A new intermediate in the photocycle of bacteriorhodopsin

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With increasing pH (6.0 to 10.5) there is an increasing discrepancy between the recovery rate of bacteriorhodopsin after a flash and the decay rates of the key photointermediates, M^{fast} and M^{slow}, which is not predicted by any model of the photocycle. However, a very slowly decaying absorbance change at 350 nm has kinetics which match the very slow part of the recovery of bacteriorhodopsin, suggesting a new intermediate. The difference spectra of the bacteriorhodopsin photocycle measured at pH 10.5, obtained from fitting 3 exponentials to the flash-induced absorbance changes in the 300–560 nm wavelength range, show maxima, at 410–420 nm corresponding to the 2 forms of M and at 350 nm corresponding to the new intermediate. We propose to call this new species R350

(Halobacterium halobium) *Bacteriorhodopsin* *Photocycle* *M photointermediate* *R350 photointermediate*

1. INTRODUCTION

Light absorbed by bacteriorhodopsin (BR) in the purple membrane of *Halobacterium halobium* initiates a photocycle in which the primary photoproduct decays through a set of intermediates until the original pigment is reformed. This photocycle leads to proton pumping across the membrane; short-wavelength intermediates absorbing at ~410 nm, called Ms, play a key role in proton pumping (reviews [1,2]). Their formation is associated with proton release and their decay with proton re-uptake. However, an exact correlation between the photochemistry and proton pumping has been lacking because the photocycle is poorly understood. Most workers have required two kinetically different Ms, a fast decaying one, M^f, and a slowly decaying one, M^s, to fit their data over a broad range of pH values (review [3]). Here we present a study of the flash-induced absorbance changes (ΔA) of BR in the near-ultraviolet and visible regions. These data can only be explained by the existence of a new intermediate in the BR photocycle which decays very slowly back to BR570, probably in parallel with the two Ms. This new intermediate has an absorbance maximum at around 350 nm ($\pm$ 20 nm) and we propose to call it R350.

2. MATERIALS AND METHODS

H. halobium cells (strain S-9) were grown and purple membrane was obtained according to standard procedures [4]. Purple membrane was suspended in 10 mM piperazine-glycylglycine buffer. The pH was constantly monitored and the given pH values are accurate to within $\pm$ 0.2 pH units. All measurements were done at 20 $\pm$ 1°C, on light-adapted samples having A = 1 at 570 nm.

The flash photolysis apparatus was similar in design to one described previously [3]. The sample was excited with 530 nm, 10 ns pulses from an Nd-YAG laser (Molectron) at a right angle to the monitoring beam (tungsten lamp plus

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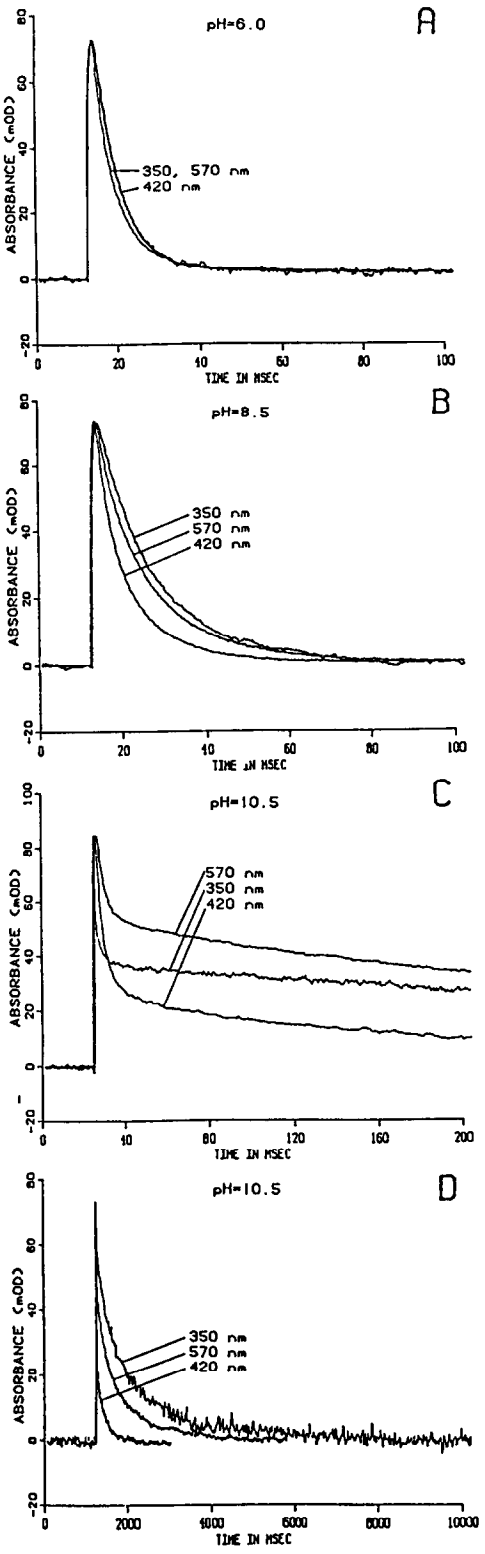

monochromator; spectral resolution, ± 5 nm). The outputs from the sample and reference photomultipliers were fed to a differential amplifier (Tektronix AM502). The resultant difference signal was then routed through a homemade 1 MΩ current amplifier and captured by a transient digitizer (LeCroy TR3837) in a CAMAC LSI-11 data acquisition system. The absorbance changes due to 20–100 flashes were averaged and stored for subsequent processing. Decreasing the exciting or monitoring light intensity by a factor of 10 did not alter the kinetics. pH titration measurements were carried out at the 'magic angle' [5] to avoid possible effects on the photochemical absorbance changes due to rotational motions of the chromophores and/or membranes [6]. At the magic angle, both the amplitude and the decay kinetics remain unchanged when the plane of polarization of the monitoring beam is rotated by 90°.

The difference spectra were taken using a xenon flashlamp (6 μs half pulse width) plus wide-band glass cut-off filters and/or interference filters on an apparatus similar to that described in [7]. Here the wavelength resolution was ± 20 nm. The kinetic traces were fitted with 3 exponentials to obtain the rate constants and amplitudes of the absorbance changes from 1 ms to 5 s in the 300–560 nm range using a nonlinear least-squares algorithm [8]. These data were not taken at the magic angle but the kinetics were identical to those taken with the laser under magic angle conditions.

3. RESULTS

According to widely accepted 'general' photocycle schemes (e.g. [5,9–11]), after a flash initiates the BR570 photocycle, the rate of recovery of BR570 should be close to the decay kinetics of the M412s. However, as shown in fig.1C,D, at pH 10.5 a portion of the recovery of BR570 (ΔA_{570}) is strikingly slower than the decay of the Ms (ΔA_{420}).

Fig.1. Flash-induced absorbance changes (ΔA) of bacteriorhodopsin (native purple membrane suspension) at 3 representative pH values, 20°C. The amplitudes are normalized to the apparent maximum of the ΔA_{420} to show the differences in kinetics (the ΔA_{570} traces are inverted). In D the normalization is only approximate.

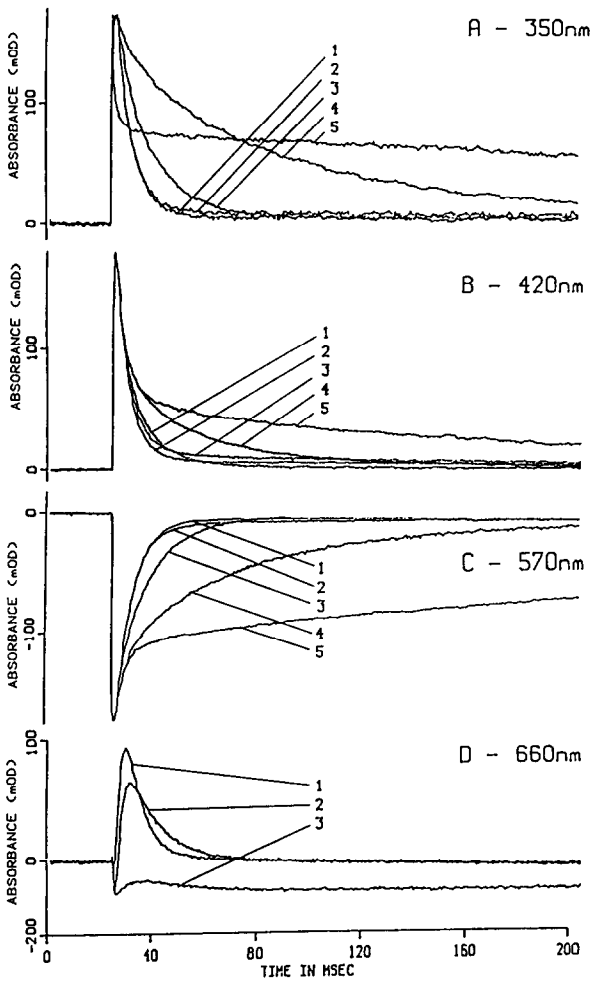

Fig.2. The pH dependence of the bacteriorhodopsin photocycle kinetics measured at 350, 420, 570, and 660 nm. The traces are normalized to the one at 570 nm. The pH in traces in A–C are: (1) 6.5, (2) 7.5, (3) 8.5, (4) 9.5, (5) 10.5. In D the traces are not normalized: (1) 6.2, (2) 8.1, (3) 10.5 (trace 3 was multiplied by 8).

The mismatch between the decay kinetics of the Ms and the kinetics of the recovery of BR570 decreases with decreasing pH but is still evident at pH 6.0 (fig.1A). In contrast to the absorbance changes at 420 nm, those at 350 nm have much slower decay kinetics and match the very slowly growing portion of the BR570 recovery (fig.1C,D). This strongly suggests the existence of a new intermediate with a near-ultraviolet absorbance maximum and a lifetime greatly in excess of M^s. The decay of this intermediate is required for a full recovery of the absorbance of BR570. We propose to call this new intermediate R350 because of its approximate absorption maximum (see below).

The pH dependence of the BR photocycle in the pH range 6–10.5 was monitored at the following wavelengths: 350, 420, 570 and 660 nm (fig.2). As the pH increases the M^f decay speeds up slightly, M^s decay slows down [12] and R350 decay and BR570 recovery slow down but much more than the Ms as measured by the ΔA_{420} (fig.2). At long times, the decay of R350 is superimposable with the recovery of the BR570 indicating that R350 decays directly to BR570. No significant amount of the photointermediate O660 is present at pH 10.5, in agreement with previous studies (e.g. [5,10,12,13]).

We measured the difference spectra of the BR photocycle at pH 10.5 where the existence of R350 is pronounced. Flash-induced absorbance changes were monitored from 1 ms to 5 s at 10-nm intervals from 300 to 560 nm and deconvoluted into 3 exponential components. In fig.3A, the rate constants of the three exponential processes associated with M^f, and M^s and R350 are plotted as a function of wavelength. The wavelength independence of all 3 rate constants provides strong evidence that the deconvolution has introduced no artifacts and thus substantiates the existence of 3 separate near-UV/blue absorbing intermediates: M^f, M^s and R350. In fig.3B the amplitudes of the 3 components are plotted vs wavelength. The positions of the M^f and M^s peaks are approximately the same (~410 nm) within our wavelength resolution (in this experiment, ± 20 nm), but the R350 intermediate has a well separated absorbance maximum around 350 nm. The crossover point for R350 (~415 nm) is also significantly shifted towards the shorter wavelengths compared with the M intermediates (~460 nm). Note that all of the 3 positive absorbance peaks correspond to recovery in the 560 nm range indicating that they decay back to BR570.

4. DISCUSSION

Our data demonstrate the existence of a new ultraviolet absorbing intermediate in the BR photocycle, R350. At least three properties distinguish R350 from M^f and M^s: its absorption

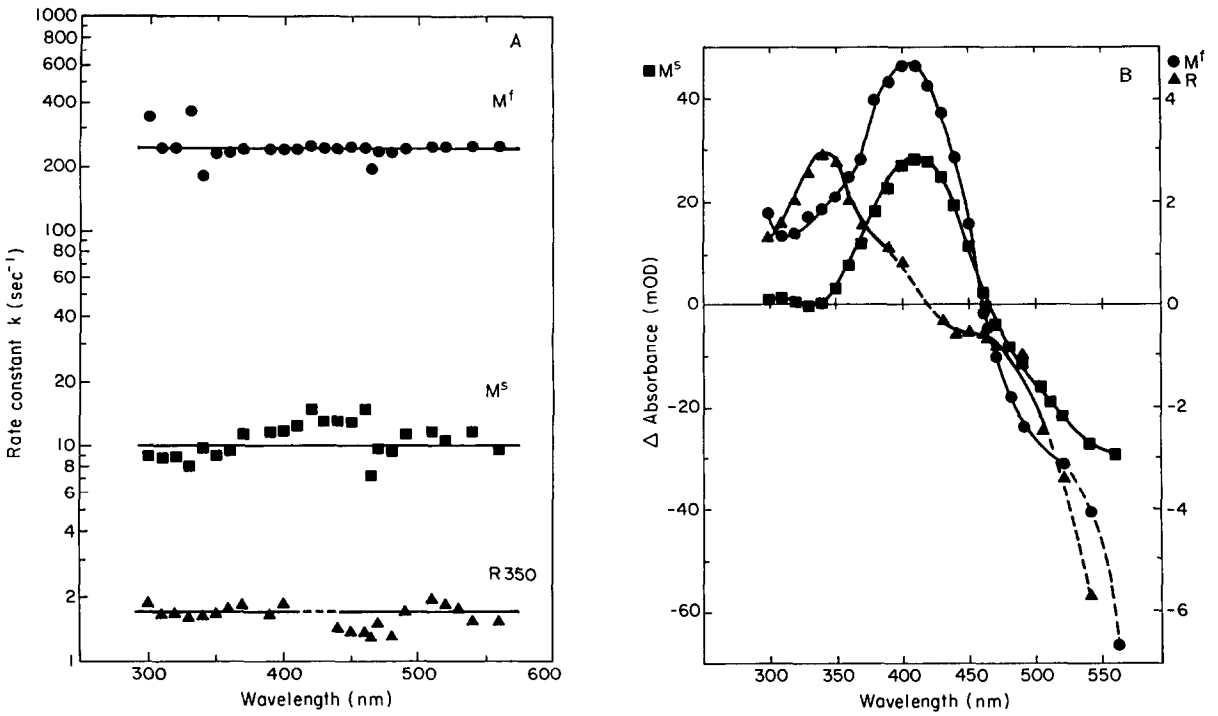

Fig.3. The flash-induced absorbance changes at pH 10.5, monitored from 1 ms to 10 s at 10-nm intervals from 300 to 560 nm, were deconvoluted into three exponentials. (A) The 3 rate constants obtained from the fit are plotted vs wavelength. The dotted lines at ~410 nm indicating the rate constant could not be reliably determined because of poor signal-to-noise ratio. The wavelength independence of the rate constants shows that there are 3 clearly resolved species. (B) The flash-induced difference spectra of M^f, M^s and R350 obtained by plotting the amplitudes of the 3 exponential components whose rate constants are given in (A). The dotted lines at ~550 nm indicate that the amplitudes could not be determined reliably.

maximum, decay rate, and pH dependence. R350 becomes more and more prominent as the pH increases from 6 to 10.5. At neutral pH values it decays rapidly and its amplitude is quite small, contributing a few percent to the total species that decay to reform the original pigment BR570; however, at pH 10.5 it decays much more slowly ($t_{½}$ = 500 ms) and about 35–40% of the recovery amplitude of BR570 is due to the decay of R350.

Recently Groma and Dancshazy [3] suggested the existence of three types of M since the absorbance changes they measured at 420 nm often required 3 exponentials for high-accuracy fits. The third M-type intermediate they postulated had a much slower decay rate than even the so-called M^s that most workers had resolved, and its weight, measured at 420 nm, was only a few percent of the total M signal. From the present study it seems likely that this third M is identical to R350. The long-wavelength portion of the R350 absorption change has a significant absorption at 420 nm (fig.3) and the kinetics of R350 seems close to that of the third 'M' [3].

The literature contains several other sets of data supporting the presence of R350. For example, Ort and Parson [12] (see their fig.4), Shkrob and Rodionov [14] (see their fig.5) and Beece, D., Eisenstein, L. and Ormos, P. (unpublished) showed that at higher pH values part of the recovery of BR570 after a flash was much slower than the decay of the Ms as measured by absorbance changes at 420 nm. Also, some difference spectra for M that have been published may contain a contribution from R350 (e.g. [14,15]). Finally, at the same FEBS conference on 'Retinal Proteins' where we first presented this work, Skulachev and co-workers [17] presented indirect evidence for a new intermediate needed to explain the slow re-uptake of

protons in BR after a flash. This new intermediate, called by them P568, may be related to R350.

At this stage we have little knowledge of either the physical nature of R350 or its physiological role in proton pumping. It seems most likely that such a short-wavelength intermediate is either an unprotonated schiff base or perhaps free retinal.

Finally, we feel we should comment on the place of this new intermediate in the BR570 photocycle. The simplest hypothesis is that since M^f, M^s, and R350 independently contribute to the recovery of BR570 after bleaching, all three species decay in parallel. This in turn suggests one more branching of the BR photocycle before the formation of the Ms.

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