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# The photocycle of the chloride pump halorhodopsin. I: Azide-catalyzed deprotonation of the chromophore is a side reaction of photocycle intermediates inactivating the pump

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**Halorhodopsin, the light-driven chloride pump of halobacteria, undergoes a photochemical cycle in the 10 ms range. Two intermediates, $HR_{640}$ and $HR_{520}$, accumulate in the photosteady state after short times (within 100 ms) of illumination. Upon prolonged illumination a third species, $HR^L_{410}$ accumulates, which is formed from $HR_{520}/HR_{640}$ by deprotonation of the chromophore in a side reaction of the photocycle. In the dark, $HR^L_{410}$ requires several minutes to reconvert thermally to $HR_{578}$. Thus, molecules in the $HR^L_{410}$ state must be inactive pumps since their maximal turnover number could only be a few per hour. Inorganic bases, such as azide, catalyze the deprotonation of $HR_{520}/HR_{640}$ as well as the reprotonation of $HR^L_{410}$. Both reactions are accelerated several hundred times by azide but the photosteady-state concentration of $HR^L_{410}$ remains unchanged.**

*Key words:* halorhodopsin/light-driven chloride pump/azide catalysis/regulation

## Introduction

The retinal protein halorhodopsin (HR) acts as an inward-directed light-driven chloride pump in halobacteria and was characterized with respect to its photochemical and transport properties in intact cells and cell-envelope vesicles (for review, see Lanyi, 1984). Recently, the isolation of the native chromoprotein as a single protein species of an apparent mol. wt. of 20 kd was reported (Steiner and Oesterhelt, 1983; Taylor *et al.*, 1983; Ogurusu *et al.*, 1984; Sugiyama and Mukohata, 1984). The protein could be incorporated into liposomes which, after attachment to black lipid membranes, demonstrated the light-driven chloride translocating activity of halorhodopsin, thus demonstrating that the pump consists of only this single protein species (Bamberg *et al.*, 1984).

Two actions of light on isolated HR were observed (Steiner and Oesterhelt, 1983; Taylor *et al.*, 1983; Ogurusu *et al.*, 1984). First, a photocycle with a turnover number of 50–100/s as analyzed by flash photolysis and second, the formation of a HR species absorbing at 410 nm ($HR^L_{410}$) upon stationary illumination which was reconverted to the original state at a much slower rate. This second reaction is accompanied by a reversible release and uptake of protons (Steiner and Oesterhelt, 1983). The reconversion to the ground state can also occur as a photochemical reaction upon absorption of blue light by $HR^L_{410}$. HR species absorbing around 410 nm were earlier observed in cell-envelope vesicles under two different conditions: (i) in the dark at alkaline pH, preferably in the absence of chloride ($HR^D_{410}$) and (ii) under constant illumination in the presence of high chloride concentrations (Ogurusu *et al.*, 1981,1982; Lanyi and Schobert, 1983; Hazemoto *et al.*, 1984). The inorganic anion azide turned out to be a useful tool for studying the formation and decay of $HR^L_{410}$ because it catalyzes the equilibration between $HR_{578}$ and $HR^L_{410}$ in light. The quantitative analysis of this reaction presented here bears significance on the photochemical cycle in HR and on a potential physiological role of $HR^L_{410}$ in intact cells.

## Results and Discussion

### *Conditions for $HR_{410}$ formation in light ($HR^L_{410}$)*

HR was isolated in 1 M NaCl containing 1% octylglucoside, 10 mM Mops pH 7 and, initially, 0.05% sodium azide. Figure 1 shows a series of spectra obtained after illumination of an HR sample with green light and demonstrates the formation of $HR^L_{410}$ and its reconversion into $HR_{578}$ in the dark. An isosbestic point occurs at 456 nm and the reaction is accompanied by release and uptake of protons (Steiner and Oesterhelt, 1983). After removal of the azide by dialysis no formation of $HR^L_{410}$ can be observed under the same conditions of illumination. This observation was made when effects of various anions on the properties of HR were studied in collaboration with J.K.Lanyi. Flash photolysis in 4 M chloride shows on a millisecond time scale the exclusive formation of the intermediate $HR_{520}$ (absolute spectrum see Figure 1) which quantitatively returns back to $HR_{578}$ within 20 ms (Oesterhelt and Hegemann, accompanying paper; Figure 1, trace 1). When azide is added back to the sample, the formation of $HR^L_{410}$ can easily be detected and Figure 2 shows a plot of the inverse initial velocity *versus* the inverse azide concentration. An affinity constant of HR for azide of 50 mM is

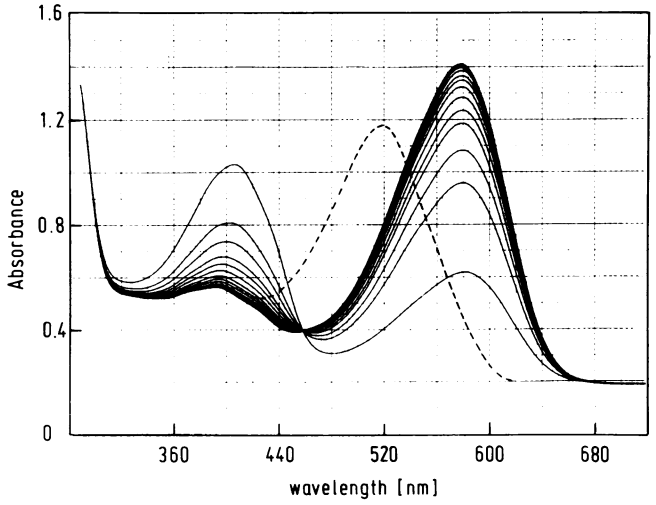


**Fig. 1.** Reversible formation of $HR^L_{410}$ upon illumination of HR with orange light. HR (24 μM) in 1 M NaCl/1% octylglucoside containing 0.05% sodium azide was illuminated with light from a 150 W projector filtered through a cut-off filter (OG 515 Schott) for 1 min and the spectra recorded continuously (20 nm/s) after turning off the light in an Aminco DW 2 spectrophotometer. The first spectrum has the most absorption at 410 nm and the least at 578 nm. The absorption band of the main intermediate $HR_{520}$ in the absence of azide is shown by the broken line. It was calculated from the difference spectrum recorded 600 μs after a flash onto an HR sample in 4 M $Cl^-$.

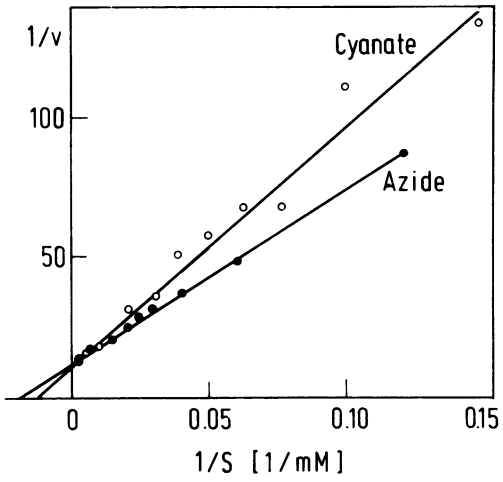


**Fig. 2.** Determination of the affinity constants of HR for azide and cyanate in the presence of 1 M NaCl at pH 7.0. The reciprocal initial velocities of $HR^{L}_{410}$ formation were plotted against reciprocal azide or cyanate concentrations. The intercept with the ordinate gives a $K_m$ value of 50 and 70 mM for azide and cyanate, respectively.

**Table I.** Formation of $HR^{L}_{410}$ in the presence of various anions (10 mM)

| Anion | p*K* of acid | formation of $HR^{L}_{410}$ |
|---|---|---|
| perchlorate | <1 | − |
| iodide | <1 | − |
| bromide | <1 | − |
| rhodanide | 0.85 | − |
| fluoride | 3.17 | − |
| nitrate | <1 | − |
| citrate | 3.13/4.76/6.4 | − |
| cyanate | 3.66 | + |
| azide | 4.72 | + |
| acetate | 4.76 | − |
| hydrogen carbonate | 6.35/3.88 | − |
| sulfide | 6.99/12.9 | + |
| cyanide | 9.31 | − |

calculated at 1 M chloride concentration and pH 7. As will be described in more detail below (Table II) $HR^{L}_{410}$ is also formed in the absence of azide but at a rate which is several hundred times slower than that at saturating concentrations of azide. The light-induced formation of a $HR_{410}$ species at alkaline pH has been seen in experiments with cell-envelope vesicles (alkaline photoreaction, Ogurusu *et al.*, 1981,1982; Lanyi and Schobert, 1983) and might well be identical to the $HR^{L}_{410}$ formation just described.

To find out whether the effect of azide on $HR^{L}_{410}$ formation is unique to this ion species we analyzed a series of strong inorganic bases which are listed in Table I with their pK values and their ability to evoke $HR^{L}_{410}$ formation. Beside azide, only hydrogen sulfide and cyanate were able to promote $HR^{L}_{410}$ formation. Cyanate has a $K_m$ value of ~70 mM (Figure 2) whereas hydrogen sulfide increases the initial velocity of $HR^{L}_{410}$ formation in a non-saturable way within the concentrations allowed for non-denaturing conditions. A common feature of the three active anions is not only their basic nature but also the high polarizability of their electronic configurations. To summarize our results up to this point, azide (or cyanate) upon light excitation of HR accelerates the formation of $HR^{L}_{410}$, which is different by its absorption maximum and its protonation state from

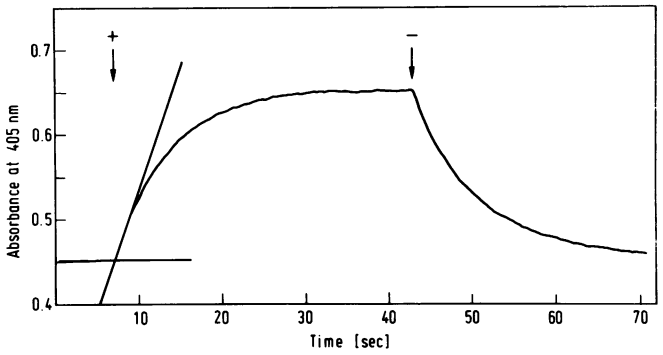


**Fig. 3.** Formation of $HR^{L}_{410}$ upon illumination (+) and its decay in the dark (−) in the presence of 1 M NaCl/1% octylglucoside/50 mM azide/10 mM Mops pH 7.14 at 13°C. HR concentration was 36 μM, the irradiance 4.8 nE/cm²/s at 553 ± 15 nm. The experiment was carried out as described under Materials and methods and initial velocities evaluated with the help of a mirror (Oesterhelt and Krippahl, 1973).

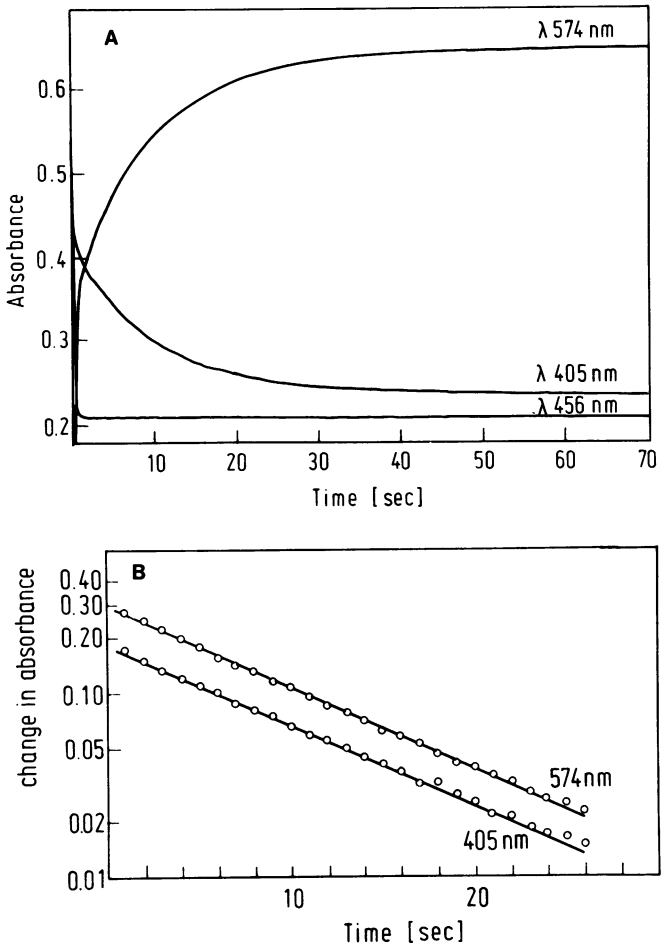


**Fig. 4.** Reconversion of $HR^{L}_{410}$ to $HR_{578}$ in the dark. The photosteady-state mixture was produced under the conditions described in Figure 3. **(A)** Time course of the absorbancies at 405, 574 and at the isosbestic point (456 nm). **(B)** Semilogarithmic plot of the data given in Figure 5A demonstrating identical half times of 7 s for both components.

all intermediates of the HR photocycle known so far.

*Characteristics of the photoequilibrium betwen $HR^{L}_{410}$ and $HR_{578}$*

Formation or decay of $HR^{L}_{410}$ is dependent on light intensity, azide concentration, pH and temperature. An example of a time course of the reaction measured at λ = 405 nm is presented in

Figure 3. Three parameters will be used for the kinetic analysis described in the following paper: the initial velocity of $HR^L_{410}$ formation, its photosteady-state concentration and the initial velocity of its decay. Figure 4 shows the time course of the decay of $HR^L_{410}$, measured as absorbance decrease at 405 nm, and of the regeneration of $HR_{578}$ measured as absorbance increase at 574 nm. An isosbestic point at 456 nm is observed (Figure 1). The decay of $HR^L_{410}$ and the regeneration of $HR_{578}$ are synchronous processes with first-order kinetics, as demonstrated by the two parallel lines of the semilogarithmic plot in Figure 4B. Although protons participate in the reaction, as has been shown by light-dependent acidification of the medium (Steiner and Oesterhelt, 1983), they do not influence the reaction order because the solutions are buffered (pseudo-first order). The data of Figure 4 further demonstrate that the conversion has a stoichiometry of 1:1 and that no other species participate to a measurable extent. A molar extinction coefficient of 31 000/M/cm for $HR^L_{410}$ can be calculated from the absorption changes at 405 nm and 574 nm in Figure 4A using the molar extinction coefficient of $HR_{578}$ (50 000/M/cm, Steiner and Oesterhelt, 1983). The value for $HR^L_{410}$ is close to that for the M intermediate of bacteriorhodopsin (BR) (Oesterhelt and Hess, 1973). The initial velocity of $HR^L_{410}$ formation is linearly dependent on light intensity (Figure 5) excluding saturation phenomena of the photochemical system under our conditions of illumination. The stoichiometry of protons participating in the reaction can be derived from the pH dependence of the photosteady-state concentrations of $HR^L_{410}$ and $HR_{578}$ on the basis of:

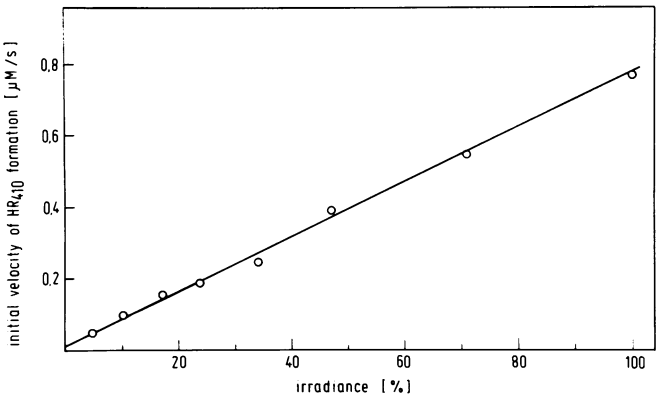


**Fig. 5.** Initial velocity of $HR^L_{410}$ formation as a function of irradiance. The HR concentration was 36 μM in 1 M NaCl/1% octylglucoside/50 mM azide at pH 7.87. Actinic light from a 150 W projector was filtered through a cut-off filter combination (SFK 553 ± 15 nm, Schott) and absorbance changes recorded at 405 nm. 100% irradiance is equivalent to 4.8 nE/cm²/s.

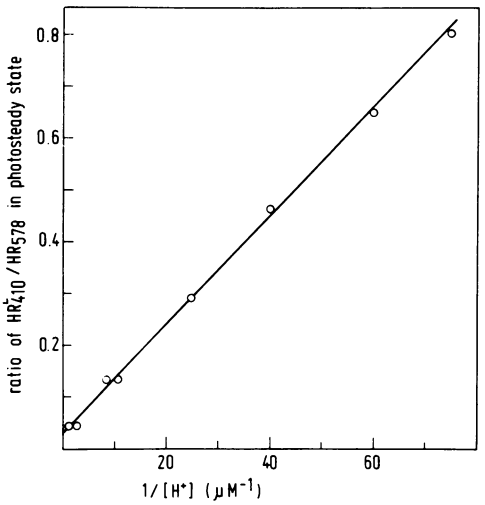


**Fig. 6.** Molar ratio of $HR^L_{410}/HR_{578}$ in the photosteady-state as a function of the proton concentration. The conditions of the experiment were as in Figure 3 but the pH was varied by the addition of acid or alkaline solutions.

$$HR_{578} - HR^L_{410} + nH^+ \quad (1)$$

$$\frac{[HR^L_{410}]}{[HR_{578}]} = K \cdot \frac{1}{[H^+]^n} \quad (2)$$

The linearity between the inverse proton concentration and the $HR^L_{410}/HR_{578}$ ratio in Figure 6 documents a stoichiometry of n = 1 (for a quantitative evaluation see Oesterhelt and Hegemann, accompanying paper). At this point the similarity of $HR^L_{410}$ and the M intermediate of the BR photocycle becomes more obvious. Both species share the same absorption maximum and the same protonation state of their chromophores and both are thermally or photochemically reconverted into their respective ground states. The main difference lies in the fact that the M state of BR is an intermediate within the photocycle and decays within 10 ms whereas $HR^L_{410}$ is produced by a side reaction during the photocycle of HR (see the following paper) and, in the absence of azide, decays over several minutes.

Azide not only accelerates the formation of $HR^L_{410}$ but also its decay, i.e., the regeneration of $HR_{578}$. Table II compares the initial rates of $HR^L_{410}$ formation and decay. Both rates increase between 0.67 mM and 320 mM azide by a factor of 55 but their ratio remains constant. This is confirmed by the ratio of the $HR^L_{410}$ and $HR_{578}$ photosteady-state concentrations measured as a function of azide concentration. At all concentrations the same

**Table II.** Azide does not influence the photosteady-state ratio of $HR^L_{410}$:$HR_{578}$.

| Azide mM | Initial rate of $HR^L_{410}$ formation M/s × $10^{-8}$ | Initial rate of $HR^L_{410}$ decay M/s × $10^{-8}$ | Ratio of forward and backward reaction | $HR^L_{410}$:$HR_{578}$ |
|---|---|---|---|---|
| 0.67 | 3.4 | 2.2 | 1.5 | n.d. |
| 4 | 14 | 9.5 | 1.5 | 0.23 |
| 9 | 33 | 22 | 1.5 | 0.28 |
| 19 | 56 | 34 | 1.6 | 0.22 |
| 39 | 77 | 50 | 1.5 | 0.22 |
| 79 | 130 | 77 | 1.7 | 0.22 |
| 159 | 175 | 100 | 1.75 | n.d. |
| 320 | 190 | 120 | 1.6 | n.d. |

HR concentration was 36.6 μM in 1 M NaCl, 1% octylglucoside and 10 mM Mops at pH 7.14 and 13°C. Illumination as in Figure 1 but with a cut-off filter OG 495 (Schott); n.d., not determined. Extrapolation to zero concentration of azide gives a value < 1.0 M/s × $10^{-8}$

steady-state level of $HR^{L}_{410}$ is reached. In the absence of azide, however, this process takes >30 min, whereas saturating amounts of azide establish the steady-state within 10 s under the same light conditions. The effect of azide on the de- and reprotonation of HR therefore is only catalytic. Without azide the reconversion of $HR^{L}_{410}$ into $HR_{578}$ is much slower than the rate-limiting step in the photocycle and is apparently not linked to a transport process (Hegemann *et al.*, 1985). Thus, constant illumination with green light shifts the HR pump molecules into an inactive state ($HR^{L}_{410}$) by deprotonation, and azide catalytically accelerates this inactivation.

Since in the absence of azide the same amount of $HR^{L}_{410}$ is formed as in its presence, $HR^{L}_{410}$ should have a physiological significance. Continuous illumination of the intact cell should accumulate $HR^{L}_{410}$ albeit slowly, and thereby decrease the pump efficiency. At the same time a photoreceptor for blue light is created.

This expected behaviour of HR as a blue light-regulated pump can indeed be observed in black lipid film experiments (Hegemann *et al.*, 1985). A detailed discussion of the connection between $HR^{L}_{410}$ and the photocycle intermediates of HR is given in the accompanying paper.

## Materials and methods

*HR isolation and purification*

HR was isolated from OD2 cells by the methods described by Steiner and Oesterhelt (1983) with the reported modifications (Bamberg *et al.*, 1984). The purification procedure was carried out in the absence of azide or in the presence of 0.05% azide.

In titration experiments the anions were added as 1 M solutions and octylglucoside as a 10% solution.

*Reversible bleaching of HR*

Spectrophotometric measurements were carried out in a double-beam spectrophotometer (Aminco DW-2) equipped with a cuvette-stirring system. The measuring beam of the spectrophotometer was passed through a fluorescence cuvette with d = 1 cm. A guard filter of 408 ± 5 cm transmittance (T = 33%, interference filter, Schott) was placed in front of the photomultiplier tube to protect it against scattered actinic light. The samples were stirred during illumination in order to ensure a homogeneous reaction.

Actinic light from a XBO 400 quartz halogen lamp (Osram) at a constant 22 V was passed through a photoshutter and focussed onto a light guide which ended at the sample cuvette, illuminating it from one side. The cuvette was thermostated at 13 ± 1°C. The pH was changed by additions of 100 mM HCl or 100 mM NaOH solution and controlled by a pH electrode (type Koax 401, Ingold) connected to a Knick pH meter.

## References

Bamberg,E., Hegemann,P. and Oesterhelt,D. (1984) *Biochemistry (Wash.)*, **22**, 6216-6221.

Hazemoto,N., Kamo,N. and Kobatake,Y. (1984) *Biochem. Biophys. Res. Commun.*, **118**, 502-507.

Hegemann,P., Oesterhelt,D. and Bamberg,E. (1985) *Biochim. Biophys. Acta*, in press.

Lanyi,J.K. (1984) in Ernster,L. (ed.), *Bioenergetics*, Elsevier, Amsterdam/NY, pp. 315-350.

Lanyi,J.K. and Schobert,B. (1983) *Biochemistry (Wash.)*, **22**, 2763-2769.

Oesterhelt,D. and Krippahl,G. (1973) *FEBS Lett.*, **36**, 72-76.

Oesterhelt,D. and Hess,B. (1973) *Eur. J. Biochem.*, **37**, 316-326.

Oesterhelt,D., Hegemann,P. and Tittor,J. (1985) *EMBO J.*, **4**, 2351-2356.

Ogurusu,T., Maeda,A., Sasaki,N. and Yoshizawa,T. (1981) *J. Biochem. Tokyo*, **90**, 1267-1273.

Ogurusu,T., Maeda,A., Sasaki,N. and Yoshizawa,T. (1982) *Biochim. Biophys. Acta*, **682**, 446-451.

Ogurusu,T., Maeda,A. and Yoshizawa,T. (1984) *J. Biochem.*, **95**, 1073-1082.

Steiner,M. and Oesterhelt,D. (1983) *EMBO J.*, **2**, 1379-1385.

Sugiyama,Y. and Mukohata,Y. (1984) *J. Biochem.*, **96**, 413-420.

Taylor,M.E., Bogomolni,R.A. and Weber,H.J. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6172-6176, 1-608.

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