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Light and GTP dependence of transducin solubility in retinal rods

Further analysis by near infra-red light scattering

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Abstract. The physical origin and functional significance of the near infra-red light scattering changes observable upon flash illumination of diluted suspensions of magnetically oriented, permeabilised frog retinal rods has been reinvestigated with particular attention paid to the degree with which transducin remains attached to the membrane. In the absence of GTP, the so called "binding" signal is shown to include two components of distinctive origins, widely different kinetics, and whose relative amplitudes depend on the dilution of the suspension and resulting detachment of transducin from the disc membrane. The fast component is a consequence of the fast interaction between photoexcited rhodopsin (R*) and the transducin remaining on the membrane. Its kinetics monitors a structural modification of the discs caused by a change in electrostatic interaction between closely packed membranes upon the formation of R*-*T* complexes. The slow component monitors the slow rebinding to the membrane and possible subsequent interaction with excess R* of *T*-GDP which, in spite of its low solubility, had eluted into solution given the high dilution of the permeated rods. In the presence of GTP, the so called "dissociation" signal includes a fast, anisotropic "release" component that specifically monitors the release into the interdiscal space of T_α-GTP formed from the membrane-bound pool, and a slower isotropic "loss" component monitoring the leakage from the permeated rod of the excess T_α-GTP which did not interact with the cGMP phosphodiesterase. The amplitudes of both components depend exclusively on the membrane bound T-GDP pool. The kinetics of the "loss" component is limited by the size and degree of permeation of the rod fragments, rather than by the dissociation rate of T_α-GTP from the membrane.

Key words: Retinal rods, phototransduction, rhodopsin, transducin, light scattering

Introduction

Near infra-red light scattering has been extensively used to investigate the light-triggered rhodopsin-transducin-cGMP phosphodiesterase cascade reactions in retinal rod outer segments (ROS). Many types of transient changes in scattering power of ROS membrane suspensions have been observed whose signs, amplitudes and time courses depend on the scattering geometry, the light flash intensity and the presence of GTP; these changes are also sensitive to the physical state of the ROS fragments and the concentration of the membrane preparation. At first, the approach was purely phenomenological, the light scattering transients were simply named for their signs: P and N "signals" for the positive (scattering increase) and negative transients observed in the absence of GTP on ROS fragments (Hofmann et al. 1976). Following an observation by Bignetti et al. (1980) on the influence of GTP on the scattering properties of ROS suspensions, Kühn et al. (1981) demonstrated by reconstitution experiments, the correlation of light scattering transients with the activation of the transducin cascade and the major role of GTP in the process. Using suspensions of cattle ROS fragments, they described three types of signals: a "binding" signal was shown to be correlated with the binding of transducin to photoexcited rhodopsin (R*), a process known to be induced by illumination in the absence of GTP (Kühn 1980). The ampli-

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Abbreviations: ROS: rod outer segment; R: rhodopsin; R*: photoactivated rhodopsin; *T*, *T*-GDP, T_α-GDP, T_α-GTP, $T_{\beta\gamma}$: transducin and its various forms; T_{mb}, T_{sol}: $T_{\alpha\beta\gamma}$ bound to membrane or soluble; PDE: cGMP-phosphodiesterase; GTP: guanosine 5′-triphosphate; GDP: guanosine 5′-diphosphate; GDPβS: guanosine 5′-O-(2-thiodiphosphate); cGMP: guanosine-3′-5′ cyclic-monophosphate; DTT: dithiothreitol; HEPES: 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid; TRIS: Tris (hydroxymethyl)aminomethane; SDS: sodium dodecyl sulfate

tude of the signal is linear with the number of rhodopsin photoexcited by the flash, but saturates when an amount of R* equivalent to the total pool of transducin has been produced; in reconstituted systems the summed amplitude of the signal is proportional to the total transducin content. As for the time course, its early rising phase is fast, i.e. in the millisecond time range, but its overall kinetics are complex: slow components are also observable whose relative contribution seems to depend on the physical state of the ROS fragment suspension, the total signal becoming slower as the ROS are more fragmented, or mistreated. This "binding" signal is equivalent to the "P" signal (Hofmann et al. 1976). Upon illumination in the presence of GTP, a "dissociation signal" of opposite sign (decrease in turbidity) was shown to relate to the R*-catalysed GDP/GTP exchange reaction on transducin. As this reaction is highly amplified (Fung and Stryer 1981), the signal is observable and saturable at very low light intensity. Its kinetics are also sensitive to the fragmentation of the rods, but get faster as the fragmentation increases. Finally, when all reactions involving transducin have been exhausted, either by total binding in the absence of GTP, or total dissociation in its presence, intense flashes elicit "rhodopsin" signals, which relate directly to the shift of the spectral absorption peak of rhodopsin upon photoexcitation. This is equivalent to the "N" signal of Hofmann et al. (1976).

Vuong et al. (1984) further analysed the "dissociation" signal in a special scattering geometry where structurally intact frog rod fragments were magnetically oriented at 45° to the incident infrared beam and scattered light observed at 90° and 270° as well as transmitted light at 0°. A fast and highly anisotropic "release" component was found superimposed on a slower, larger and isotropic "loss" component which is equivalent to the former "dissociation" signal. The interpretation is that both components are due to the solubilisation of the T_α-GTP subunit of transducin which is released from the disc membrane after the GDP/GTP exchange step: the early "release" signal corresponds to the release of transducin from the disc surface into the interdiscal cytoplasmic space, and the "loss" signal to its subsequent loss from the leaking, fragmented rod into the bulk solution.

The "release" component must not be taken as a small "binding" signal even though they are of the same polarity. Indeed, in the absence of GTP, a "binding" signal large enough to be observed requires a photolysis level of at least 0.5% R*; this is about 100 times more R* than is needed to elicit a "release" signal. In other words, without GTP, a small flash (e.g. 0.1% R*/R) does not elicit a "binding" signal, or any measurable signal. Furthermore there is a tremendous difference between the left/right anisotropy of the "release" signal and that of the "binding" signal; the former anisotropy is at least 10 while the latter is at most 2. Finally, the kinetics of the two signals are also very dissimilar. While the "release" signal has a rise time in the 100-ms range which depends on the photolysis level, the "binding" rises in 10–20 ms and this is independent of the level or R*/R.

Other types of fast signals elicited by weak flashes in the presence of GTP have since been observed by Kamps et al. (1985), Kamps and Hofmann (1986) and Wagner et al. (1987). We will discuss later these other experiments in the context of our new investigations on the "release" component.

In all our experiments, the samples are diluted suspensions of rods or rod fragments. The dilution is such that the average distance between the scattering objects is much larger than the wavelength of the probing light. The waves scattered from different objects add up incoherently and the scattered intensity is not sensitive to possible changes in interaction between distant rod fragments. Interference effects between fragments being negligible, the total scattered intensity of the sample is the scalar sum of the intensities scattered by each individual fragment and is therefore linear with the concentration of the suspension. Changes of total scattered intensity can result only from a gain or loss of scattering material between the solution and the particles (i.e. proteins that bind to the membrane or go into solution), or from a change in index of refraction due to a spectral absorption shift of a component of the particle (i.e. anomalous dispersion effects). Each rod or rod fragment includes an array of closely stacked and strongly interacting discs whose packing and ordering may be slightly modified upon changes in electrostatic interaction between the membranes caused by changes in surface charges. These slight changes in the internal structure of the particles should not change the total scattered intensity but may modify slightly the pattern of scattering, and can be observed by measuring scattered light at non zero angles (see Chabre 1985, for a general discussion). In concentrated suspensions of disc membrane vesicles, much larger and slower light scattering changes have been observed under various conditions (Lewis et al. 1984; Caretta and Stein 1985, 1986). In these preparations, light scattering is clearly dominated by interparticle interferences between scattered waves and is very sensitive to variations in the correlation distance between particles. These light scattering changes result from a change of interaction, probably electrostatic in origin, between the membrane fragments upon formation of the R*-T complex and from the ensuing partial aggregation of the membrane vesicles. However, the amplitude of these light scattering changes is not linear with the sample concentration and is therefore very difficult to analyse quantitatively. Other kinetically

complex and slow light scattering changes have also been observed by Carretta and Stein (1985, 1986). These depend on the presence of GTP as well as cGMP and on the vesicular state of the preparation. They must therefore be related to the onset of the cGMP PDE activity and will not be discussed here.

In dilute suspensions of cattle rod membrane vesicles, Schleicher and Hofmann (1987) recently documented a "binding" signal that was significantly slower than that observed on ROS fragments: its final level is proportional to the light flash intensity and to the amount of transducin that binds to R*, but its time course is clearly slower than that of the R*-*T* complex formation which can be monitored independently by spectroscopic measurement of the extra Metarhodopsin II formed from the interaction of *T* and R* (Emeis et al. 1982). Their interpretation is therefore that in this preparation the slow "binding" signal (which they denote P_D) is indeed caused by the rebinding to the vesicles of transducin that has previously been released from the membrane and whose concentration in solution is in equilibrium with the surface concentration of membrane bound transducin. Upon illumination, the transducin that binds very strongly to R* becomes non-exchangeable with the solution. This depletes the population of weakly membrane-bound transducin and causes rebinding from the solution. In this preparation of separated disc membrane vesicles, the fast R*-*T* association does not lead to any fast light scattering changes. This is in apparent contradiction with the case of ROS or ROS fragment suspensions.

Some confusion thus seems to have arisen concerning the physical origin and significance of the "binding" signal. This may be due in part to the adopted nomenclature: in dark, intact ROS, essentially all the transducin is bound to the disc membrane and the fast component of the "binding" signal is originally assumed to be an indirect effect of the light-induced binding to R* of transducin molecules that are already present on the disc membrane, and not to arise from binding to the membrane of transducin molecules previously in solution. Such an interpretation cannot account for the slow component. It is now realised that when rods are fragmented and diluted, a significant fraction of the transducin dissolves into the solution even though the concentration there remains much lower than the concentration on the membranes. The recent work of Schleicher and Hofmann (1987) suggests that re-equilibration between membrane-bound and free transducin also plays an important role in the generation of the "binding" signal. In short, "binding" in this revised context must refer to two interrelated processes: *i*) the binding of membrane-associated *T*-GDP to R* and *ii*) the re-binding of soluble transducin to the membrane surface.

The present work further investigates the physical origins and the structural as well as the functional significance of the "binding" and "dissociation" signals. In particular, we analyse the signals in relation to the binding and solubilisation of transducin to and from the discs. The samples are diluted suspensions of permeabilized frog ROS or ROS fragments. These large photoreceptor cells are structurally much more rigid than the cattle ROS commonly used in other studies. They can be fragmented and made sufficiently permeable to allow fast equilibration of soluble components between the cytoplasmic space and the external solution while preserving the native, ordered stacking of the disc membranes, as was shown in previous neutron diffraction experiments conducted on the same types of preparations (Vuong et al. 1987).

Materials and methods

All experiments were performed under dim red light. Unless otherwise stated, the suspension buffer contained: NaCl 102 m*M*, KCl 2.7 m*M*, $CaCl_2$ 1.9 m*M*, $NaHCO_2$ 2.0 m*M*, NaH_2PO_4 0.36 m*M*; pH 7.4. Alternatively, the phosphate carbonate buffer may be replaced by a TRIS acetate buffer (10 m*M*).

GTP (5 m*M*) was prepared in suspension buffer (pH 7.4); GTPγS (1 m*M*) was prepared in HEPES (5 m*M*, pH 7).

a) ROS preparation

Frog rod outer segments (ROS) were prepared according to the usual procedure (Chabre 1975): freshly dissected retinae are shaken in buffer and filtered through gauze. ROS were separated from small membrane debris by a mild (280 g, 2 min) centrifugation and resuspended in buffer at a concentration of about 4 retinae/ml. This stock suspension was stored on ice and used within 2 h.

b) ROS permeation

Two techniques were used: mechanical fragmentation or electroporation.

Mechanical fragmentation: passing a ROS suspension a few times through a constricted hypodermic needle (21 gauge, 0.8 mm ID) yields 10 μm long fragments which are leaky to GTP and transducin.

Electroporation: it is known that brief (10 μs) electric pulses (1,000 V/cm) can irreversibly permeabilize the plasma membrane of cells 10 μm in size (Zimmermann 1982). We install between the pole pieces of an electromagnet (0.8 T) a 300 μl cuvette equipped with two par-

allel flat electrodes 5 mm apart. The magnetic field insures that all ROS have the same orientation with respect to the electric field. A high voltage generator (0–2,000 V), a capacitor and a mechanical mercury switch complete the system. Routinely, we apply three electric pulses (2,000 V/cm) 20 s apart to the suspension; the ROS are oriented with their axes parallel to the electrode plates. Electroporated ROS were used within 15–30 min of their preparation. Light microscopic observation and use of the fluorescent dye *N–N'* di DANSYL cystine (Yoshikami et al. 1974) reveal that the suspension contains mainly 50 μm long permeable ROS. A more extensive study of electroporation on oriented ROS will be published elsewhere.

c) Extraction of partially purified transducin

The extraction was done as described by Kühn (1981). ROS from a concentrated (10 retinae/ml) suspension were mechanically fragmented (see above) and sedimented at 400,000 *g* for 3 min (Beckman TL100, München, FRG). The supernatant contains soluble proteins and was removed; the pellet was illuminated and resuspended at the same ROS concentration in low ionic strength buffer (HEPES 5 m*M*, DTT 1 m*M*). Centrifugation separates the membranes from a "crude PDE" extract that was also removed. The pellet was resuspended again in low ionic strength buffer at the same ROS concentration with 60 μ*M* GTP; centrifugation (400,000 *g*, 5 min) yields a protein extract containing mainly transducin. After hydrolysis of the GTP bound to T_α (10 min at room temperature), the solution was kept on ice. We have checked that this transducin still binds to membranes at moderate ionic strength and is released in the presence of R* and GTP (data not shown, see Table 2).

d) Light scattering set-up

All measurements were performed using the apparatus described by Vuong et al. (1984) (see also Fig. 1). ROS suspended in a 1 cm-path-length quartz cuvette were magnetically oriented at 45° to the incident infrared beam (900–1,000 nm). The light scattered at right angles (90°: right side; 270°: left side) was monitored by photovoltaic diodes whose output was amplified, digitized and stored in 512 channels. Fast and slow recordings were filtered with RC filters of time constants 2.5 and 50 ms respectively. A photographic flash unit fitted with a 500 nm interference filter and a series of neutral density filters provides 0.5 ms light pulses to excite the sample via a light guide from below the cuvette. The sample volume is 1.3 ml.

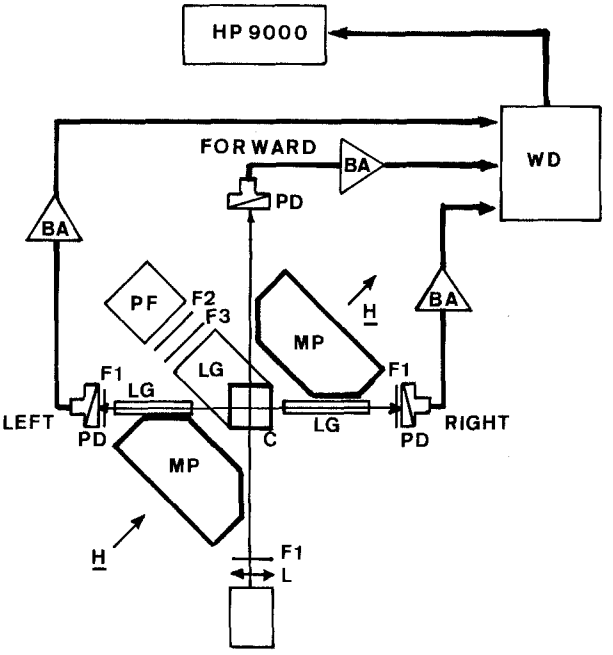

Fig. 1. Experimental set-up. The infra-red light source is an Osram 64625 quartz-halogen lamp (*TL*). A 75 mm focal length plano convex lens (*L*) collimates the incident beam. An Oriel 5155 long pass filter (*F1*) selects the infra-red light. The source of actinic light is a Sunpack 36DX photographic flash (*PF*) fitted with a 500 nm interference filter (*F2*) and appropriate neutral density filters (*F3*). The actinic light illuminates the 1 cm × 1 cm fluorescence cuvette (*C*) from below via a light guide (*LG*). The two magnet pole pieces (*MP*) are oriented to provide a field of 0.3 T, directed at 45° to the incident beam. Scattered light is collected on the left and right by two PIN 10DP (United Detector Technology, Hawthorne, California, USA) photodiodes (*PD*) each fitted with a light guide (*LG*) and an Oriel 5515 long pass filter (*F1*). Transmitted light is collected by a PIN 6DP photodiode (United Detector Technology Hawthorne, California, USA). The current output from each photodiode is converted to voltage and amplified by a UDT 101A (United Detector Technology, Hawthorne, California, USA) preamplifier. The UDT 101A output is fed into one differential input of an Analog Device (AD 521) instrumentation amplifier (*BA*), while a constant voltage source is presented to the other differential input. The output of the AD 521 instrumentation amplifier (which measures the variation in scattered light ΔI_{sc}) is digitized by a multi-sector, dual time base waveform digitizer (*WD*). The constant voltage source (which gives the initial scattering intensity I_{sc}) is continously monitored by a digital voltmeter. Data (ΔI_{sc} and I_{sc}) are sent to an HP 9000 computer for storage and analysis

e) Recording of light scattering transients

An adequate volume of a permeated suspension was diluted with buffer in the cuvette, the sample was then kept in the dark at room temperature for 5 min to insure equilibration between membrane bound and soluble transducin. For dissociation signals, 0.5 m*M* GTP was then added. The sample was then put into the cuvette holder, orientation of ROS and stabilization of the suspension take from 3 to 5 min. The recording was made 2 to 3 min after this stabilization is reached.

f) Titration of membrane bound and soluble transducin

DTT (1 m*M*) was added to the degassed suspension buffer. An aliquot (100 µl) of the sample used for the light scattering recording was set aside. As a light scattering signal is recorded with the sample, the aliquot is centrifuged at 436,000 *g* for 1 min. The supernatant (S_{sol}) contains the soluble transducin. The pellet was then fully photolyzed and resuspended in low ionic strength buffer (5 m*M* HEPES, 1 m*M* DTT) in the presence of 40 µ*M* GTPγS. Centrifugation (436,000 *g*, 5 min) extracts the membraneous transducin from the discs (supernatant S_{mb}). The two supernatants (S_{sol} and S_{mb}) were analyzed by SDS-polyacrylamide gel electrophoresis (10%). The relative amount of T_α in each supernatant was assayed by scan densitometry of the Coomassie Blue-stained bands.

Results

I) Binding signal: evidence for a fast and a slow component of different origins

Figure 2 shows a typical "binding" signal as observed at 90° and 270° on a suspension of oriented frog ROS after a flash bleaching a few percent of the rhodopsin *in the absence of GTP*. These signals clearly result from the superposition of two components of comparable amplitudes, but whose kinetics differ by nearly three orders of magnitudes: a fast component whose rise time is 10–20 ms, and a slow one whose progression lasts for about 20 s. No components of intermediate kinetics can be detected. The rise time of the fast component is highly invariant: it does not depend on the concentration of the ROS fragment suspension, nor on its degree of fragmentation by shearing, nor on the flash intensity. By contrast, the rise time of the slow component appears to vary slightly with different preparations and seems to depend on the degree of fragmentation and the dilution of the sample. The dramatic difference in kinetics allows an unambiguous resolution of the total amplitude A_T into a fast and slow component, A_F and A_S. In agreement with the previous results on cattle ROS suspensions (Kühn et al. 1981), the total amplitude $A_F + A_S$ is approximately linear with the amount of rhodopsin photolysed per flash, and saturates at a photolysis level of about 10%. However the relative amplitudes of the two components depends on the sample concentration at a given flash intensity and for aliquots of the same sample, on the flash intensity. This results from very different saturation behaviours for the amplitudes of A_F and A_S. When aliquots of a given ROS suspension are submitted to flashes of increasing intensity, one observes that A_F increases linearly with the flash intensity, but only up to 2 to 4% photolysis, depending on the sample concentration, and saturates sharply above this limit (Fig. 3). In contrast, the slow component A_S is barely detectable at the lowest flash intensity but increases non-linearly at the larger flash intensities and saturates at about 10% photolysis. The two components differ also in their left/right anisotropy. All these differences point to different origins for these two components of the "binding" signal.

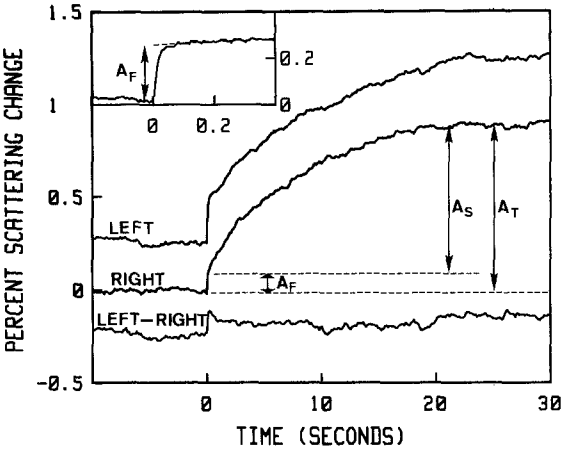

Fig. 2. A typical "binding" signal. The conditions are: R*/R = $6.4 \cdot 10^{-2}$; 0.1 mg rhodopsin/ml; no GTP added; temperature 23 °C. Both right angle detectors record a fast as well as a slow component; the difference between left and right signals reveals the anisotropy of the fast component. *Insert:* The same signal seen by the right detector on a faster time scale: linear extrapolation of the slow component to $t = 0$ defines the amplitude of the fast component A_F. The amplitude of the slow component (A_S) is obtained by subtracting A_F from the total amplitude A_T

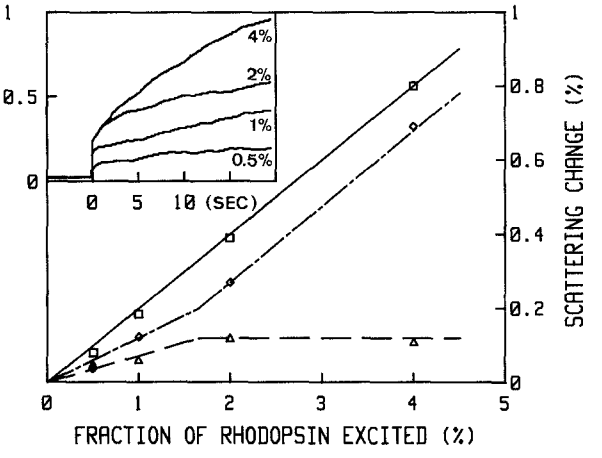

Fig. 3. The fast component of the "binding" signal saturates at 2% photolysis. The total amplitude (*solid line*) of the "binding" signal, amplitudes of the fast (*dashed line*) and slow (*dashed-dotted line*) component are plotted as a function of photolysis level. These data are from the right side detectors. *Insert:* Four "binding" signals were obtained on aliquots of the same suspension (0.05 mg rhodopsin/ml) with increasing photolysis levels: 0.5, 1, 2, 4% R*/R. These data are from the left side detectors

As suggested by the work of Schleicher and Hofmann (1987) one can hypothesize that the two components of the "binding" signal are related to two different pools of transducin: the membrane bound transducin, T_{mb} would give rise to the fast component, and a solubilised transducin pool, T_{sol} would be responsible for the slow component. In a suspension medium of ionic composition comparable to that of the cytoplasm, T-GDP ($T_{\beta\gamma}$-T_{α}-GDP) is sparingly soluble in the dark. An equilibrium exists between the surface concentration of T_{mb} and the volume concentration of T_{sol}. Inside the intact rod, the concentrations of transducin and disc membrane are very high: 500 μM and a few hundred mg/ml; from the binding equilibria (Liebman and Sitaramayya 1984) one can estimate that practically 100% of the transducin is on the membrane. In our samples, the proteins on the disc membrane equilibrate with a volume of solution that is about three orders of magnitude larger than that of an intact ROS and although the concentration in solution remains very low, a significant proportion of the transducin elutes into the medium in the dark. Upon illumination, the photoexcited rhodopsin acquires a very high affinity for transducin and will bind to a membrane-associated transducin molecule located in its vicinity. The transducin that is trapped in R*-T complexes is very tightly bound and can no longer take part in the equilibrium between T_{mb} and T_{sol}: the depletion of the T_{mb} pool will therefore induce a rebinding of previously solubilised transducin to the membrane and eventually to R* if the flash has produced R* in excess of the initial T_{mb} pool. This is a simple case of mass effect where the equilibrium $T_{mb} \rightleftharpoons T_{sol}$ is shifted to the left as the T_{mb} population is depleted due to the binding to R*. The kinetics of the rebinding is necessarily slower than that of the initial R*-T_{mb} interaction and the subsequent depletion of the T_{mb} pool which causes the rebinding. One also expects these kinetics to depend on the physical state (i.e. disc stacking, disc diameter and outer membrane leakiness) of the ROS fragments. To test the hypothesis that the fast and slow components of the binding signal correlate with the T_{mb} and the T_{sol} pools respectively, we analysed the dependence of the two components not only on the flash intensity, but also on the sample concentration.

The fast component is related to the R-T interaction on the membrane.* A first indication that the fast binding component only involves the fast interaction between R* and the pool of transducin already present on the membrane was the observation that A_F saturates sharply at photolysis levels between 2% and 4%, depending on the sample concentration: the higher the sample concentration, the higher the saturation level. In Fig. 3, with a membrane concentration of about

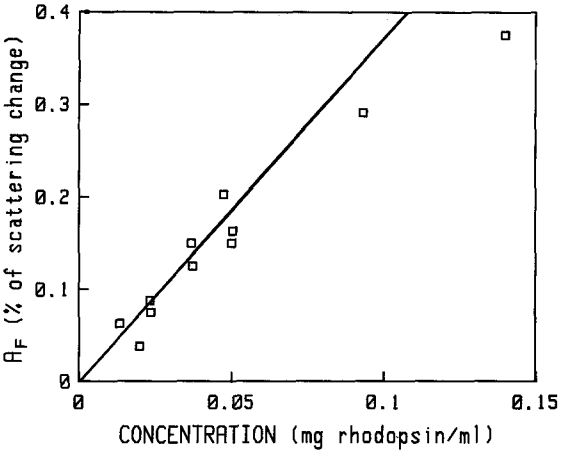

Fig. 4. Effect of dilution on the amount of bound transducin. The amplitude of the fast component of the binding signal (no GTP added; R*/R = 6.4%) is plotted as a function of rhodopsin concentration; data are those of the right detector. Titration of the samples shows that 3 V in initial scattering intensity correspond roughly to 0.1 mg rhodopsin/ml; this calibration is linear up to 5 V in initial scattering or about 0.2 mg rhodopsin/ml. The first 10 points are fitted to a straight line passing through zero

0.05 mg rhodopsin/ml, saturation already occurs for a flash which activates 2% of the rhodopsin. Assuming a native R : T-stoichiometry on the disc membrane of ten to one, this implies that only 20% of the transducin had remained membrane bound in this preparation. This is consistent with the previous estimate of about 30% of membrane-attached transducin on samples of permeabilised frog ROS of comparable concentrations (Liebman and Sitaramayya 1984). When the same flash of saturating intensity for A_F (6.4% photolysis) was applied to suspensions of various concentrations, the amplitude of the saturated fast component increases linearly with the sample concentration (Fig. 4). Furthermore, addition of purified transducin to a ROS suspension increases the amplitude of the fast component obtained on a first saturating flash. The linearity of the saturation level with the amount of membrane bound transducin was checked by using the GTP-requiring "release" signal, and will be discussed later (Fig. 7).

Evidence that the fast component does not monitor a net binding of protein to the particle, but only a rearrangement of internal structures comes from the anisotropy of its angular distribution. A gain or loss of mass by a scattering particle induces a variation in scattered light $\Delta I_{sc}/I_{sc}$ that must be essentially independent of the angle if the proteins gained or lost are distributed uniformly within the particle: in particular it should be identical on the left and right detectors. Here, the left/right anisotropy of the fast component is about 2 to 1. If $\Delta I_{sc}/I_{sc}$ is the same at all angles, a variation of similar amplitude should also be observed

on the integral $S = \int I_{sc} d\sigma$ of the scattered light over all angles. S can be estimated by measuring of the transmitted light I_T on the forward detector: $S = I_0 - I_T$, I_0 being the incident beam intensity. For a mass gain or loss $\Delta S/S = \Delta I_{sc}/I_{sc}$. By contrast, for a change in particle structure without gain or loss of mass, the very intense forward angle scattering which is quantitatively predominant in S, is comparatively less affected since at vanishingly small scattering angles the scattered intensity depends only on the total scattering mass. Therefore in such cases $\Delta S/S$ is probably different, and usually smaller than $\Delta I_{sc}/I_{sc}$ measured at a large angle (see Chabre 1985). For the fast component of the binding signal, $\Delta S/S$ measured on the forward detector was much smaller than $\Delta I_{sc}/I_{sc}$ measured on either of the side detectors, thus indicating that $\Delta I_{sc}/I_{sc}$ must be of vanishing amplitude at small angles. This is in agreement with measurements by Hofmann et al. (1981) of the angular variation of the "binding" signal's amplitude on intact cattle ROS where the fast component was the only one detected.

Table 1. Recovery of the fast component of the "binding" signal after one flash. Samples at roughly the same concentration were flashed twice at 2% R*/R, intervals between flashes were 1, 4, 20 and 90 s. The table summarizes the fast component A_F (in %) for each flash as well as the amplitude of the slow component A_S in between. These data are from the left detector

Interval between the two flashes (s)		1	4	20	90
Amplitude of the fast component (A_F)	first flash (*F1*)	3.6	2.9	2.5	2.1
	second flash (*F2*)	0.92	0.59	1.1	1.8
Recovery (A_{F2}/A_{F1})		0.26	0.20	0.44	0.86
Amplitude of the slow component between the flashes (A_S)		0.4	1.1	3.6	3.5
Fraction of the slow component reached at time of the second flash (A_S/A_{Smax} where $A_{Smax} = 3.5$)		0.1	0.3	1.1	1

The slow component comes from a rebinding to the membranes of solubilised transducin. An important clue as to the origin of the slow component comes from studying the response to pairs of flashes separated by a variable time interval. When the second flash is applied within a few seconds of a first flash which has saturated the fast component, i.e. early during the rise of the slow component, this second flash elicits no significant new fast component and only the slope of the slow component increases (Fig. 5). However if the delay before the second flash is increased, a second fast component is recovered whose amplitude may become comparable to that of the first one (see Table 1). This suggests that the pool of membrane-bound transducin is replenished during the slow phase. This can be only at the expense of the T_{sol} pool, since R*-T complexes do not decay significantly on this time scale. In Fig. 5, the final level reached upon a succession of two distant 2% flashes both eliciting a fast component, is comparable to that reached after a single 4% flash which elicits a fast component similar to that of the first 2% flash. The slow component must therefore monitor both the net binding of transducin to the membrane and its eventual interaction with R* if R* has been formed in excess over the initial T_{mb} pool. This conclusion is supported by the analysis of the flash intensity dependence of A_F and A_S (Fig. 3): at low flash intensity when A_F is not saturated, A_S is small and increases slowly: it monitors only the rebinding of T_{sol} to the membranes. Beyond the saturation of A_F, A_S levels out at higher plateaus; it must now monitor also the formation of R*-T complexes. These form very fast but only after the binding of transducin to the membrane. The kinetics of the slow component is therefore always that of transducin rebinding to the membrane.

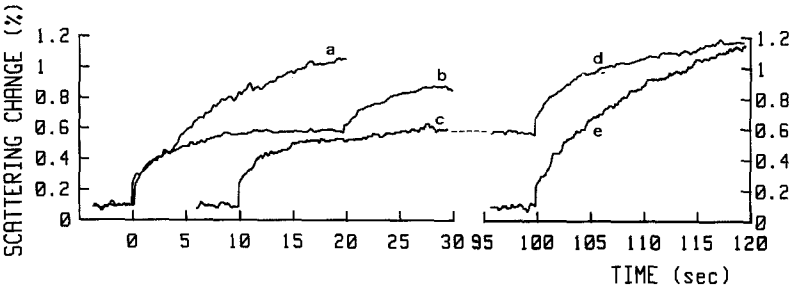

Fig. 5. Regeneration of the fast component of the "binding" signal upon a delayed second flash after a first saturating flash. Curves a, b, c and d illustrate the recovery of the fast component of the "binding" signal after a saturating flash (2% R*/R): a second flash 4 s later (curve a) elicits no fast component but alters the slope of the slow component, whereas a small fast component is clearly recognizable when the second flash is applied 20 s later (curve b). Moreover, if both flashes are separated by 90 s (curves c and d), the fast components are quite similar. A 2% R*/R flash saturates the fast component because curves c (2% R*/R) and e (4% R*/R) have the same fast component. *Note:* Curves a and b are aliquots of one sample while c, d and e are aliquots of another sample; curves c, d and e were normalized to a and b by way of the fast component ($\times 1.26$)

The low level of anisotropy of the slow component and its variability give further support to this interpretation: at low flash intensity that is below or around the saturation level of the fast signal, the slow component is practically isotropic which is the expectation for a mass gain. For higher flash intensities some left/right anisotropy can be detected but always remains lower (1.3) than that of the fast signal (1.7). Transmission measurements on the forward detector (data not shown) also indicate that the slow component is much less attenuated at forward angles than the fast one, in accordance with the idea that it is mainly due to a mass gain which gives an isotropic signal.

II) Dissociation signal: a fast, anisotropic "release" component and a slower, isotropic "loss" component

Figure 6 shows a typical "dissociation" signal as observed by the two side detectors upon a low intensity flash *in the presence of GTP*. A fast and transient positive component seen only on the left side is superimposed on a slower decrease of scattered light which is of equal amplitude on both sides and which levels out in a few seconds. By substracting the signal on the right side from that measured on the left side, one isolates the fast component which we previously called "release" signal (Vuong et al. 1984), the slow isotropic component being termed "loss". This terminology was based on lines of evidence suggesting that these signals relate respectively to the release of T_α-GTP from the disc membrane surface into the interdiscal cleft and to the subsequent loss of this highly soluble form of transducin into the external medium (Vuong et al. 1984; Chabre 1985). As in these previous studies the possibility of transducin solubilisation before the flash was neglected, we here provide further evidence that these signals monitor the fast release and loss of only the T_{mb} pool.

The saturation amplitudes of both the release and loss components correlate with the T_{mb} pool. Dilution of a given sample reduces by the same factor the maximum amplitudes of both components of the dissociation signal obtained with a saturating (0.1% photolysis) flash (Fig. 7a). The signals being expressed on a relative scale $\Delta I_{sc}/I_{sc}$ and I_{sc} being linear with the ROS fragment concentration, the most likely explanation is that these signals depend on the T_{mb} pool which decreases upon dilution of the sample. This is reminiscent of the fast component A_F of the "binding" signal. To document quantitatively this point, exogenous transducin is added to the ROS suspension; its attachment to the membrane in the dark is assessed by biochemically titrating dark aliquots of illuminated samples, the amount of soluble transducin present in solution before the flash (see Materials and methods). Comparison of Fig. 7b with Table 2 shows that both "loss" and "release" components increase in exact proportion to this artificial enrichment of the T_{mb} pool. This linearity was verified up to an enrichment of 2.7×. In the preceding section we propose that the saturation amplitude of the fast binding signal is a measure of the T_{mb} pool. A linear correlation is therefore expected between the saturation amplitude of the "binding" fast component and that of the "dissociation" loss component obtained from aliquot suspensions. This is dem-

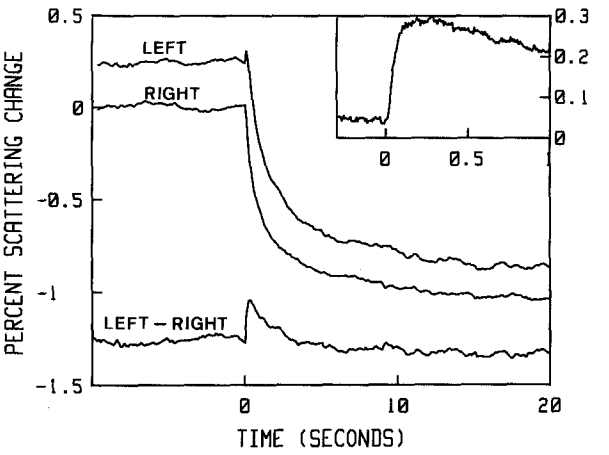

Fig. 6. The two components of the "dissociation" signal. The two upper curves are recordings on the slow time scale from left and right sides of a typical "dissociation" signal (photolysis $R^*/R = 5 \cdot 10^{-3}$; 0.1 mg rhodopsin/ml; 0.5 m*M* GTP; temperature = 23 °C). The "loss" signal is the component common to both detectors. The "release" signal appears only on the left side: subtracting right recording from left brings out the "release" signal (*bottom curve*). The insert shows the release signal on the fast time scale. The time constants ($T_{1/2}$) are 700 and 40 ms for the "loss" and "release" component respectively

Table 2. Addition of exogenous transducin increases the amount of membrane-bound transducin. Preparation of the sample and isolation of the various pools of transducin are explained in the text (Materials and methods). Determination of the amount of T_α contained in the extracts (arbitrary unit) was achieved by scanning the appropriate SDS gel bands

	Soluble T_α pool	Membrane-bound T_α pool	Total T_α
Transducin-enriched sample (percent of total)	96 (43)	126 (57)	222
Control sample (percent of total)	32 (29)	80 (71)	112
Enrichment of the pool (ratio of T_α in the transducin-enriched sample to that in the control sample)	3.00	1.58	1.98

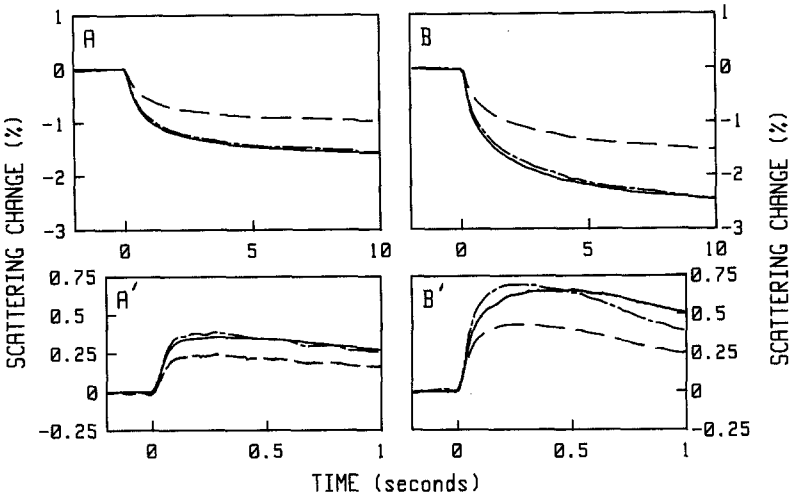

Fig. 7 A and B. Varying the amount of bound transducin changes the amplitude of the "release" and "loss" signals without modifying their kinetics. The conditions are: mechanically fragmented ROS; 0.5 m*M* GTP; photolysis level R*/R = 0.2%. *Upper panels (A and B):* "loss" signals (right side detector; slow time scale). *Lower panels (A' and B'):* "release" signal (left minus right; fast time scale). **A and A'.** Effect of dilution. The proportion of bound transducin is varied by dilution of a given suspension. *Solid line:* 0.15 mg rhodopsin/ml (control). *Dashed line:* 0.075 mg rhodopsin/ml (test). *Dashed-dotted line:* normalization of the diluted sample (test) to the control with a factor of 1.70. **B and B':** Addition of exogenous transducin. The proportion of bound transducin is varied by supplementing the suspension medium with a transducin extract (see Materials and methods). *Dashed line:* control 0.15 mg rhodopsin/ml. *Solid line:* aliquot of the same suspension supplemented with a transducin extract corresponding to 0.3 mg rhodopsin/ml. *Dashed-dotted line:* normalization of the control to the transducin enriched sample with a factor of 1.60. The amount of membrane-associated transducin in the test sample was determined (Table 2) to be 1.57 time that found in the control

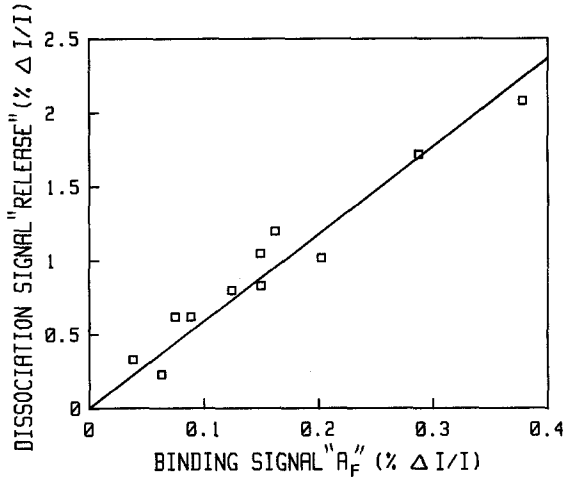

Fig. 8. The saturated fast component A_F of the "binding" signal monitors the transducin bound to discs. "Binding" signals (R*/R = 6.4%, no GTP added) and "dissociation" signals (R*/R = 0.2%, 0.5 m*M* GTP) were obtained on pairs of aliquots of the same suspension at various ROS concentrations. The correlation coefficient between the saturated fast component A_F of the "binding" signal and the "release" signal is 0.98. *Solid line:* the 11 points are fitted to a straight line going through zero. *Note:* the fast component of the binding signal is saturated because a second flash 7 s later does not trigger another fast component (data not shown)

onstrated in Fig. 8 using pairs of aliquots of various dilution, and hence with variable proportion of T_{mb}/T_{sol}.

The kinetics of the loss component, but not that of the release step, depend on the permeation state of the ROS. As seen in Figs. 7a and 7b, dilution of the ROS suspension or addition of transducin does not measurably affect the kinetics of either component of the dissociation signal. It has been noticed that the kinetics of the loss component seemed to be sensitive to the degree of ROS fragmentation (Vuong et al. 1984). To confirm this point, aliquots of an ROS suspension were subjected to either fragmentation by shearing or electroporation (see Materials and methods) and diluted to exactly the same concentration. Electroporation does not significantly fragment the ROS, hence giving less scattering per mass of ROS than mechanical shearing. Although the initial scattered intensity is different for the two samples, the relative amplitudes ($\Delta I_{sc}/I_{sc}$) of the two components of the dissociation signal at saturation are identical in both preparations (Fig. 9). This proves that these amplitudes are strictly related to the amount of membrane and hence to the

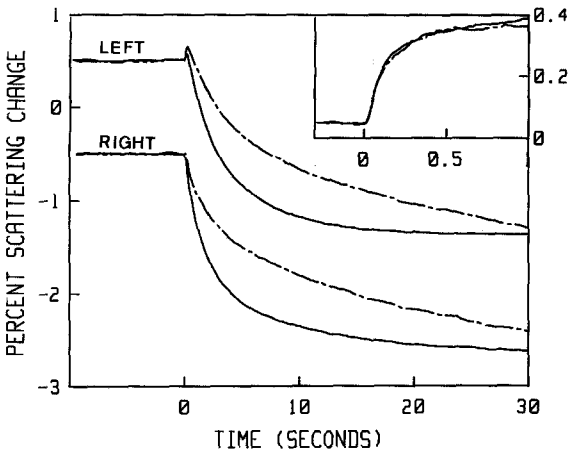

Fig. 9. Sensitivity of the "loss" signal but not of the "release" signal to the permeation state of the ROS plasma membrane. An electroporated (see Materials and methods) ROS suspension was diluted in buffer to a concentration of 0.15 mg rhodopsin/ml. Half was further mechanically fragmented (see Materials and methods). Dissociation signals of the fragmented (*solid line*) and electroporated (*dashed-dotted line*) suspension were obtained with 0.5 m*M* GTP and 0.2% R*/R. The kinetics but not the amplitudes of the "loss" components are widely different, the "release" component (insert) are identical. *Note:* to prevent sedimentation of the large intact ROS, the suspension buffer was prepared with D_2O instead of H_2O

proportion of bound transducin and do not depend on the physical state of the ROS fragments. The electroporated ROS are expected to be less permeable than the mechanically sheared ROS and are shown to be indeed so by the fluorescent dye penetration test (see Materials and methods). This should be appreciated in the context of Fig. 9 where the kinetics of the loss component is notably slower for the electroporated ROS than for the mechanically sheared, more permeable ROS fragments. The fastest "loss" component obtained with highly sheared frog ROS had a time constant ($T_{1/2}$) of the order of 0.35 s. This value may be compared to the average time of 0.13 s that a perfectly soluble protein of the size of transducin would take to diffuse freely over a distance of 3 μm (radius of a frog ROS). The thickness of the aqueous layer between two discs being only three or four times the size of the diffusing protein, it is not surprising that the diffusion time is notably increased (in this case, from three to five times: see Kuffler and Nicholls 1976).

Unlike the "loss" component, no sensitivity of kinetics on the ROS permeation state can be detected for the rise time of the fast, anisotropic "release" component (Fig. 9, insert), thus confirming that it monitors an early structural event, internal to the ROS fragment and insensitive to the degree of permeation of the outer membrane as long as the permeability is sufficient to allow the entry of GTP.

Discussion

Using suspensions of frog ROS fragments we have clearly identified two origins for the two components of the "binding" signal. This helps shed some light on the confusion associated with the interpretation of various light-scattering signals obtained with different types of ROS membrane preparations. Indeed, the differing forms of scattering transients that accompany the R*-*T* binding reaction exhibit dissimilar kinetics because of important differences in sample preparation and concentration. Photolysis in the absence of GTP brings about two related but different processes: *i*) the binding of *T*-GDP to R* and *ii*) the re-binding of soluble transducin to the ROS membrane. Both processes contribute to the "binding" signal but by different mechanisms, with different kinetics and to different extents depending on the type of ROS membrane preparation and on the observation geometry. In our case, resolving the two components of the "binding" signal is made easy in two ways. First, the large frog ROS diameter and the preserved tight disc stacking must have helped slow down the rate of re-entry of soluble transducin to bring about the pronounced kinetic difference displayed by the two components. Second, the relative contribution by the fast component is enhanced at the large scattering angles (90° and 270°) where our "binding" signal is recorded.

The fast component A_F only monitors what occurs instantly on the disc membrane where in the dark adapted, intact ROS essentially all of the transducin is located. Its rise time at 20 °C is 22 ms which is markedly slower than the time course for the formation of R* (Metarhodopsin II) which can be measured on the same preparation using the "rhodopsin" signal to directly monitor the spectral shift of the chromophore. A time of 22 ms is also certainly slower than the time it takes transducin to bind to R* since one knows from the analysis of the dissociation signal (Vuong et al. 1984) and from the kinetics of PDE activation (Liebmann and Pugh 1979, 1982) that one R* processes about one transducin per millisecond. This fast component A_F must therefore arise from delayed pertubations of the overall membrane organisation within the ROS fragment as a result of the R*-*T* complex formation, rather than being directly related to structural changes at the molecular level. Time-resolved neutron diffraction experiments on the same type of oriented frog ROS suspensions (Vuong et al. 1987) have shown that photolysis in the absence of GTP (i.e. conditions to obtain a "binding" signal) results in a small (0.3%) shrinkage of the disc repeat distance which is completed within the 4-second time resolution of this technique and which is sustained for at least several minutes thereafter. In the scattering geometry used here, a purely axial compression of the ROS fragment

should ideally give no signal on the right detector (Vuong et al. 1984; Chabre 1985). The left/right anisotropy observed for the fast component is thus suggestive of a predominantly axial change. A reasonable interpretation is that the formation of R*-T complexes on the highly packed disc surfaces changes the electrostatic interaction between them. The absence of a fast component in the "binding" signal from isolated disc vesicle suspensions (Schleicher and Hofmann 1987) suggests that this effect is short ranged. This is consistent with the existence of large effects and membrane aggregation processes in very concentrated suspensions (Lewis et al. 1984).

The exact physical origin of the pertubation giving rise to the fast component of the "binding" signal remains, however, unknown: it has to do with the transconformation induced in R* and in transducin upon their mutual interaction. A well documented effect of the formation of an R*-T complex is the opening of the nucleotide site in the T_α subunit of the bound transducin. This allows the release of GDP if no nucleotides have been added to the suspension and thus the total concentration of GDP in solution is below its dissociation constant $K_d = 20\ \mu M$ (Bennett and Dupont 1985). We have however checked that this release of nucleotide from the membrane bound protein is not in itself the cause of the perturbation: the fast component of the binding signal was not reduced when a 100 μM concentration of GDPβS (an analogue of GDP that cannot be phosphorylated to GTP by endogenous enzymes) was added to the ROS suspension before the flash.

The slow component of the binding signal mainly follows the return of T-GDP ($T_{\beta\gamma}$-T_α-GDP) to the membrane from the solution. Its rise time is indicative of a binding rate that is much slower than one obtained in the diffusion-controlled limit, in agreement with the values given by Liebman and Sitaramayya (1984) and by Schleicher and Hofmann (1987). Taken together with an equilibrium constant of about $10^6\ M^{-1}$ (Liebmann and Sitaramayya 1984) for the "reaction" $T_{sol} + site \rightleftharpoons T_{mb}$, this slow binding rate implies a very low dissociation rate in the range of tens of seconds. More than 99% of the transducin must be membrane-associated in the dark, intact rod. This excludes the possibility that solubilisation of T-GDP could play any role in the physiological response which only depends on collisions of R* with the membrane bound transducin.

As for T_α-GTP, the vector of PDE activation, its interaction with the membrane is totally different. T_α-GTP or its stable analog T_α-GTPγS is fully soluble in media of ionic composition close to that of the cytoplasm (Kühn 1981; Pfister et al. 1983) and does not show any significant affinity for $T_{\beta\gamma}$ which remains partially membrane bound. Moreover it has been shown (Deterre et al. 1986) that T_α-GTP alone interacts with the PDE. This paper presents further evidence that the loss component of the "dissociation" signal is due to the leakage from the ROS fragment of T_α-GTP formed by GDP/GTP exchange from the pool of initially membrane bound T-GDP. Previous experiments from this group (Deterre et al. 1986) indicate that only the excess of T_α-GTP which could not interact with the membrane bound PDE inhibitor becomes soluble and is monitored by the light scattering signal. The kinetics of this "loss" component is mainly controlled by the size and the permeation state of the ROS. The good correlation between the amplitude of the early, anisotropic "release" component with that of the "loss" component (Fig. 7) further supports our original interpretation (Vuong et al. 1984) that the former monitors the fast dissociation of T_α-GTP from R*, from $T_{\beta\gamma}$ and from the membrane. T_α-GTP may then take an aqueous route between R* and the PDE. Liebman et al. (1987) contested this suggestion on the grounds that the dissociation of T_α-GTP from the membrane would be too slow a process. However, the experiment they report in support of their argument does not concern T_α-GTP as it involves the activation of the PDE in bleached but transducin depleted membranes by dark adapted ROS membranes. The kinetics of this activation is limited by the transfer through the solution of $T_{\beta\gamma}$-T_α-GDP from the dark adapted membrane to the bleached and transducin stripped membrane which we have shown in this work to be a slow process; it does not depend at all on the solubilisation of T_α-GTP, which we have shown to be very fast.

The fast kinetics, the GTP dependence and the high degree of left/right anisotropy of our "release" component indicate an origin in the light triggered interactions of transducin with membrane bound components at the disc surface. This does not exclude "a priori" that this signal might also depend in part on the interaction of transducin with the cGMP phosphodiesterase. Such an interpretation has been proposed by Kamps and Hofmann (1985, 1986) for an analogous signal, also observed in the presence of GTP. The preparations used by these authors differ from ours by the fact that a fraction of the ROS are resealed, and no significant loss component is observed. Kamps and Hofmann called AT the fast positive signal observed under such conditions and attributed its origin to the light-induced activation of the cGMP phosphodiesterase. This interpretation was contested by Wagner et al. (1987) which claim that the AT signal is related to the activation of transducin and might be influenced by the turning over of the PDE only in the presence of cGMP. Since we have not added cGMP in our preparations, we cannot fully dispute this line of work here. However, the correlation of the "release" signal amplitude with that of the fast compo-

nent of binding (Fig. 8) demonstrate a strict linearity of the "release" signal with the amount of membrane-bound transducin and this up to 3-fold the native amount (Fig. 7). The transducin : PDE stoichiometry being 10 : 1 in situ and 30 : 1 with added transducin one hesitates to give too much credence to the importance of the transducin-PDE interaction on the amplitude of the release signal.

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