Phase-Lifetime Spectrophotometry of Membranes from Ion Flux Mutants of *Halobacterium halobium*

John Krupinski, John L. Spudich, and Gordon G. Hammes

From the Department of Chemistry and the Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853 and the Department of Anatomy, Albert Einstein College of Medicine, Bronx, New York 10461

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Phase-lifetime spectrophotometry has been used to study the rate processes associated with intermediates in the photocycling pigments in membrane vesicles of mutant strains of *Halobacterium halobium*. Vesicles deficient in bacteriorhodopsin, but containing halorhodopsin, were monitored with light at 490 nm. Two relaxation processes, with kinetic parameters largely independent of pH over the range 6.2–7.8, were found to be associated with halorhodopsin photocycling in 4 mM NaCl, 10 mM buffer at 23 °C. The average relaxation times are 0.94 and 11.4 ms. When vesicles deficient in both bacteriorhodopsin and halorhodopsin were monitored at 370 nm, a single relaxation process with an average relaxation time of 168 ms was detected. This process is independent of pH over the range 4.7–8.8. Examination of vesicles from ion flux mutants showed this slow process to be unrelated to halorhodopsin content and to derive from another photoactive retinal pigment, possibly the recently described slow cycling pigment s-rhodopsin.

The proton pump, bacteriorhodopsin (cf. Stoeckenius and Bogomolni, 1982), the chloride or sodium ion pump, halorhodopsin (Lindley and Macdonald, 1979; Schobert and Lanyi, 1982), and s-rhodopsin (Bogomolni and Spudich, 1982) appear to be functionally distinct membrane-bound proteins found in *Halobacterium halobium*. All three are photobiologically reactive retinal-containing proteins. This characteristic has made them particularly suitable systems for investigation by flash photolysis. Such studies reveal that bacteriorhodopsin is physiologically than those of flash photolysis. Experiments with illumination, the experimental conditions are more nearly independent of pH over the range 6.2–7.8, were found to be associated with halorhodopsin photocycling in 4 mM NaCl, 10 mM buffer at 23 °C. The average relaxation times are 0.94 and 11.4 ms. When vesicles deficient in both bacteriorhodopsin and halorhodopsin were monitored at 370 nm, a single relaxation process with an average relaxation time of 168 ms was detected. This process is independent of pH over the range 4.7–8.8. Examination of vesicles from ion flux mutants showed this slow process to be unrelated to halorhodopsin content and to derive from another photoactive retinal pigment, possibly the recently described slow cycling pigment s-rhodopsin.

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2. The *H. halobium* proteins are designated as: bR, bacteriorhodopsin; hR, halorhodopsin; sR, s-rhodopsin.
3. The abbreviation used is: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
In the experiments in which the amplitude was measured as a function of the chopping frequency, both the monochromator setting and the maximum transmission wavelength of the interference filter were either 490 or 370 nm. The data were fit to the equation

$$A_{obs} = \sum_{i} \frac{A_i}{1 + \omega^2 r_i^2}$$

where $A_{obs}$ is the observed amplitude, $A_i$ is the maximum contribution to the observed amplitude made by the process characterized by the relaxation time $r_i$, $\omega$ is the chopping frequency in radians/s, and $n$ is the total number of processes. The data fitting was done on a Digital 11/24 computer by a general nonlinear least squares analysis.

To obtain amplitude spectra, the wavelength of the monitoring beam and the interference filter or the photomultiplier were changed in 10 nm increments, and the amplitude was measured with the lock-in amplifier while chopping the actinic light source at a constant frequency of either 5 or 10 Hz. Measurements were not made above 500 nm since scattering of the actinic beam resulted in a frequency-dependent signal. All amplitude spectra were corrected for the variation in transmission of the narrow band-pass filters, the wavelength-dependent variation in the intensity of the measuring light, and the wavelength dependence of the photomultiplier sensitivity.

RESULTS

An amplitude spectrum for L33 vesicles (1.2 mg/ml of protein) in 10 mM HEPES, 4 mM NaCl (pH 6.98) at 23 °C is presented in Fig. 1. Two prominent peaks occur in the near ultraviolet and a third emerges at 500 nm. Repetition of the experiment while chopping the actinic light source at 10 Hz (data not shown) revealed that the entire spectrum was frequency-dependent. In particular, the peaks in the near ultraviolet showed a greater percentage decrease in amplitude upon increasing the chopping frequency than did the peak at 500 nm. This indicates that at least one of the processes occurring in the near ultraviolet is on a much slower time scale than that at 500 nm.

Initial measurements at pH 7.0 monitored at 490 nm indicated that some bleaching of the sample occurs (~25%) if the sample is exposed to the actinic and monitoring light during the entire experiment in which a frequency spectrum is measured. However, if exposure to the light is limited only to the time during which the actual measurement of signal intensity is made, as was done for all data presented here, the photobleaching is negligible. Photobleached samples fully regenerated if left at 4 °C overnight.

The frequency spectrum of L33 vesicles was measured over the pH range 6.2–7.8. A typical spectrum is shown in Fig. 2 where the signal amplitude is plotted versus the chopping frequency. In all experiments, the chopping frequency was varied in a nonsequential order and several frequencies were checked twice to be certain photobleaching was negligible. Multiple data sets at a given pH were fit to Equation 1. In all cases, two relaxation processes were required to fit the data. The parameters obtained over the range of pH studied are summarized in Table I. The sum of the amplitudes was normalized to $A_1 + A_2 = 1.00$. At pH 7.00, $A_1 + A_2 = 1042 \text{ mV}$. 

![Normalized amplitude spectrum (in arbitrary units) of L33 vesicles at a constant chopping frequency of 5 Hz. The membrane vesicles (1.2 mg of protein/ml) were in 10 mM HEPES, 4 mM NaCl (pH 7.0) at 23 °C. Each point was obtained as described under "Experimental Procedures," and a smooth curve was drawn through the points.](image)

**Fig. 1.** Normalized amplitude spectrum (in arbitrary units) of L33 vesicles at a constant chopping frequency of 5 Hz. The membrane vesicles (1.2 mg of protein/ml) were in 10 mM HEPES, 4 mM NaCl (pH 7.0) at 23 °C. Each point was obtained as described under "Experimental Procedures," and a smooth curve was drawn through the points.

![Plot of amplitude versus frequency for L33 vesicles (1.2 mg of protein/ml) monitored at 490 nm in 10 mM HEPES, 4 mM NaCl (pH 7.0) at 23 °C. Three experiments were performed to fit simultaneously to Equation 1 with $n = 2$. The relaxation times used to calculate the curve were 0.65 and 11.2 ms, with relative amplitudes of 0.06 and 0.94, respectively. The total signal amplitude was 1042 mV.](image)

**Fig. 2.** Plot of amplitude versus frequency for L33 vesicles (1.2 mg of protein/ml) monitored at 490 nm in 10 mM HEPES, 4 mM NaCl (pH 7.0) at 23 °C. Three experiments were performed and fit simultaneously to Equation 1 with $n = 2$. The relaxation times used to calculate the curve were 0.65 and 11.2 ms, with relative amplitudes of 0.06 and 0.94, respectively. The total signal amplitude was 1042 mV.

**Table I**

<table>
<thead>
<tr>
<th>pH</th>
<th>$r_1$ (ms)</th>
<th>$r_2$ (ms)</th>
<th>$A_1$</th>
<th>$A_2$</th>
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</thead>
<tbody>
<tr>
<td>7.80</td>
<td>1.1</td>
<td>0.09</td>
<td>11.7</td>
<td>0.91</td>
</tr>
<tr>
<td>7.40</td>
<td>1.8</td>
<td>0.16</td>
<td>12.8</td>
<td>0.90</td>
</tr>
<tr>
<td>7.00</td>
<td>0.65</td>
<td>0.06</td>
<td>11.2</td>
<td>0.94</td>
</tr>
<tr>
<td>6.55</td>
<td>0.78</td>
<td>0.05</td>
<td>11.9</td>
<td>0.95</td>
</tr>
<tr>
<td>6.45</td>
<td>0.57</td>
<td>0.04</td>
<td>11.2</td>
<td>0.96</td>
</tr>
<tr>
<td>6.20</td>
<td>0.71</td>
<td>0.07</td>
<td>9.8</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Average $0.94 \pm 0.02$, $0.07 \pm 0.02$, $11.4 \pm 0.7$, $0.93 \pm 0.02$.
maximum at 380 nm decays with a time constant of 10 ms, while the rise to that state has a time constant of 0.4 ms. The faster component ($\tau = 5.1$ ms) observed in this experiment may represent a mean relaxation time for these two halorhodopsin processes, or perhaps only the decay process found with flash photolysis is being observed. These two possibilities can not be distinguished within the error of the experiment. On the other hand, the slower process ($\tau = 129$ ms) occurs on a very different time scale. Experiments with the halorhodopsin-deficient mutant, Flx37, reveal that the fast component is related to the presence of halorhodopsin, while the slow component is not.

As shown in Fig. 3, the amplitude spectrum for Flx37 vesicles shows a small peak at 490 nm, while the peaks in the ultraviolet are still prominent, although present in different proportions than for L33 (Fig. 1). When samples of Flx37 and L33 vesicles at the same protein concentration in 10 mM HEPES, 4 mM NaCl (pH 7.0) were examined at a chopping frequency of 5 Hz, the amplitude of the Flx37 sample was found to be 8.1% that of the L33 sample at 490 nm. This is in excellent agreement with the value of 8% given by Spudich and Spudich (1982) for the relative amount of halorhodopsin in the two strains as determined by light-driven proton influx. Relaxation spectra of Flx37 vesicles were determined following the procedure outlined above for the L33 vesicles, except that the monitoring light was at 370 nm. A typical frequency spectrum for the Flx37 vesicles with the monitoring beam at 370 nm is presented in Fig. 4. The frequency spectra again were fit to Equation 1. In all cases, a single relaxation process was sufficient to describe the data. The curve in Fig. 4 represents the best fit to Equation 1 with the best fit parameters as given in the figure legend. If the data were fit to more than one relaxation process, the computer either ignored the extra parameters, or the precision of the fit did not improve. Apparently, the amplitude of the fast process (due to halorhodopsin) is decreased to such an extent that it can no longer be observed at 370 nm. The slower time constant that remains agrees within experimental error with that found for the slow process observed at 370 nm with L33 vesicles.

The relaxation times and amplitudes over the pH range 4.7–8.8 are given in Table II. Again, no significant pH dependence is observed. The increase in the time constant at pH 8.8 can probably be accounted for by a slight buffer effect (compare borate (pH 8.1) to HEPES (pH 8.2)). The addition of 10 $\mu$M carbonyl cyanide m-chlorophenylhydrazone had no appreciable effect on the slow relaxation time. Reduction of the light intensity by a factor of 2 also did not alter the relaxation time.

To test whether the halorhodopsin-independent slow process depends on retinal, the relaxation spectrum of Flx3R vesicles was examined. Flx3R is a retinal-deficient derivative of a halorhodopsin-deficient strain. With Flx3R membranes in the absence of retinal, no signal above the background noise level (<20 $\mu$V) is observed at pH 6.95 with the monitoring light at 370 or 500 nm. With vesicles prepared from Flx3R cells reconstituted with retinal (as in Fig. 4; Bogomolni and Spudich, 1982), a single relaxation process with a relaxation time of 121 ms is observed with the monitoring beam at 370 nm.

Experiments with OD2 vesicles were performed at pH 7.0. These vesicles contain substantial amounts of carotenoids along with an even greater amount of halorhodopsin than is found in L33 vesicles. However, the added carotenoids considerably increase the background absorbance, and a smaller signal resulted. The decrease in amplitude and photoinstability of the carotenoids prevented extensive experiments; however, the time constants associated with halorhodopsin were significantly faster. The slower halorhodopsin component had a time constant of about 3 ms, while the faster component was too fast to be resolved.

**TABLE II**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Time Constant (ms)</th>
<th>Amplitude (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borate</td>
<td>8.8</td>
<td>227</td>
<td>4048</td>
</tr>
<tr>
<td>HEPES</td>
<td>8.8</td>
<td>176</td>
<td>3511</td>
</tr>
<tr>
<td>Borate</td>
<td>8.1</td>
<td>204</td>
<td>4090</td>
</tr>
<tr>
<td>HEPES</td>
<td>8.1</td>
<td>143</td>
<td>2944</td>
</tr>
<tr>
<td>HEPES</td>
<td>7.7</td>
<td>146</td>
<td>2878</td>
</tr>
<tr>
<td>HEPES</td>
<td>7.1</td>
<td>151</td>
<td>2391</td>
</tr>
<tr>
<td>HEPES</td>
<td>6.3</td>
<td>139</td>
<td>2347</td>
</tr>
<tr>
<td>Succinate</td>
<td>6.3</td>
<td>140</td>
<td>2446</td>
</tr>
<tr>
<td>Succinate</td>
<td>4.7</td>
<td>186</td>
<td>2429</td>
</tr>
</tbody>
</table>

Average: 168 ± 27

* Plus 10 $\mu$M carbonyl cyanide m-chlorophenylhydrazone.
DISCUSSION

The results obtained indicate two kinetic processes are coupled to the intermediate with an absorption maximum at 490 nm that is associated with ion pumping by halorhodopsin in L33 vesicles. The average relaxation times observed for halorhodopsin photocycling (0.9 and 11 ms) are in reasonable agreement with the values of 0.6 and 14 ms found by flash photolysis (Weber and Bogomolni, 1981). A halorhodopsin-independent process with a relaxation time of 168 ms and an absorption maximum in the near ultraviolet also is observed. This relatively slow process, which depends on retinal, may derive from the photocycle of s-rhodopsin, which is present in the vesicles used here (Bogomolni and Spudich, 1982). However, the relaxation time observed is considerably less than the 800-ms value found for s-rhodopsin by flash photolysis (Bogomolni and Spudich, 1982). Possibly the 168-ms relaxation time derives from a faster intermediate of the s-rhodopsin photocycle not detected by flash photolysis.

The ion being pumped by halorhodopsin is probably Cl⁻ (Schobert and Lanyi, 1982), although it was initially thought to be Na⁺ (Lindley and MacDonald, 1979). The proton gradient clearly is not directly coupled to the pumping process since the proton ionophore carbonyl cyanide m-chlorophenylhydrazone has no influence on the rates. This confirms the similar conclusion reached by Lindley and MacDonald (1979) based on ion flux measurements. The finding of two kinetic processes indicates that the intermediate with an absorption maximum at 500 nm is coupled to at least two other species in the photocycling of halorhodopsin. None of the relaxation times display a significant dependence on pH over the limited range tested. This is in contrast to bacteriorhodopsin where a specific ionizable group can be identified with one of the relaxation processes.

A detailed mechanistic interpretation of the relaxation times is not possible. However, the nature of the mechanisms can be somewhat defined. In the case of halorhodopsin-independent process, the minimum mechanism consistent with the data is

\[
A \xrightarrow{k_1} B \xrightarrow{k_2(I)} C
\]

where the \(k_1\) and \(k_2\) are rate constants, \(k_2(I)\) is a function of the light intensity, \(I\), and \(A\) is the species with an absorption maximum at 370 nm. Since the relaxation time is not dependent on light intensity, \(1/\tau = k_1\). If \(k_2\) is proportional to the light intensity, the amplitude is proportional to the light intensity as observed. In the case of halorhodopsin, a mechanism consistent with the observation of two relaxation times is

\[
A \xrightarrow{k_1} B \xrightarrow{k_2} C
\]

Since the relaxation times are independent of the light intensity, \(1/\tau_1 \approx k_1\) and \(1/\tau_2 \approx k_2\). However, if the species with an absorption maximum at 500 nm is assumed to be \(A\) and \(B\) is assumed to have negligible absorbance at 500 nm, the experimentally observed ratio of amplitudes is not predicted \(\frac{A_1}{A_2} \approx \frac{k_2}{k_1} (1 + k_2/k_1(I))\). Moreover, this ratio is predicted to be light intensity-dependent. Therefore, either both \(A\) and \(B\) have appreciable light absorption at 500 nm, or a complex mechanism must be invoked. An alternative mechanism also consistent with the data is the occurrence of two parallel paths, each of which generates an intermediate with appreciable absorption at 500 nm. The rate constants characterizing the decay of these intermediates to the ground state would be \(1/\tau_1\) and \(1/\tau_2\). These two mechanisms cannot be distinguished by the kinetic measurements. An analogous linear mechanism has been proposed on the basis of flash photolysis experiments, but only \(A\) has been proposed to have appreciable light absorption at 500 nm (Weber and Bogomolni, 1981). Our results suggest this mechanism must be modified.

REFERENCES

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