SHIFT IN THE RELATION BETWEEN FLASH-INDUCED METARHODOPSIN I AND METARHODOPSIN II WITHIN THE FIRST 10% RHODOPSIN BLEACHING IN BOVINE DISC MEMBRANES

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1. Introduction

In the light-induced reaction chain of vertebrate rhodopsin, the transition from metarhodopsin I (M I)[+] to metarhodopsin II (M II) is the last reaction which is fast enough to be involved in visual transduction. This reaction, on the other hand, is the first which is drastically influenced by intermolecular interactions of the rhodopsin molecule. The reaction only takes place in the presence of water [1], rhodopsin becomes protonated [2] and detergents have a pronounced effect on the reaction kinetics [3]. After thorough bleaching, M II is in a pH- and temperature-dependent equilibrium with M I [4]; equal amounts of M I and M II are found at pH 7, $T = 3°C$. High temperature and low pH favour M II. A similar pH- and temperature-dependent equilibrium is also found in rod outer segments (ROS) [5]. The equilibrium between the species is also dependent on the outer pressure [6] and the membrane surface potential [7]. It can be shifted by pressure jumps [8] and by T-jumps [9]. The kinetics of the shift is then comparable to the light-induced rise of M II.

The equilibrium can be taken as a further monitor of the intermolecular sensitivity of the rhodopsin molecule in the metarhodopsin stages.

The light-induced production of M II shall be used in this study as an indicator of the state of the disc membrane when only a few percent of light-transformed rhodopsin are present in it (bleaching <10%). Evidence will be provided that the 'normal' dependence of the equilibrium on pH and temperature is only found for bleachings >10%.

Abbreviations: M I, metarhodopsin I; M II, metarhodopsin II; ROS, rod outer segments

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2. Materials and methods

Cattle rod outer segments were prepared according to a standard procedure. Briefly, the retinae are shaken in isotonic saline [130 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM (ethylene dinitrilo)–tetraacetic acid (EDTA), 1 mM dithiothreitrol (DTT), 10 mM Piperazine–1,4-diethane sulfonic acid (Pipes)], the suspension is then filtered through a nylon mesh, layered on a discontinuous sucrose gradient and washed in saline. In the experiments of this study fresh and rapidly N_2-frozen samples yielded the same results. Isolated discs were prepared according to [10]. 2.5% (w/v) Ficoll was used instead of 5% to minimize side effects [11].

The apparatus for the simultaneous measurement of pure absorption changes and of near-infrared scattering changes is described in more detail in [12]. It consists of a fast two-wavelength absorption spectrophotometer and a near-infrared scattering device arranged around the same sample. Using this system, scattering artefacts in the absorption signals are no longer detectable in the case of ROS. In the case of isolated discs, some scattering difference remains, due to the considerable scattering at higher angles, which cannot be compensated completely by high aperture optics.

The dependence of the M II-yield on illumination will be investigated by stepwise photolysis of the system (exhaustion experiment [12]). The M II indi-

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cating 387/417 nm absorption difference is thereby measured in series of flashes. Every flash serves to evoke a signal and bleaches the system one further step. The use of exhaustion curves will be briefly explained.

A flash of the intensity I_f transforms a certain amount of rhodopsin which is proportional to the amount of unbleached rhodopsin R:

$$\Delta R = C_R \,.\, R \,.\, I_f \qquad (1)$$

From eq. (1) it follows that in a series of equal flashes of intensity I_f the unbleached rhodopsin content decays exponentially:

$$R = R_0 \,.\, \exp\,(-c_R \,.\, N \,.\, I_f) \qquad (2)$$

where R_0 is the initial rhodopsin concentration and N the flash number. The validity of eq. (2), that means especially the constancy of the excitation factor c_R, was shown for bovine ROS suspensions in [13]. The amount of M II produced by one flash will be proportional to the amount of rhodopsin still present:

$$\text{M II} = \epsilon_{\text{M II}} \,.\, Y_{\text{M II}} \,.\, R \qquad (3)$$

For the absorption coefficient $\epsilon_{\text{M II}}$, no dependence on physicochemical parameters is described in the literature. For $Y_{\text{M II}}$, the yield of M II, however, a dependence on pH and temperature is expected according to the equilibrium between M I and M II [4,5].

Under the assumption that the quantities $Y_{\text{M II}}$ and $\epsilon_{\text{M II}}$ are not dependent on illumination, all exhaustion curves will be linear in the semilogarithmic representation according to the exponential dependence of R on the flash number (eq. (2)). The straight lines for different pH and temperature are expected

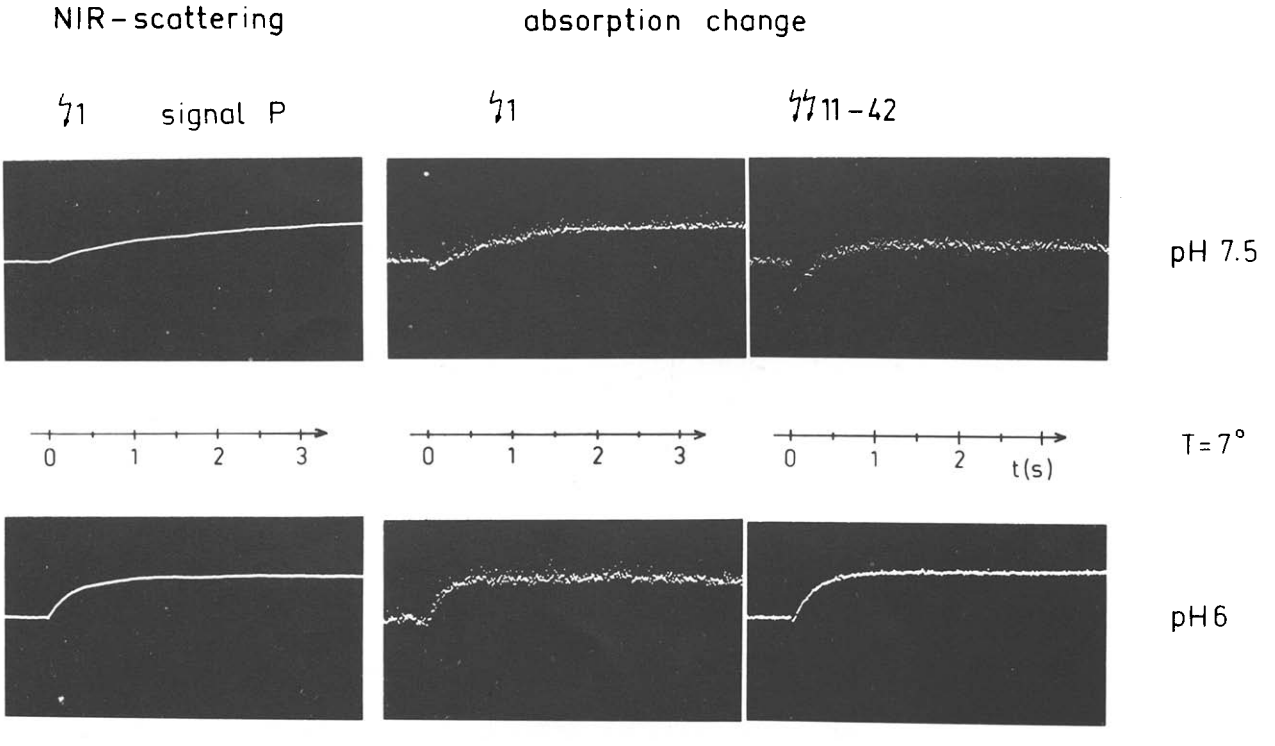
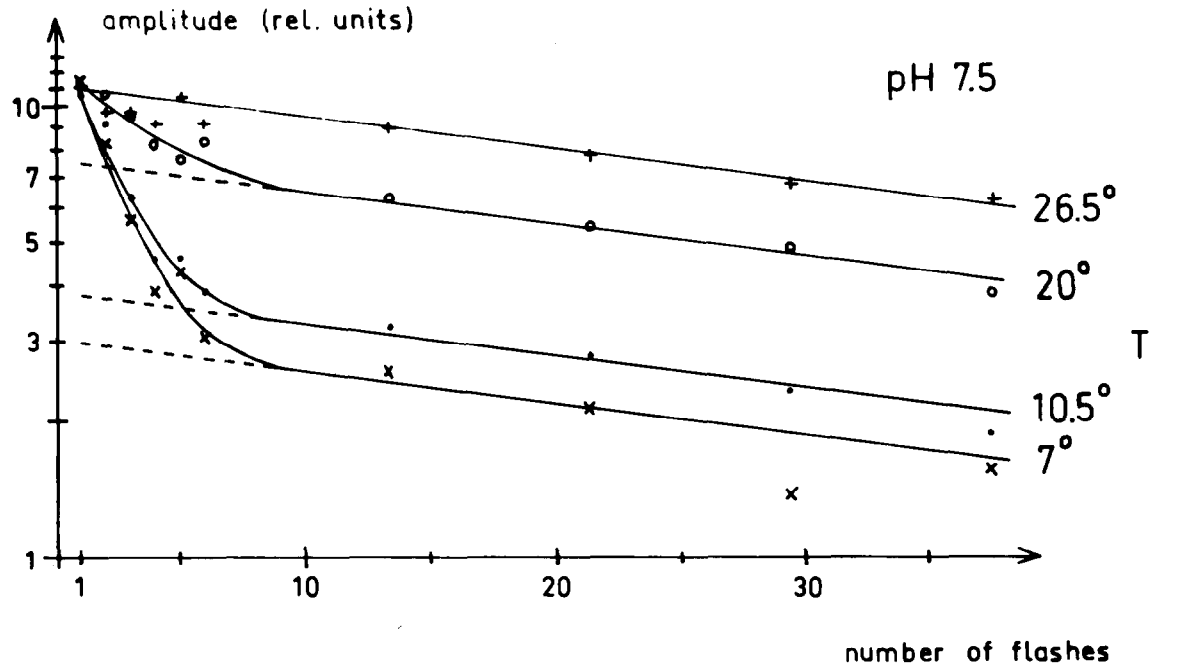


Fig.1. Flash-induced near-infrared scattering and absorption signals in rod outer segments. Flash applied at $t = 0$, amplitudes are normalized to the same value (amplitudes are plotted in fig.2). Scattering signals are the relative intensity changes at the measuring wavelength $\lambda = 800$ nm and in an angular range $6.5° < \theta < 23°$; absorption signals are the absorption changes, i.e., the negative difference between the relative intensity changes at 387 nm and 417 nm. The signals in the first and in the second line belong to 2 measurements on 2 different samples; the scattering signal P and the left absorption signal are a simultaneous measurement from the first flash, the absorption signal at the right is an average of flashes no. 11–42. See text for the kinetic comparison of the signals. Measuring parameters as indicated in the figure; relative rhodopsin turnover by one flash $\rho = 0.02$.



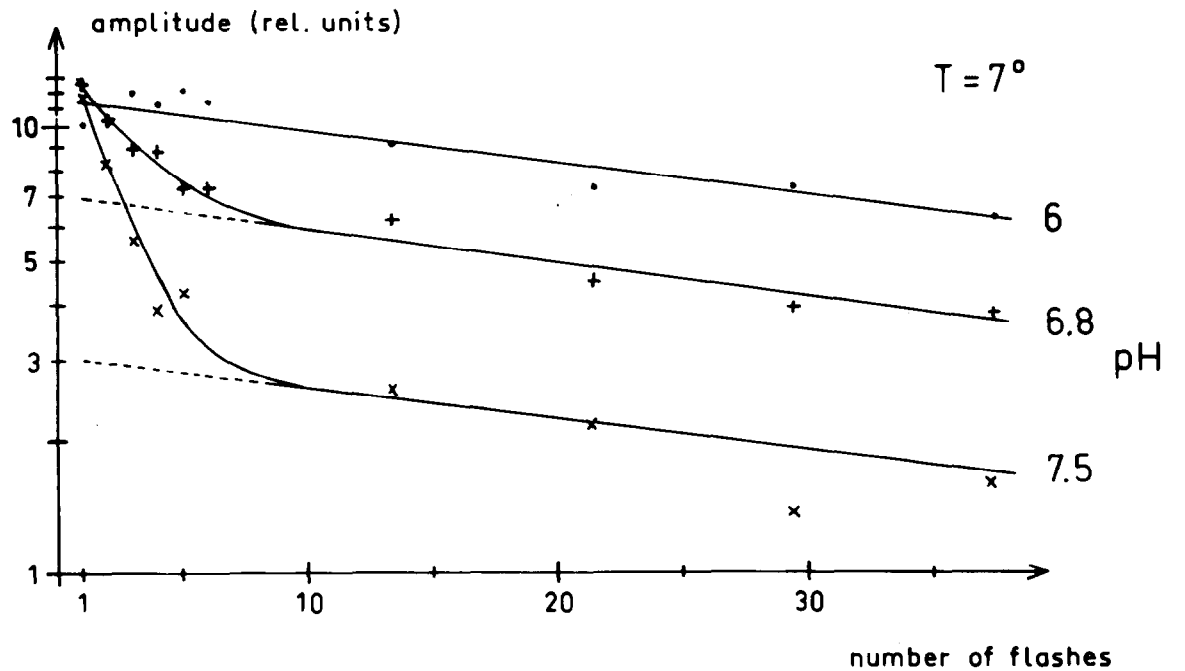


Fig.2. Successive photolysis of rod outer segment (ROS) suspensions in series of flashes (exhaustion curves). Every flash bleaches the system a further step; the amplitude of the flash induced signals are plotted as single recordings (flash no. 1–7) resp. as the average of groups of 8 flashes (higher bleaching). Signals are defined as in fig.1, measuring parameters as indicated in the figure; relative rhodopsin turnover by one flash $\rho = 0.02$.

to be parallel, shifted to the lower amplitudes, the smaller the yield of M II. If, however, the M II-yield is dependent on illumination, a curved exhaustion must be found.

3. Results

The flash-induced change in the absorption difference between 387 and 417 nm and the scattering change at 800 nm were measured simultaneously in series of flashes. Every flash bleaches the system a further step (exhaustion experiment, section 2).

In fig.1, scattering and absorption signals are shown for 7°C, for two different pH-values and in different stages of bleaching. It is seen that, at pH 6, the scattering signal *P* and the absorption signals for the first and the later flashes are kinetically similar. At pH 7.5, however, the scattering and absorption signals from the first flash are comparable to each other but they are both significantly slower than the absorption signal in the higher bleaching stage. This latter signal displays the normal kinetics of the M I/II transition at this pH and temperature [14].

The amplitude exhaustion curves (section 2) are shown in fig.2. All curves start at the same amplitude value and approximate to a linear slope at ~10% bleaching. For all pH-values and temperatures, the slope in the non-linear part of the curves is similar to that of the simultaneously measured scattering signal (not shown). A measure for the deviation of the exhaustion curves from the linear slope (the non-linear absorption superposition) is given by the intercept on the ordinate; it is the smaller the more the bulk milieu favors M II (low pH, high temperature).

The kinetic similarity and the parallel exhaustion of the scattering signal *P* and of the non-linear absorption superposition could suggest that (in spite of the instrumental precautions) scattering changes contribute to the latter. All known scattering signals, however, when measured in the near-infrared, display dependencies on temperature and pH which are in contrast to the non-linear absorption superposition; there is especially no signal which totally disappears at high temperature and low pH (compare the signal *P* in fig.1 under these conditions).

As a more direct proof, the spectral dependence of the absorption signals was measured. In fig.3, the non-linear absorption superposition is plotted as a function of the measuring wavelength for pH 7.5,

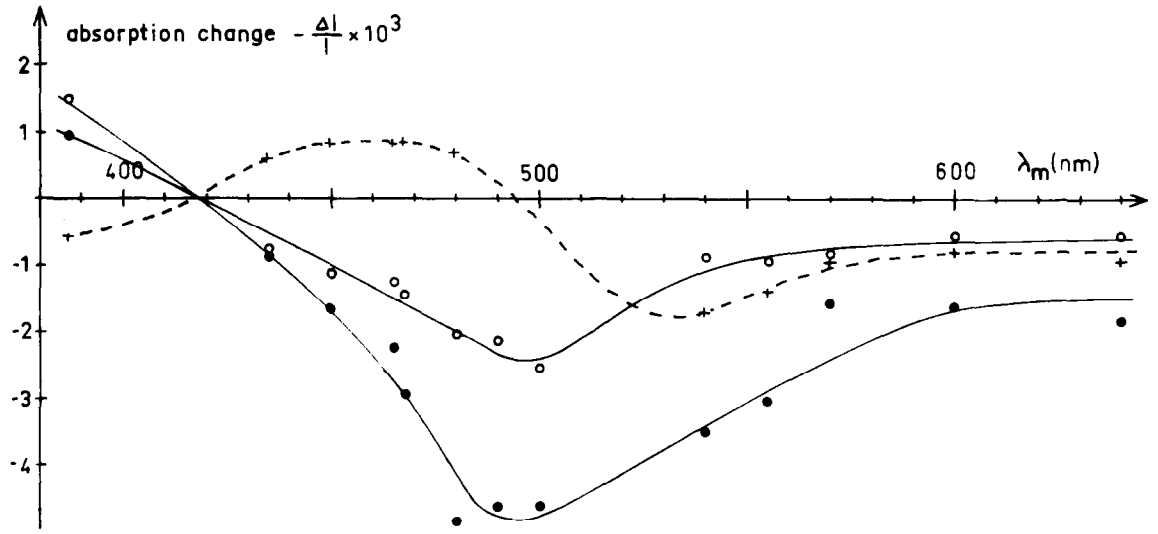


Fig.3. Spectral dependence of absorption signals in rod outer segments. The plot represents the absorption changes, i.e., the negative difference between the relative intensity changes at the wavelength indicated on the abscissa and at 417 nm. Three different signals are plotted: (+) Initial jump, due to the (not time-resolved) rise of M I; according to the absorption difference measurement with the reference beam at 417 nm, the jump is normalized to zero at this wavelength. The maxima and minima coincide with the normal difference spectrum; (○) averaged signal amplitide for flashes number 11–42; (●) signal amplitude for the first flash. Measuring parameters: 10°C, pH 7.5; optical bandwidth 10 nm.

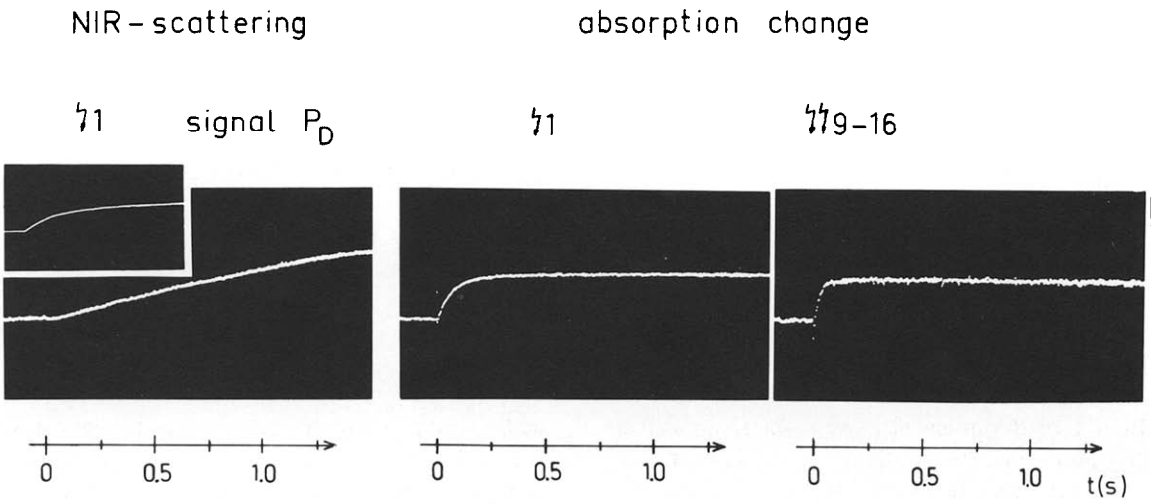


Fig.4. Flash-induced near-infrared scattering and absorption signals in isolated disc vesicles. Flash applied at $t = 0$, all amplitudes are normalized to the same value (amplitudes are plotted in fig.5). Scattering and absorption signals are defined as in fig.1. In analogy to fig.1, the scattering signal P_D and the first absorption signal are a simultaneous measurement from the first flash of a series, the absorption signal at the right is an average of the flashes no. 9–16. Measuring parameters as in fig.1. It is seen that, in contrast to fig.1, the scattering signal is much slower in this system (full time scale of P_D in the inset: 8 s).

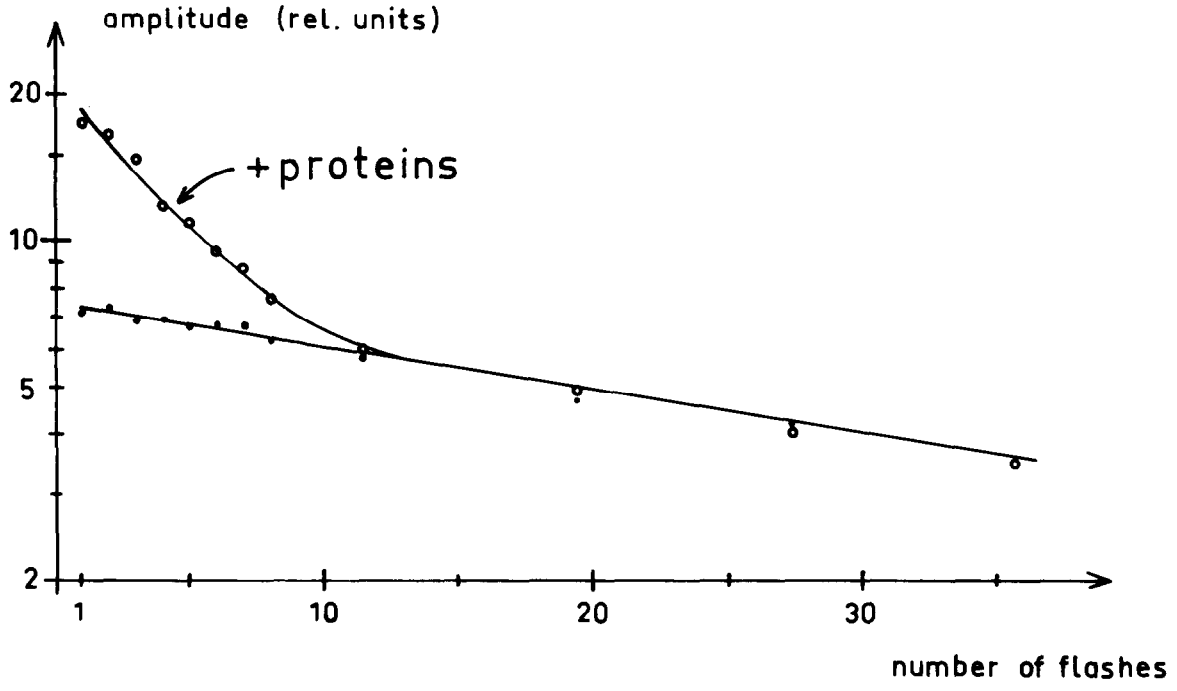


Fig.5. Successive photolysis of isolated disc suspension in series of flashes (exhaustion curve). Representation as in fig.2. Measuring parameters: pH = 8, $T = 19°C$. The lower curve shows a measurement on pure isolated discs. In the upper curve, the total fraction of peripheral proteins (extracted during the disc preparation at low ionic strength) is added to the sample after reconstitution of the isotonic saline.

$T = 10°C$. One of the absorption beams thereby remained at 417 nm, the other was adjusted to the wavelength indicated on the abscissa. It is seen that the spectral dependence of the non-linear absorption superposition matches the M I/II difference spectrum. The kinetics of the non-linear superposition as well as that of the signals in the linear range are spectrally invariant. An essential contribution of scattering artefacts and an effect solely on $\epsilon_{M\ II}$ (eq. (3)) is thereby excluded.

The same measurement as with ROS (fig.1,2) was also performed on isolated discs, prepared as above and recombined with the peripheral proteins [15] (fig.4). As seen in fig.5, qualitatively the same non-linear exhaustion as in the intact disc stacks of ROS is observed in this system. Without recombination of the protein fraction, only a very small or no deviation from the linear slope is found. Like in ROS, the exhaustion of the simultaneously measured near-infrared scattering signal (P_D in our nomenclature, [16]) is similar to that of the non-linear absorption superposition. As in ROS, the absorption signals in the non-linear exhaustion range are considerably slower than those in the linear range. In this case, however, all absorption signals are much faster than the scattering signal P_D (fig.4).

4. Discussion

The results show that the first light flash applied on disc membranes prepared under dim red light produces essentially only metarhodopsin II (M II). With increasing bleaching of the system, more metarhodopsin I (M I) is formed at the expense of M II, until the normal equilibrium fraction of M II is reached for a bleaching of >10%. The present data cannot decide whether the entity of M II molecules produced in the non-linear part of the exhaustion curve participates in a (totally shifted) equilibrium with M I. The alternative would be that the M II formed in excess of the normal equilibrium amount (extra M II) represents a separate population. The kinetics can, so far, not be measured with sufficient precision to recognize two eventual different populations by the slow and fast kinetic components which they must contribute to a signal at $T = 7°C$, pH 7.5 and a bleaching of a few percent.

Notwithstanding this question, the data show that the light-induced transition to M II is a practically irreversible step in the physiologically relevant low bleaching state of the disc membrane.

A screening of the extra-M II molecules against the influences forcing them to M I could be caused by tight binding of M II to another molecule. Support for this explanation seems to come from the similar exhaustion of the extra-M II and of the near-infrared scattering signal P_D. This signal is interpreted by a polarisability change [16] and indicates most probably a binding of M II to the GTPase [16,17]. It is true that the extra-M II is drastically diminished in the absence of recombined proteins (fig.5) and the protein fraction therefore must contain some determining factor for the existence of extra-M II. The binding seen in the signal P_D can, however, not be the actual reason for the extra-M II, because there is a kinetic contradiction: the formation of the extra-M II is much faster than the signal P_D (fig.4). Thus, the screening of the extra-M II must be established before the binding reaction observed in P_D takes place.

An additional indicator for the screening mechanism might be given by the signal P (fig.1) which is comparable (not only in its exhaustion but also in its kinetics) to the formation of extra-M II. This signal is caused by an axial and radial shrinkage of the disc stack [16]. The protein factors possibly involved in these shrinkage effects are currently under investigation.

The practical conclusion of this study is that data on M II must be specified with respect to bleaching.

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