. The difference signal is detected by a PAR 128 or 126 lock-in amplifier (minimum response time, 1 ms) whose amplified reference output was the excitation source for the bridge ($\nu = 100$ kHz). Off-balance signals after dye laser flashes (xenon-flash-pumped rhodamine 6G; 590 nm, 1 μs, $\approx$1 mJ) were stored in a Nicolet 1170 signal averager. The laser trigger is locked to the PAR reference signal and, by means of a variable delay, the position of the flash can be placed at any phase angle relative to the phase detector. The PAR detector phase was set to maximize the thermal signal from a solution of crystal violet in KCl. This phase setting corresponds to that giving the maximum signal when the bridge is put off balance resistively. Single-shot sensitivity for signal/noise = 1:1 corresponds to a conductivity change of 1 to 2 ppm.

The purple membranes were the gift of W. Stoeckenius and were kept in 40% sucrose and stored frozen at −20°C. Small aliquots of the stock bacteriorhodopsin were thawed and washed by three cycles of dilution in glass-distilled water followed by centrifugation (Sorvall SS-34 rotor, 13,000 rpm, 0.5 hr). The final suspension gave an absorbance of about 0.6. These solutions were stored at 4°C and used within 2 wk. Similar results were obtained with membranes stored in 3 M NaCl and treated as above.

RESULTS AND DISCUSSION

The conductance of the sample is proportional to $\sum_i c_i\Lambda_i$, where c_i is the concentration of ion i and Λ_i is its equivalent conductance. The steady value of conductance becomes null when the bridge is balanced. Changes in either the concentration or equivalent conductance for any ion will give a change in conductance and generate an off-balance signal. With a photochemically active sample, the laser flash will cause changes proportional to the quantum yield and the total absorbed light in the concentration of one or more ions. After sufficient time, in a cyclic system, the absorbed flash energy is degraded to heat. If there is a net chemical reaction, the heat of reaction must be included. This heating increases the Λ_i for each ion because it is a strong function of temperature via solvent viscosity. The heating also causes increases in volume (and hence decreases in all concentrations), but these are neglected because $(1/V)(dV/dT)$ is 1% of $(1/\Lambda)(d\Lambda/dT)$. The thermal step allows an internal calibration of the apparatus that takes into account the cell constant, inhomogeneous illumination, etc. If we take the ratio of the photoinduced transient conductance to

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the thermal step, these factors and integrals cancel out, and we get the quantum yield directly from the ratio and well-known physical constants:

$$\phi\Lambda = \left(\frac{S_{photo}}{S_{thermal}}\right) \cdot \frac{N_0}{1{,}000} \cdot \sum_i \frac{\varepsilon \Lambda_i C_i}{C\rho}\left(\frac{1}{\Lambda_i}\frac{d\Lambda_i}{dT}\right), \qquad [1]$$

where Λ is the equivalent conductance of the ion produced photochemically with quantum yield ϕ, N_0 is Avogadro's number, ε is the energy of the photon, C and ρ are the specific heat and density of the solution, and $(1/\Lambda_i)(d\Lambda_i/dT)$ is the temperature coefficient of Λ_i (equal to 0.022 K^{-1} for most ions except H^+ and OH^-). This treatment assumes that the light and electrical responses are in the linear region and that the change in conductivity due to bacteriorhodopsin itself is negligible.

A transient conductance obtained at pH 8 is shown in Fig. 1. The thermal effect appears as a base-line shift because thermal relaxation is very long compared with the experimental sweep time. Experiments with photochemically inactive dyes showed that the thermal step is complete within the instrumental response time ($t_{1/2}$ = 1 ms). The transient signal is well described by two consecutive first-order processes: a rise limited by the instrument and a single exponential decay with $t_{1/2} \approx 7$ ms.

A series of traces obtained at pH 4 with various concentrations of sodium acetate buffer is shown in Fig. 2. With no buffer, there is a large negative signal that we interpret as uptake of protons by the purple membrane prior to proton release, in agreement with time-resolved (4) and steady-state measurements at low pH (11). As the acetate buffer concentration increases, the signal amplitude diminishes in magnitude, goes through zero, and becomes positive.

Within our time resolution, all ionic buffer reactions will certainly be equilibrated. At low acetate concentration, H^+ is taken from solution and the conductance decreases. As the acetate concentration increases, the buffer reactions lead to dissociation of acetic acid, giving a net generation of acetate ion instead of a loss of H^+. Hence, the signal behaves as expected for bacteriorhodopsin taking up H^+ from solutions of increasingly strong acetate buffers. Further, the ratio of the amplitude at high acetate concentration to that with no buffer should be the ratio of the equivalent conductances: $\Lambda_{AcO}/-\Lambda_H = -0.12$. We observe -0.16. Aspartate and glutamate side chains in the purple membrane could act as buffers, reducing the signal amplitude even with no acetate present. This would tend to make the ratio higher than the theoretical value.

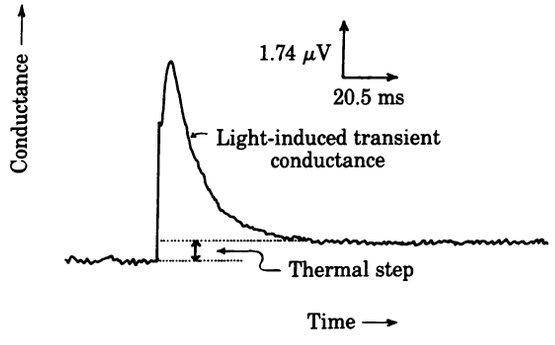

FIG. 1. Laser flash-induced transient conductance of 10 μM bacteriorhodopsin/40 mM $N(C_2H_5)_4ClO_4$/9 mM $N(CH_3)_4HCO_3$/1 mM NH_3, pH 8. The trace shown is the mean of 64 flashes. The discontinuity in the fast rise of the transient is due to an electrical artifact from the laser discharge. The thermal step relaxes with a time constant of many seconds, so it appears as a dc base-line shift on the time scale of the conductance measurements.

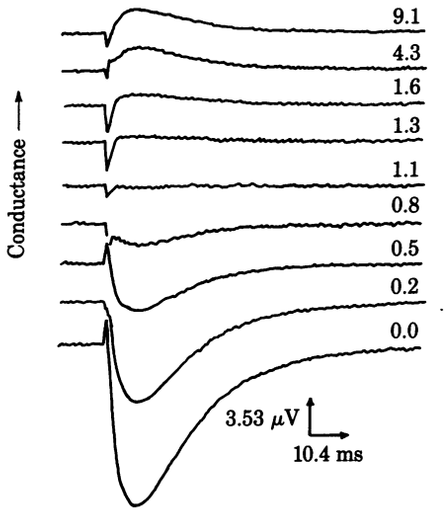

FIG. 2. Effect of increasing the sodium acetate buffer concentration on transient conductance of bacteriorhodopsin at pH 4. Total concentrations of buffer (given above each trace) are in mM, and the ionic strength varied from 0.1 mM to 1.6 mM throughout the series. The sharp transient conductance in the first millisecond after the flash is an electrical artifact that appears whether or not bacteriorhodopsin is present. It can be suppressed by adjustment of the phase shift between the laser firing and the detector phase setting. However, because of "jitter" in the ignition of the spark gap, there is some residual artifact that appears with variable sign. Each trace is the mean of 128 flashes.

The curves are well fit by two consecutive first-order processes, a fast rise ($t_{1/2}$, 3.1–3.3 ms) and a slower decay with a $t_{1/2}$ that decreases slightly, from 12 ms at the lowest acetate concentration to 10 ms at the highest. From the observed maxima of the traces at highest acetate concentration, an average quantum yield of 0.2 is obtained for acetate production (i.e., H^+ uptake). From the results of the curve fitting, the quantum yield rises to about 0.4. The main source of error (±20%) is the small size of the thermal signal because of the low ionic strength (0.1–1.6 mM). Ort and Parson (7) report a value of 0.25 at 15 mM ionic strength and pH 7.8; Govindjee *et al.* (6) found $\phi = 0.27$ in 10 mM KCl (pH 6.6). These measurements are not directly comparable because our data at pH 4 refer to fast H^+ uptake followed by release while those at pH 6–8 refer to the opposite process.

The situation is quite different in alkaline solution. A series of traces like those of Fig. 2 are summarized in Fig. 3, in which increasing amounts of $N(CH_3)_4HCO_3$ buffer were added to a 1 mM NH_3-buffered bacteriorhodopsin suspension. (Fig. 1 shows the response at $[HCO_3^-] = 9$ mM.) In the absence of HCO_3^-, protons released by bacteriorhodopsin will be consumed by NH_3, giving NH_4^+, and a net increase in conductivity. As HCO_3^- is added to swamp the NH_3 buffer, most of the H^+ release will be consumed by HCO_3^-, neutralizing the charge and giving a net loss in conductivity. The dashed curve shows the calculated behavior as the buffer composition changes normalized to the point $[HCO_3^-] = 0$. In particular, we expect the signal to go through zero and change sign between 1 and 10 mM HCO_3^-. But the observed data are essentially constant. The quantum yield is calculated to be about 1 for a typical univalent ion (e.g., Na^+ with $\Lambda = 50$ ohm^{-1}·cm^2·equiv^{-1}). At higher ionic strength (above about 100 mM), the amplitude increases, corresponding to a quantum yield of 2 to 3. If the 10% decrease from $[HCO_3^-] = 0$ to 10 mM is due to H^+, it would correspond to a quantum yield of about 0.2. The kinetics of conductivity change are almost constant from $[HCO_3^-] = 0$ to 100 mM (ionic strength, 40–250 mM): a rise time ($t_{1/2}$ = 1 ms) and a single exponential decay

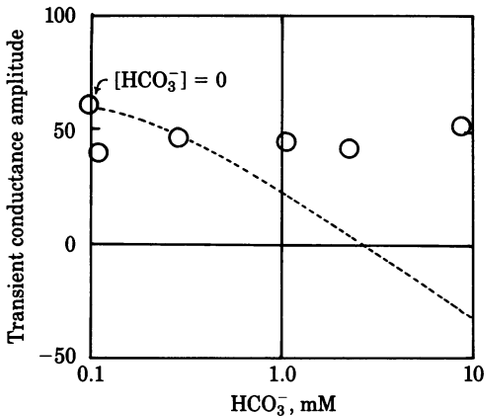

FIG. 3. Amplitude of transient conductance in bacteriorhodopsin at pH 8 as a function of bicarbonate concentration. Samples have the composition of the sample of Fig. 1, only the total $N(CH_3)_4HCO_3$ concentration was varied. ----, Signal amplitudes if the signal is due to H^+ release; ○, observed data. These data imply that most of the observed ionic movements are *not* due to H^+. The vertical scale is proportional to the conductance change expressed in units of equivalent conductance.

whose $t_{1/2}$ rises from 6.8 ms at the lowest to 11 ms at the highest ionic strength.

These data are for suspensions of bacteriorhodopsin—from solution conductivity alone, we cannot distinguish ions that are translocated from those that are released and rebound at the same surface. The behavior in alkaline solution could be explained by a light-induced change in the surface charge of the purple membrane, which is large and negative (9). A change in the surface charge could release cations from the double layer that later rebind. This would have the effect of producing a positive conductance signal. Alternatively, the mobility of the surface-bound ions could be changed because of rearrangement of the negative charges on the membrane. The charge displacement seen by Kimura *et al.* (12) in membrane suspensions oriented by the polarizing field cannot explain these results because the degree of orientation under our experimental conditions will be $\approx 10^{-5}$ assuming that the same half-polarizing voltage applies at 100 kHz as at 770 Hz. Dc voltage-induced orientation (Keszthelyi and Ormos, ref. 13) cannot occur because our excitation source is transformer coupled to the bridge. We have also determined that the quantum yields at pH 4 and pH 8 are independent of frequency between 10 and 100 kHz. Thus, frequency-dependent polarizations are unlikely to contribute to the measured conductivity change.

The fact that the kinetics of these widely different effects (i.e., at pH 4 and pH 8) are similar shows that the rate-determining step involves the protein and not H^+ or buffer ions *per se*. Similar experiments must be done under ion-translocating conditions, but it is evident that large ion movements may be significant in the working of bacteriorhodopsin under natural conditions.

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