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Kinetic Study on the Equilibrium between Membrane-Bound and Free Photoreceptor G-Protein

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Summary. Formation of the complex between photoreceptor G-protein (G) and photoactivated rhodopsin (R_M) leads to a change in the light scattering of the disk membranes (binding signal or signal P). The signal measured on isolated disks (so-called P_D signal) is exactly stoichiometric in its final level to bound G-protein but its kinetics are much slower than the R_MG binding reaction. In this study on isolated disks, recombined with G-protein, we analyzed the P_D-signal level and kinetics as a function of flash intensity and compared it to the R_MG-complex formation monitored spectroscopically (by extra metarhodopsin II). The basic observation is that the initial slopes of the P_D signals decrease with flash intensity when the signals are normalized to the same final level. This finding prevents an explanation of the scattering signal by a slow postponed reaction of the R_MG complex. We propose to interpret the scattering change as a redistribution of G-protein between a membrane-bound and a solved state. The process is driven by the complexation of membrane-bound G to flash-activated rhodopsin (R_M). The experimental evidence for this two-state model is the following: (1) The intensity dependence of the initial rate of the P_D signal is explained by the model. Under the assumption of a bimolecular reaction of free G with sites at the membrane, equal to rhodopsin in their concentration, the measured rates yield a K_D of 10^{-5} M. (2) Evaluation of the extra MII kinetics yields a biphasic rise at saturating flashes. The measured rates fit to the supply of free and membrane-bound G-protein for the reaction with R_M. (3) Quantitative estimation of the expected scattering intensity changes gives a comprehensive description of binding signal and dissociation signal by the gain and loss of G-protein scattering mass. (4) The temperature dependence of the P_D-signal rate leads to an activation energy of the membrane-association process of E_a = 44 kJ/mol.

Key Words rod outer segment · rhodopsin · time-resolved light scattering · G-protein

Introduction

Light absorption by rhodopsin leads to the activation of an enzymatic cascade, which results in the rapid hydrolysis of cytosolic cGMP by a membrane-bound phosphodiesterase (PDE) (Wheeler & Bitensky, 1977; Yee & Liebman, 1978; Fung, Hurley & Stryer, 1981).

An early step of this activation process is the interaction of photoactivated rhodopsin with the peripheral membrane-bound G-protein (GTP-binding protein, transducin) (Kühn, 1980). During this interaction rhodopsin catalyses GDP/GTP exchange at the nucleotide binding site of the G-protein (Fung & Stryer, 1980). When GTP is bound, the rhodopsin-G complex dissociates (Kühn, 1980). In the absence of GTP, G-protein remains persistently bound (Kühn, 1980) in its inactive GDP-binding form.

Two optical monitors are available for measuring the light-induced rhodopsin-G interaction *in situ*. The first is provided by the spectroscopic behavior of rhodopsin itself. Photoexcited rhodopsin relaxes after msec in a temperature and pH dependent equilibrium between the 480-nm intermediate metarhodopsin I (MI) and the 380-nm intermediate metarhodopsin II (MII) (Matthews et al., 1963). While being in the spectroscopic state MII, rhodopsin adopts an enzymatically active conformation R_M which can bind G-protein. By the interaction with G, the MI/MII equilibrium is shifted strongly towards MII when G-protein is bound (Emeis & Hofmann, 1981; Bennett, Michel-Villaz & Kühn, 1982; Emeis et al., 1982). Under conditions (e.g., 10°C, pH 7.5) that normally favor (in the absence of G-protein) the MI conformation, excess G-protein leads to an enhanced formation of the 380-nm photoproduct MII ("extra MII"). The extra MII formation is a direct real-time monitor of the complex formation. However, the application of this monitor is naturally restricted to measuring conditions where the normal MI/MII equilibrium favors the MI conformation, i.e., low temperature and high pH.

ROS or disk membrane suspensions respond to light by changes in near-infrared light scattering (Hofmann et al., 1976). One of these effects (P signal, binding signal) was shown to be stoichiometrically related to the G-protein binding to rhodopsin (Kühn et al., 1981). Using this monitor, Kühn et al. (1981) could demonstrate the 1 : 1 stoichiometry of the G-protein interaction. The saturation of the P signal as a function of rhodopsin conversion obeys

normal hyperbolic saturation (Bennett & Dupont, 1985). The fit of the data yields reasonable values for the dissociation constant and the G-protein concentration G_o (Bennett & Dupont, 1985).

The P signal is sensitive to the structure of the ROS or disks used (Emeis & Hofmann, 1981; Hofmann et al., 1981; Kühn et al., 1981). In isolated ROS or disk stacks the scattering change is nearly as fast as its biochemical trigger, the formation of the R_MG complex. However, in disrupted and osmotically shocked ROS or isolated disks it is considerably slower. In such preparations the rate of formation of extra MII can exceed to up to 50-fold the rate of development of the scattering change. This kinetic discrepancy shows clearly that the scattering change does not reflect the binding itself but a following structural process.

The physical nature of this structural transformation is still unknown. However, a characteristic difference between intact disk stacks and isolated disks has been derived from the angular dependence of the scattering change: ROS respond by a shape change (Hofmann et al., 1981), whereas the isolated disks appear to change their integral polarizability, which was previously interpreted as an apparent refractive index change (Hofmann et al., 1981). Reichert (1984) has already discussed a binding of dissolved G-protein to the membrane, with diffusion as the rate-limiting step. Michel-Villaz, Brisson and Chapron (1984), however, interpreted the scattering change of both ROS and isolated disks as a membrane thickness change accompanying the light-induced RG-complex formation.

This study is restricted to isolated disks recombined with peripheral proteins. The slow scattering change in this system was termed P_D-signal (Hofmann et al., 1981).

We measured both light-scattering changes and spectroscopic changes to compare the time course of appearance of MII with the kinetics of P_D. Our data suggest a model that accounts for the scattering change in terms of a relatively slow light-induced shift in the equilibrium between dissolved and membrane associated G-protein, with the membrane association as the rate-limiting step.

Materials and Methods

Preparation Procedure

Bovine rod outer segments (ROS)[1] were prepared according to a standard procedure (Emeis & Hofmann, 1981). The retinae were shaken in isotonic saline (buffer A: 130 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM EDTA, 1 mM DTT, 10 mM PIPES, pH 7) and filtered through a nylon mesh. The resulting crude suspension was layered on a discontinuous sucrose gradient and washed in buffer A.

The extraction of the *peripheral proteins* was done as described by Kühn (1980). The ROS were osmotically shocked in a low ionic strength buffer (1 mM DTT, 1 mM EDTA, 5 mM PIPES, pH 7.0), gently homogenized and sedimented. The supernatant was centrifuged again and yielded the extracted peripheral proteins. This protein extract was stored in liquid nitrogen.

Isolated disks were prepared from ROS. The procedure used was similar to that described by Smith, Stubbs and Litman (1975) except that (i) 2.5% Ficoll was used instead of 5% to reduce side effects caused by Ficoll (Bauer & Mavromatti, 1980); and (ii) Ficoll was not removed by a separate washing step. The resulting Ficoll concentration after dilution to the final measuring concentration was abut 0.25% (wt/vol). Isolated disks were used within 24 hr after preparation without freezing.

Prior to the measurement the isolated disks were recombined with protein extract and diluted to a final concentration of 4 μM rhodopsin. All measurements were done in buffer A. For measurements at pH 6 the sample was buffered with 20 mM MES instead of 10 mM PIPES.

NIR-Scattering and Absorption Measurements

All measurements were performed using the apparatus described by Hofmann and Emeis (1981). This instrument is a fast two-wavelength spectrometer which allows a simultaneous measurement of absorption and near infrared light (NIR) scattering changes. A Neodym-YAG Laser YG580 (Quantel, Les Ulis, Orsay, France) equipped with a frequency doubler provided an actinic light flash (530 nm).

The angular range of scattering detection was adjusted to θ = 10–30°. MII formation was measured by comparing light-induced changes in the difference of absorption at 380 and 417 nm (Hofmann & Emeis, 1981). In such measurements the photocurrent at 417 nm (isosbestic point of MI to MII) serves as a reference for determining the level of MII (λ_{max} = 380 nm).

The use of stepwise photolysis with a series of flashes, "exhaustion curves," has been described in detail by Emeis and Hofmann (1981). Briefly each flash photoexcites a fixed mole fraction ρ of rhodopsin. The absolute amount of rhodopsin photoexcited per flash thus decreases exponentially. An enhanced formation of MII in the presence of excess G-protein at the first flash appears as an enhancement over the normal exponential course of the signal amplitude. After a rhodopsin turnover of about 10% all G-protein is complexed, further flashes yield a normal exponential decrease. In Fig. 4, the response to a flash late in the sequence (normal exponential region) was normalized by exponential extrapolation to the first flash to allow a comparison with the measured MII formation at the first flash.

Results and Discussion

I. Basic Observation

The near infrared light scattering change, P_D-signal, has been measured on suspensions of isolated disks recombined with "protein extract." Typical signals (pH 6, 20°C) after light flashes of different intensi-

[1] *Abbreviations:* ROS, rod outer segment; G, G-protein; R, rhodopsin; R_M photoactivated enzymatically active R; NIR, near infrared; MI, metarhodopsin I; MII, metarhodopsin II; ρ, mole fraction of photoactivated rhodopsin per flash.

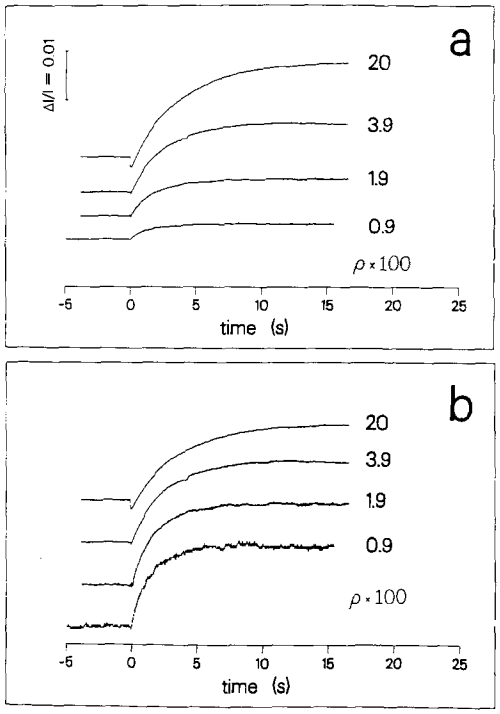

Fig. 1. (*a*) Light-scattering signals, P_D signals, of isolated disks reconstituted with peripheral G-protein. The signals correspond to the first flash (applied at $t = 0$) of different intensities. ρ is the mole fraction of photoactivated rhodopsin per flash. (*b*) As *a* but scaled by an appropriate factor to obtain the same amplitude (final level) for all signals in order to allow a better kinetic comparison. Measuring conditions: $[R] = 4\ \mu$M, pH 6, $T = 21$°C. The flash was applied at $t = 0$. The fast negative component is the N signal (Hofmann et al., 1976)

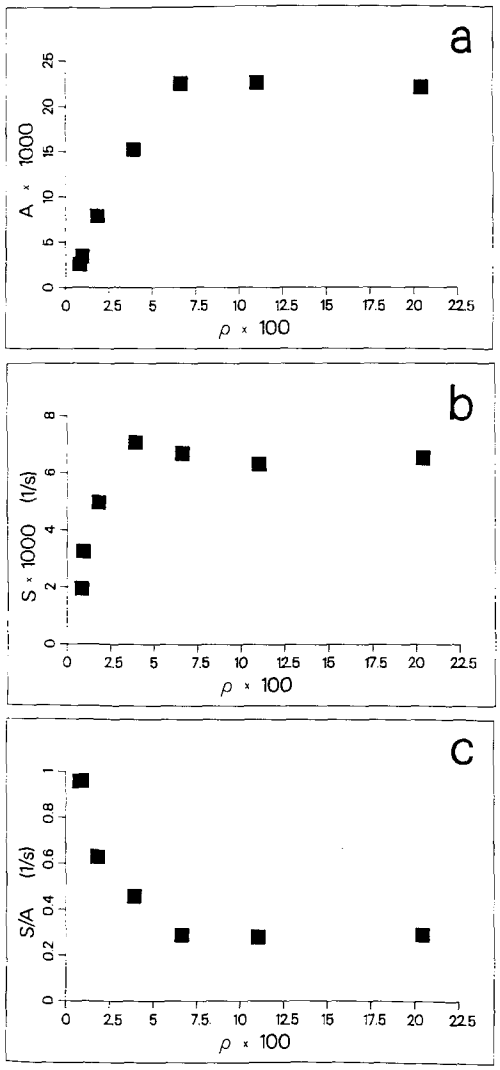

Fig. 2. (*a*) Signal amplitude A of the P_D signal plotted against the mole fraction of photoactivated rhodopsin per flash ρ. (*b*) Initial slope S ($d(\Delta I/I)/dt$) of the P_D signal plotted against ρ. (*c*) Initial slope S of the P_D signal divided by the signal amplitude A, plotted against ρ

ties are shown in Fig. 1*a*. Each signal is from the first light flash on a dark-kept sample of the same preparation. The fast negative component of the signal waveform represents the N signal described by Hofmann et al. (1976). The amplitude of this component is proportional to the amount of photoactivated rhodopsin (Hofmann et al., 1976). The subject of this paper is the positive-going component (P_D signal) which follows the N signal.

The saturation curve of the P_D signal (final signal level as function of the flash intensity) is plotted in Fig. 2*a*. The signal amplitude A is defined as the maximal scattering change $(\Delta I)_{max}$ normalized to the nominal intensity (I) that is, $A = (\Delta I)_{max}/I$. The flash intensity is expressed in terms of the mole fraction ρ of photoexcited rhodopsin produced by the flash. The signal amplitude saturates at $\rho = [R_M]/[R] = 0.05$. In agreement with previous studies (Kühn et al., 1981; Bennett & Dupont, 1985), this mole fraction corresponds to the concentration of added G-protein.

For better kinetic comparison the signals of Fig. 1*a*, normalized to the same final amplitude, are shown in Fig. 1*b*. This figure shows a clear dependence of the P_D signal on ρ. The signals are strikingly faster at low ρ than at saturation.

This basic observation rules out two simple interpretations of the P_D signal (section II, below). In section III we develop an alternative explanation for the P_D signal and test it in detail in section IV.

II. Previous Interpretations

A. P_D-Signal as an Indicator of the R_MG-Complex

Disregarding the known kinetic difference of the P_D signal and the extra MII monitor, the simplest model for the P_D signal would be that the scattering change is a direct real-time monitor of complex for-

mation. This assumption would imply that after the fast formation of the active rhodopsin conformation R_M the signal kinetics follows a bimolecular reaction scheme:

$$R_M + G \xrightarrow{k} R_M G. \tag{1}$$

Under homogeneous conditions the initial rate of such a reaction depends on both initial concentrations $[R_M]_o$ and $[G]_o$:

$$d[R_M G]/dt|_{t=0} = k[R_M]_o \cdot [G]_o. \tag{2}$$

As $[G]_o$ and k are constant for all signals at the first flash, the initial slope of the signal $S = d(\Delta I/I)/dt$ should be proportional to ρ. However, experimental data for S as a function of ρ (Fig. 2*b*) do not follow the predicted linear dependence of S on ρ expressed in Eq (2). Rather, the data show a pronounced saturation of S as ρ increases.

B. P_D-Signal as a Subsequent Reaction of the $R_M G$-Complex

An obvious explanation for the slowed but stoichiometric expression of the complex in the scattering signal is a subsequent slower reaction, which is seen in the scattering of the sample as proposed by Bennett and Dupont (1985). We consider the following reaction sequence

$$R_M + G \underset{\text{fast}}{\rightarrow} R_M G \underset{\text{slow}}{\xrightarrow{k}} P. \tag{3}$$

In this hypothetical scheme, the formation of $R_M G$ includes the appearance of a product P on a relatively slow time scale. The scattering signal is assumed to reflect the (time dependent) level of P. Since the first reaction is assumed to be much faster than the second one, the initial rate of such a reaction depends linearly on the concentration of the complex $[R_M G]_f$ formed by the flash.

$$d[P]/dt|_{t=0} = k[R_M G]_f. \tag{5}$$

Since the final concentration of the complex $[R_M G]_f$ is proportional to the final signal amplitude A (Kühn et al., 1981), the normalized initial rate S/A of all signals should be independent of ρ. The experimental values of this ratio are shown in Fig. 2*c*. This plot shows a strong dependence of the ratio S/A on ρ in contrast to the prediction.

III. Interpretation of the P_D-Signal as a Transition of G from Solution to a Membrane-Bound State

The models considered so far assumed homogeneous reaction conditions with the same reaction rate for all G-proteins. The data of others (Liebman & Sitaramayya, 1984) showed that only a part of G-protein is membrane bound. Thus a model with inhomogeneous reaction conditions might be more adequate to describe the observed process. The membrane binding of G is not complete even in an isotonic reaction medium at the relatively low $[R]$ and $[G]$ concentrations used. Liebman and Sitaramayya (1984) investigated the membrane-binding equilibrium of G-protein by centrifugation experiments. Their results show that under the conditions of our study ($[R] = 4\ \mu$M, $[G] = 0.2\ \mu$M) only 70% of the total G-protein is bound to unphotolysed membranes. The exact percentage varies, however, with different types of disk and G-protein preparations. At saturating light flashes all G-protein is complexed with rhodopsin (Kühn, 1980). The previously nonmembrane-bound G-protein therefore must migrate from the solution to the membrane. The association of G-protein with the membranes is expected to enhance the scattering of the disks by an enhancement of the scattering mass. We propose therefore to interpret the P_D-signal by this membrane association process of previously soluble G-protein. Data of Liebman and Sitaramayya (1984) show that such a scattering change has to be expected. They investigated the transition of G-protein to the membrane by mixing activated and dark-adapted membranes. For the $[G]$ and $[R]$ concentrations used in our study, they found that the kinetics of transition is in the time range of sec, and therefore in the same time range as the P_D signal.

According to Liebman and Sitaramayya (1984), the equilibrium of G-protein association to unphotolysed membranes can be formulated by

$$G_{\text{free}} + M \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} G_{\text{memb}} \tag{6}$$

where M is a hypothetical binding site on the membrane, G_{free} free G-protein in solution and G_{memb} membrane-bound G-protein. The binding constant of this equilibrium K_A can be written

$$K_A = [G_{\text{memb}}]/([G_{\text{free}}][M]). \tag{7}$$

The membrane association of G-protein saturates at a mole fraction of $[G]/[R] = 0.25$ (Liebman & Sitaramayya, 1984). At $[G]/[R] = 0.05$ used in this study, no saturation occurs and the concentration

of the free binding site $[M]$ is approximately constant, $[G_{\text{memb}}]/[G_{\text{free}}]$ is therefore a constant ratio

$$K_{MB} = [G_{\text{memb}}]/[G_{\text{free}}] \tag{8}$$

where the constant K_{MB} is related to K_A by

$$K_{MB} = [M]K_A. \tag{9}$$

The formula of the equilibrium reaction between both states of G-protein can be simplified to

$$G_{\text{free}} \rightleftharpoons G_{\text{memb}}. \tag{10}$$

When rhodopsin is photoactivated a part of G-protein is complexed with rhodopsin. The total G-protein $[G_{\text{tot}}]$ is then subdivided in the three states

$$[G_{\text{tot}}] = [G_{\text{free}}] + [G_{\text{memb}}] + [R_MG]. \tag{11}$$

The membrane-binding equilibrium is coupled to the complex formation equilibrium. We propose a concatenation of both equilibria as in the following reaction scheme

$$G_{\text{free}} + R_M \overset{1}{\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}} G_{\text{memb}} + R_M \overset{2}{\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}} R_MG. \tag{12}$$

The light-induced formation of R_M shifts equilibrium 2 to the right side and thereby induces a rearrangement of the membrane binding equilibrium 1. Reaction 2 can be monitored by the extra MII formation. Reaction 1 in our model is supposed to be seen in the P_D signal.

IV. Testable Predictions of the Model

A. Saturation

Membrane-bound and complexed G-protein $\{[G_{\text{memb}}] + [R_MG]\}$ both contribute to the scattering mass of the disks. Thus the scattering change depends on the net light-induced change in the concentrations of the species $\{[G_{\text{memb}}] + [R_MG]\}$. The proportionality between mass change and relative scattering intensity change will be derived below.

To analyze the light-induced changes of the three G-protein states we indicate by indices 0 and ∞ the initial and final concentrations before and after the equilibrium shift, and by Δ the difference of them. The total amount of newly associated G-protein can be expressed using Eq. (11) and (8) by

$$\begin{aligned}\{\Delta[G_{\text{memb}}] + \Delta[R_MG]\} &= -\Delta[G_{\text{free}}] = [G_{\text{free}}]_o \\ &- [G_{\text{free}}]_\infty = ([G_{\text{tot}}] - [R_MG]_o)/(1 + K_{MB}) - ([G_{\text{tot}}] \\ &- [R_MG]_\infty)/(1 + K_{MB}) = \Delta[R_MG]/(1 + K_{MB}).\end{aligned} \tag{13}$$

Equation (13) shows that the mass change and thus the amplitude of the scattering signal is proportional to $\Delta[R_MG]$.

B. Kinetics

The kinetic differential equations for the proposed reaction scheme can be written following chemical kinetic theory. Because the signal waveform is determined by the change in time of $\{[G_{\text{memb}}] + [R_MG]\}$, the rate of this sum is of interest. Using the definition of the rate constants as in formula (12) the rates of formation of the three forms of G obey the following equations

$$\begin{aligned}d[G_{\text{free}}]/dt &= -k_1[G_{\text{free}}] + k_{-1}[G_{\text{memb}}] \\ d[G_{\text{memb}}]/dt &= k_1[G_{\text{free}}] - k_{-1}[G_{\text{memb}}] \\ &\quad - k_2[G_{\text{memb}}][R_M] + k_{-2}[R_MG] \\ d[R_MG]/dt &= k_2[G_{\text{memb}}][R_M] - k_{-2}[R_MG] \\ d([G_{\text{memb}}] + [R_MG])/dt &= k_1[G_{\text{free}}] - k_{-1}[G_{\text{memb}}] = \\ &\quad -d[G_{\text{free}}]/dt.\end{aligned} \tag{14}$$

In addition the following equations are valid under equilibrium conditions

$$k_1/k_{-1} = [G_{\text{memb}}]/[G_{\text{free}}] = K_{MB} \tag{15}$$

$$k_2/k_{-2} = [R_MG]/[G_{\text{memb}}][R_M] = K_{LB} \tag{16}$$

where K_{MB} is the equilibrium constant of the membrane association and K_{LB} the binding constant of the complex formation of R_MG.

The initial concentrations $[G_{\text{memb}}]_o$ and $[G_{\text{free}}]_o$ are in equilibrium as described by Eq. (8). The short light flash at time $t = 0$ is assumed to produce instantaneously a certain amount of photoactivated rhodopsin $[R_M]_o$.

A complete analytical solution of the differential equations is not possible. Due to the large kinetic difference between reactions 1 and 2, however, good approximations can be found for the two extremes of (i) very small rhodopsin turnover or (ii) saturating flashes.

In both cases the initial rate of reaction 1 is zero ($d([G_{\text{memb}}] + [R_MG])/dt = 0$ for $t = 0$) because in the first instance only equilibrium 2 is shifted. On the time scale of the membrane association, however,

the binding reaction on the membrane is so fast that the resulting delay of the signal can be neglected. It is sufficient to consider the time domain of the fastest slope of the signal after some msec.

1) In the case of a first flash with *very low rhodopsin turnover* ($[R_M]_o \ll [G_{tot}]$ the initial concentration of membrane bound G-protein $[G_{memb}]_o$ is in sufficient excess to form complexes with all $[R_M]_o$ within a few msec. After this short time delay τ we obtain

$$\begin{aligned}
&[G_{free}] = [G_{free}]_o \\
&[G_{memb}] = [G_{memb}]_o - [R_M]_o \\
&d([G_{memb}] + [R_MG])/dt|_{t=\tau} = k_1[G_{free}]_o \\
&\quad - k_{-1}[G_{memb}]_o + k_{-1}[R_M]_o . \qquad (17)
\end{aligned}$$

$[G_{free}]_o$ and $[G_{memb}]_o$ are the equilibrium concentrations before the flash, and satisfy Eq. (15). Equation (17) is thus reduced to

$$d([G_{memb}] + [R_MG])/dt|_{t=\tau} = k_{-1}[R_M]_o . \qquad (18)$$

As all R_M is complexed under these conditions, $[R_M]_o$ is equal to the final complex concentration and can be substituted by $[R_MG]_\infty$.

$$\begin{aligned}
&d([G_{memb}] + [R_MG])/dt|_{t=\tau} = k_{-1}[R_MG]_\infty \\
&\quad (\text{for } [R_M]_o \ll [G_{tot}]). \qquad (19)
\end{aligned}$$

To obtain an expression for the experimental ratio S/A this equation is divided by the final signal amplitude $\Delta[G_{memb}] + \Delta[R_MG]$ from Eq. (13).

$$S/A = k_{-1}(1 + K_{MB}) = k_1 + k_{-1} . \qquad (20)$$

2) In the case of *a saturating flash* ($[R_M]_o \gg [G_{tot}]$) the membrane-bound G-protein is not sufficient to complex all R_M. The concentration of noncomplexed G-protein on the membrane is reduced to zero in a few msec. Thus the "initial" conditions after this short time delay τ are

$$\begin{aligned}
&[G_{free}] = [G_{free}]_o \\
&[G_{memb}] = 0 \qquad (\text{for } [R_M] \gg [G_{tot}]) \\
&[R_MG] = [G_{memb}]_o .
\end{aligned}$$

As "initial" rate of the scattering change $d([G_{memb}] + [R_MG])/dt$ we get from Eq. (14):

$$d([G_{memb}] + [R_MG])/dt|_{t=\tau} = k_1[G_{free}]_o . \qquad (21)$$

Using Eq. (8) $[G_{free}]_o$ can be expressed in the form

$$[G_{free}]_o = ([G_{free}]_o + [G_{memb}]_o)/(1 + K_{MB}).$$

$[G_{memb}]_o + [G_{free}]_o$ is the total amount of G-protein and corresponds in the case of saturating flashes to the final amount of formed complexes $[R_MG]_\infty$. Eq. (21) can therefore be written in the form

$$\begin{aligned}
&d([G_{memb}] + [R_MG])/dt|_{t=\tau} = k_1[R_MG]_\infty/(1 + K_{MB}) \\
&\quad (\text{for } [R_M]_o \gg [G_{tot}]) \qquad (22)
\end{aligned}$$

The value S/A is again obtained by dividing by the final amplitude expressed by Eq. (13)

$$S/A = k_1 \quad (\text{for } [R_M]_o \gg [G_{tot}]. \qquad (23)$$

Equation (23) concerns the behavior upon presentation of a saturating flash (relatively low R_M), where the formation of R_MG complex suppresses the reaction $G_{memb} \rightarrow G_{free}$. Equation (20) describes the behavior for $G_{free} \leftrightarrow G_{memb}$ occurring upon presentation of a weak flash (relatively low R_M).

V. Experimental Test of the Model

A. Kinetics of the P_D-Signal

The theoretical considerations of the last paragraph could not give a complete expression for the signal kinetics expected on the basis of the proposed model. However, Eqs. (20) and (23) account for the initial rise of the scattering change under both extreme conditions of low rhodopsin turnover and saturating flashes. These equations explain the relatively rapid development of the P_D signal (S/A) for small ρ shown in Figs. 1*b* and 2*c*. This striking behavior of relatively rapid response to small stimuli is not explained by the earlier models.

It is easily seen that Eq. (23) is not only valid for the first flash but for all saturating flashes independent of R_M and R_MG prior to the flash. To test this result experimentally, a second and saturating flash ($\rho = 0.2$) was applied after the first flash. The final amplitudes of the resulting P_D signals are plotted against ρ of the first flash in Fig. 3*a*. The S/A ratio of these signals is plotted in the same way in Fig. 3*b*. Although all these signals correspond to different initial conditions and thus have different amplitudes, the S/A ratio exhibits a dependence on ρ consistent with Eq. (23).

The experimental values of the S/A ratio at low rhodopsin turnover and saturating flashes of Figs. 2*c* and 3*b* can be evaluated by Eqs. (20) and (23) and give $K_{MB} = 0.42$ and $k_1 = 0.28$ sec^{-1}. This value of K_{MB} corresponds to a membrane binding of 30% of the total G-protein.

The obtained values for K_{MB} and the apparent

rate constant of the membrane association reaction k_1 depend on the concentration of G-protein. To obtain the concentration-independent values of rate constant and binding constant we have to return to Eq. (6). This equation described the membrane association as a bimolecular reaction of G-protein with excess membrane binding sites (M). The rate constant of the forward reaction in this scheme is related to k_1 by

$$k_{on} = k_1/[G_{free}]_o .$$

The rate constant of the backward reaction of this equilibrium (k_{off}) is independent of the concentration and identical to k_{-1}. The values of k_{on} and k_{off} are determined as $k_{on} = 2 \times 10^6$ mol^{-1} s^{-1}, $k_{off} = 0.67$ sec^{-1}.

When the concentration $[M]$ of membrane binding sites in Eq. (9) is tentatively equated with the rhodopsin concentration $[R]$, a value of $K_A = 10^5$ M^{-1} is obtained.

B. Extra MII Formation in the proposed Model

The assumption of two states of noncomplexed G-protein $\{G_{memb}$ and $G_{free}\}$ concatenated in the proposed reaction scheme (12) has testable consequences for the formation of extra MII. At low ρ the complex formation (and therefore the extra MII formation) is fast, and independent of the subsequent membrane association process seen in the P_D signal. This kinetic difference between extra MII formation and scattering signal was observed on disks at low rhodopsin turnover by Emeis and Hofmann (1981). After saturating flashes, however, the complex formation is not terminated when all the previously membrane associated G-protein $[G_{memb}]_o$ is complexed with R_M. Further conversion of the previously free G-protein $[G_{free}]_o$ into the R_MG state is rate limited by the membrane association reaction. The extra MII formation measured after saturating light flashes is therefore expected to be biphasic and to consist of a fast and a slow component. The latter must be kinetically equal to the P_D signal.

The extra MII formation measured at 10°C and pH 7.5 after a saturating flash is shown in Fig. 4*a*. For comparison the extra MII formation measured under the same conditions at low rhodopsin turnover is shown in Fig. 4*b*. The signal corresponding to the saturating flash shows the expected biphasic kinetics in contrast to the fast MII formation at low rhodopsin turnover.

To test for the presence of a scattering artifact on the absorption measurement of extra MII which could explain the slow signal component, a measurement was made at $T = 10$°C and pH 6.0 (Fig. 5*b*) and compared to a control measurement at $T = 10$°C and pH 7.5 (Fig. 5*a*). At pH 6.0 all photoactivated rhodopsin forms MII independent of the complex formation and no enhancement of MII is observed. Figure 5*b* shows that under these conditions the MII signal lacks the slow component. However, under conditions of MII enhancement (Fig. 5*a*) the slow component occurs, with the same kinetics as the scattering signal. This observation excludes the contribution of a scattering artifact.

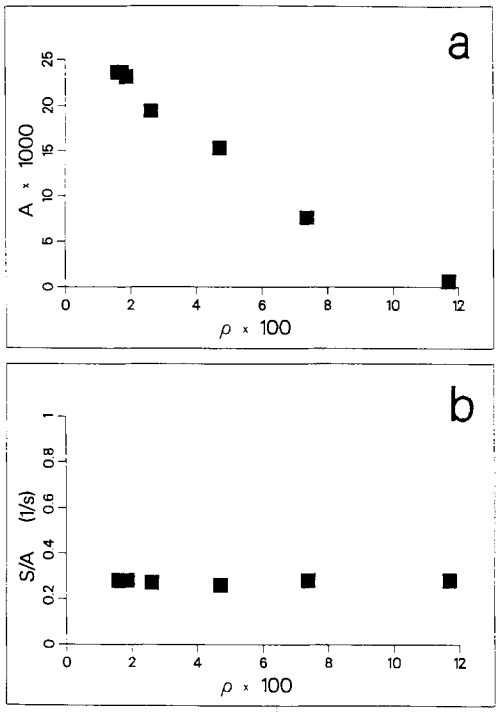

Fig. 3. (*a*) Signal amplitude A of the P_D signal induced by a second and saturating flash 5 min after the first flash. Values are plotted against the mole fraction ρ of rhodopsin photoactivated by the first flash. The second flash photoexcited $\rho = 0.2$ of the remaining rhodopsin. (*b*) Initial slope S of the signals corresponding to *a* divided by the corresponding signal amplitude plotted in *a*. The values S/A are plotted against the mole fraction ρ of photoactivated rhodopsin at the first flash

C. Quantitative Estimation of the Scattering Change $\Delta I/I$ by means of the Scattering Theory

The scattering intensity of a particle with size and shape of disks can be described by the Rayleigh-Gans theory (e.g. van de Hulst, 1957). Following this theory, the scattering intensity at the scattering angle θ is proportional to

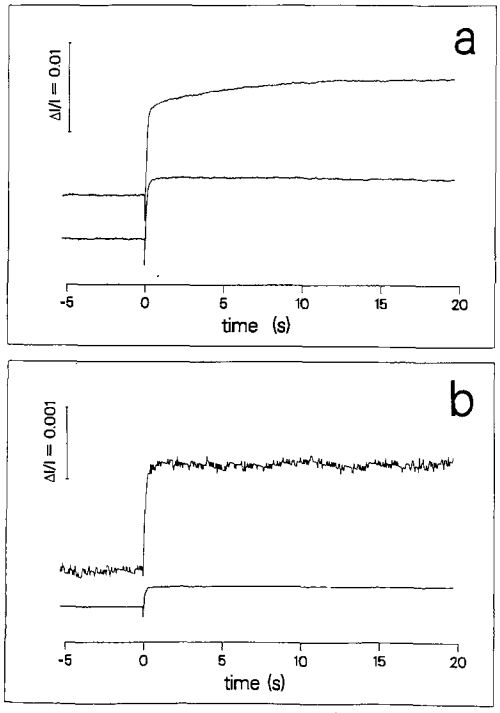

Fig. 4. MII formation after stepwise photolysis of rhodopsin with different flash intensities. (*a*) Upper trace: signal from flash 1 ($\rho = 0.2$); lower trace: sum of signals from flashes 3 ($\rho = 0.2$) and 4 ($\rho = 0.2$) normalized by exponential extrapolation to the same absolute amount of $[R_M]$ formed by flash 1 (*see* Materials and Methods). (*b*) Upper trace: signal of flash ($\rho = 0.009$); lower trace: flash 3 ($\rho = 0.2$) after flash 1 ($\rho = 0.009$) and flash 2 ($\rho = 0.2$) normalized to the first flash like the lower signal in *a*. Measuring conditions: pH 7.5, $T = 10°C$. The flash was applied at $t = 0$. MII formation was measured as difference of the absorption changes at 380 and 417 nm. Comparison of upper and lower traces shows the enhanced MII formation (extra MII)

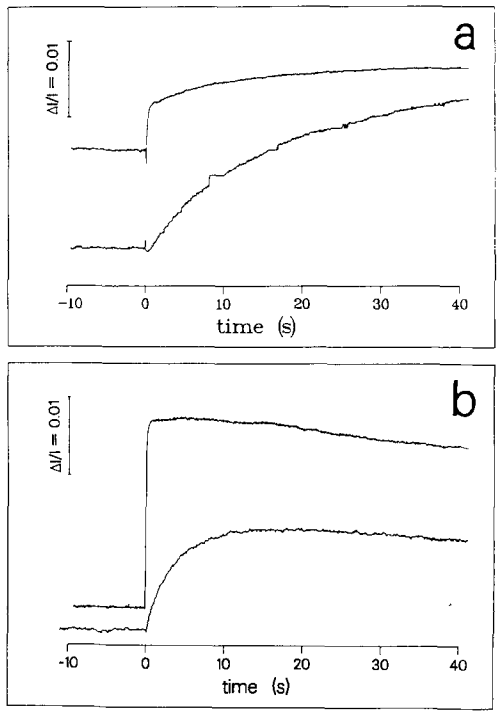

Fig. 5. MII formation and P_D signal measured simultaneously. Saturating flash ($\rho = 0.2$). (*a*) pH 7.5; $T = 10°C$. (*b*) pH 6.1; $T = 10°C$. In both *a* and *b*, the upper trace is the MII formation and the lower trace the P_D signal. MII measurement as in Fig. 4. The flash was applied at $t = 0$

$$I(\theta) \sim ((n/n_o)^2 - 1)^2 \cdot V^2 \cdot P^2(\theta) \cdot (1 + \cos^2\theta) \quad (24)$$

where n is the refractive index and V the volume of the particle and n_o the refractive index of the surrounding medium. $P(\theta)$ is the particle-scattering function, a term dependent on the particle shape. We consider the scattering intensity change when G-protein is associated to the membrane surface of flat disks. The $P(\theta)$ function is in the case of flat disks (thickness $\ll \lambda$) only dependent on the disk radius and nearly independent of the disk thickness (van de Hulst, 1957; Hofmann et al., 1981). The relative scattering intensity change $\Delta I/I$ is therefore independent of the scattering angle when G-protein is associated. This result is in agreement with the experimentally determined angular dependence of the relative intensity change described elsewhere (Hofmann et al., 1981; Schleicher & Hofmann, 1984).

On the assumption that G-protein and other portions of the disk have equal refractive indices, G-protein association has an effect only on the volume V. When we further assume a homogeneous density of membrane and G-protein, V can be replaced by the particle mass M in Eq. (24). This substitution leads to an equation relating the relative mass change with the scattering intensity change

$$dI/I = 2dM/M. \quad (25)$$

For small changes, the relative intensity change $\Delta I/I$ is predicted to be twice the relative mass change $\Delta M/M$.

Rhodopsin (mol wt = 40 kD) contributes 31% of the dry weight of disk membranes (Daemen, 1973). The G-protein has a molecular weight of 80 kD (Kühn, 1980). At a mole fraction of $[G_{tot}]/[R] = 0.05$ the mass of G is about 3.1% of the disk mass. 70% of G is free prior to illumination as shown above. The complete binding of G after saturating illumination results in a relative mass change of the disks of 2.2%. Using Eq. (25) a scattering intensity change of $\Delta I/I = 0.044$ is obtained. The estimated scattering change is seen to be of the same order of magnitude as the measured scattering signal ($\Delta I/I = 0.026$). The deviation by a factor of 1.7 of the theoretical

estimation from the experimental value suggests that the assumption of a homogeneous density and refractive index of G-protein and membrane may not be valid. A lower refractive index of G-protein than of the membrane can explain the discrepancy obtained. Using the value of the refractive index of the membrane $n_{memb} = 1.46$ (Liebman et al., 1974) and $n_o = 1.334$, the measured $\Delta I/I$ can be fitted with a refractive index of G-protein $n_G = 1.41$ when homogeneous density is assumed.

D. Comparison with the Dissociation Signal

Further support for the proposed model comes from a comparison of the signal amplitude of the P_D signal and the dissociation signal which corresponds to the complete release of the G_α subunit and a partial release of the $G_{\beta\gamma}$ subunit from the membrane in the presence of GTP (Kühn et al., 1981; Michel-Villaz et al., 1984; Vuong, Chabre & Stryer, 1984). Under the measuring conditions 30% of G is membrane-bound before illumination. The mass change seen in the saturating P_D signal is due to the complete binding of previously free molecules of G (70% of G_{tot}). In the presence of GTP every individual G-protein transiently interacts with photoactivated rhodopsin. After the fast GDP/GTP exchange the G_α subunits are completely released from the membrane (Kühn, 1980). The $G_{\beta\gamma}$ subunits are not released to the same extent as G_α but are less associated to the membrane after the release of G_α than before illumination (Kühn, 1980; Fung, 1983). The loss of mass indicated by the dissociation signal therefore corresponds to the release of previously associated G_α subunits (30% of $G_{\alpha\,tot}$) and a small part of the previously associated $G_{\beta\gamma}$ subunits. As both subunits have approximately the same molecular weight, the dissociation signal is expected to have a sign opposite to that of the P_D signal and an amplitude of 1/3- to 1/4-fold that of the P_D signal.

Both signals, a P_D signal measured in the absence of GTP and a dissociation signal measured at a saturating GTP concentration [GTP] = 80 μM are shown in Fig. 6. The final amplitude of the P_D signal is seen to be about three times higher than the amplitude of the dissociation signal, which is in agreement with the prediction.

The dissociation signal shown in Fig. 6 is faster than the P_D signal measured under the same conditions in the absence of GTP. However, this fact presents no contradiction to the sequence hypothesized above for membrane binding and dissociation of G-protein. At relatively high GTP concentrations both the complex formation and dissociation of the previously membrane-bound G_α, are very fast and

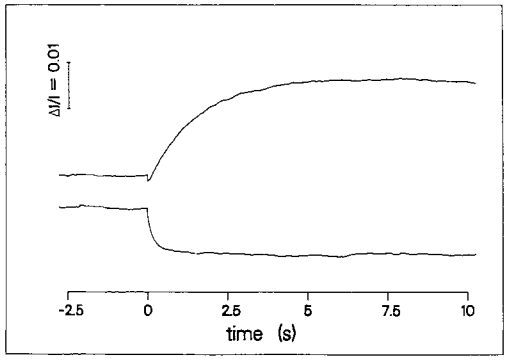

Fig. 6. P_D signal and dissociation signal. Upper trace: P_D signal in the absence of GTP. Lower trace: Dissociation signal in the presence of 80 μM GTP. Measuring conditions as in Figs. 1 and 2. Saturating flash ($\rho = 0.2$). The fast negative component seen in the upper trace is the N signal (Hofmann et al., 1976). The same negative component is also superimposed on the dissociation signal in the lower trace. For comparison of the amplitudes it has to be subtracted. The flash was applied at $t = 0$. Measuring conditions: pH 6, $T = 21$°C

give rise to a fast dissociation signal. The previously free G-protein molecules are slowly bound and quickly released from the membrane. This transiently bound protein mass contributes only very little to the total scattering mass. The dissociation signal therefore reaches its final amplitude before all of the G-protein is activated.

Bennett and Dupont (1985) have interpreted the P_D signal as a conformational change of the G-protein during the R_MG interaction, necessary for the GDP/GTP exchange. The present finding, that the P_D signal can be slower than the dissociation signal, argues against this interpretation. That is, according to the interpretation of Bennett and Dupont the P_D signal should always be rate limiting for the development of the dissociation signal.

E. Determination of the Activation Energy of the Membrane Association

Diffusion of soluble G-protein to the membrane cannot be the rate-limiting step for the binding of G to the membrane since a diffusion limited reaction of a molecule with the diameter of G-protein would be at least by two orders of magnitude faster than the observed signal (Wedler, 1982). The kinetics of the P_D signal has therefore to be determined by the mechanism of the association itself. Our results cannot elucidate the binding mechanism of the G-protein to the membrane, but we can determine the activation energy of the association reaction.

The activation energy of the binding reaction can be determined from the temperature depen-

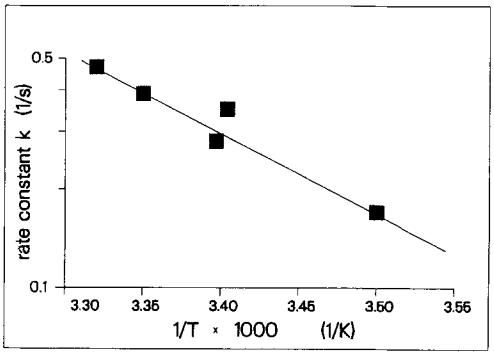

Fig. 7. Arrhenius plot of the rate constant of the P_D signal. The rate constant k was obtained by a least squares fit of the scattering signal by a single exponential function $a \cdot (1 - \exp(-k \cdot t))$. All measuring points correspond to signals from a first and saturating flash ($\rho = 0.2$)

dence of the kinetics of the P_D signal. The signal waveforms, recorded at different temperatures with saturating flashes, were fitted by a first-order reaction using a computer fit program. The activation energy can be determined from the P_D signal kinetics by an Arrhenius representation of the rate constant as shown in Fig. 7. The activation energy obtained from the analysis is $E_a = 44$ kJ/mol. The obtained value is by a factor of 3 higher than the activation energy of a diffusion-limited reaction (Wedler, 1982).

Conclusion

In this kinetic study on isolated disk membranes we have compared the light-induced formation of the complex between rhodopsin and G-protein with its stoichiometric light-scattering monitor (binding signal or P_D signal). The main objective has been to explain the slow kinetics of the P_D signal.

The proposed model interprets the scattering change by the gain of scattering mass arising from the transition of G-protein from a free state (G_{free}) into a disk membrane-bound state (G_{memb}). The equilibrium between both these states is shifted by the binding of G_{memb} to photoactivated rhodopsin R_M

$$G_{\text{free}} + R_M \leftrightarrow G_{\text{memb}} + R_M \leftrightarrow R_M G.$$

The analysis of the signal kinetics as a function of flash intensity has especially shown that the P_D signal is neither a real-time monitor of the $R_M G$-complex formation itself, nor a subsequent structural reaction of the formed complex.

Bennett and Dupont (1985) have proposed a model of concatenated reactions involving the formation of $R_M G$ complex, a resulting conformational change of the bound G-protein and GDP/GTP exchange. They interpreted the P_D signal as a conformational change of the G-protein during the $R_M G$ interaction as a necessary step preceding GDP/GTP exchange. However, comparison of the observed kinetics of P_D signal and dissociation signal shows that the dissociation signal can, at least under certain conditions, develop much faster than the P_D signal. Thus the step responsible for the P_D signal cannot be rate limiting for the dissociation signal, and it can be concluded that the process seen in the scattering change is not involved in the rhodopsin/G-protein/phosphodiesterase signal transduction chain.

The time course of the membrane-association process of G-protein is not rate limited by the diffusion time of G-protein to the membrane. There are two distinct states, the membrane-bound and free G-protein, which are separated by an activation energy barrier of 44 kJ/mol. These states might correspond to two different configurations of the G subunits.

The kinetic analysis of the P_D signal yielded the rate of the membrane association. The rate constants of forward (k_{on}) and backward (k_{off}) reaction were determined as $k_{\text{on}} = 2 \times 10^6\ \text{M}^{-1}\ \text{sec}^{-1}$ and $k_{\text{off}} = 0.67\ \text{sec}^{-1}$. These values of k_{on} and k_{off} are in the same order of magnitude as those determined by Liebman and Sitaramayya (1984) by mixing activated and dark-adapted membranes. The differences between these values are most probably due to the differences in the preparation. These rate constants demonstrate that the transition of G-protein to and from the membrane are extremely slow processes compared to the very fast complex formation of membrane-bound G-protein with photoactivated rhodopsin (as seen in the extra MII formation at low rhodopsin turnover; Fig. 4*b*). In agreement with Liebman and Sitaramayya (1984) this excludes a so-called "hopping" mechanism, i.e., a transition of membrane-bound G-protein into solution and back to photoactivated rhodopsin.

Consequently the interaction of G-protein on the membrane with photoactivated rhodopsin does not appear to involve any dissociation of G from its dark membrane-binding site. This argues strongly for a diffusible anchor on the membrane surface. G-protein would remain persistently bound to this site while interacting with R_M.

The question of the molecular nature of the dark-binding site could not be elucidated in this study. Current investigations indicate that monoclonal antibodies against the G_α subunit are able to

inhibit G-protein activation by affecting a counterpart of the dark-binding site at G_α (H.E. Hamm, D. Deretic, K.P. Hofmann, A. Schleicher & B. Kohl, to be *published*). This effect is observed on membranes as well as in detergent, suggesting that the dark-binding site is located on rhodopsin.

This study is restricted to the investigation of the scattering signal on isolated disk membranes. In nonfragmented ROS, the observed *P* signal is much faster and might arise from structural processes (Hofmann, et al., 1981) that are basically different from the transition of G-protein from the soluble state to a membrane-associated condition.

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