EFFECT OF TRITON X-100 AND OF DEUTERATION ON THE AMPLITUDE OF THE O_{640}-INTERMEDIATE IN THE BACTERIORHODOPSIN PHOTOCYCLE

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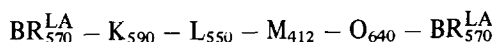
1. Introduction

Bacteriorhodopsin, the only protein of the purple membrane of *Halobacterium halobium*, acts as a light-driven proton pump [1,2]. Light absorption of bacteriorhodopsin (BR) leads to a cyclic reaction over at least 5 spectroscopically distinguishable intermediates (including BR) denoted by their absorption maxima [3]:



$$BR^{LA}_{570} - K_{590} - L_{550} - M_{412} - O_{640} - BR^{LA}_{570}$$

Proton pumping only occurs in the BR^{LA} cyclic reaction. The coupling of the ejection of protons on one side and proton uptake on the other side of the membrane to the intermediates of the photocycle as well as the stoichiometry of the pumping, i.e., the ratio of protons pumped to BR-molecules cycling, have been investigated repeatedly [4–8]. Experiments which have shown deprotonation of the protonated Schiff base linkage of all-*trans* retinal to bacteriorhodopsin at the M_{412}-intermediate [9] have led to the assumption that the Schiff base proton might be involved in the pumping, thus leading to a maximum stoichiometry of 1. The stoichiometry of the pumping depends strongly on external parameters such as salt concentration and pH. At physiological conditions, i.e., at high [salt], stoichiometries of ~2 have been reported [6,8]. At lower [salt] the H^+/M_{412} ratio observed is generally smaller and was found to be 0–1 [6]. The formation of M_{412}, however, does not depend on [salt].

This indicates that another pathway may be involved in the proton pumping. The pH-dependence of the stoichiometry of proton extrusion from *Halobacterium* cells and cell envelopes suggests that this might be a pathway of the O-intermediate, since it shows the same increasing yield for lower pH [5,7,10]. Again, the formation of M_{412} is not influenced by variation of pH in this pH-range. Here, we report the influence of deuteration and of solubilisation on the amplitude of the O_{640}-intermediate. The influence of deuteration is not the result of an isotope effect on the kinetics, but rather indicates an isotope-depending branching of the photocycle. A corresponding influence on the M-intermediate could not be detected. An influence of deuteration was also found on a fast absorbance change, which chronologically appears in the range of the L_{550}-intermediate. No corresponding influence on the L_{550}-intermediate as measured at its maximum absorbance was found. These aspects are discussed in relation to current models of the bacteriorhodopsin photocycle. The influence of solubilisation on the O_{640}-intermediate was investigated for another reason also. The exceptional position of O_{640} among the other intermediates concerning its dependence on pH, temperature and ionic strength, have led us to the presumption that O_{640}, in contrast to other intermediates, might be influenced by the membrane integrity.

The desintegration of the membrane and its influence on the O_{640}-intermediate, however, could show that its main features are still present in solubilized purple membrane.

2. Materials and methods

For the experiments purple membrane from *Halobacterium halobium* (mutant R_1M_1), isolated as in [23], was used. Experiments were also performed with purple membrane kindly given to us by M. P. Heyn and N. A. Dencher (Biozentrum Basel). Flash photometric measurements were done with purple membranes suspended in distilled water or in 2 M NaCl. pH-Dependence of O_{640} was measured in buffered solutions (25 mM) in various buffers. Bacteriorhodop-

sin monomers were obtained as in [24], using Triton X-100 as a detergent. In the solubilized membrane the protein–detergent ratio was 1:5 (w/w), and Triton X-100 was 0.5%.

For the investigation of the effect of deuteration on the amplitude and kinetics of O_{640} thin purple membrane films were used, which could either be hydrated or deuterated. The use of such films for the investigation of the bacteriorhodopsin photocycle was described in [11]. We have also used such films for infrared spectroscopy and kinetic infrared investigations. Their preparation is described in [12]. We were thus able to get kinetic signals of the same purple membrane film either in the hydrated or in the deuterated state, which proved to be very useful for the correct analysis of signal amplitudes.

A conventional flash photolysis system was used, equipped with a semiconductor diode detector for better performance at longer wavelengths. Since fluorescence of the BR-samples was superimposed on fast absorbance changes when measuring at wavelengths between 600 nm and 700 nm, interference filters were used in addition to cutoff filters to minimize the intensity changes caused by a broad fluorescence background. As a source for excitation light a dye laser (Molectron DL 100) pumped by a nitrogen laser (Molectron UV 300) was used for excitation at 578 nm or 515 nm, respectively. Temperature of the sample was regulated within 0.2°C.

Kinetic signals were stored in a NIC 1070 signal averager, where up to 100 signals were added to improve signal-to-noise ratio. Kinetics of the signals were analyzed with a semilogarithmic plot of the reaction time course as well as with a standard computer procedure [13]. Evaluation with up to 3 exponentials could be performed.

Absorbance changes due to the intermediate O_{640} are usually monitored between 620 nm and 660 nm. A considerable amount of the L-intermediate is superimposed on the rise and decay of the O_{640}-intermediate in this spectral region, which makes a correct evaluation of its amplitude and kinetic data very complicated, since it introduces at least 3 exponentials for a correct description. For a detailed evaluation of the kinetics and the amplitude of the 'O'-intermediate we have, therefore, measured its time course in the wavelength region between 680 nm and 720 nm, where the overlap of the absorption bands of the different intermediates is smaller.

3. Results and discussion

3.1. *Effect of hydrogen/deuterium-exchange on the intermediate O_{640}*

Fig.1a shows the time course of the O_{640}-intermediate at 706 nm in H_2O and D_2O. The amplitude of the signal in D_2O is about twice the amplitude measured in H_2O. One possible explanation of this effect would be a strongly increasing decay time of this intermediate, thus leading to a higher apparent amplitude. We have, however, analyzed the kinetic traces by the computer program mentioned above. To get information about the changes of kinetics as well as of the amplitude of the O_{640}-intermediate upon deuteration, several linear reaction schemes were used for evaluation. Guided by the characteristical sigmoidal form of the signal time course observed at higher time resolution (fig.1b), we chose a linear reaction scheme L–X–O–BR, i.e., we evaluated the O_{640}-intermediate as a product of a consecutive reaction scheme arising from L and decaying to BR. X denotes an intermediate which does not absorb around 700 nm. Neglecting the very fast absorbance increase occurring in a few microseconds, which we are not able to resolve yet, this reaction scheme yielded much better agreement between calculated and measured data over the whole time scale than the simple reaction scheme M–O–BR. The calculation yielded the isotope effect on the rise and decay time of the O-intermediate to be a factor of 1.5–2. The actual (not only the apparent) amplitude given by the computer evaluation is about twice as high in D_2O as in H_2O. Another effect that might account for an increase of the amplitude of the O_{640}-intermediate would be pH-shift. Since, however, H_2O–D_2O exchange leads to a pH ~0.5 pH-units higher [14], thus leading to a decreasing O-intermediate, and as this shift is accompanied by a corresponding shift in the pK-values of the protein, this effect can be ruled out.

Fig.1b shows the time course of the O_{640}-intermediate at a higher time resolution. A fast absorbance increase can be seen, influenced by deuteration in the same way as the amplitude of the O_{640}-intermediate. Such an absorbance increase cannot be derived from low temperature spectroscopy, where an absorbance decrease from BR_{570} to L_{550} in the wavelength region from 640–720 nm is observed [15,16]. Kinetic measurements, however, performed at room temperature or at moderate cooling, show stronger absorbance for the L-intermediate than for BR [17]. Deuteration

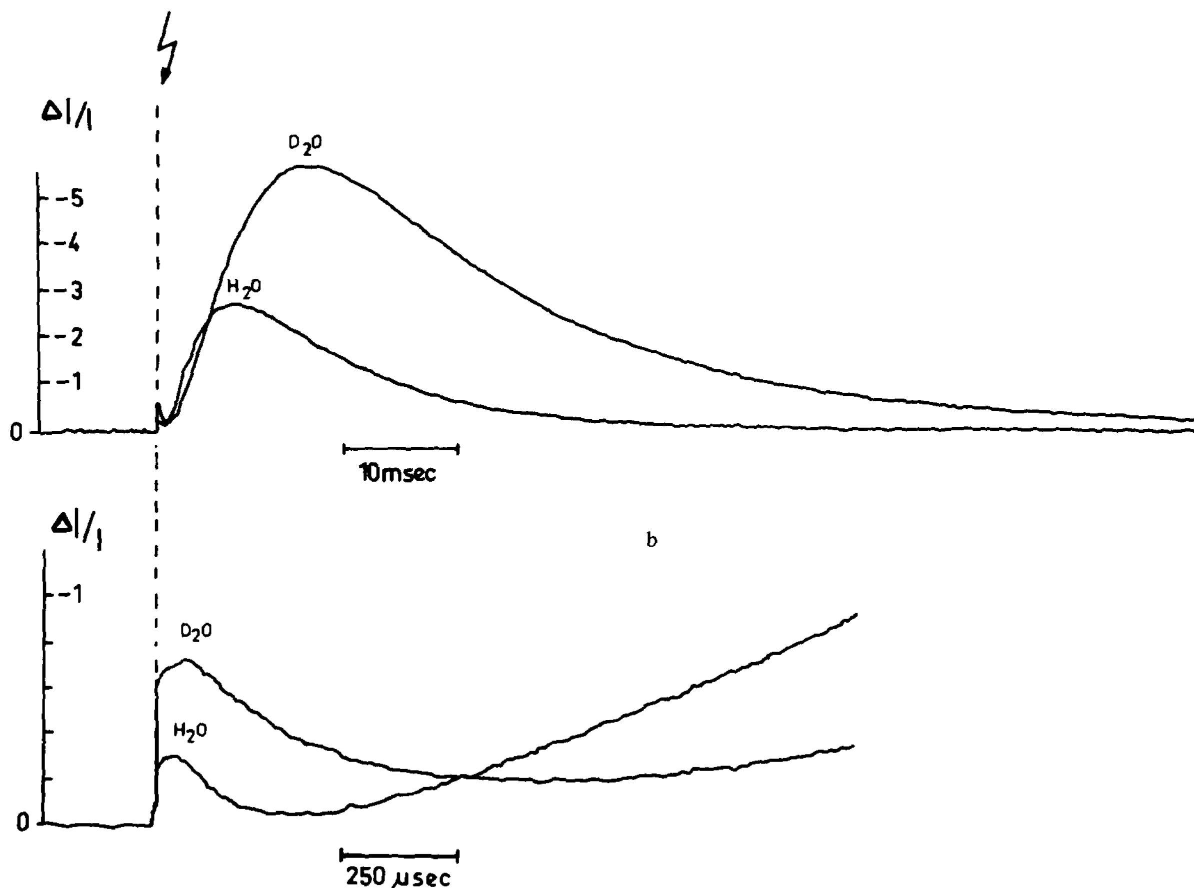


Fig.1.(a) Kinetic signals of the intermediate O_{640} measured at 706 nm of the same purple membrane film in the hydrated and deuterated state, respectively. T = 20°C, Laser flash at t = 0 is at 578 nm, 10 signals were averaged to improve SNR. (b) Signals shown in (a) at higher time resolution.

increases this fast absorbance change by a factor of ~2. In that wavelength region, however, there is some absorbance change due to the BR–L transition superimposed on the signal. We therefore measured the BR–L transition at its maximum absorbance change and found no deuteration effect on the amplitude of this intermediate. These arguments thus suggest that an intermediate, different from L, manifests at the wavelength region considered.

In fig.1b the fast absorbance change is followed by a slower component. This subsequent slower absorbance increase does not occur in the time range of the M-intermediate, also, it does not exhibit the kinetic deuteration effect of the M-intermediate, whose risetime is slowed down by a factor of ~6 upon ^1H–^2H exchange [18]. Both, the fast and the slow absorbance increase, decay down to almost the base-line absorbance within ~1 ms. In the visible spectral range no corresponding rise of an intermediate, called 'X' in our calculations, can be seen, excluding that this decay is caused by the formation of M_{412}. In the infrared spectral range, however, we have evidence for changes in the protein in the millisecond time scale. Our method of kinetic infrared spectroscopy (KIS) allows measurement of very small transmission changes in the mid-infrared spectral region in the time scale from microseconds to seconds [12]. Many of the transmission changes can be assigned to chromophore molecular changes. In addition, there are molecular changes that cannot be assigned to vibrations of the retinal chromophore. As an example, a signal we denoted 'slow component', with a risetime of ~1 ms, appears superimposed on chromophore signals at several wavelengths [19]. The fact that it considerably increases its ampli-

Fig.2.(a) Kinetic signals at 706 nm of solubilized purple membrane (B) as compared to the intact purple membrane (A). Amplitudes were normalized for direct comparison. Assuming constant M_{412}-amplitude, the signals are ~4-times smaller in monomers. In the solubilized membrane the protein–detergent ratio was 1:5 (w/w), and Triton X-100 concentration was 0.5%. T = 20°C, laser flash at 578 nm, 10 signals were averaged to improve SNR. (b) Signals shown in (a) at higher time resolution.

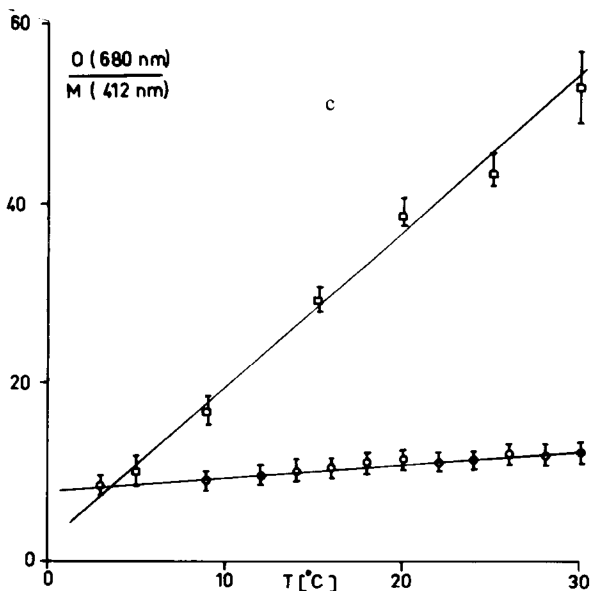


Fig.2.(c) Temperature dependence of the amplitude of O_{640}. Measuring wavelength was 680 nm. Measuring conditions for monomers and PM were as in (a). Amplitudes were normalized to the maximum amplitude of M_{412}. (□) Intact purple membrane; (○) solubilized purple membrane.

tude upon deuteration suggests its possible relation to the O_{640}-pathway. Measurements are in progress to clarify the meaning of this component.

3.2. *Effect of Triton X-100 on the intermediate O_{640}*

Fig.2a shows the time course of the O_{640}-intermediate of purple membranes solubilized in Triton X-100 as compared to the intact membranes. The signals are drawn in the same scale for direct comparison: the calculated amplitude of the O_{640}-intermediate in monomers is ~25% the amplitude measured in purple membranes, based on constant M_{412}-amplitude. As can

be seen, the fast absorbance increase observed at higher time resolution is slightly affected by solubilisation. The blue shift of the absorption of bacteriorhodopsin in monomers [25] may, at least in part, account for its increasing amplitude, since it implies a smaller negative contribution of the BR–L transition to the sum of absorbance changes observed at 700 nm. Since the principal features of the absorbance changes at 640–720 nm have not changed as compared to the intact membrane, we conclude that O_{640} is actually a photolysis product. It can therefore be excluded that O_{640} represents an unphotolyzed bacteriorhodopsin molecule whose absorbance spectrum has been changed by the interaction with its photolyzed neighbours. The most striking effect, however, can be seen on the temperature dependence of the O_{640}-amplitude as shown in fig.2c. While in intact purple membrane sheets the amplitude of O_{640} decreases continuously with decreasing temperature and becomes almost undetectable at ~0°C, in solubilized purple membrane it remains practically unaffected over 3–30°C. This shows that besides pH, temperature, ionic strength and deuteration the membrane integrity adds to the parameters that influence the O_{640}-intermediate.

4. Discussion and conclusions

In the bacteriorhodopsin photocycle several reaction schemes can be applied to describe the time course of the absorbance changes of the intermediates. A linear, unbranched reaction scheme is a fairly good approximation for conditions where neither 'O'-intermediate nor biphasic decay of M_{412} are observed. The pH- and temperature-dependence of the O_{640}-intermediate have led to the assumption that a branched pathway exists for the regeneration of bacteriorhodopsin, one branch leading directly from M_{412} to BR_{570}, the other via O_{640}. Temperature-, pH- and deuteration-dependence might be responsible to influence the branching [21,22].

The existence of 2 forms of M_{412} was reported in [20], with an inhomogeneous M-spectrum at its decay from steady state conditions. In addition, 2 forms of BR in a temperature-dependent equilibrium have been suggested in [21]. These 2 forms, decaying on 2 different pathways, would account for the biphasic decay of the M-intermediate as well as for the temperature-dependence of the O_{640}-intermediate.

Under certain conditions, however, where clearly monophasic decay of M_{412} is accompanied by a considerable amount of O, these models cannot give a satisfying explanation. To clarify this point, the decay of the M_{412}-intermediate and the rise of the O_{640}-intermediate were measured parallel under various conditions and their time course was analyzed with a computer program. The results of the kinetic evaluation can be summarized as follows:

(i) At low salt concentration or in distilled water the decay of the M_{412}-intermediate was found monophasic, i.e., no fast component of M_{412}-decay leading to O could be observed under conditions where high [O] are prevailing;
(ii) The monophasic decay of M_{412} is ~2-times slower than the rise of O_{640};
(iii) In solubilized purple membrane, where M_{412} decays with 2 distinct components, the risetime of O is still 2-times faster than the fast decay component of the M_{412}-intermediate;
(iv) At high [salt] (2 M NaCl) and high pH, where distinct biphasic decay of the M_{412}-intermediate is found, the intermediate O_{640} appears at a negligible concentration.

These are mostly kinetic arguments and are therefore subject to further discussion. A reaction scheme for the bacteriorhodopsin photocycle with a branching point for the pathway of the O_{640}-intermediate at M_{412} does, however, not satisfy these arguments, since an increase of O_{640} upon deuteration should result in a decrease of some M-component. If a branching is assumed to take place at any intermediate of the thermal reaction sequence before M_{412}, an increase of O_{640} should result in a decrease of M_{412}.

Since, within a range of pH and temperature, where the amplitude of M_{412} stays unaffected, the O_{640}-amplitude is changed tremendously, and since deuteration, which doubles the O_{640}-yield, does not influence M_{412}, a branching in the thermal reaction sequence is unlikely. We conclude that the O_{640}-intermediate is generated by another pathway than that via K_{590} from the excited state. This conclusion relies heavily on the assumption that the absorbance changes assigned to O_{640} observed with solubilized purple membrane are due to bacteriorhodopsin monomers and therefore represent photolysis products.

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