PICOSECOND EVENTS IN THE PHOTOCHEMICAL CYCLE OF THE LIGHT-DRIVEN CHLORIDE-PUMP HALORHODOPSIN

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ABSTRACT The early events in halorhodopsin after light excitation are studied with picosecond time resolution. Absorption and fluorescence measurements show that the electronically excited state of the incorporated retinal has a lifetime of 5 ps. Within that time a red-shifted photoproduct is formed that remains stable for at least 2 ns.

INTRODUCTION

Halobacterium halobium contains three different retinal proteins (1): bacteriorhodopsin (BR) (2), halorhodopsin (HR) (3, 4), and slow cycling rhodopsin (5). The most extensively studied protein is BR (6–8). It acts as a light energy transducing pigment by the generation of a proton gradient across the cell membrane (9). Less information is available on HR, which is identified as an inwardly directed chloride pump (10, 11). Electrical measurements on black lipid membranes demonstrated that the chromoprotein alone exhibits the transport activity and, therefore, the chloride pump is identical with HR (12). Little is known about the intermediates that are formed after light excitation. Two different photocycles occur for high chloride and low chloride concentrations, respectively (13, 14). Only the high chloride photocycle is associated with transport activity (10) and a hypsochromically shifted long-lived intermediate has been reported (13). It has an absorption maximum at $\lambda = 520$ nm and decays to the initial state of HR within several milliseconds (14). At temperatures of liquid nitrogen, a red-shifted intermediate could be trapped (13), but the time scale of its occurrence under physiological conditions is unknown. Here we report on spectroscopic experiments with purified HR on a picosecond time scale that identify an early intermediate in the HR photocyle.

GLOSSARY

- η quantum yield
- τ_r intrinsic radiative lifetime
- τ_s fluorescence lifetime
- t_D time delay between exciting and probing pulse.

MATERIALS AND METHODS

HR was isolated from OD2 cells by the method described in reference 15 with reported modifications (12). After detergent exchange on the second phenylsepharose column, the sample was concentrated in an Amicon cell (YM-10 filter; Amicon Corp., Scientific Sys. Div., Danvers, MA) up to 40 μmol/l, dialyzed against 1% octylglucoside, 1 M NaCl, 10 mM MOPS (pH 7) for 16 h at 4°C, and concentrated further to 150 μmol/l by the same procedure.

Picosecond experiments were performed using single, frequency-doubled picosecond pulses ($\lambda = 526.5$ nm) of a mode-locked Nd: glass-laser system. The green input pulse was divided by a beam splitter. One part was frequency shifted by transient stimulated Raman scattering to $\lambda = 540$ nm (pulse energy 100 nJ, pulse duration 2.5 ps, peak intensity 10^2 MW/cm^2). The second part generated a picosecond light continuum in water. The level of excitation was kept small, ~10% of the molecules absorbed one photon per pulse at 540 nm, to prevent nonlinear effects or heating of the sample. The low irradiance led to a linear response of the sample, i.e., the observed fluorescence signal or the absorption changes were proportional to the laser energy. The light path through the sample was 1 mm and the absorbance $A(578\text{ nm}) = -\lg I/I_0 = 0.75$. The monitoring pulse was produced by selecting a wavelength interval ($\Delta\lambda \simeq 10$ nm) from the light continuum. The intensity ratio of probe pulse to excitation pulse was small: $I_{pr}/I_{ex} < 0.1$. Parallel polarization of excitation and probe pulses was chosen. The improved electrooptical detector system (16) made it possible to measure changes in the energy of the probe pulses with high accuracy of $\Delta E/E_0 < 5 \times 10^{-4}$.

After each run, the cross-correlation curve of the excitation and probe pulses was recorded. This experiment yielded the time resolution (<1 ps) and the zero point ($t_D = 0$), which is determined by the temporal coincidence of the two pulses. The dashed line in Fig. 3 gives an example of such a cross-correlation curve between the exciting pulse at 540 nm and the probe pulse at 675 nm. From the half-width of the cross-correlation curve of 4.5 ps, a pulse duration of the two experimental pulses of 3 ps was deduced.

The fluorescence spectrum (unpolarized, corrected for spectral sensitivity) and the streak data were measured with excitation pulses at 526.5 nm (only 5% of the molecules were excited). The spectral investigations were made with the help of a 25-cm monochromator-photomultiplier combination (spectral resolution 3.5 nm). The time dependence of the fluorescence emission ($\lambda > 570$ nm) was measured by a streak camera

with a time resolution of 3 ps. The results are corrected for nonlinear instrumental response of the camera. We ascertained that stray light or fluorescence from impurities did not falsify our results (i.e., by measuring control samples without HR). The absorption spectrum of the samples was controlled before and after each experiment. No irreversible bleaching of the specimen was observed.

RESULTS

Absorption and Fluorescence

The absorption and fluorescence spectra of HR are presented in Fig. 1. The broad unstructured absorption band with λ_{max} = 578 nm (light adapted) resembles that of BR. The extinction coefficient of 50,000 $M^{-1}cm^{-1}$ at 578 nm is somewhat smaller than that of BR (63,000 $M^{-1}cm^{-1}$). The fluorescence spectrum is approximately the mirror image of the absorption spectrum (Fig. 1). A large Stokes shift of ~4,000 cm^{-1} is found when the absorption and fluorescence are plotted in the form of $\epsilon(\nu)/\nu$ and $F(\nu)/\nu^3$, respectively.

The fluorescence quantum yield is determined to be $(5 \pm 2) \times 10^{-4}$ by comparing the emission of HR with that of oxaine 1 in ethanol ($\eta = 0.11$) (17). From the absorption spectrum we calculate (18) an intrinsic radiative lifetime of $\sim\tau_r = 8$ ns and predict a fluorescence lifetime of $\tau_s \sim 4$ ps for the first excited singlet state of retinal in HR ($\tau_s = \eta \times \tau_r$).

Fluorescence Lifetime of Halorhodopsin

The time dependence of the fluorescence ($\lambda > 570$ nm) was measured with a streak camera using an excitation pulse at 526.5 nm with a pulse duration of 4 ps. The fluorescence intensity rises rapidly to a peak value and decays exponentially (Fig. 2). Deconvolution with the time dependence of the excitation pulse gives a fluorescence lifetime of $\tau_s = 5 \pm 2$ ps, in agreement with the estimate mentioned above.

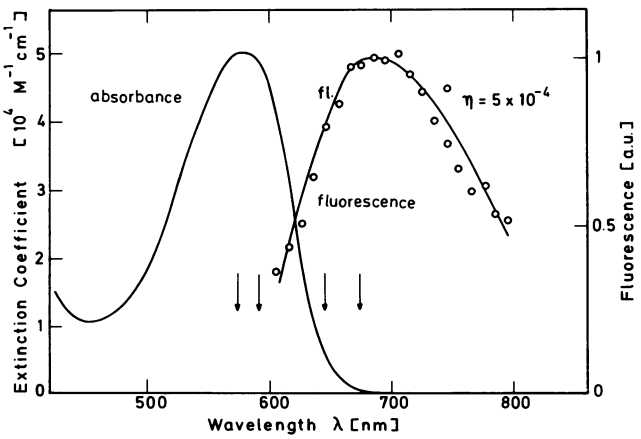


FIGURE 1 Absorption (—) and fluorescence spectrum (O—O) of HR. We find a fluorescence quantum yield of 5×10^{-4} and a Stokes shift of 4,000 cm^{-1}. The wavelengths of the probe pulses in Figs. 3 and 4 are marked by arrows, the fluorescence intensity is given in arbitrary units (*a.u.*).

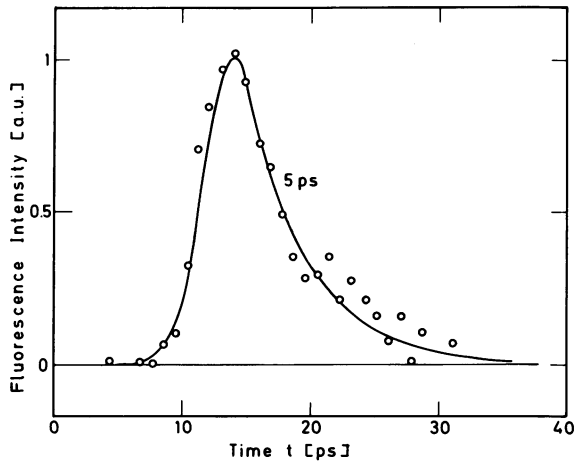


FIGURE 2 Fluorescence intensity as a function of time ($\lambda > 570$ nm) measured with a streak camera. A relative time scale is used. We deduce a fluorescence lifetime of 5 ± 2 ps.

Transient Absorption and Amplification

The transmission of probe pulses at various frequencies was carefully measured during and after excitation at 540 nm. Four interesting probing frequencies at 675, 646, 592, and 573 nm were selected and are marked by arrows in Fig. 1. Strong fluorescence with negligible absorption occurs at 675 nm but strong absorption with small fluorescence occurs at 573 nm. We observed three different physical processes when the sample was excited by a light pulse: (*a*) an additional signal at the probing frequency, which is induced by the monitoring pulse and emitted from the populated excited state (amplification), (*b*) a reduced absorption (bleaching of the initial state), and (*c*) an increase of absorption due to a photoproduct.

First, the data obtained with a probe pulse at 675 nm are discussed in Fig. 3. A transient amplification is followed by an increase in absorption. The amplification rises quickly during the excitation of the sample and reaches a maximum value of 2% at t_D = 1.5 ps. Later when the exciting pulse has left the specimen, the amplification decays with a time constant of 5 ps and, finally, for $t_D > 2$ ps, the probe pulse indicates absorption. The amplification is caused by the fast population of the first excited singlet state. The decay time of amplification of 5 ps agrees with the fluorescence lifetime discussed above. The generated absorption for $t_D > 20$ ps belongs to an intermediate photoproduct that persists during the observation time of 300 ps.

The results obtained with the three other monitoring wavelengths at 573, 592, and 646 nm are presented in Fig. 4. In all three cases, the probe pulse experiences changes of absorption of different sign and magnitude during and after the excitation process. At 573 nm (Fig. 4 *a*), a strong and rapid decrease of absorption is seen as a result of the passage of the exciting pulse. This observation is due to the build-up of a population in the first excited singlet state, S_1,

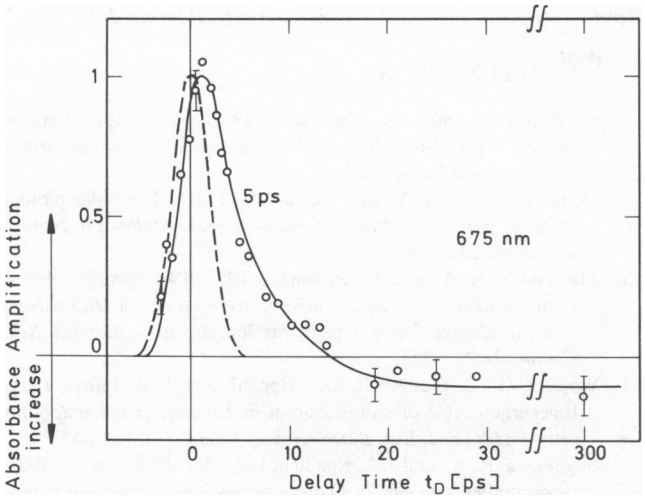


FIGURE 3 Change of the probe intensity at λ_{pr} = 675 nm. The probe pulse is amplified (≈2%) during the population of the excited singlet state. The small absorption increase for $t_D > 15$ ps is caused by the excess absorption of a photoproduct. The dashed line gives the cross-correlation curve between the exciting and probing pulse at 540 and 675 nm, respectively.

and the depopulation of the ground state (see Fig. 1). The bleaching disappears with a time constant of 5 ps, which is the same value as found for the lifetime of the S_1 state (see above). For times 300 ps > t_D > 20 ps, the absorption does not return to its original value at all four monitoring wavelengths (Figs. 3 and 4). We therefore suspect that a

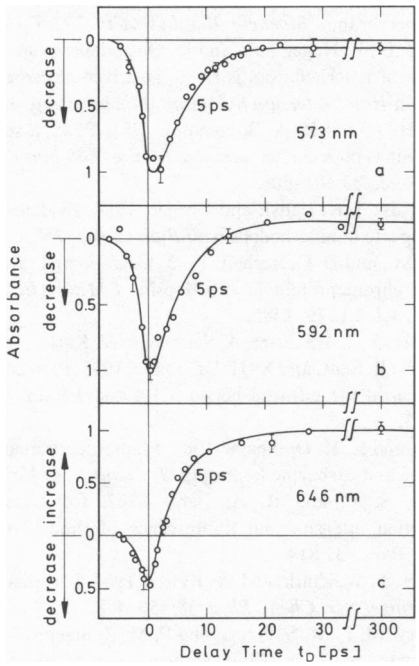


FIGURE 4 Absorption changes of the probe pulse as a function of time delay between exciting (λ_{ex} = 540 nm) and probing (λ_{pr} = 573, 592, 646 nm) pulse. The rise of the excess absorption at 646 nm reflects the growth of the first photoproduct. It remains constant during the experimental delay time of 300 ps. Absorption (absorbance) changes are normalized.

red-shifted photoproduct, which has a smaller absorption cross section at 573 nm than the initial nonirradiated HR, exists.

At 592 nm (Fig. 4 *b*), the same early bleaching is found as was found at 573 nm. A small excess absorption for times $t_D > 20$ ps appears, i.e., the generated photoproduct has a larger absorption at 592 nm than the original HR, and the isosbestic point of the two species must occur between 573 and 592 nm.

At 646 nm (Fig. 4 *c*), the early decrease of absorption is small, but for $t_D > 20$ ps the transient absorption of the photoproduct is large. The absorption change after initial bleaching has a time constant of 5 ps at all four wavelengths, i.e., the photoproduct seems to rise with a time constant of 5 ps. We recall that the lifetime of the S_1 state measured by fluorescence (Fig. 2) and by decay of amplification (Fig. 3) is 5 ps. The photoproduct is found to persist for at leat 2 ns (within the 300 ps of observation <10% variation was measured.

Our finding may be summarized as follows. (*a*) The fluorescence spectrum of HR is quite broad with a large Stokes shift of 4,000 cm^{-1}. (*b*) The fluorescence quantum efficiency is small with $\eta = 5 \times 10^{-4}$. (*c*) The fluorescence lifetime is found to be 5 ps, which is in good agreement with the value calculated from the radiative lifetime and the quantum efficiency. (*d*) At 300°K, a red-shifted photoproduct is observed. (*e*) The build-up of the intermediate proceeds within a time of 5 ps, the lifetime of the S_1 state.

It is interesting to compare the present data of HR with our recent investigations on BR (19). We found evidence for a very short-lived (0.5 ps) excited singlet state in BR with a smaller fluorescence quantum efficiency (1×10^{-4}). Furthermore, the first red-shifted intermediate (19, 20, 21) in BR appears very rapidly within 1 ps (19, 21, 22), whereas it seems to be 5 ps in HR (Fig. 4). The slow formation of the first photoproduct in HR may lead to a lower efficiency of the photoreaction in HR than in BR.

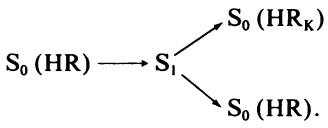
DISCUSSION

Our results clearly demonstrate the conversion of the excited state of HR into a product with red-shifted absorption. The maximum is between 590 and 670 nm and might well be similar to the absorption maximum of the intermediate K in the photocycle of BR. Furthermore, the photoproduct could be identical with HR_{632} found at low temperature (13). A detailed description of the photoproduct that we refer to as HR_K will be possible if transient difference spectra and the quantum yield of the primary process become available. Preliminary experiments indicate a smaller quantum yield in HR than in BR.

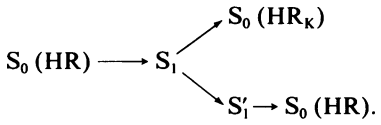
The lifetime of the excited state obtained from fluorescence and amplification measurements is 5 ps. A fluorescence lifetime of 5 ps was also calculated from the radiative lifetime and the quantum efficiency. The large Stokes shift

is similar to that in BR and indicates a considerable difference in the geometric arrangement of retinal in the ground and the excited state.

The time of formation of the first photoproduct, HR_K, appears to be 5 ps. At present it is not possible to decide whether the time of formation is even faster. Nevertheless, we try to explain our results with the most simple model. Both processes, the repopulation of the ground state S_0 (HR) and the formation of the intermediate HR_K, occur in parallel from the excited singlet state S_1 with a time constant of 5 ps.



A faster build-up of the intermediate HR_K may be explained by a more complicated model. After excitation immediate branching forms the photoproduct HR_K and a longer-living excited state S_1' (lifetime τ = 5 ps) that differs from the optically excited state S_1 in its electronic or conformational structure.



Thus, the transition of S_1' to S_0 (HR) would determine the measured dynamics (τ = 5 ps) of absorption and fluorescence. The present experimental data do not decide between the two models.

The red-shifted absorption of the intermediate K in the photocycle of BR may be interpreted as an increased distance of the positively charged nitrogen of the Schiff's base and the negative counterion in the protein (23). In BR this is very likely due to a rotation of the retinal molecule around the 13th double bond and the 14th single bond (24). It seems unlikely that within a few picoseconds the protein structure could respond to the excited state of retinal with molecular movements increasing the distance between the charges. We, therefore, prefer the interpretation that the red-shift in HR is also due to a rotation of bonds in retinal molecule (isomerization). Presently, whether this rotation includes the 13th double bond is not known. However, an increased 13-*cis* retinal content was found upon extraction of illuminated HR-containing envelope vesicles, which indicates the possibility of a *trans* to *cis* isomerization during the photocycle in HR.

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