

Sulfhydryl group modification of photoreceptor G-protein prevents its light-induced binding to rhodopsin

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The effect of sulfhydryl modification on the light-induced interaction between rhodopsin and the peripheral GTP-binding protein of the photoreceptor membrane (G-protein) has been investigated by time-resolved near-infrared light-scattering and polyacrylamide gel electrophoresis. It has been found that the modification of rhodopsin with the alkylating agent *N*-ethylmaleimide (NEM) does not affect its light-induced interaction with the G-protein. Modification of G-protein with NEM or other sulfhydryl agents prevents any light-induced binding to rhodopsin. Dark-association of G to the membrane as well as the light-induced complex with rhodopsin (once formed) is insensitive to NEM.

<i>Sulfhydryl modification</i>	<i>Photoreceptor</i>	<i>Peripheral membrane protein</i>	<i>GTP-binding protein</i>
	<i>Rhodopsin</i>	<i>Protein-protein interaction</i>	

1. INTRODUCTION

Signal transduction in light-activated as well as in hormone-activated receptors appears to include 3 components. Namely, a specific receptor unit, a GTP-binding transmitter unit (G/F-protein, transducin) and a catalytic unit [1–4]. The compatibility of components [5] and the structural homology of subunits [6] have been demonstrated. The relationship between the systems and the known effects of sulfhydryl modification on the hormone receptor proteins [7] has led to this study on the photoreceptor G-protein. Recent work has opened possibilities to investigate specifically the interaction between photoexcited rhodopsin and G-protein [3,8,9]. We report that this interaction is prevented by the alkylating agent NEM and other SH-blocking agents. The relevant SH group is

located at the G_{α} -subunit.

After light absorption, the visual pigment rhodopsin (R) interacts in an active state R^* with a peripheral membrane protein (G-protein or transducin). The kinetics and stoichiometry of the interaction can be analysed in situ by a change of the NIR light-scattering via linked shifts of the ROS scattering mass [8,9] (binding signal or signal P). The 1:1 stoichiometry of the interaction and its preference for and retroaction on a certain photoproduct (metarhodopsin II [9–13]) suggest a specific R^* –G complex. The interaction lasts only milliseconds in the presence of millimolar GTP [8], because the exchange of GTP for GDP leads to the dissociation of R^* –G. In the absence of GTP, however, the complex is stable (~30 min at 20°C, ~2 h on ice, standard buffer; see below), not dissociating until after the decay of metarhodopsin II [13]. During this time, in the R^* binding mode, the G-protein is, in contrast to its looser membrane association in the dark, not released from the membranes at low ionic strength [3,14]. This provides another test for the R^* –G complex which is used here in addition to the NIR scattering signal of complex formation (here termed NIR signal).

Abbreviations: ROS, rod outer segments, G, G-protein or GTP-binding protein or transducin; NIR, near-infrared; R^* , photoexcited rhodopsin; GTP- γ S, guanosine 5'-O-(3-thiotriphosphate); DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; 2-PDS, 2,2'-dithiodipyridine; DTT, dithiothreitol; Pipes, piperazine-1,4-diethanesulfonic acid

2. MATERIALS AND METHODS

The apparatus for the measurement of the NIR signal was as in [11]. To prepare isolated ROS, bovine retinas were shaken in a preparation buffer (i.e., standard buffer (fig.1) + 1 mM DTT to prevent oxidation of the SH groups) and then filtered through a nylon mesh. The filtrate was layered on a discontinuous sucrose gradient and the resultant crude ROS suspension was washed twice in standard buffer.

As shown in fig.1, one sample of this ROS preparation was divided into two portions. NEM was added to one of the portions before incubation

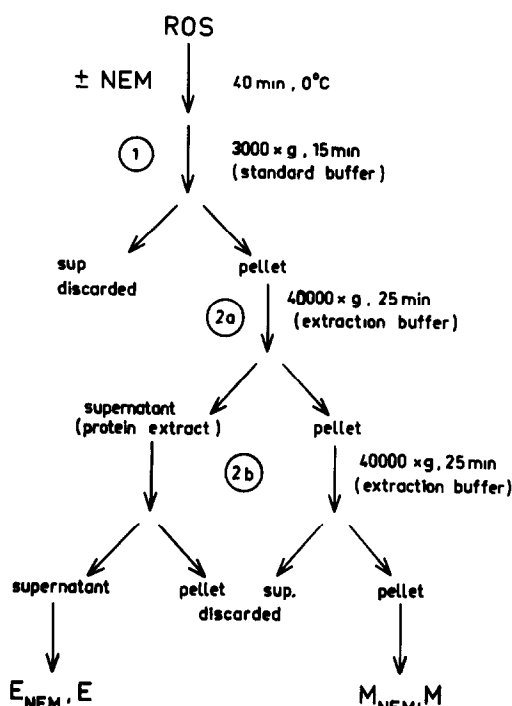


Fig.1. Preparation scheme for separating the rhodopsin containing membranes (M) and extractable proteins (E). The alkylating reaction with NEM lasts for 40 min at ice temperature (not shown); $C_{NEM} = 100 \mu M$, rhodopsin concentration (C_R) = $70 \mu M$. Centrifugation step 1 serves for eliminating the excess NEM, steps 2a and 2b for extraction of proteins and for removing by the remaining membrane material in the E-fraction. Standard buffer: 130 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM EDTA, 1 mM DTT, 10 mM Pipes (pH 7.0). Extraction buffer: 5 mM Pipes (pH 7.0), 1 mM EDTA (pH 7.0).

of both. A washing step at moderate ionic strength (step 1 in fig.1) eliminating the excess NEM was then applied. Even after freeze-thawing of the sample, the supernatant contained no significant amounts of G-protein (which was, however, extractable by GTP- γS [9]), indicating that NEM had no effect on the dark association of G to the membranes. Two further centrifugation steps at low ionic strength (steps 2a,b in fig.1) separated the dark extract (containing G-protein) from the membranes (containing rhodopsin) [3]. Four different components were thereby obtained: untreated and NEM-treated membranes (M and M_{NEM}) and untreated and NEM-treated extracts (E and E_{NEM}). Note that the alkylation was performed prior to the extraction steps.

Polyacrylamide gel electrophoresis was performed as in [9]. SH groups of rhodopsin were assayed by the 412 nm absorption of DTNB in 1% SDS.

3. RESULTS

Fig.2 shows NIR signals for each of the 4 possible recombinants of M, E, M_{NEM} and E_{NEM}. It is seen that M_{NEM} with E leads to the same NIR signal as observed without any modification (M + E). This demonstrates the undisturbed interaction of photoexcited rhodopsin (R*) with G-protein contained in the E-fraction. However, both the

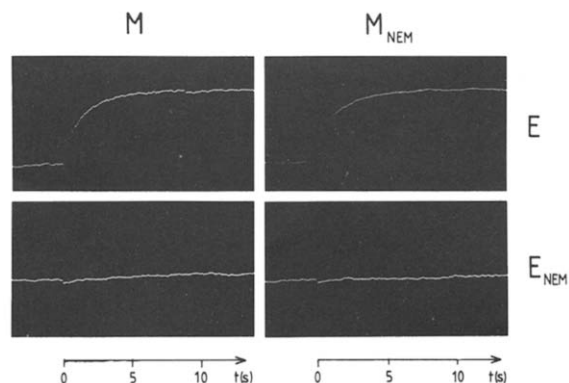


Fig.2. NIR signals obtained with M and M_{NEM}, recombined with E and E_{NEM}, respectively. A 4% bleaching flash ($\lambda > 515$ nm) is applied at $t = 0$. The samples are suspended in standard buffer ($T = 20^\circ C$, cuvette $d = 1$ cm, $C_R = 2 \mu M$, scattering angle $\theta = 20 \pm 3^\circ$, $\lambda_m = 800$ nm). Note that binding only occurs with the untreated extract E.

recombinants of the untreated or NEM-treated M-fractions (M or M_{NEM}) with E_{NEM} fraction do not display any scattering signal. Apparently, the alkylation of some relevant group(s) of the G-protein (contained in E_{NEM}) is sufficient to prevent

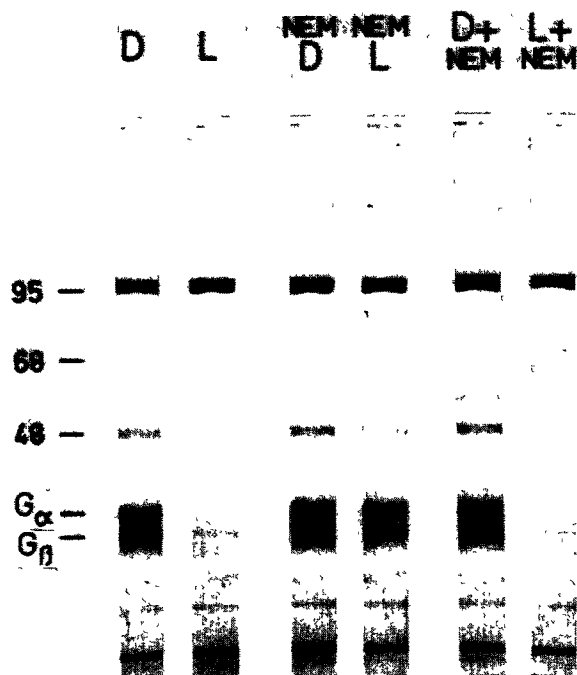


Fig.3. Extractability of G-protein with NEM and/or light. An ROS suspension was divided into 6 equal portions, which were subjected to different treatments before extraction of soluble proteins. D: corresponds to fraction E in fig.1a. L: ROS fully bleached (2 min at 20°C), cooled down to ice temperature and held in the dark for 40 min, then the extraction was carried out. NEMD: corresponds to fraction E_{NEM} in fig.1. NEML: at the end of the 40 min incubation period (fig.1) the ROS were bleached at 20°C, followed by the extraction procedure at ice temperature. D + NEM: dark control, as E_{NEM}. L + NEM: at first the ROS were bleached (2 min, 20°C), then NEM was added and kept on ice for 40 min in the dark before the extraction was carried out. Seventy μ l (i.e., 30 μ g protein in D) of the fractions above were applied to 10% SDS-polyacrylamide gels. Numbers on the left are M_r values ($\times 1000$). Traces D and L serve as a dark-light control. The G-protein is bound by light [3] and, therefore, not found in the light extract L. NEMD and NEML show that the light binding of G-protein is prevented by NEM. It is found in the light extract NEML. In contrast, L + NEM shows that G-protein, once bound by light, is not solubilized by subsequent NEM-treatment.

its binding to R*. This is further supported by the following sedimentation experiments.

Fig.3 shows polyacrylamide gels of extracts after treatments of the ROS with NEM and/or light in varied order. After these treatments, all samples were extracted at low ionic strength. The first two traces show the expected extractability of G α and G β in the dark (D) which is prevented by light (L) [3]. However, after previous NEM treatment (traces NEM + D and NEM + L), light no longer prevents the extraction of G, thus indicating once again the lack of light-induced binding (cf. fig.2). In the last two traces, (D + NEM) merely serves as a dark control, showing the expected agreement with NEM + D. The trace L + NEM, however, shows that G protein bound by light remains unextractable after subsequent NEM, indicating insensitivity of the R*-G complex, once formed, to NEM. Note that the known pattern of dark- and light-binding for the other proteins displayed in the gels [3] is not influenced by NEM treatment.

Fig.2,3 shows in summary that the alkylation of G prevents the formation of the R*-G complex but is not able to split the complex.

As with NEM, 2-PDS [15] or DTNB (addition of 100 μ M to 10 μ M rhodopsin and incubation for 1 h at 22°C) suppresses the NIR signal of R*-G binding. The effect of these was reversible (the NIR signal was fully restored) by a subsequent application of 10 mM β -mercaptoethanol. These findings identify the relevant groups as sulfhydryl groups.

4. DISCUSSION

The experiments show that alkylation of highly accessible SH groups at the G-protein prevents its interaction with photoexcited rhodopsin. This must also prevent all subsequent events first of all GTP-GDP exchange at the G-protein and phosphodiesterase activation. We have indeed verified that the 'dissociation signal' [8] is not observed after alkylation of G, indicating the absence of nucleotide exchange.

This work opens up the possibility of blocking the enzymatic cascade rather specifically at its very beginning, without extensive modifications of the other proteins including rhodopsin. The mechanism that is ultimately responsible for preventing R*-G interaction remains unresolved.

At present it is possible that a conformation change of the G-unit is induced by the SH-modification (thereby preventing the interaction taking place somewhere in the protein) or that the SH groups in question are near the interacting region of G. In the latter case they could either be directly involved in the R*-G interaction or they could block the interaction by steric hindrance when alkylated.

These questions are currently under investigation. Using a radioactivity assay we have already found that an average alkylation of one SH group at the G α -subunit is sufficient to suppress its binding to R*; under these conditions, only 0.2 SH groups of rhodopsin are modified. To modify all accessible SH groups of rhodopsin we alkylated the M-fraction alone, after separating E and M (in contrast to fig.1). Also, this treatment ($C_{NEM} = 1 \text{ mM} = 10 \times C_{Rhod}$, incubation for 2 h on ice, final average alkylation of 2.4 mol SH/mol rhodopsin) did not result, however, in any measurable influence on either the dark- or light-binding of G.

Thus the 3 cysteines on the cytoplasmic surface of rhodopsin [16,17] seem to be irrelevant for binding. The question remains if the binding conformation of rhodopsin (metarhodopsin II [9-13]) exposes one or more additional SH groups [18,19] which could be essential for the interaction with G but are not accessible to modification during the lifetime of the photoexcited state.

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