Volume 175, number 2 FEBS 1826 October 1984

Light-dependent *trans* to *cis* isomerization of the retinal in halorhodopsin

Janos K. Lanyi

Department of Physiology and Biophysics, University of California, Irvine, CA 92717, USA

Received 6 August 1984

Flash-induced absorption changes in the near UV were determined for bacteriorhodopsin and halorhodopsin on a millisecond time scale. The difference spectrum obtained for bacteriorhodopsin was comparable to model difference spectra of tyrosine (aromatic OH deprotonated *vs* protonated), as found by others. The flash-induced difference spectrum for halorhodopsin, in contrast, resembled a model spectrum opbtained for *trans* to 13-*cis* isomerization of retinal in bacteriorhodopsin. A model for chloride translocation by halorhodopsin is presented, in which the retinal isomerization moves positive charges, which in turn modulate the affinity of a site to chloride.

Halorhodopsin *Retinal isomerization* *Tyrosine deprotonation* *Chloride transport* *Flash spectroscopy*

1. INTRODUCTION

Bacteriorhodopsin and halorhodopsin are retinal proteins in the cytoplasmic membrane of ***Halobacterium halobium***, similar in several spectroscopic [1,2] and molecular [3–7] properties, but different in amino acid composition [8,9] and, as far as has been determined (D. Oesterhelt, personal communication), amino acid sequence. Most important, they are different in their functional roles: bacteriorhodopsin is a light-driven proton pump (review, see [10]), while halorhodopsin acts as a light-driven chloride pump [11,12]. In halorhodopsin, as in bacteriorhodopsin, the functional entity is the chromoprotein [13]. Thus, a comparison of those molecular events in the retinal proteins which accompany the light-driven translocations should be informative in attempts to construct a model for the functioning of halorhodopsin as a pump.

Upon illumination both pigments undergo characteristic spectroscopic transformations with 5–10 ms overall recovery kinetics, which are conveniently followed in single-turnover experiments (flash spectroscopy). The bacteriorhodopsin photocycle has been described in detail [10], and contains 5 distinct intermediates (distinguished by their absorption bands in the visible region) which are usually placed in a sequential pathway. Of these, M_{412}, which corresponds to the deprotonated Schiff's base [14], accumulates in the greatest quantity during the photocycle. In addition to the absorption changes in the visible, flash-induced absorption changes were observed also in the UV region. These reflect (a) *trans* to 13-*cis* isomerization, which occurs on a μs time-scale [15] and (b) the state of protonation and environment of tyrosine and tryptophan residues [15–17], as well as changes in UV fluorescence [16–18], which reflect mostly the state of tryptophan residues and which occur on a ms time scale. The light-dependent effects involving the aromatic residues are presumably linked to the movement of the transported protons through the protein. It has been suggested that this transport occurs via the reversible deprotonation of a chain of residues arranged across the membrane barrier [19,20]. Tyrosine (and perhaps tryptophan) may be

Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; $C_{12}E_9$, polyethylene glycol lauryl ether

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elements of this chain, and indeed it has been shown that chemical modification of tyrosines [21], and particularly tyr_{26} [22] and tyr_{64} [23] has specific effects on the pigment.

The photocycle of halorhodopsin is not fully described, but it is known [24–27] that the principal intermediate observed in the presence of halides absorbs near 500 nm (P_{520}), while in the absence of chloride it absorbs near 640 nm (P_{660}). It would be very helpful to know, for the construction of a functional model for halorhodopsin, if a *trans* to 13-*cis* isomerization took place in this pigment also. The kinetics of chloride uptake and release, events which are presumed to take place analogously to those for protons in bacteriorhodopsin, have not been determined. Various spectroscopic measurements [25,28,29], as well as a binding assay [30], have suggested [31], however, that chloride binds to 3 distinct sites (Sites I, II and III) in halorhodopsin. The involvement of Site II in transport seems clear because its binding constant and anion specificity agree with those determined for the transport itself [11,29]. The involvement of Sites I (which alters the Schiff's base p*K*) and III in transport is less clear, because the specificity of these binding sites is much broader than that of the transport. Since the affinity of binding sites to chloride would be modulated if the number of positive charges in their vicinity were altered, cyclic deprotonation of strategically located residues during the illumination could be part of the chloride translocation mechanism.

We report here on flash-induced absorbance changes measured in the near UV. We use bacteriorhodopsin as reference material, and halorhodopsin to explore the possibility that *trans* to 13-*cis* isomerization and/or reversible deprotonation of tyrosine might play a role in this system also.

2. MATERIALS AND METHODS

Halorhodopsin was prepared as in [9], different from the procedure of [5] in that polyethylene glycol lauryl ether ($C_{12}E_9$, Nakarai Chemicals, Japan) was used instead of Lubrol PX, and the Tween solubilization was in 2 M NaCl instead of diluted salt. Bacteriorhodopsin was prepared as described in [31]. When solubilized, bacteriorhodopsin was incubated overnight with $C_{12}E_9$. A 7 nm blue-shift in the visible absorption maximum confirmed that the pigment was solubilized [33]. Light-adaptation was by extensive illumination with yellow light until no further absorption changes were observed. All solutions were buffered with 0.05 M MOPS, pH 7.0.

For flash spectrometry 1 × 1 cm quartz cuvettes were used at 22°C, in a cross-beam instrument described previously [11]. For measurements in the UV a 50 W deuterium lamp was used as the light source. The flash (PRA 6100B pulsed light source) was through a 530 nm long-pass filter, while the measuring beam wavelength was set with two monochromators, one before and the other after the sample. Additional filters, placed between the sample and the second monochromator, were used to further protect the photomultiplier. Signal averaging was for 512 flashes.

3. RESULTS

Absorbance changes in the near UV (260–400 nm) were measured after flash illumination of both bacteriorhodopsin and halorhodopsin. The time scale of the measurements was 35 ms after the flash, with a 100 μs time-constant, which allowed the resolution of the decay but not the rise of the absorption changes. Both pigments exhibited distinct wavelength-dependent absorption changes in the UV region (not shown). From the traces it was evident that the decay kinetics of the absorption changes in the UV corresponded well to the kinetics in the visible, i.e., to rapid (unresolved) rise, and a decay $t_{1/2}$ of 4.6 ms for bacteriorhodopsin and 16.5 ms for halorhodopsin.

The maximal amplitudes of the flash-induced absorption changes were used to construct difference spectra. As reported previously [15–17], bacteriorhodopsin is seen to exhibit a minimum near 275 nm and a maximum near 297 nm (fig.1). The large increase in absorption at wavelengths longer than these corresponds to the production of M_{412}. The absorption change at 275 nm is $-7600\ M^{-1}\cdot cm^{-1}$ for the photocycling fraction. The amount photocycling was calculated from the absorption change at 400 nm, using $23000\ M^{-1}\cdot cm^{-1}$ as differential extinction coefficient [34]. Halorhodopsin is seen to exhibit a somewhat similar maximum and minimum in the

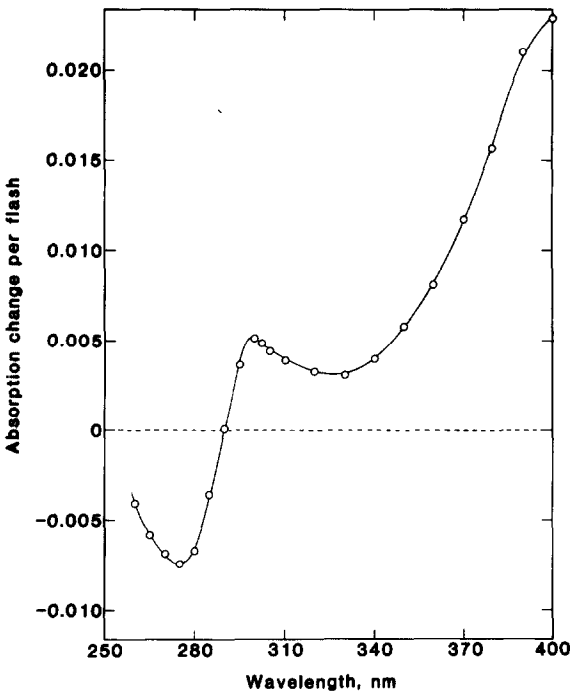

Fig.1. Flash-induced difference spectrum for bacteriorhodopsin. Conditions: bacteriorhodopsin (2.9 nmol/ml) in 0.05 M MOPS, pH 7.0; flash intensity 4 kV. The maximal amplitudes of the flash-induced absorption changes (within 1 ms after the flash) are plotted *vs* the measuring beam wavelength.

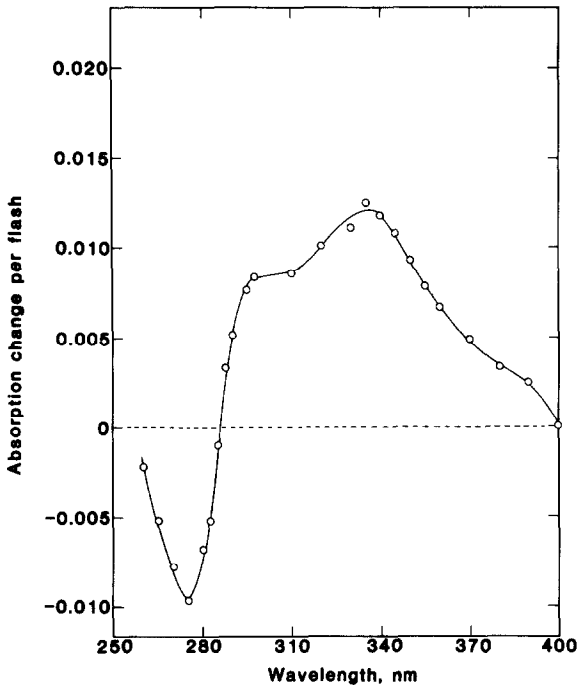

Fig.2. Flash-induced difference spectrum for halorhodopsin. Conditions: halorhodopsin (7.0 nmol/ml) in 2 M NaCl plus 0.05 M MOPS, pH 7.0, 0.5% $C_{12}E_9$; flash intensity 5 kV. The maximal amplitudes of the flash-induced absorption changes (within 1 ms after the flash) are plotted *vs* the measuring beam wavelength.

UV, but with a prominent maximum at ~335 nm (fig.2). No absorption band at 380 nm [24] is evident. The absorption change at 275 nm is $-4400\ M^{-1}\cdot cm^{-1}$ for the photocycling fraction. The amount photocycling was estimated from the absorption change at 500 nm, using $18800\ M^{-1}\cdot cm^{-1}$ as differential extinction coefficient (our unpublished approx. value, from the relative absorption changes at 500 and 570 nm).

Alkaline *vs* acid (pH 12 *vs* pH 5) difference spectra were obtained for tyrosine in various solvents, ranging from polar (water), medium non-polar (ethanol–water, 1:1), to non-polar (1-butanol). As shown in fig.3A, deprotonation of the phenolic OH is accompanied by decrease in absorption between 270 and 280 nm, and a larger increase between 290 and 300 nm. The positions of the minimum and maximum shift to shorter wavelengths with increasing solvent polarity, and the magnitude of the absorption difference (measured between the maximum and the minimum) becomes less. The latter effect corresponds to a general decrease in absorption at all pH values as solvent polarity is increased (not shown). A difference spectrum between light- and dark-adapted bacteriorhodopsin is shown in fig.3B. Such a spectrum was reported by authors in [15], who used it as a model spectrum for *trans* to 13-*cis* isomerization of the retinal. From fig.3B the differential extinction coefficient at 275 nm was calculated as $-2750\ M^{-1}\cdot cm^{-1}$ for 100% conversion of *trans* to 13-*cis*.

It is generally agreed [15–17,35] that the absorption changes observed for bacteriorhodopsin in the near UV originate from *trans* to 13-*cis* isomerization (on a μs time scale) and from perturbation of aromatic amino acids (on a ms time scale). However, unambiguous interpretation and quantitation of the effects involving the aromatic residues is difficult. Authors in [36] argued that

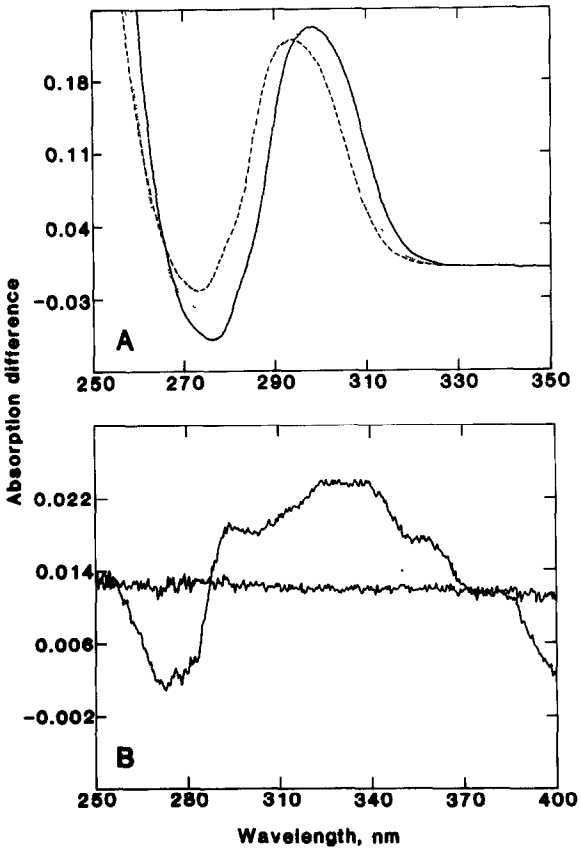

Fig.3. Model difference spectra for the deprotonation of the phenolic OH in tyrosine (A), and for *trans* to 13-*cis* isomerization of retinal (B). A: L-tyrosine (92 μM) in water (– – –), in ethanol–water (1:1) (···), and in 1-butanol (——). Sample cuvette at pH 12, reference cuvette at pH 5. B: bacteriorhodopsin (8.7 μM) in 0.05 M MOPS, pH 7.0. After recording the baseline the contents of the reference cuvette were illuminated to light-adapt the bacteriorhodopsin, and the difference spectrum was recorded.

while the ms difference spectrum resembled the model spectrum for the deprotonation of the phenolic OH in tyrosine, a better fit could be made from a combination of deprotonation and a shift from a hydrophobic to a polar environment. The present data for bacteriorhodopsin also do not quite fit the model spectra for the deprotonation of tyrosine, because the maximum at 297 nm is somewhat smaller than the minimum at 275 nm (fig.1), rather than much larger, as in the model spectra (fig.3A). Another model spectrum, which resembles the obtained difference spectrum (fig.1) quite well, can be obtained by considering 5 tyrosine residues undergoing hydrophobic to polar shift plus a single tyrosine deprotonated in a butanol-like environment. This combination of events gives an extinction difference ($\Delta A_{297-275}$) of 5510 $M^{-1} \cdot cm^{-1}$ per deprotonation. Thus, for the sake of comparison with previously published data, a rough calculation can be made for the number of deprotonations per photocycling bacteriorhodopsin. The data for purple membrane in 0.05 M MOPS, pH 7.0, yield 2.2 ± 0.09 tyrosines deprotonated (fig.1), and in 2 M NaCl plus 0.05 M MOPS, pH 7.0, 2.3 ± 0.09 tyrosines deprotonated per photocycle. For detergent-solubilized bacteriorhodopsin, comparable to the halorhodopsin sample, these numbers are 1.9 ± 0.09 and 1.8 ± 0.05, respectively. Authors in [36] estimated from steady-state illumination experiments that either two tyrosines or a tyrosine plus two tryptophans are deprotonated per photocycle.

The absorption changes we observe for halorhodopsin, on the other hand, must originate largely from *trans* to 13-*cis* isomerization, because they resemble quite well the model light-adapted *vs* dark-adapted difference spectrum of bacteriorhodopsin. The absorption change for halorhodopsin at 275 nm is -3450 ± 400 $M^{-1} \cdot cm^{-1}$ for the photocycling fraction (fig.2). If the assumption is made that the *trans* to 13-*cis* isomerization in halorhodopsin causes the same magnitude of absorption changes as in bacteriorhodopsin, this agrees reasonably well with -2750 $M^{-1} \cdot cm^{-1}$, the value expected if 100% of the retinal were isomerized in the photocycling fraction. The difference between the two values allows the deprotonation of no more than about 0.1 tyrosine. I conclude that in halorhodopsin a *trans* to 13-*cis* isomerization occurs before or concurrently with the formation of P_{520}, and decays together with this intermediate. If deprotonation is to be part of a model for the translocation of chloride by halorhodopsin, it cannot involve tyrosine. A deprotonation reaction may involve histidine and/or cysteine, however, residues found in this protein [8] but not in bacteriorhodopsin. Histidine, in particular, might participate in proton transfer by a tautomerization mechanism.

Using the idea that the light-induced *trans* to 13-*cis* isomerization of the retinal is the key event

in the chloride translocation mechanism, and taking into account the lack of an M-like intermediate with a deprotonated Schiff's base in the normal halorhodopsin photocycle (D. Oesterhelt, personal communication, [31]), I present a functional model of halorhodopsin in fig.4. The retinal protein is shown with a 'high affinity' binding site (upper circle in each species), e.g., Site II, and a 'low affinity' binding site (lower circle in each species). The former is to communicate with the cell exterior, the latter with the cytoplasmic side. Isomerization of the retinal, induced by the illumination, places the positively charged Schiff's base nitrogen near a movable positive charge (e.g., a histidine residue, as mentioned above). The positive charge, displaced to a location near the low affinity site, converts the latter into a high affinity site. This is shown, for illustrative purposes, as an increase of the positive charges of the site from two to three. Species designated as I and VI are in an equilibrium dependent on external chloride, an interconversion which is accompanied by a small absorption change [27,31]. Illumination causes the formation of species II and VII, which are also interconvertible in a chloride-dependent manner. In species II the alternative exists for the chloride to migrate to the newly created high affinity binding site. The resulting species III either loses chloride to the cytoplasmic side (with the net translocation of a chloride and regeneration of halorhodopsin through the III–VII–VI–I pathway), or gains chloride from the cell exterior (and accomplishes the chloride translocation through the III–IV–V–I pathway). The relative rates of these reactions are dependent on the affinities of the sites and the internal and external chloride concentrations, and these parameters are unknown at this time. We have demonstrated earlier [25], however, that in cell envelope vesicles the decay rates of both halorhodopsin photointermediates, P_{520} and P_{660}, are decreased at increasing extravesicle chloride concentrations, consistently with this model. The model presented here is influenced by our findings of chloride binding sites in halorhodopsin. It does not take into account retinal bond-energy considerations. Another

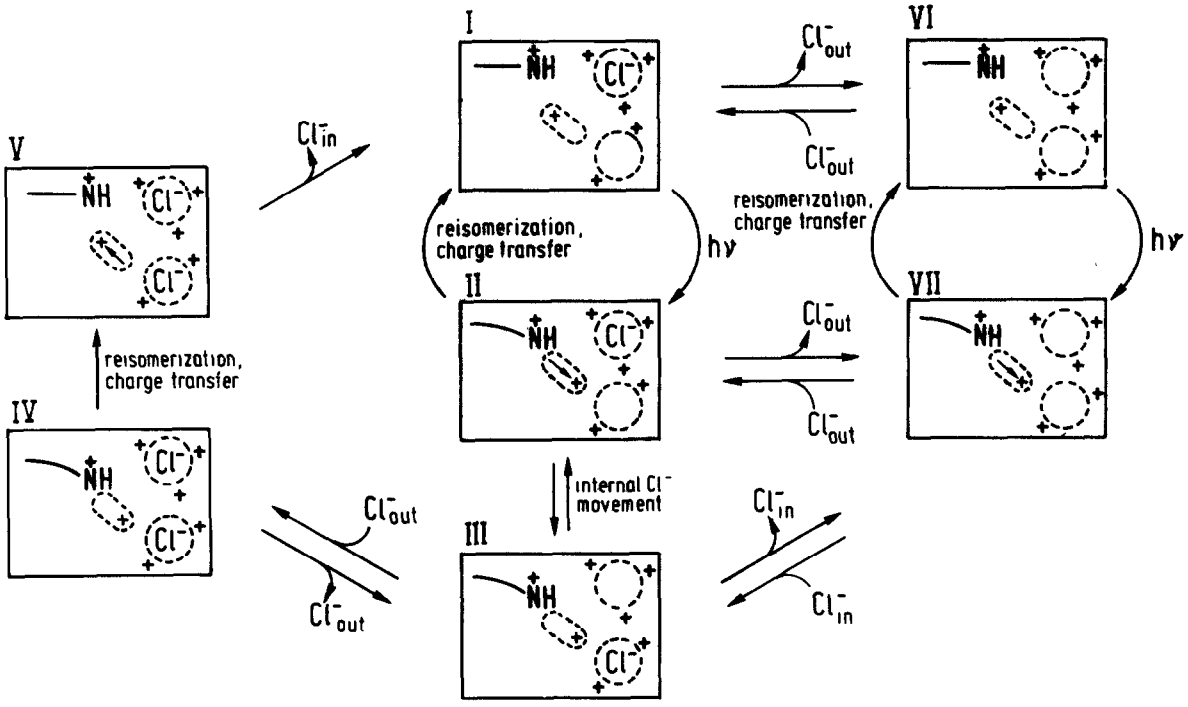

Fig.4. Proposed model for the light-induced chloride translocation by halorhodopsin. The retinal is shown either in the *trans* form (straight chain) or in isomerized form (bent chain). Details of the model are explained in the text.

model, based upon the latter consideration, has been formulated (D. Oesterhelt, personal communication).

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Institutes of Health (NIH GM 29498). The author is very grateful to Dr B. Schobert, for discussions and Drs D. Oesterhelt and P. Hegemann for sharing their ideas on the photochemical interconversions of halorhodopsin.

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