

Proton Uptake by Light Induced Interaction between Rhodopsin and G-Protein

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The light-induced proton uptake of rod outer segment disc membranes has been investigated in the absence and presence of G-protein. Proton uptake was measured as the alkalisation of the suspending medium using a pH electrode and/or the indicator dye bromocresol purple. It was found that besides the known proton uptake of photolysed rhodopsin additional uptake of one proton accompanies formation of the complex between rhodopsin and G-protein. No measurable proton uptake was found under conditions of rapid redissociation of the complex indicating an only transient protonation during its lifetime. Proton uptake was the same in washed membranes recombined with G-protein and in ordinarily stacked rod outer segments. The additional proton uptake reported here is not due to enhanced formation of the protonated photoproduct metarhodopsin II.

Introduction

Light absorption in vertebrate rhodopsin is followed by very rapid (ps) isomerisation of the chromophore 11 *cis* retinal, and subsequent shifts of the absorption spectrum (ps to ms) related to the thermal decay over a number of photoproducts [1]. The blue-shifted product metarhodopsin II (MII, 380 nm) which arises in a few ms is the one in which the Schiff base linkage of retinal to lysine becomes deprotonated [2]. MII remains in a pH and temperature dependent equilibrium with MI [3], while slowly decaying to MIII. In the time domain of the internal deprotonation of the Schiff base, rhodopsin performs proton uptake from the external aqueous phase [4–7]. There are several studies comparing the kinetics and stoichiometry of the protonation with the formation of MII (as spectrally defined) [7–14].

A maximal uptake of one proton per bleached rhodopsin molecule was found in digitonin solutions [12], whereas the reported stoichiometries of bound protons for membrane bound rhodopsin are still in disagreement [7–10, 14].

At the time of these studies the interdependence between photochemical reactions and peripheral proteins was still unknown. In the meantime it has been established that G-protein is bound to photoexcited

rhodopsin with a stoichiometry of 1:1 [15] and that the binding conformation of rhodopsin coincides with the photoproduct MII [16]. Interaction with the G-protein is strong enough to shift the MI/II equilibrium considerably towards MII [16–18]. Further studies dealt with the stabilisation of the spectrally defined MII [19, 20] and showed that the chromophore microenvironment and binding site for G remain in mutual interaction during the lifetime of the R-G complex.

These recent results stimulated the present reinvestigation of the light-induced rhodopsin protonation which takes into account the influence of G-protein binding and GTP-induced dissociation.

The measurements were made on ROS and on isolated disc membranes, reconstituted with purified G-protein.

Materials and Methods

Bovine *rod outer segments* (ROS) were prepared according to a standard procedure [17]. The retinæ were shaken in isotonic saline (113 mM KCl, 0.5 mM MgCl₂, 1.5 mM CaCl₂, 1 mM dithiothreitol (DTT), 0.5 mM ethylen-diamine tetraacetic acid (EDTA), 15 mM phosphate buffer (pH 7.2)) and filtered through a nylon mesh. The resulting crude suspension was layered on a discontinuous sucrose gradient and washed in buffered saline (124 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 1 mM DTT, 0.5 mM EDTA, 15 mM 2-morpholino ethane sulphonic acid (MES)). After a second washing step in unbuffered saline (140 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 1 mM

Abbreviations: ROS, rod outer segments; G-protein (G), GTP-binding protein; MI, metarhodopsin I; MII, metarhodopsin II; NIR, near-infrared; R, rhodopsin; R*, photoexcited rhodopsin.

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DTT, 0.5 mM EDTA) the suspension was frozen in liquid nitrogen.

"Washed membranes" (WM), divested of G-protein [21], were obtained by gently homogenising and twice washing fresh ROS in hypotonic saline (5 mM KCl, 1 mM DTT, 0.5 mM EDTA). The supernatant of the first centrifugation step contains the G-protein among other polypeptides [15] and is termed "total extract".

Purified G-protein was obtained from ROS using the method of Kühn [22]. Excess GTP was removed on a Sephadex G-25 column. Buffer was replaced by unbuffered saline (5 mM KCl, 1 mM DTT, 1 mM EDTA) by dialysis (12 h, 4 °C). Purity of the G-protein was established by SDS gel electrophoresis as in [16].

WM suspensions and G-protein solutions were stored in liquid N₂.

The pH variations were measured by means of a pH electrode or by indicator dye absorption changes.

For the *electrode measurements*, the pH electrode (Amagross pH-C-1018) amplified output fed into to a digital oscilloscope (Nicolet 2090-IIIa) controlled by a microcomputer (Commodore CBM 8032). All electrode measurements were made in isotonic saline (140 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 1 mM DTT, 0.5 mM EDTA).

The *optical measurements* (indicator and MII absorption and scattering signals respectively) were performed using the same apparatus as in [16, 17]. This instrument is a fast two wavelength spectrometer which allows a simultaneous measurement of scattering changes. It has been described in detail in [23]. Indicator absorption changes were measured as absorption differences between 595 nm and 650 nm using the two wavelength system. To obtain pure Δ pH-records free of contaminations by photoproduct absorption changes, two indicator measurements were performed on an unbuffered and on a pH-buffered aliquot, the difference representing the pH changes. All spectroscopy on ROS was done in hypotonic saline (35 mM KCl, 0.125 mM MgCl₂, 0.25 mM CaCl₂, 0.25 mM DTT, 0.125 mM EDTA) to obtain osmotically swollen ROS and a better access for the pH indicator. At this ionic strength G already recombines completely with the membrane. Bromocresol purple was used as pH indicator dye in a concentration of 10 μ M. Buffered samples were obtained by adding 25 mM MES (2-morpholino ethane sulphonic acid). The ROS samples which had lost part of the

peripheral proteins due the necessary washing steps were enriched with "total extract". G-protein was determined as ca. 10% (mol/mol) of rhodopsin via the saturation of the NIR scattering signal [24, 16].

All Δ pH measurements were performed under conditions (pH 6, 22 °C) where the MI/II equilibrium is shifted virtually completely towards MII. This avoids interference of the present measurements with enhanced formation of the protonated photoproduct MII [17].

Calibration of the indicator dye and electrode measurements was performed by titration with HCl or KOH to obtain the effective concentration changes of H⁺. Thereby the pH remained within 0.2 pH units, a range in which the buffer capacity can be considered to be constant. The buffer capacities of unbleached and bleached samples showed no detectable difference.

The flash intensity was measured for each flash and deviated less than $\pm 5\%$.

The use of stepwise photolysis (exhaustion) with a series of flashes was described in detail in [17]. Briefly, each flash transforms a fixed relative amount of rhodopsin. Thereby the amount of rhodopsin which is photoexcited per flash decreases exponentially and the rhodopsin turnover per flash is linear when plotted semilogarithmically against the flash number. Such a presentation of flash induced effects provides a sensitive test of whether they occur in a fixed proportion to rhodopsin turnover.

Results

The flash induced pH-change (Δ pH) in washed membranes (WM) and rod outer segment (ROS) suspensions was measured in a series of flashes, each bleaching stepwise 3.6% (in one case 6.7%) of the rhodopsin.

A typical Δ pH-record is shown in Fig. 1. The signal rises with the time constant of the apparatus. The level which is reached after 10 s corresponds to a persistent light induced alkalinisation.

In Fig. 2a the signal amplitudes after 10 s measured on the WM fractions (22 °C, pH 6) are plotted on a logarithmic scale against the flash number (exhaustion curve, see Materials and Methods). The linear slope of the exhaustion curve indicates a proton uptake proportional to rhodopsin turnover.

The proton uptake of photolysed rhodopsin is calibrated as follows: The absolute rhodopsin turnover

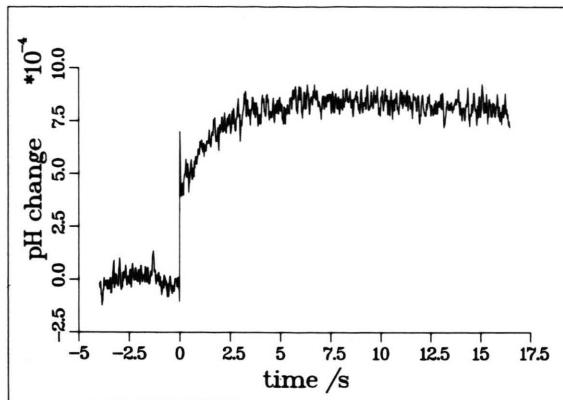


Fig. 1. Light induced pH change in a suspension of washed disk membranes reconstituted with purified G protein. The flash was applied at $t = 0$ and bleached 3.6% of rhodopsin (pH 6, $T = 22^\circ\text{C}$). The record is an average of 4 signals from a pH electrode.

of flash 1 was $0.036 \cdot c_{\text{Rhod}} = 0.036 \cdot 1.6 \cdot 10^{-6} \text{ mol/l} = 5.7 \cdot 10^{-8} \text{ mol/l}$. The related pH-change was $6.7 \cdot 10^{-4}$. Titration of the sample with $1 \mu\text{M}$ HCl increased the pH by $1.04 \pm 0.1 \cdot 10^{-2}$ units. Thus the netto proton uptake per photolysed rhodopsin is $1.1 \pm 0.1 \text{ H}^+/\text{R}^*$. It should be noted that transient higher protonation as described by Bennett [9] for WM would escape our observation due to the limited time resolution of the method.

The same protocol was applied to the recombined WM-G system. In this sample G protein was ca. 10% of rhodopsin (mol/mol) as determined by NIR scattering control measurements. The exhaustion of the ΔpH in this system (Fig. 2b) displays a non-linear slope, indicating an enhanced or additional and superimposed alkalisiation at the first two flashes. At later flashes the ΔpH per flash approximates a linear exhaustion curve with the same slope as that seen on the WM sample.

The stoichiometry of proton uptake per bleached rhodopsin was determined in the same way as described for WM samples. For flashes 3–20 a normal ratio of $0.95 \pm 0.1 \text{ H}^+/\text{R}^*$ was obtained. For flash 1, the calibration yields an uptake of 1.7 protons per bleached rhodopsin. A rough extrapolation yields an uptake of two protons per photoexcited rhodopsin at very low bleaching levels. ΔpH seen on the WM-G samples was generally lower than that in the WM samples, in agreement with the measured higher buffer capacity.

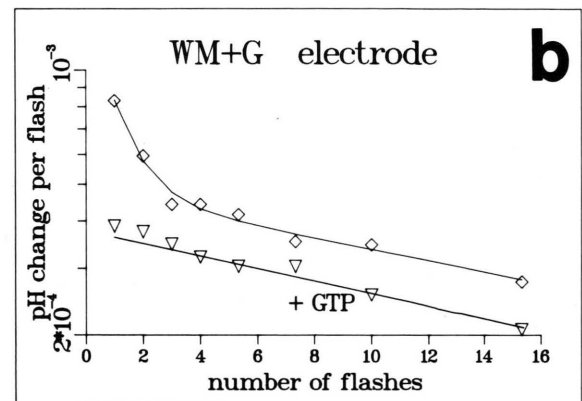
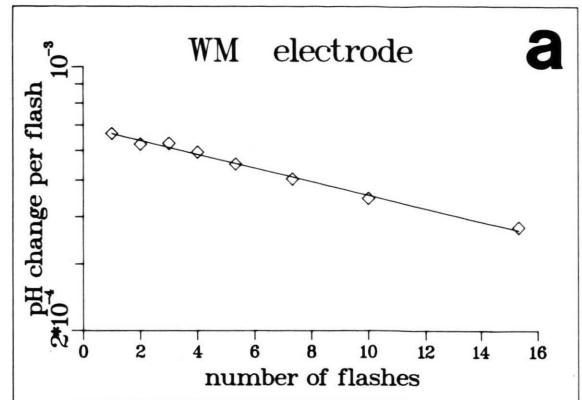


Fig. 2. pH changes from a succession of flashes on washed disk membranes (WM) using pH electrode. Each flash transformed 3.6% of rhodopsin into metarhodopsin II.

a) WM suspension

b) WM reconstituted with purified G protein.

(\diamond) without nucleotides,

(∇) with $100 \mu\text{M}$ GTP and $100 \mu\text{M}$ GDP. Each point represents the average of 4 measurements.

The range in which additional proton uptake occurs coincides with that of stable R-G complex formation. Thus it is strongly suggested that the protonated site is located in one of the interacting proteins.

In the presence of GTP the R-G complex is not stable [15]. The question arises as to whether or not the additional bound proton rapidly returns into solution, leading to transient or even immeasurable ΔpH . To answer this question ΔpH was measured on WM-G samples containing $100 \mu\text{M}$ GTP and $100 \mu\text{M}$ GDP. Under these conditions the R-G complex is formed and redissociates in ms [24]. This was verified by monitoring the dissociation signal [24] from an

aliquot (signal not shown). At later flashes neither dissociation signal nor binding signal was observed.

The exhaustion curve of this alkalinisation measurement is shown in Fig. 2b (lower trace). The linear slope of the curve shows the lack of enhanced proton uptake.

A control measurement with 100 μM GDP and without GTP displayed the undisturbed enhanced proton uptake (not shown).

In the presence of the protonophore dinitrophenol (500 μM , no nucleotides) the WM-G system showed a normal enhanced proton uptake as in Fig. 2b. This argues against a release of the proton into the disk lumen.

For comparison ΔpH was also measured on ROS using the electrode and the dye indicator bromocresol purple.

Indicator absorption changes measured on the buffered and unbuffered sample, and the difference between them are shown in Fig. 3. It is obvious that the optical method yields a much higher signal to noise ratio and time resolution than the electrode. However, one has to be very careful in applying the absorption method to WM suspensions because of the enormous scattering artefacts. In spite of the precautions built into the apparatus (two wavelength method, high apertures collecting the scattered light), the very broad scattering pattern of WM suspensions causes the relative intensity changes to be dominated by scattering signals. The indicator ΔpH signals from ROS, however, are only slightly contaminated by scattering artefacts. These are reliably eliminated by subtracting records of the unbuffered and buffered samples from each other.

The resulting exhaustion curves are shown in Figs. 4a and b. Nonlinear exhaustion curves are obtained by both methods, these are similar to that obtained with the reconstituted system.

The calibration of the measured ΔpH was done by analogy to the electrode measurements. The absorption changes were calibrated by titration with HCl and related to the signal amplitudes. A proton uptake of $0.9 \pm 0.1 \text{ H}^+/\text{R}^*$ was obtained at the last flashes whereas at the first flash 1.7 ± 0.2 protons are bound per photolysed rhodopsin molecule. These results are in agreement with the results obtained with the reconstituted system.

Control measurements of MII formation were carried out in the presence of indicator dye. The relative amount of MII formed was measured by absorption

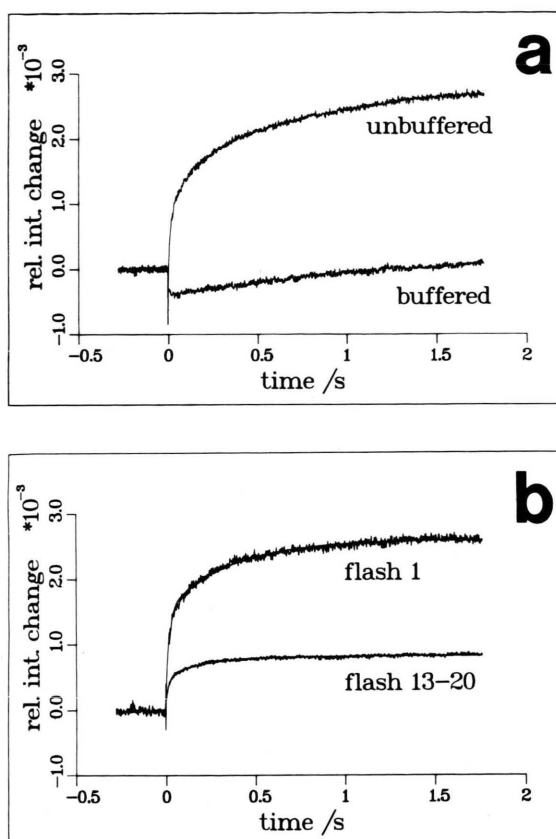


Fig. 3. a) Absorption changes of pH indicator dye (bromocresol purple 10 μM) evoked by the first flash on buffered and unbuffered rod outer segment suspensions (measured as absorption difference change (595/650 nm) by means of a two wavelength system [23]). b) Upper trace: difference between both signals in (a); lower trace: corresponding difference signal as average of the flashes 13–20. The solid line within the noise is the fit to a sum of two first order reactions.

signals (380/417 nm) and also plotted as an exhaustion curve in Fig. 4c. The linear slope of the curve indicates the lack of enhanced MII formation at pH 6 and 22 $^{\circ}\text{C}$, in agreement with previous investigations [17]. This allows the conclusion that the proton uptake reported here is not due to an enhanced MII formation.

The kinetics of the ΔpH indicator signals deviate considerably from first order, in agreement with observations of others [7–11]. Both the records from the first flash and from flashes 13–20 (Fig. 3b) can be fitted by a sum of two first order processes with slightly different time constants. Obviously the en-

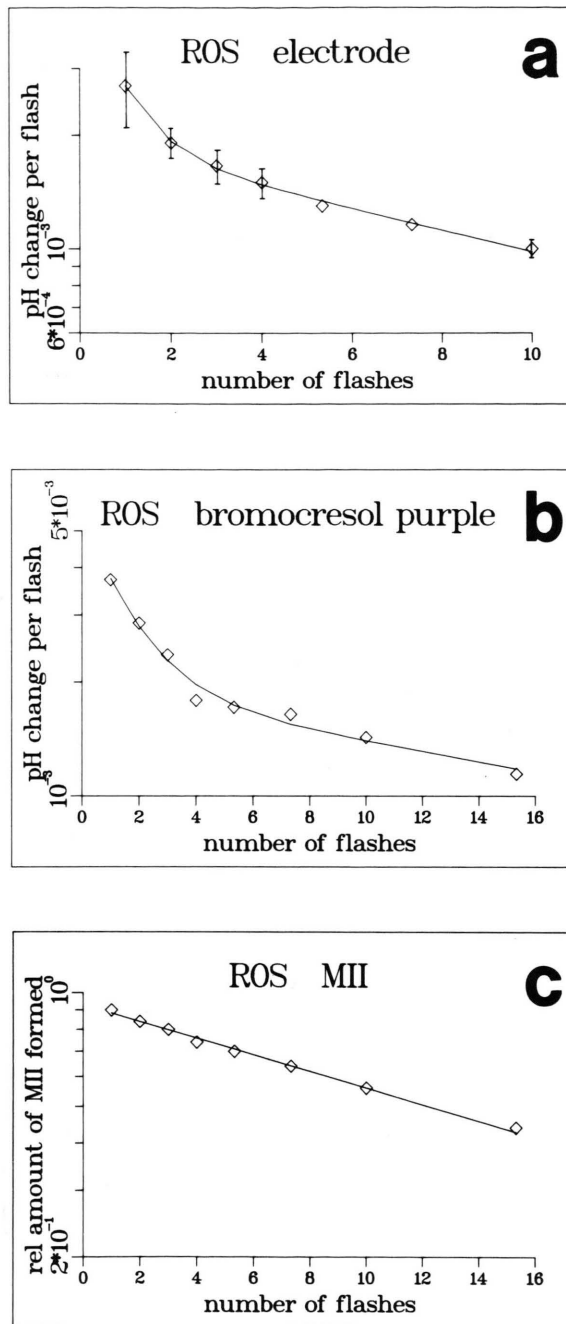


Fig. 4. pH changes and MII formation from a succession of flashes on rod outer segments (pH 6, $T = 22^\circ\text{C}$).

a) pH change using pH electrode. Each flash bleached 6.7% of rhodopsin. Error bars refer to electrode voltage drift.

b) pH change by indicator absorption change. Each flash bleached 4% of rhodopsin.

c) MII formation measured as absorption difference change at 380/417 nm; flash as in b.

hanced protonation does not express itself in a distinct uptake rate. This may be subsumed under the more general observations [7, 11] that the measured ΔpH in ROS is rather rate limited by external factors than by proton uptake itself.

Discussion

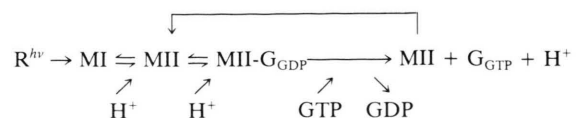
The present study shows that complex formation between rhodopsin and G-protein is accompanied by proton uptake. This protonation is additional to the well-known rhodopsin protonation which is coupled to the transition from metarhodopsin I (MII) to metarhodopsin II (MII) [4–14]. It should be noted that under the conditions of this study enhanced MII formation does not occur [17].

The main characteristic of R-G complex formation and protonation was its exclusive occurrence under excess free G-protein i.e. at low levels of bleached rhodopsin. Emrich and Reich already described a relatively enhanced alkalinisation at low rhodopsin bleaching [7]. At that time G-proteins were unknown and it was obvious to seek an explanation in some kind of rhodopsin cooperativity.

Under the conditions of this study (pH 6, 22°C) and the absence of GTP one proton per R-G remains persistently segregated from the external aqueous phase during the lifetime of the complex. Under conditions where the R-G complex is rapidly redissociated ($100\ \mu\text{M}$ GTP) [24] no enhanced alkalinisation was found, indicating an only transient protonation. Direct recording of this protonation as a transient wave form could however not be performed due to the limited time resolution of the electrode. The optical method could in principle provide enough time resolution, but so far we were not able to suppress the superimposed GTP induced scattering artefact (dissociation signal [24]).

The additional proton uptake may be understood as a probe for a conformational state of the R-G system which exposes a proton acceptor. This conformation persists during interaction and is terminated by GTP/GDP exchange.

The following reaction scheme inserts this new conformation in the known succession of events [24] leading to G-protein activation.



These results do not allow determination of the locus of the proton acceptor, which may be in rhodopsin, in the G-unit or in the narrow space [25] between the interacting proteins.

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