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EVIDENCE FOR DIFFERENTLY PROTONATED FORMS OF METARHODOPSIN II AS INTERMEDIATES IN THE DECAY OF MEMBRANE-BOUND CATTLE RHODOPSIN

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SUMMARY

Suspensions of vesicles from cattle rod disc membranes are flash illuminated at varying pH and temperature ; the light induced proton uptake and spectral change associated with the formation of metarhodopsin II are measured. Metarhodopsin II is shown to be diprotonated at 3°C, and to exist in at least two forms at higher temperature : the diprotonated form observed at 3°C(mainly at acid pH), and an unprotonated form (mainly at alkaline pH), which is found to be in temperature dependent equilibrium with metarhodopsin I and/or protonated metarhodopsin II.

INTRODUCTION

The metarhodopsin I - metarhodopsin II transition has been the subject of much interest, since it is the last step in the bleaching sequence of rhodopsin, which has a lifetime compatible with the transduction of the photic signal. Metarhodopsin I and metarhodopsin II have been shown to be in a stable pH dependent equilibrium at 3°C, both in digitonin solution (1) and in sonicated membranes (2). The rapid light-induced proton uptake observed in digitonin (3) and in rod outer segment membranes (4) (5) has been shown by kinetic studies to be associated with the metarhodopsin I - metarhodopsin II transition (6) (4), thus explaining the pH dependence of the equilibrium. The proton uptake and the spectral shift between metarhodopsin I (λ max 480 nm) and metarhodopsin II (λ max 380 nm) are however two distinct processes, since it is established that the spectral shift is produced by deprotonation of the Schiff base linking the retinal to the protein (7). These two processes may therefore differ in their sensitivity to temperature : although the influence of temperature on the spectral transition has been studied in digitonin solution (1) and in rod outer segments (4), the influence of temperature on the proton uptake has not previously been reported. Besides, while metarhodopsin II is reported to bind one proton in digitonin solution (6), there is some disagreement between the different reports on membrane-bound rhodopsin (4) (5) (8).

In this paper, we report parallel studies of the light-induced proton uptake and of the spectral change associated with the formation of

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Metarhodopsin II, in vesicles from cattle rod disc membranes, at varying pH and temperature (3°C to 37°C).

METHODS

Rod disc membranes are prepared according to (9), suspended in 100 mM Kcl pH 7 and kept frozen in liquid nitrogen. Electron micrographs (10) show that a large fraction of the vesicles thus obtained is about 0.5 µm diameter.

Bleaching is achieved by means of an electronic flash (Sunpak Auto Zoom 3400), having a lifetime of 3 ms.

Proton uptake measurement - The light-induced pH rise of samples containing 10 to 30 nmol rhodopsin (100 mM KCl, 0.5-2mM Tris-acetate) is recorded with a Radiometer pHM 64 pHmeter and REC 61 servograph recorder equiped with the REA 112 high sensitivity unit. After bleaching, several injections of 10 nmol HCl (10 µl of a 1 mM solution) are made in order to calibrate the signal . Total pH variation is in the order of a few hundredths of a pH unit. The low buffer concentration used allows small pH changes to be easily measured : the signal produced by 10 nmol HCl is at least 20 times larger than the noise. The number of rhodopsin molecules bleached is determined for each experiment from the 500 nm absorption (ε = 40 000) of aliquots of the bleached and unbleached suspension solubilised in 3 % Ammonyx LO (20°C.).

Absorption spectra and kinetic measurements at fixed wavelength are recorded with a Jobin-Yvon Duospac 203 spectrophotometer, using a suspension of fully bleached membranes as reference. The OD at 500 nm of the sample suspension (100 mM KCl, 2 mM Tris-acetate) is between 0.15 and 0.3 ; pH is measured in the cell at the temperature of the experiment. The amount of rhodopsin remaining after the flash is measured at the end of each experiment by adding 5 M hydroxylamine (pH 7) to a final concentration of 50 mM ; the cell is left at room temperature for at least 15 min before recording the spectrum, which is considered to be correct if the isosbestic point between rhodopsin and retinal oxime is situated at 405 ± 5 nm. When absorption changes are recorded during the flash, the photomultiplier is protected by incorporating crossed filters.

Rapid spectral measurements are carried out using a HARRICK rapid scan spectrophotometer connected to a HITEK A_1 signal averager as a transient recorder (11)

RESULTS

Protonation of Metarhodopsin II at 3°C

When membrane suspensions are bleached at 3°C, a rapid proton uptake is observed between pH 5 and pH 8 (Fig 1) ; no further change occurs after the initial pH rise.

Absorption spectra (Fig. 2) of membrane suspensions recorded from 5s to 30 min after the flash are identical, confirming that at 3°C metarhodopsin II does not decay into the subsequent intermediates.

The number of protons bound per rhodopsin bleached, and the proportion of metarhodopsin II formed at the equilibrium are plotted in

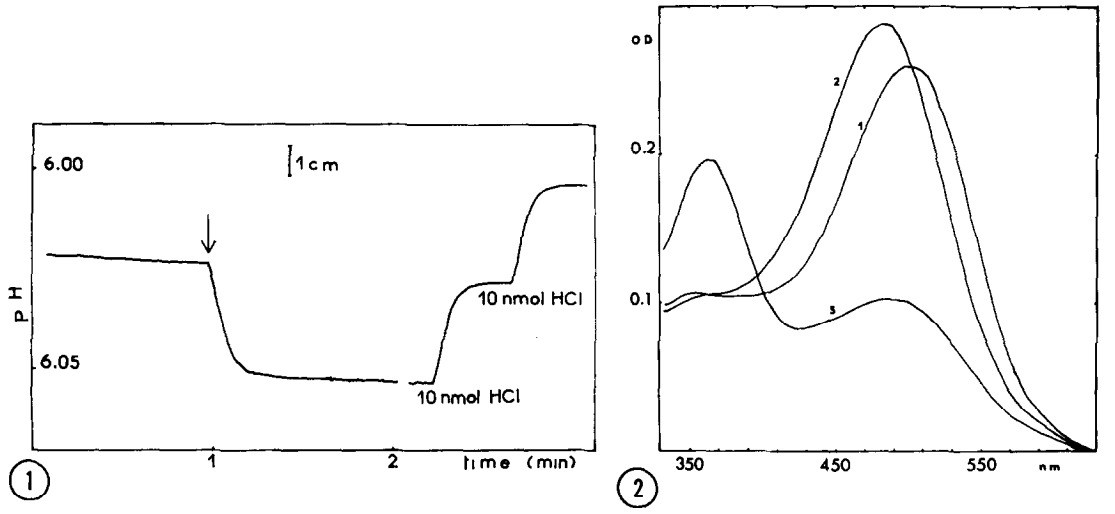


FIG. 1 : PHmeter recording of the light-induced proton uptake by rhodopsin in a suspension of rod disc membranes (100 mM KCl ; 1 mM Tris-acetate ; 3°C). The arrow indicates illumination by a 3 msec flash which bleached 9,4 nmoles rhodopsin. HCl injections are used to calibrate the proton signal.

FIG. 2 : Absorption spectra of a rod disc membrane suspension at 3°C, pH 8. A suspension of fully bleached membranes is used as reference. (1) unbleached membranes, (2) spectrum recorded after bleaching with a 3 msec flash (stable for at least 30 min.), (3) spectrum recorded after degradation of all intermediates by hydroxylamine.

This spectrum is considered to be the spectrum of metarhodopsin I with no metarhodopsin II, since Yoshizawa et al (20) have shown that metarhodopsin I has a β band at 350 nm, and the extinction coefficient measured on their absorption spectrum (ε^{MI}_{max} = 1.16 $\varepsilon^{Rhod.}_{max}$) is very close to the mean value obtained from 5 spectra of membranes bleached at pH 8 - 8.5 : ε^{MI}_{max} = 1.15 (± 0.02) ε^{Rhod}_{max} (maximum deviation).

Fig. 3 as a function of pH. Comparison of the two curves shows that 2 protons are bound per metarhodopsin II between pH 6 and pH 8. The divergence between the two curves below pH 6 might be due to partial protonation of rhodopsin before bleaching, since the isolectric point of bovine rhodopsin has been reported to be situated between pH 5.2 and pH 5.67 (3). The reason why the two curves again coincide at pH 5 is however unclear.

Protonation of metarhodopsin II at 20°C and 37°C

It has been shown that above 3°C in digitonin solution, metarhodopsin II decays into metarhodopsin III (absorbing at 465 nm) with proton release (12). In order to correct possible interference between the formation and the decay of metarhodopsin II, we have also carried out spectral and pH studies of metarhodopsin II decay ; complete results will be reported in a subsequent paper (13).

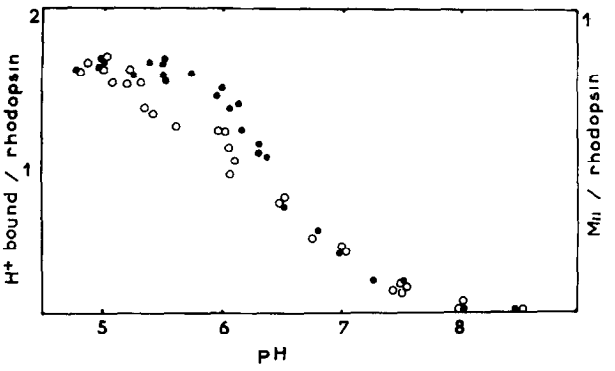


FIG. 3 : pH dependance of the light-induced proton uptake and of the metarhodopsin I - metarhodopsin II equilibrium at 3°C.
(o) number of protons bound per rhodopsin bleached
(•) proportion of metarhodopsin II : MII/rhodopsin bleached = 1-MI/rhodopsin bleached. The absorption at 480 nm due to metarhodopsin I is obtained by subtracting the spectrum recorded after addition of hydroxylamine (i.e. the amount of rhodopsin remaining after the flash) from the spectrum recorded after bleaching, and the proportion of metarhodopsin I is calculated using $\varepsilon^{MI} = 1.15\ \varepsilon^{rhod}$ (Fig. 2).

The proton release process associated with the decay of metarhodopsin II is too slow at 20°C to modify the initial proton uptake associated with its formation (response time of the electrode at this temperature : 6 ± 1 s) ; the experimental points are therefore plotted in Fig. 4 without correction. Corrected values of the proton uptake measured at 37°C are calculated by exponential extrapolation of the proton release process back to the time at which the flash was triggered.

The amount of metarhodopsin II formed at the equilibrium as a function of pH, at 20°C and 37°C, is calculated from absorption changes at 480 nm or 380 nm. In order to assure that the amount of metarhodopsin III formed during the dead time of the spectrophotometer (2 s) can be neglected, a series of experiments has been carried out at 37°C, using a rapid spectrophotometer : between pH 5 and pH 9, the absorbance at 380 nm is found to be constant within 2 % from 10 ms to 2 s after a flash of 1 ms duration.

Fig. 4 shows that, whereas the number of protons bound per rhodopsin is similar at 3°C, 20°C and 37°C, the amount of metarhodopsin II formed at the equilibrium increases with temperature. Comparison of the two parts of the Fig. 4 demonstrates that at 20°C and 37°C, metarhodopsin II is mainly diprotonated at pH 5, while it is mainly unprotonated above

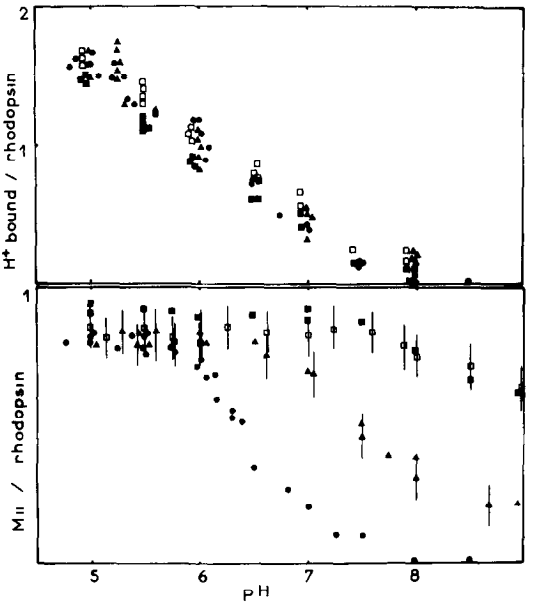


FIG. 4 : Influence of temperature on the light-induced proton uptake and on the metarhodopsin I - metarhodopsin II equilibrium.

Number of protons bound per rhodopsin bleached : (●), 3°C ; (▲), 20°C ; (■), 37°C, measured value ; (□), 37°C, corrected value calculated by extrapolating the proton release process associated with the decay of metarhodopsin II back to the trigger of the flash. Proportion of metarhodopsin II formed at the equilibrium : (●), 3°C, and (▲), 20°C, from absorption spectra as described in Fig. 2 ; (■), 37°C, from the absorption decrease at 480 nm using $\varepsilon^{MI}_{max} = 1{,}15\ \varepsilon^{Rhod}_{max}$; (Δ), 20°C, and (□), 37°C, from the absorption increase at 380 nm as described below (error bars mainly correspond to the uncertainty in the value of ε^{MII}_{max}).

The proportion of metarhodopsin II formed is :

$$\text{MII/rhodopsin bleached} : b \times \varepsilon^{Rhod}_{max}/R \times \varepsilon^{MII}_{max}$$

where R is the decrease at 500 nm corresponding to bleached rhodopsin, and b is the absorption at 380 nm corresponding to metarhodopsin II formed ($b = A^{total}_{380} - A^{MI}_{380} - A^{remaining\ rhodopsin}_{380}$).

b is expressed as a function of R and a (absorption increase measured at 380 nm between the bleached and unbleached sample), using the following approximations : $\varepsilon^{MI}_{380} \# 1.15\ \varepsilon^{Rhod}_{380}$ (from spectra of metarhodopsin I), and $\varepsilon^{Rhod}_{380} = 0.19\ (\pm\ 0.01)\ \varepsilon^{Rhod}_{max}$ (from 12 different preparations of delipidised rhodopsin (21)).

A mean value of ε^{MII}_{max} has been estimated from 15 spectra of membrane suspensions bleached at 3°C and pH 5-6 (the proportion of metarhodopsin II being calculated from the proportion of metarhodopsin I) :
$\varepsilon^{MII}_{max} = 1.23\ (\pm\ 0.07)\ \varepsilon^{Rhod}_{max}$ (maximum deviation).

Replacing b and ε^{MII}_{max} gives :

$$\text{MII/rhodopsin bleached} = 0{,}99\ \frac{a}{R}\ (1\text{-}0{,}03\ \frac{R}{a})\ \pm\ 0{,}08\ \frac{a}{R}\ (1\text{-}0{,}03\frac{R}{a})$$

pH 8 ; it is present in at least these two forms at intermediate pH.

Ability of the protonated and unprotonated metarhodopsin II to revert to metarhodopsin I

It has been previously reported (1) that in digitonin solution, raising the pH or lowering the temperature shifts the metarhodopsin I - metarhodopsin II equilibrium back towards metarhodopsin I.

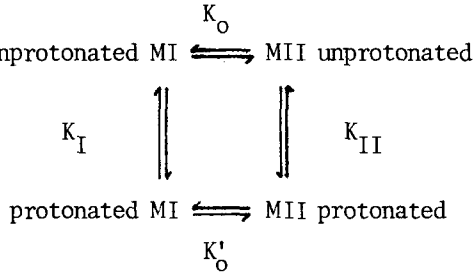
Indeed, when the pH of a sample bleached at 3°C and acid pH (intermediates observed : metarhodopsin I and diprotonated metarhodopsin II) is raised to pH 8, a spectrum similar to that of pure metarhodopsin I is recorded. When the temperature of a sample bleached at 20°C or 37°C at pH 8 (intermediates observed : metarhodopsin I and unprotonated metarhodopsin II) is rapidly lowered to 3°C, cooling being initiated ten seconds after the flash, a spectrum of pure metarhodopsin I is also obtained. Both protonated and unprotonated metarhodopsin II are therefore directly or indirectly in reversible, pH or temperature dependent, equilibrium with metarhodopsin I.

If membranes bleached at pH 8 and 37°C are incubated several minutes at 37°C before being cooled, in order to allow a certain amount of retinal to be formed, then an absorption band at 380 nm is present in the spectrum recorded at 3°C. This indicates that retinal + opsin does not revert to metarhodopsin I, and therefore demonstrates that retinal does not contribute to the absorption measured at 380 nm until at least ten seconds after a flash at 37°C.

DISCUSSION

We have shown that, at 3°C, metarhodopsin I and metarhodopsin II are in a stable pH dependent equilibrium ; two protons are bound per metarhodopsin II formed, with apparent pK 6.1-6.25. At 20°C and 37°C, metarhodopsin II also exists in an unprotonated form, which is in temperature dependent equilibrium with metarhodopsin I and/or protonated metarhodopsin II ; the metarhodopsin I - metarhodopsin II equilibrium is not completely shifted towards metarhodopsin II, even at 37°C. Since all measurements have been made at the equilibrium, it is not possible to derive from our data which of the two changes (protonation and spectral shift) is the first to take place. In both cases, the unprotonated form of metarhodopsin II is expected to be favoured at high temperature, since both the decay of metarhodopsin I and the dissociation of protons from acid-base groups of amino acids (14) are endothermic reactions ; using the diagram below as a reaction model, it is indeed possible to reproduce the effect of temperature, i.e. to increase the amount of metarhodopsin II formed at a given pH while

the number of protons bound per rhodopsin remains approximately constant, by decreasing K_o or K'_o, or increasing K_{II} (with $K_I > 10^{-5}$).



$K_o = \frac{MI}{MII}$ (unprotonated)

$K'_o = \frac{MI}{MII}$ (protonated)

K_I, K_{II} : acid dissociation constants

It is usually assumed that metarhodopsin II only binds one proton ; however, quantitative measurements of the proton uptake have not always been accompanied by measurements of the amount of metarhodopsin II formed in the same conditions, and when they have, the possibility that metarhodopsin II could exist in differently protonated forms was not considered. The proton uptake was first observed by Radding and Wald (3) upon bleaching digitonin solubilised rhodopsin, at a time when metarhodopsin II had not yet been characterized : they report that the proton uptake per rhodopsin bleached at 20°C as a function of pH corresponds to the titration of one proton site per rhodopsin. Wong and Ostroy (6) also report a value of 0.96-1.04 proton per metarhodopsin II in digitonin at pH 4.5, -15°C. The digitonin micelle does not however provide the rhodopsin molecule with exactly the same environment as the lipidic membrane, as is suggested by the fact that the decay of metarhodopsin I into metarhodopsin II in digitonin micelles is slowed down by complete removal of phospholipid (15) ; it seems therefore possible that solubilisation induces modifications of the equilibrium constants, which can result in a modification of the number of protons bound per rhodopsin bleached or per total metarhodopsin II at a given pH. Similarly, the difference between the various reports on the proton uptake by membrane-bound rhodopsin could be due to the influence of the experimental conditions (in particular : ionic strength and ionic gradients across the membrane) on the equilibrium constants. Mc Connel (5) reports that the number of protons bound per rhodopsin bleached in rod disc fragments varies from 0.7 to 1.7 at pH 5.5, depending on the composition of the medium ; under conditions similar to ours (100 mM KCl or NaCl, pH 5.5, 25°C), 1.2 protons are taken up per rhodopsin (compared to 1.3). The apparent discrepancy between the calorimetric measurement reported by Cooper and Converse (8) (1 proton per rhodopsin at 3°C,pH 5.4) and our results (1.3 protons per rhodopsin at 3°C ; pH 5.4) might therefore be due to the use of a different medium (100 mM acetate instead of 100 mM KCl, 0.5 mM

tris-acetate). Another measurement has been made by Emrich (4) on intact rods ; although the temperature of the experiment is not indicated, it is interesting to note that in some cases he found an uptake of 2-3 protons per metarhodopsin II.

Although the existence of several forms of metarhodopsin II might be apparent from kinetic studies, published reports on metarhodopsin I decay are somewhat conflicting : while some authors find two rate constants (15) (16), others describe metarhodopsin I decay as a simple first order process (17) (18). It is however possible that under some experimental conditions, one of the rate constants is too small to be detected. In agreement with our work, Emrich (4) finds two rate constants in rod outer segments at 23°C while only one at 2°C from which he deduces the existence of an activated form of metarhodopsin II, postulated as being unprotonated, as an intermediate between metarhodopsin I and protonated metarhodopsin II.

Finally, it can be suggested that the existence of a diprotonated and an unprotonated form of metarhodopsin II seems to exclude the possibility that the proton associated with the Schiff base linkage in metarhodopsin I be released during the metarhodopsin I - metarhodopsin II transition. It seems more likely that the Schiff base proton is transferred to another proton site within the protein, as already suggested (19) (8).

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