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The photocycle of the chloride pump halorhodopsin. II: Quantum yields and a kinetic model

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The quantum yield of the primary photoreaction of HR_{578} was determined as $\phi_{green} = 0.34 \pm 0.02$ and that of the photochemical reconversion of HR^{L}_{410} into HR_{578} as $\phi_{blue} = 0.01 \pm 0.004$. From steady-state illumination and flash-photolysis experiments, a quantitative description of HR^{L}_{410} formation and decay was made leading to a model of the photocycle in isolated, light-adapted HR. This model satisfies all known facts about HR including its photochromic behaviour.

Key words: halorhodopsin/photocycle/light-driven chloride pump/regulation

Introduction

In the preceding paper (Hegemann *et al.*, 1985) a quantitative description of a reaction of the light-driven chloride pump halorhodopsin (HR) was given, which leads to a deprotonated and long-living species, HR^{L}_{410}. Here the analysis of HR photochemistry is extended by measurements of the quantum yields for both HR_{578} and HR^{L}_{410}. Flash-photolytic data and photostationary measurements were necessary to develop a model of the photocycle of HR which defines HR^{L}_{410} not as an intermediate but as a side product and further can serve as a basis for detailed studies on the chloride dependence of the HR photochemistry.

Results

The quantum yields in halorhodopsin

The photochemical behaviour of HR in the presence of azide allows a determination of the quantum yield, ϕ, of the HR photocycle under conditions of stationary illumination. The approach is similar to that taken for bacteriorhodopsin (BR) in the salt/ether system of purple membranes (Oesterhelt and Hess, 1973). The linear correlation betwen initial velocity of HR^{L}_{410} formation and irradiance in the presence of defined concentrations of azide was verified as follows: an HR-containing cuvette was placed into a spectrophotometer and illuminated by actinic light from the side. Knowing the molar extinction coefficient of HR_{578}, the optical density of the sample and the quantum-flux intensity of the actinic light, the quantum yield for HR^{L}_{410} formation can be determined. The quantum flux was measured by replacing the HR solution with an actinometric solution of equal volume under the same geometric conditions in the spectrophotometer. The HR concentration was varied from 48.6 μM to 6.9 μM by dilution with the same buffer. Initial velocity values of HR^{L}_{410} formation upon illumination divided by (1 − transmission) of the sample varied only by 10% (see Materials and methods), indicating that the measured quantum yield values were independent of the HR concentration under the conditions of the experiment (Table I). The ratio of HR^{L}_{410}/HR_{578} reached in the photosteady state was constant up to optical densities of 2.43 in the sample.

The ϕ value obtained yields the quantum efficiency for HR^{L}_{410} formation at the azide concentration used in the experiment and has to be extrapolated to an infinite azide concentration using a Lineweaver-Burk plot (Figure 2 in Hegemann *et al.*, accompanying paper) to obtain the quantum yield for the photocycle. Under these conditions all molecules excited must end in HR^{L}_{410}, a fact which is demonstrated in flash-photolytic experiments (Figure 1). In order to exclude effects of azide on the quantum yield of the primary photoreaction the extent of HR_{600} formation with and without azide under otherwise identical conditions was measured and found to be the same (H. Polland and W. Kaiser, unpublished results). Summary of all measurements gives a ϕ value of 0.34 ± 0.02 for the HR photocycle. Under identical conditions we determined the quantum yield for the BR photocycle in the salt/ether system. A value close to 0.6 was found, which is the lower limit because the significant light scattering of the salt/ether system was not taken into account. This value is therefore comparable with the 0.79 reported by Oesterhelt and Hess (1973). The quantum yield of the photochemical conversion of HR^{L}_{410} into HR_{578} was measured as described above. An HR sample was converted to a photosteady-state mixture of HR^{L}_{410} and HR_{578} by illumination with yellow light ($\lambda > 495$ nm) at 1 mM azide. When a ratio of ~1:1 was reached, the yellow light was turned off and the initial velocity of HR_{578} formation was measured as a function of blue light intensity. A quantum yield, ϕ, of 0.01 ± 0.004 was determined.

Flash photolysis of halorhodopsin

Intermediates of the photocycle of HR have been described (Weber and Bogomolni, 1981; Tsuda *et al.*, 1982; Schobert *et al.*, 1983; Steiner *et al.*, 1984; Hazemoto *et al.*, 1984). After formation of the first photoproduct, designated as HR_{600} (Figure 3), which rises within 5 ps after excitation (Polland *et al.*, 1985), HR_{520} is formed with a rise time of ~5 μs (J. Vogel, unpublished data). It decays in the absence of azide and in 4 M NaCl (1% octylglucoside, pH 7) with $t_{1/2} = 20$ ms. Under these conditions the same half time is found for regeneration of HR_{578}. In 1 M NaCl (Figure 1A, trace 1), HR_{578} reappears with $t_{1/2} = 15$ ms, but HR_{520} disappears at a faster rate ($t_{1/2} = 14$ ms in the absence of azide and $t_{1/2} = 9$ ms at 160 mM azide, Figure 1C). A further species, HR_{640}, can be observed in 1 M NaCl during the photosteady-state (Figure 2a) and upon flash photolysis. Spectra recorded in the 100 μs range after flash excitation demonstrate the rise of HR_{520} to be faster than the time resolution and also show a slower rise of HR_{640} to a steady state which then decays somewhat slower than HR_{520} (H.Desel and H.Uhl, unpublished results). We use this information only to justify the description of the HR photocycle shown in Figure 3 and mainly focus here on the reaction conditions for HR^{L}_{410} formation. Figure 1A shows that in the presence of azide the reformation of HR_{578} occurs with at least two time constants, the fast component being that of the azide-free system and the slow component showing $t_{1/2} = 1.2$ s at 160 mM azide. The slow component corresponds

Table I. Dependence of the formation of HR^L_{410} on the HR concentration

Optical density at 552 nm	Initial concentration of HR_{578} (μM)	Photosteady concentration of HR_{410} (μM)	Ratio of HR_{410}/HR_{578} at photosteady-state
2.43	48.6	10.4	0.27
1.64	32.8	7.3	0.29
1.11	22.1	5.2	0.30
0.73	14.7	3.3	0.29
0.51	10.1	2.2	0.28
0.35	6.9	1.5	0.28

The measurements were carried out in 1 M NaCl, 1% octylglucoside, 150 mM azide and 19 mM Mops at a pH of 7.63. Irradiance was 4.8 nE/cm²/s. Light was filtered through an SFK 21 (552 ± 15 nm) filter (Schott).

to the decay time of HR^L_{410} formed in the presence of azide. The half time of 1.2 s found at 22°C and 160 mM azide for HR_{578} formation compares well with the 7 s half time of HR^L_{410} decay from the photosteady state at 13°C and 50 mM azide (Figure 4B in Hegemann *et al.*, accompanying paper). Furthermore, an estimation of the affinity constant for azide can be obtained by a plot of the inverse amplitude of the plateau values in Figure 1A *versus* the inverse azide concentration. A value similar to that found from the initial velocity measurements (Hegemann *et al.*, accompanying paper) is obtained.

Following the decay of HR_{520} at $\lambda = 490$ nm in the presence of 160 mM azide, the half time of 9 ms agrees with the half time of formation of HR^L_{410} measured at $\lambda = 410$ nm (10 ms) (Figure 1B and C). Since, however, two intermediates, HR_{520} and HR_{640}, are in equilibrium, it is unclear which one of these is converted into HR^L_{410}. At $\lambda = 490$ nm not only HR_{520} (with $\epsilon_{490} = 31\,000$/M/cm) but also HR_{578} (with $\epsilon_{490} = 13\,000$/M/cm) absorbs. The absorbance decrease at 490 nm remaining after the decay of HR_{520} is therefore caused by the decreasing concentration of HR_{578} (see spectra in Figure 1 of Hegemann *et al.*, accompanying paper). It should be emphasized that the measuring light for the experiment in Figure 1 established various concentrations of HR^L_{410} before the flash excited the sample, leading to smaller flash amplitudes at high azide concentrations. This is shown in Figure 1D for two azide concentrations and is also evident from the experiment in Figure 2B. In Figure 1A the initial amplitudes of all traces are normalized.

Without azide no measurable quantitites of HR^L_{410} are found in flash experiments (Figure 1A, trace 1). However, continuous illumination produces the same photosteady-state level of HR^L_{410} with and without azide, although very slowly in the latter case (Hegemann *et al.*, accompanying paper). An example is presented in Figure 2A where spectra are recorded every millisecond after turning on the light. The photosteady-state mixture of HR_{520} and HR_{640} is established well within 50 ms (Figure 2A and spectrum 1 in Figure 2B), whereas HR^L_{410} continues to increase for seconds until it reaches the photosteady state (Figure 2B).

Determination of the equilibrium and kinetic constants for HR^L_{410} formation

The measured initial velocities of H^L_{410} formation and decay, the steady-state concentrations of HR^L_{410} and HR_{578}, the measured quantum fluxes used for excitation of HR, the quantum yield and the flash-photolytic results allow the derivation of some of the rate constants for the scheme depicted in Figure 3. Furthermore, the results of flash-photolytic experiments can be compared quantitatively with data obtained from steady-state illumination experiments.

First, a short description of the reactions relevant for the calculations will be given. Excitation of HR molecules in the ground state (S_0) leads to the excited state HR^* (S_1) from which the first photoproduct HR_{600} is formed with a time constant $k_1 = 2 \times 10^{11}$/s (see Figure 3) (Polland *et al.*, 1985). HR_{520} presumably is formed directly from HR_{600} with $k_2 > 2 \times 10^5$/s. It should be noted, however, that it is not important whether or not another intermediate exists between HR_{600} and HR_{520} for our calculations because the rate constants involved would cancel out in the equations for steady-state concentrations. HR_{520} is converted into HR_{640} with rate constant k_3 and concomitant loss of a chloride ion or into HR^L_{410} and a proton plus a chloride ion. At present, no evidence exists to show whether HR^L_{410} is formed from HR_{520} and/or from HR_{640}. The scheme in Figure 3 includes the two possibilities that HR_{520} or HR_{640} releases the proton to form HR^L_{410}, described in the following as model I and II. The rate constants k_5 and k_6 connecting HR^L_{410} and HR_{520} and/or HR_{640} are apparent rate constants depending on azide concen-

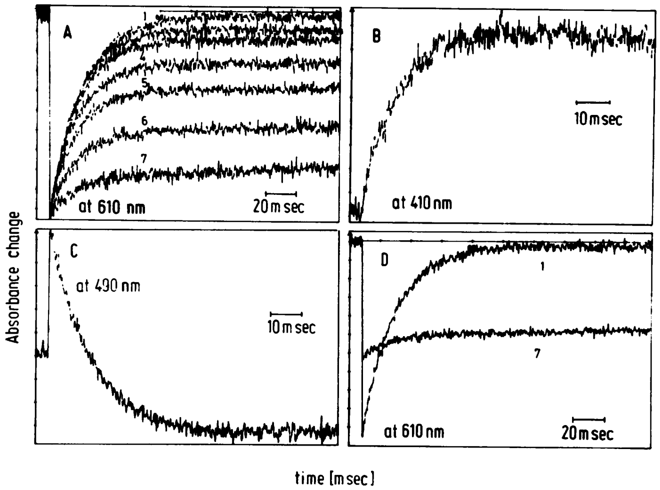

Fig. 1. Flash photolysis of HR in the presence and absence of azide. Laser-induced absorbance changes (downward deflection corresponds to decreased absorbance) of HR (36 μM) in 1 M NaCl/1% octylglucoside/10 mM Mops, pH 7.15 were recorded at various wavelengths; T = 22°C. **(A)** At 610 nm in the absence of azide (full reconversion with a half time of 22 ms, **trace 1**) or in the presence of 5, 10, 20, 40, 80 and 160 (**trace 7**) mM azide. An increasing portion of the molecules returns to HR_{578} with a slow decay time seen as an enlarged absorbance change at 610 within the time of observation (200 ms). The curves are normalized with respect to their maximum absorbance change (see **D**). **(B)** At 410 nm in the presence of 160 mM azide and within the first 100 ms after flash. **(C)** At 490 nm in the presence of 160 mM azide and within the first 100 ms after the flash. **(D)** At 610 nm in the absence of azide (**trace 1**) and in the presence of 160 mM azide (**traces 2–7**). The data are the same as in **A** (**traces 1** and **7**) but were not normalized.

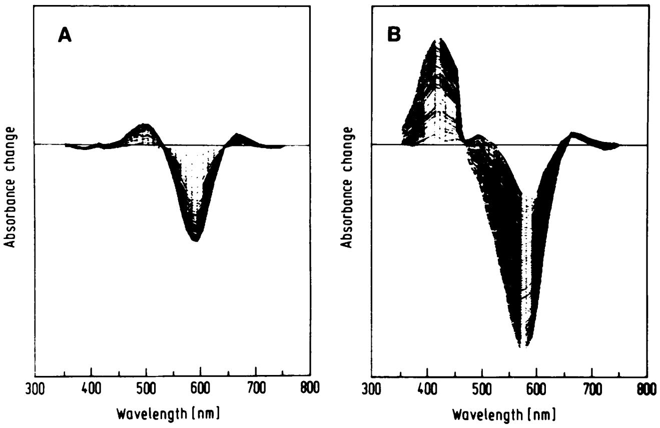

Fig. 2. Formation of HR_{520}/HR_{640} and HR_{410} with azide upon continuous illumination of HR. Difference spectra were recorded in 1 ms intervals between 1 ms and 50 ms after turning on the measuring light at pH 7.06 **(A)** and in 10 ms intervals between 50 ms and 660 ms after turning on the measuring light at pH 9.0 **(B)**. The light passed through a cut-off filter (λ >340 nm). The experimental set up is described in Uhl *et al.* (1985). HR (12 μM) was used under the same conditions as in Figure 8. The spectra were recorded by H.Desel.

tration according to the following relationship:

$$k_5 = k^0_5 + \frac{k^\infty_5}{1 + \frac{K_M^{520}}{[\text{Azide}]}} \qquad \text{with } k^\infty_5 >> k^0_5 \qquad (1)$$

and

$$k_6 = k^0_6 + \frac{k^\infty_6}{1 + \frac{K_M^{410}}{[\text{Azide}]}} \qquad \text{with } k^\infty_6 >> k^0_6 \qquad (2)$$

The superscripts 0 and ∞ indicate rate constants at zero and infinite azide concentration, respectively. The data presented in Table II of Hegemann *et al.* (accompanying paper) document the independence of the HR_{578} and HR_{410} photosteady-state concentrations from azide. This is only possible if the ratio k_5/k_6 is azide-independent and the affinities of HR_{520} and HR^L_{410} for azide are equal.

In the 'flash photolysis' section, evidence for the existence of the various intermediates and the effect of azide on both formation and decay of HR^L_{410} has already been presented. Equations for the steady-state concentrations of photocycle intermediates using steady-state kinetics (Table II) can be derived. The stationary illumination applied leads to a steady-state concentration of HR^* which can be expressed as:

$$HR^* = \frac{k_0}{k_{-0} + k_1} \cdot I \cdot HR_{578} \qquad (3)$$

The amount of the first intermediate, HR_{600}, in the steady state is:

$$HR_{600} = \frac{k_1}{k_2} \cdot \frac{k_0}{k_{-0} + k_1} \cdot I \cdot [HR_{578}] \qquad (4)$$

or

$$HR_{600} = \frac{\epsilon \cdot \phi}{k_2} \cdot I \cdot [HR_{578}] \cdot d \qquad (5)$$

Under conditions of a strongly absorbing system (optical densities of the samples are ~2, transmittance <2%) equation 5 is simplified:

$$HR_{600} = \frac{\phi \cdot I_{abs}}{k_2} \qquad \text{(see Table II)} \qquad (6)$$

HR_{578} is the steady-state concentration of the ground state, the *k*s are first-order rate constants in s^{-1}, I is the quantum flux in E/s, I_{abs} is the quantum flux multiplied by (1 −transmission), ϵ is the molar extinction coefficient in M/cm, d is the light-path length (1 cm) and ϕ is the quantum yield in mol/E.

Initial rates of formation and steady-state concentrations of intermediates other than HR_{600} can be derived by standard equations of steady-state kinetics and are listed in Table II for the two possible cases included in Figure 3. In model I, LR^L_{410} formation occurs from HR_{520} with the concomitant release of a Cl^- ion, whereas in model II, HR^L_{410} is formed from HR_{640} without Cl^- release. We do not consider here the possibility that HR^L_{410} is formed from both intermediates.

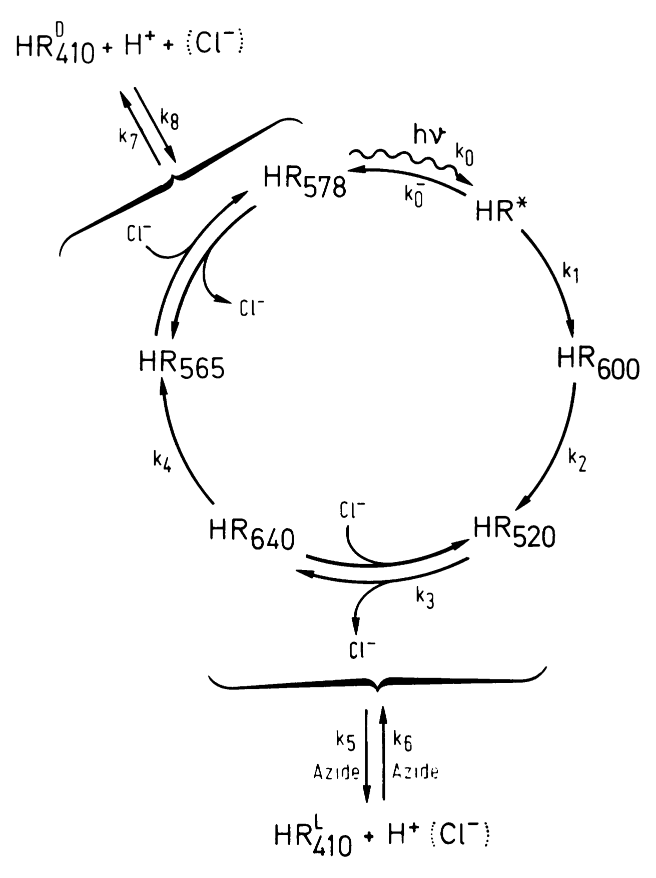

Fig. 3. Photocycle of HR connected with chloride transport and the reaction leading to HR^L_{410} and HR^D_{410}. HR_{578} is the dominant ground-state species at low Cl^- concentrations. Its photochemical activity (not considered here) apparently does not lead to Cl^- transport.

Both models can be tested with the experimental results obtained at 1 M Cl^- concentrations and described above. Most importantly, both models explain an azide-dependent rate of formation and decay of HR^L_{410} but also a steady-state concentration, which is independent of azide (Hegemann *et al.*, accompanying paper). The experimental quantitites which can be used for further evaluation are the following. (i) The ratio of the steady-state concentrations of HR_{520} and HR_{640} has a value of 2 at 1 M NaCl, pH 7.06 and room temperature (Figure 2A). (ii) The initial rate of HR^L_{410} formation under steady illumination is proportional to irradiance.

$$\frac{dHR^L_{410}}{dt} = \text{const}_A \cdot \phi \cdot I_{abs}$$

const_A is a proportionality constant and not a rate constant. From the experiment in Figure 5 of Hegemann *et al.* (accompanying paper) a value for const_A of 0.5 is obtained. (iii) The rise time of HR_{410} formation at 160 mM azide (Figure 1B) yields $k_5^{160\ mM}$ to be 67/s, k^∞ to be 88/s and $k^{50\ mM}$ to be 44/s. (iv) In the steady state where HR^L_{410} and HR_{578} are the dominant species (Figure 2) the dependence of the HR^L_{410}/HR_{578} equilibrium on the pro-

Table II. Initial velocities of formation and steady-state amounts of photocycle intermediates

Species	Model I ($HR_{520}-HR^L{}_{410}$)		Model II ($HR_{640}-HR^L{}_{410}$)	
	initial velocity	steady-state	initial velocity	steady-state
HR_{600}	$k_1 \cdot HR^*$	$\frac{1}{k_2} \cdot I_{abs} \cdot \phi$	$k_1 \cdot HR^*$	$\frac{1}{k_2} \cdot I_{abs} \cdot \phi$
HR_{520}	$k_2 \cdot HR_{600}$	$\frac{k_4 + k_{-3} \cdot Cl^-}{k_4 \cdot k_3} \cdot I_{abs} \cdot \phi$	$k_2 \cdot HR_{600}$	$\frac{k_4 + k_{-3} \cdot Cl^-}{k_4 \cdot k_3} \cdot I_{abs} \cdot \phi$
HR_{640}	$k_3 \cdot HR_{520}$	$\frac{1}{k_4} \cdot I_{abs} \cdot \phi$	$k_3 \cdot HR_{520}$	$\frac{1}{k_4} \cdot I_{abs} \cdot \phi$
$HR^L{}_{410}$	$k_5 \cdot HR_{520}$	$\frac{(k_4 + k_{-3} \cdot Cl^-) \cdot k_5}{H^+ \cdot Cl^- \cdot k_6 \cdot k_4 \cdot k_3} \cdot I_{abs} \cdot \phi$	$k_5 \cdot HR_{640}$	$\frac{k_5}{H^+ \cdot k_6 \cdot k_4} \cdot I_{abs} \cdot \phi$

ton concentration can be expressed as:

$$\frac{HR^L{}_{410}}{HR_{578}} = \text{const}_B \cdot \frac{I}{[H^+]}$$

From the experiment in Figure 6 of Hegemann *et al.* (accompanying paper) carried out at 50 mM azide and 1 M NaCl, the proportionality constant was evaluated with $\text{const}_B = 2.3$. (v) The initial rate of $HR^L{}_{410}$ decay is given for both models at 1 M chloride concentration as:

$$-\frac{dHR^L{}_{410}}{dt} = [H^+] \cdot [HR^L{}_{410}] \cdot k_6$$

$$\frac{HR_{520}}{HR_{640}} = \frac{k_4 + k_{-3}\,[Cl^-]}{k_3} = 2 \qquad \text{[compare (i)]} \qquad (7)$$

The experimentally-determined value for this ratio at 1 M chloride is 2.

$$\frac{dHR^L{}_{410}}{dt} = k_5 \,.\, \frac{k_4 + k_{-3}\,[Cl^-]}{k_4 \cdot k_3}\, I_{abs} \cdot \phi \qquad (8)$$

(Table II, calculated from initial velocity $HR^L{}_{410}$ and steady state of HR_{520}.) This expression is only valid if k_5 is at least one order of magnitude smaller than k_4, k_3 and k_{-3}, which holds true for the experimental conditions without azide (Figure 2).

$$\text{const}_A = k_5{}^{50\ \text{mM}}\, \frac{k_4 + k_{-3}\,[Cl^-]}{k_4 \cdot k_3} = 0.5 \ \text{[compare (ii)]}$$

$$(7) + (8) \qquad k_4 = 4\, k_5{}^{50\ \text{mM}} = 176/\text{s} \qquad \text{[compare (iii)]}$$

Since the equation $I_{abs}\, \phi = \epsilon\, \phi\, d\, I\, HR_{578}$ follows from (5) and (6), the values from Table II predict the relationship:

$$HR^L{}_{410} = \frac{k_5(k_4 + k_{-3} \cdot Cl^-)}{k_4 \cdot k_3}\, \epsilon \cdot \phi \cdot d \cdot \frac{1}{k_6} \cdot \frac{1}{Cl^-} \cdot \frac{I}{[H^+]} \cdot HR_{578}$$

Therefore with the value of $\text{constant}_B = 2.3$:

$$2.3 = \text{const}_A \cdot \epsilon \cdot \phi \cdot d\, \frac{1}{Cl^-} \cdot \frac{1}{k_6{}^{50\ \text{mM}}}$$

With the known values for const_A (0.5), ϵ (50 000/M/cm) and d (1 cm) at 1 M Cl^- concentration:

$$k_6{}^{50\ \text{mM}} = 3.6 \times 10^6/\text{s/M}$$

or

$$k_6{}^{\infty} = 7.2 \times 10^6/\text{s/M}^2$$

This value for $k_6{}^{\infty}$, calculated for model I, is in excellent agreement with the experimentally-determined value $7 \times 10^6/\text{s/M}^2$ [compare (v)].

It should be pointed out that for this calculation the experimental values of ϕ and I, as well as flash-spectroscopy data and data from steady-state illumination experiments, had to be combined. This not only confirms the scheme of the photochemical cycle in Figure 3 as very likely, but also strengthens the validity of the various experimental results.

If the same calculations are carried out for model II with equations (5) – (7) and the equation for the $HR^L{}_{410}$ steady-state concentration from Table II, two differences from model I are found. The rate constant k_4 is obtained as 88/s instead of 176/s in model I. This result favours model I because flash-photolytic data (Steiner *et al.*, 1984) in the absence of Cl^- show that the decay rate of HR_{640} is more closely related to the k_4 value of model I rather than to that of model II. On the other hand, model I predicts a chloride dependence of the $HR^L{}_{410}$ steady-state amplitude (Table II) and the decay rate. Experiments as in Figure 3 of the accompanying paper were carried out at different Cl^- concentrations (0.5 – 400 mM Cl^-, see Materials and methods) in the presence of nitrate, and no chloride dependence of HR_{410} steady-state concentration was found. This result favours model II where the $HR^L{}_{410}$ steady-state concentration (Table II) and the decay rate are independent of Cl^-. Whether the formation rate is Cl^- dependent or not under constant illumination depends on light intensity and HR concentration. In experiments with high HR concentrations and low light intensities (fully absorbing system) the formation rate of the HR_{410}/HR_{578} equilibrium is also independent of Cl^- because the turn-over number is not rate limiting.

The pK value of the Schiff's base for HR as HR_{520} was calculated as the ratio of k_6 to k_5. A pK value of 4.8 is obtained, which has to be compared with the pK value of 9.6 (unpublished data) for HR_{578} under the same conditions. This dramatic change in pK reflects the fact that HR stores energy by rearranging groups within the protein domain and that this conformationally-stored energy could account for the 5.8 kcal/mol seen with the pK change from HR_{578} to HR_{520}.

Discussion

The photocycle of HR as presented in Figure 3 begins with a picosecond reaction to form HR_{600}. The quantum yield is 0.34 which is lower than that for BR. We found under the same conditins, i.e., identical geometry and the same actinometer solution, a ϕ value of 0.6 for BR when measured from the initial rate of formation of the M intermediate in the basal salt/ether system. This value is closer to the 0.79 reported earlier for the same system (Oesterhelt and Hess, 1973) than to values of ~0.3 reported for purple membrane suspensions in water (Goldschmidt *et al.*, 1976; Becher and Ebrey, 1977; Hurley and Ebrey, 1978; Govindjee *et al.*, 1980). Picosecond experiments with purple membranes agree better with a ϕ value of 0.6; no differences in the primary reaction kinetics between the basal salt/ether system and the aqueous purple-membrane system were observed (Polland, 1984). The difference in the quantum yield between BR and HR is paralleled by the apparent lack of a J-like very fast intermediate in HR (Polland *et al.*, 1985). Only femtosecond experiments will definitely settle the question of whether the excited state of HR exists for 5 ps and not for 0.5 ps, as does the excited state of BR.

HR_{520} formed from HR_{600} should contain a protonated chromophore species because: (i) the reaction to HR^{L}_{410} releases protons with a stoichiometry of 1; and (ii) the blue shift to 410 nm absorption maximum is typical for a deprotonated Schiff's base. The most important difference between the photocycles in HR and BR therefore is that the HR chromophore is not reversibly deprotonated. Formation and decay of HR^{L}_{410} are not part of the photocycle reaction pathway because: (i) the rates are very slow in the absence of azide compared with the photocycling time of ~ 10 ms; (ii) the azide independence of the photosteady-state concentration of HR^{L}_{410} is only compatible with a model of the photocycle where HR^{L}_{410} returns to the same intermediate as it branches off from. The experiments on HR^{L}_{410} were all carried out in 1 M Cl^- and no attempt was made to demonstrate a Cl^- dependence of the reaction. This will be the subject of a more detailed study. A drastic difference is found between HR_{578} and HR_{520} when comparing their respective pK values. The pK of 9.6 for the Schiff's base in HR_{578} is not as high as that in BR (pK >12, Fischer and Oesterhelt, 1980) but higher by five units when compared with HR_{520}. This species has the expected pK value for a standard Schiff's base in solution and apparently the proton can react with exogeneously added base. In contrast, the HR_{578} conformation shields the Schiff's base from such an interaction.

The quantitative description of the rate constants connected with HR^{L}_{410} formation and decay do not depend on the assumption that HR_{640} is the intermediate which follows HR_{520} and which equilibrates with it in a Cl^--dependent fashion. We prefer the model in Figure 3 over the model with two separate photocycles for low and high Cl^- concentrations (Stoeckenius and Bogomolni, 1982) because HR_{640} at 1 M Cl^- concentration is formed more slowly than HR_{520} but decays with nearly the same rate constant as HR_{520}. At low Cl^- concentrations, HR_{640} decays more slowly than HR_{520} with a half-life time of ~3–4 ms and makes the HR_{520} concentration negligible. This rate constant corresponds to k_4 in Figure 3, and the calculated value of 176/s obtained for model I fits the experimental observation quite well. Model II would predict a value of 125/s and does not fit as well. An estimation of the values for k_3 and k_{-3} of the HR_{640}/HR_{520} equilibrium can be obtained by considering the fact that at 1 M Cl^-, $(176 + k_{-3}\,[Cl^-])/k_3$ must equal 2. At low Cl^- concentrations the formation of HR_{640} is much faster than 1 ms (Stoeckenius and Bogomolni, 1982) and $k_{-3}\,[Cl^-]$. Therefore k_3 must have at least a value of 1000/s. This condition narrows the possible range of the equilibrium constant k_{-3}/k_3 for the HR_{520}/HR_{640} equilibrium to ~2, i.e., close to unity. A more detailed discussion of the photocycle, especially including the equilibrium of the HR_{565} species with HR_{578} will be presented elsewhere (Tittor *et al.*, in preparation). However, it should be mentioned that at low Cl^- concentration the photocycle is predicted to and actually found to run faster than at high Cl^- concentrations. At the same time, transport activity goes down (Schobert *et al.*, 1983; Bamberg *et al.*, 1984). Since HR_{565} accumulates under these conditions it can be predicted that this species must be either photochemically less active or, if it reacts with similar quantum efficiency, its photochemical activity is not connected to transport of chloride ions.

In contrast to BR, HR has several possible opportunities to become inactivated: (i) in the dark by the formation of HR^{D}_{410} at high internal pH, preferably at low Cl^- concentrations; (ii) by formation of HR_{565} in the absence of any Cl^-, conditions not very likely to be encountered by the living cell; (iii) in the light, however, HR^{L}_{410} formation also leads to inactivation at pH 7 and 4 M Cl^-. Inactivation is faster with azide, but the extent of inactivation is independent of azide. Most interestingly, blue light will influence the steady-state concentration of HR^{L}_{410} and thus the pump activity if the properties of HR are the same in the intact cell as under the conditions of the experiments presented here. This prediction is born out by transport experiments (Hegemann *et al.*, 1985) and can be quantified by equations 11–13 which describe the thermal and photochemical decay rate of HR^{L}_{410}:

$$-\frac{dHR^{L}_{410}}{dt} = k_6 \cdot HR_{410} \cdot H^+ + \phi^B \cdot \epsilon^B \cdot I^B \cdot HR^{L}_{410} \qquad (9)$$

$$+\frac{dHR^{L}_{410}}{dt} = k_5 \cdot HR_{520} = \frac{2k_5}{k_4} \cdot I^{G}_{abs} \cdot \phi^G \qquad \text{(see eq. 7)} \qquad (10)$$

$$= \frac{2k_5}{k_4} \cdot \epsilon^G \cdot \phi^G \cdot d \cdot I^G \cdot HR_{578} \qquad \text{(see Table II)}$$

In the steady state, equation 11 follows from 9 and 10:

$$\frac{HR^{L}_{410}}{HR_{578}} = \frac{(2 \cdot k_5/k_4) \cdot \epsilon^G \cdot \phi^G \cdot I^G \cdot d}{k_6 \cdot H^+ + \epsilon^B \cdot \phi^B \cdot I^B \cdot d} \qquad (11)$$

where ϕ, ϵ, d and I have their usual meaning (cf. equations 1–8) and G and B stand for green and blue light, respectively. Since the activity of HR is proportional to the number of molecules in the HR_{578} state, the blue light activation of HR and its inactivation by green light becomes immediately evident from equation 11. Three testable predictions can be made: (i) Increasing irradiance of green light in the absence of blue light increasingly inactivates the pump, and azide accelerates the process of inactivation more and more until k_6 H^+ becomes small compared with ϵ^B ϕ^B dI^B. (ii) In white light (constant ratio of green and blue light intensities) increase of light intensity does not inactivate the pump. (iii) At constant blue and green light intensities, decreasing pH and increasing azide diminish the blue light effect. These predictions were confirmed experimentally (Hegemann *et al.*, 1985). Thus, HR acts as a photochromic pigment in *Halobacterium* and regulates its own activity via its photochemical properties and becomes completely deactivated at saturating yellow light intensities. Comparison with BR clearly shows that the M state of BR, although able to photochemically reconvert into BR, is not able to serve the same role as HR^{L}_{410} does for HR. Only a reaction connecting an intermediate with a side product, which is slow in both directions, is able to fulfill this role. Even at the low quantum efficiency of HR^{L}_{410} conversion into HR_{578}, an efficient blue light regulation is the result.

Materials and methods

Isolation of HR and its reversible photobleaching were carried out as described (Hegemann *et al.*, accompanying paper; Steiner and Oesterhelt, 1983; Bamberg *et al.*, 1984). The Cl^- dependence of HR^{L}_{410} formation and decay was measured with HR dialysed twice against 1000 vol of 1 M KNO_3, 0.5 mM NaCl, 10 mM Mops pH 7.0. Octylglucoside (0.5% final concentration) was added to keep the pK of the HR^{D}_{410} transition high. Different amounts of 4 M NaCl were added up to 500 mM final concentration. The light quality was selected by a cut-off filter combination (SFK 21 = 552 ± 15 nm, SFK 4 = 380 ± 13 nm, Schott).

Determination of quantum efficiency

For the determination of quantum efficiency a cuvette filled with 1.5 ml of an actinometer solution (Actinochrome N, Amco) was illuminated 12 times for 15 s every 30 s with light from a Xenon lamp (λ_{ex} 552 ± 15 nm). The geometry for illumination was exactly the same as used for the dark intervals. The increase in absorption at 429 nm was plotted against time and its initial value of 0.077/15 s used for further calculations with equation 12.

$$I\ [E/s] = \frac{F \cdot E_{429} \cdot V}{t \cdot d} \qquad (12)$$

I = quantum flux entering the cuvette, expressed in E/s; E_{429} = extinction difference at 429 nm; t = time of irradiation; V = volume; d = optical length; F = actinometric calibration factor in M/cm [F = $(2.44 \pm 0.06) \times 10^{-4}$ M/cm].

For all experiments carried out with the SKF 1 (552 ± 15 nm) filter (Schott) and at 100% light intensity, a quantum flux of 7.2 nE/s ± 0.4 was calculated. This is the amount of light absorbed by a 1.5 ml solution within 1 s and results in a quantum flux intensity of 4.8 nE/cm^2/s (1 ml). During the measurement the illuminated area was nearly homogeneous because (i) stirring of the solution had only very little influence on the results and (ii) lowering the irradiance by calibrated neutral glass filters yielded a proportional decrease of quantum-flux intensity. HR samples containing a constant (50 mM) concentration of azide were placed into the spectrophotometer under exactly the same geometric conditions as the actinometer solution. The HR^L_{410} formation was followed at 405 nm with the photomultiplier protected by an interference filter (405 ± 15 nm). The initial velocity of HR^L_{410} formation (v_0) is proportional to the absorbed light and the quantum yield. It can be shown that v_0 divided by (1 − transmission) must be constant (for constant azide concentration). This relationship was verified with sample concentrations between 7 and 36 μM HR.

For the quantum-yield determination of the photochemical reaction of HR^L_{410} an SFK 4 filter combination (380 ± 13 nm) was used. The quantum flux intensity was determined by comparison with green light (SFK 21 = 552 ± 15 nm, see above) with the help of a calibrated radiometer detector (EG & G, Electro-Optics Division, Salem, MA). The HR sample contained 1 mM azide. After the photosteady state was reached, green light was turned off and blue light turned on. Reformation of HR_{578} was followed.

Flash photolysis

Flash photolysis experiments were carried out with apparatus described by Vogel *et al.* (1983). The measuring light source was white light. The following light wavelengths; at 610 nm, light with $\lambda > 530$ nm; at 490 nm, light with $\lambda > 460$ nm and at 410 nm, light with a maximum at λ 410 ± 10 nm (interference filter, Schott, T at 410 nm = 30%) from selected regions were used.

For recording at 610 and 490 nm, the light intensity was decreased to 25% by neutral interference filters. The transient absorption spectra were recorded by a polychromatic flash apparatus described by Uhl *et al.* (1984).

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