THE TRANSDUCIN CASCADE IS INVOLVED IN THE LIGHT-INDUCED STRUCTURAL CHANGES OBSERVED BY NEUTRON DIFFRACTION ON RETINAL ROD OUTER SEGMENTS

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ABSTRACT Time-resolved neutron diffraction on retinal rod outer segments are performed to reinvestigate the origin of the light-induced structural change observed by Saibil et al. (Saibil, H., M. Chabre, and D. L. Worcester, 1976, *Nature (Lond.)*, 262:266–270). Photoactivating rhodopsin triggers in rods a cascade of GTP-dependent and transducin-mediated reactions controlling cyclic-GMP hydrolysis. Infrared light-scattering studies (Kühn, H., N. Bennett, M. Michel-Villaz, and M. Chabre, 1981, *Proc. Natl. Acad. Sci. USA*, 78:6873–6877; Vuong, T. M., M. Chabre, and L. Stryer, 1984, *Nature (Lond.)*, 311:659–661) demonstrated the existence of structural changes that correspond to this cascade rather than to rhodopsin photoactivation. We thus look for neutron diffraction changes of similar origins. With 1-min time resolution, intensity changes are observed mainly for orders 2 and 4. The illumination and GTP dependence of these changes indicates an involvement of transducin. Without GTP, they are linear with the amount of photoexcited rhodopsin, saturate at 10% photolysis, and thus correlate well with the light-scattering "binding signal." With GTP, light sensitivity is higher and saturation occurs below 0.5% photolysis, as for the "dissociation signal" of light scattering. In both cases, lattice compressions of 0.2–0.3% are observed. With 4-s time resolution the intensity change with GTP present precedes the lattice compression. The fast intensity change is probably due to the displacement of transducin α-subunits away from the disc membrane and the slower lattice shrinkage to an osmotic readjustment of the rod.

INTRODUCTION

The ordered disc stack which fills up the retinal rod outer segment (ROS) has been extensively studied by x-ray and neutron diffraction on intact retinas or isolated but structurally intact ROS (Gras and Worthington, 1969; Blaurock and Wilkins, 1972; Corless, 1972; Chabre and Cavaggioni, 1973, 1975; Chabre, 1975; Saibil et al., 1976; Yeager et al., 1980). In this structure, rhodopsin makes up for more than 60% of the total protein mass and more than 95% of the disc membrane's intrinsic protein content. Until now analysis of ROS diffraction data has always neglected the possible contribution of molecules other than rhodopsin to the scattering density of the disc membrane and the intervening cytoplasmic space. With this abbreviated analysis it was nevertheless possible to characterize rhodopsin as a transmembrane protein with at least 50% of its mass located in the membrane's hydrophobic layer (Saibil et al., 1976). Studies using other physical methods (Chabre, 1985) also lend credence to this overall model. It has been confirmed by sequence data analysis and is now generally accepted as valid. As for rhodopsin mass in the hydrophilic phase, previous analysis of x-ray and neutron diffraction data (Blaurock and Wilkins, 1972; Corless, 1972; Chabre, 1975; Schwartz et al., 1975; Saibil et al., 1976) suggested there was more protein protruding into the cytoplasmic spacing than into the intradiscal compartment. Such distribution, however, is not consistent with the sequence-derived α-helical model which assigns similar sizes to the two hydrophilic portions of rhodopsin.

In some of the previous x-ray diffraction experiments (Corless, 1972; Chabre and Cavaggioni, 1973; Chabre, 1975), small light-induced structural changes in the vicinity of the disc membrane's cytoplasmic surface were also detected. Taking advantage of the method of contrast variation, analysis of the neutron diffraction data indicated that these changes were indeed caused by a displacement of protein mass (Saibil et al., 1976). As rhodopsin was the only protein considered, this displacement was interpreted as due to a light-dependent conformational change of the photosensitive protein.

It is now well documented that many other proteins also take part, along with rhodopsin, in the process of visual transduction. Upon photoexcitation rhodopsin triggers a cascade of reactions whose end result is a massive hydroly-

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sis of the cyclic-GMP inside the ROS (Woodruff and Bownds, 1978; Stryer, 1983). This cascade involves transducin, a GTP-binding protein, as well as several other peripheral and soluble species. Transducin by itself constitutes nearly 20% of the total ROS protein complement (Kühn, 1980, 1981, 1984; Stryer, 1986). It is made up of three subunits: Tα (40 kD), which is the guanyl nucleotide binding subunit, Tβ (37 kD), and Tγ (6 kD). In the dark, with GDP bound, transducin is peripherally associated to the cytoplasmic disc surface. In the first step of the light triggered cascade, photoactivated rhodopsin (R*) forms a transient complex with transducin and catalyzes on it a GDP/GTP exchange (Fung et al., 1981). Replacing the GDP by a GTP has dramatic consequences on the structure of transducin. It dissociates into two separate components: Tα-GTP, which is released into the aqueous cytoplasmic space, and T$\beta\gamma$, which remains preferentially membrane-bound (Kühn, 1981, 1984). In the delay between the light flash and the electrophysiological response, one R* can sequentially interact with several hundred transducins (Kühn et al., 1981). Without GTP in the medium, the R*–transducin complex persists indefinitely, and upon sufficient illumination one can achieve binding of the total pool of available transducin to R* (Kühn, 1980; Kühn et al., 1981).

In using near infrared (IR) light-scattering to understand the kinetics and stoichiometry of this light-induced cascade of reactions, two types of ROS suspensions have proved very useful: cattle ROS suspensions and suspensions of magnetically oriented frog ROS fragments where the stacking order of the disc membranes is preserved. Besides their presentation in the original works, these near IR light-scattering studies have already been discussed extensively in a Ph.D. dissertation (Vuong, 1984) and in recent reviews (Kühn, 1984; Chabre, 1985). With the first type of preparation, reconstitution experiments allowed to correlate small changes in the scattered light intensity with various steps of the cascade (Kühn et al., 1981). A "binding signal" observed in the absence of GTP can be used to monitor the amount of one-to-one complex formed between R* and transducin. Its amplitude is linearly related to the amount of transducin that binds semipermanently to photoexcited rhodopsin after flash illumination. But its physical origin is still debated and is not, contrary to what is often misunderstood, due to a light-induced attachment of transducin to the ROS membrane. Transducin is already membrane-bound in the dark under the conditions of these experiments, and the light-scattering change is but a secondary consequence of the R*–T interaction. In the presence of GTP, another type of transient called "dissociation signal" correlates with the amplified GDP/GTP exchange catalyzed by R*:

$$T_{GDP} + R^* \rightarrow R^* - T_{GDP} \quad GTP \qquad GTP \quad R^* - T_{GTP}$$
$$\rightarrow R^* + T\alpha_{GTP} + T\beta\gamma.$$

The observation by biochemical techniques of the solubilization of $T\alpha_{GTP}$ upon this exchange reaction suggested that the dissociation signal is directly related to this release of a protein component from the membrane fragment. With oriented frog ROS preparations (identical to the one used in the present study), the "dissociation" signal could be further analyzed in an early and highly anisotropic component related to the release of the α-subunit in the interdiscal space and a later isotropic component related to the subsequent loss from the leaky ROS (Vuong et al., 1984). The light- and GTP-dependent release of transducin has been also demonstrated by sedimentation technique and biochemical analysis on the same type of frog ROS sample (Pfister et al., 1983).

Upon strong illumination when all the reactions involving transducin have been exhausted (by total binding or release), quantitative photoactivation of rhodopsin elicits a "rhodopsin signal." It arises mainly from the change in index of refraction (anomalous dispersion effect) due to the shift in the spectral peak of absorption of rhodopsin when it is photoexcited to meta-II rhodopsin.

These near IR light-scattering changes cannot, however, be quantitatively interpreted in terms of structural changes at the molecular level: the probing wavelength is much too large (800–1,000 nm). It is comparable to the coherence length of the quasi-crystalline disc stack, and within an order of magnitude of the ROS fragments' overall dimension. That is why interpretation of these light-scattering data were first empirically derived from reconstitution experiments and dose response curves for the un-oriented bovine ROS preparations (Kühn et al., 1981) and then further refine by examining the angular anisotropy of the signal obtained from the magnetically oriented sample (Vuong et al., 1984).

All these recent advances in vision biochemistry and biophysics call for a more elaborate set of neutron diffraction experiments, in particular, to reinvestigate the light-induced diffraction changes observed before. They should now be understood in terms of structural changes involving not only rhodopsin alone but also transducin; correlation with results from the light scattering work should be instructive. Several improvements over previous neutron diffraction experiments are necessary. (*a*) The level of photoexcitation must be precisely controlled (i.e., complete "bleaching" of 90–100% is no longer adequate). This will require preparing samples of low optical densities (and therefore of large areas) to ensure homogenous illumination throughout the whole volume of the sample. (*b*) Reproducible and controllable amounts of guanine nucleotides must be introduced into the ROS, requiring their permeabilization. (*c*) The time resolution must be adequately fast.

A first series of experiments of slow time resolution (1 min per spectrum) demonstrated that light-induced changes in the diffraction pattern are not only related to rhodopsin photolysis but also to the interaction between R*

and transducin and, with GTP present, to the onset of transducin activation. By improving the time resolution to 4 s per spectrum we were able to discriminate between fast object changes related to molecular rearrangements and slower lattice changes due probably to osmotic readjustments.

MATERIALS AND METHODS

All experiments were performed on the D11 small angle scattering instrument of the Institut Laüe-Langevin (Grenoble, France).

Sample Preparation, Orientation, and Illumination

Frog ROS isolated and purified as described elsewhere (Chabre, 1975) were suspended in a D_2O Ringer's solution containing (in mM): 102 NaCl, 2.7 KCl, 1.9 $CaCl_2$, 2.1 $MgSO_4$, 2 $NaHCO_3$, 0.36 NaH_2PO_4, pH 7.5. The suspension was forced three times through a constricted 22-gauge hypodermic needle. This treatment broke the ROS into fragments 10–15-μm long and made them permeable to small molecules as checked by a fluorescent dye assay (Yoshikami et al., 1974). Our previous experience with this kind of treatment indicates that the thus fragmented ROS are also leaky to soluble proteins (Vuong et al., 1984). However, this fragmentation procedure does not seem to perturb the regular stacking and ordering of the disc membranes: diffraction patterns obtained with these fragments are identical to those obtained with intact ROS. In contrast to previous experiments, ours were performed on dilute suspensions. Sedimentation was not a problem for ROS fragments are nearly at equilibrium density in D_2O.

It was critical for these experiments that all ROS fragments over the whole sample volume got illuminated roughly to the same extent. Given the large cross-section of the neutron beam, we designed a quartz sample cell with a large area (5-cm high, 1.5-cm wide, 2-mm thick), which could be illuminated from both sides. Light from a photographic flash fitted with suitable filters was delivered to both front and back faces of the sample cell via a collection of light guides and neutron-transparent aluminum mirrors (Fig. 1). When a sample of optical density (OD) 1 is illuminated from one side, the light intensity varies by a factor of 10 from front to back. This variation is reduced to 1.6 between the faces and the center when the same sample is sandwiched between the two light beams coming from both directions. In our sample cell, a suspension of ROS extracted from about 24 frog retinas has an OD of 1 and can thus be adequately illuminated by this method. Unfortunately as discussed below, the data acquisition electronics could not handle the counting rate generated from such a sample. Most experiments were performed using four retinas per sample, guaranteeing a very uniform illumination. To fully cover the area of the sample the neutron beam was collimated only in the horizontal direction by two vertical slits. The ROS fragments were very well oriented by the horizontally directed 12-kG magnetic field, and the lamellar diffraction pattern appeared on the detector as an array of vertically elongated spots (Figs. 1 and 2).

Detector Geometry and Data Acquisition

The 64 × 64 cell detector of the D11 instrument at the Institut Laüe-Langevin was positioned 2.5 m from the sample. The neutron wavelength was adjusted to 7.87 Å so that only the first four orders of diffraction were recorded. The wavelength dispersion ($\Delta\lambda/\lambda$) was 10%. Data acquisition followed two procedures.

Standard Two-Dimensional X-Y Acquisition. Two-dimensional diffraction patterns are recorded in the usual manner on the 64 × 64 area detector and stored in the 4,096-channel buffer memory. After each accumulation this memory must be transferred to disk. The transfer time being ~20 s, this collection procedure does not permit fast sequential measurements. The accumulation time was set at 40 s per spectrum, giving an overall time resolution of 1 min per point. This acquisition mode, however, is not without merit: as full two-dimensional diffraction patterns were obtained, the orientation of the diffraction lines as well as the background level away from them could be checked.

Time Driven, One-Dimensional X-t Acquisition. Given the nearly perfect vertical orientation of the diffraction pattern, the information is completely contained in the horizontal, *X*-direction. Thus, one needs to only record the *X*-coordinate values of the diffracted neutron flux, and this can be easily achieved using the 64 vertical anode wires of the area detector. Diffraction patterns are now reduced to linear, one-dimensional arrays of 64 points, each point being the total intensity integrated in the vertical direction. The *Y*-index can now be replaced by a timing index and up to 64 spectra can be sequentially accumulated and stored in the buffer memory. The accumulation time for each linear spectrum is set by the pulse duration of a clock. There is practically no dead time between successive accumulations in each group of 64 spectra. The actinic light flash is triggered at the appropriate moment by the acquisition electronics. In this *X-t* mode, counting time per spectrum is

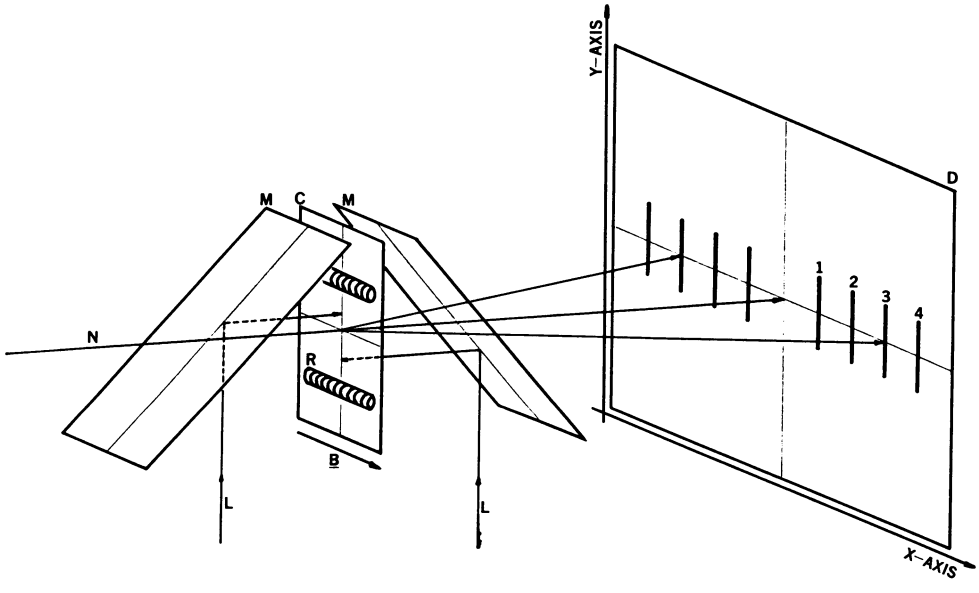

FIGURE 1 Schematic diagram of the sample holder. C, quartz sample cuvette (5 cm × 1.5 cm ×2 mm); M, neutron-transparent aluminum mirror; R, ROS fragment; N, neutron beam; L, actinic light beam; B, 12-kG magnetic field; D, 64 cm × 64 cm area detector.

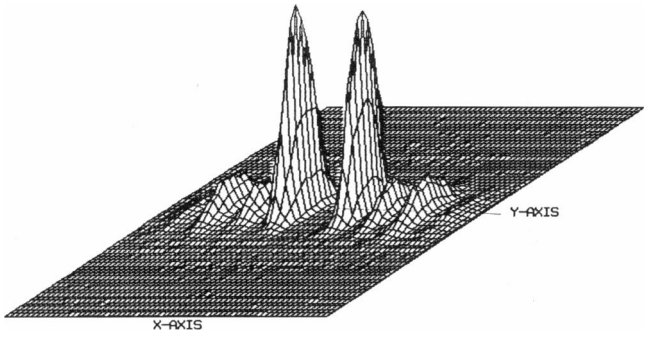

FIGURE 2 A two-dimensional neutron diffraction pattern obtained in 4 s, at 6°C with ROS fragments from four dark-adapted frog retinas in the standard D_2O saline solution without GTP.

determined only by the required counting statistics. As stated above samples containing ROS fragments from up to 24 retinas could be adequately illuminated and such samples generated counting rates of $\sim 2.5 \times 10^5$ neutrons/s. Unfortunately the acquisition electronics could not handle rates beyond 3×10^4 neutrons/s. At this lower counting rate it took 4 s of accumulation time per spectrum to achieve satisfactory statistics.

Experimental Procedures

Each sequence of measurements on a freshly obtained, dark-adapted sample of ROS fragments began with the recording of a full two-dimensional, *X-Y* diffraction pattern whose overall quality and orientation could be fully assessed. Then depending on the experiment, successive spectra were recorded either in the *X-Y* mode with a 1-min time resolution or in the *X-t* mode with a 4-s resolution. The first few spectra were recorded in the dark to provide a measure of the reproducibility and stability in time. For the first three diffraction orders, the counting statistics were sufficient to yield noise levels of 0.7% for the peak intensity and 0.1% for the lattice spacing (Figs. 4 and 8). There was more noise for the fourth order, especially in the *X-t* mode.

After recording a preset number of spectra, the light flash (1-ms duration) was triggered at the beginning of the following spectrum. Subsequent flashes of different intensities might be eventually triggered. In the *X-Y* mode up to 16 spectra were collected per sample, whereas in the *X-t* mode the number was always 64 spectra/sample.

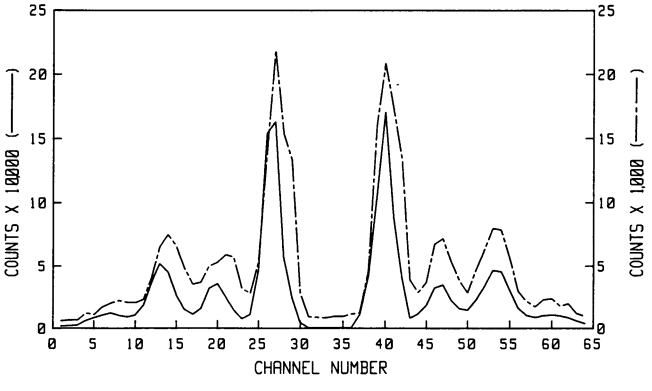

FIGURE 3 Linear diffraction patterns from dark adapted ROS in D_2O saline solution without GTP. *Solid line,* obtained in 1 min, at 20°C, using the *X-Y* mode of acquisition (see text). *Dashed lined,* obtained in 4 s at 6°C, using the *X-t* mode of acquisition (see text). The higher uniform background seen in the 4-s pattern is due to diffuse scattering above and below the diffraction lines (see Fig. 2).

Data Reduction and Analysis

Only the first four orders of diffraction were recorded. The peaks are well resolved and background is essentially negligible thanks to the high contract in D_2O. There is thus no ambiguity in the evaluation of peak intensities. In the *X-Y* mode, these peak intensities were summed over the vertical extent of the lines. In the *X-t* mode this integration was achieved over the whole vertical direction of the detector by the vertical anode wires. As the background was not excessive above and below the diffraction lines, this latter procedure did not significantly reduce the quality of the data (Fig. 3). Integrated peak intensities were plotted versus time to look for light-induced changes. Lattice spacing was computed as the distance between the centers of gravity of symmetrical diffraction lines. Light-induced changes in this quantity were again studied by plotting it as a function of time. Whenever possible, data from several comparable experiments were summed to improve upon the signal-to-noise ratio.

RESULTS

Experiments with 1-min Time Resolution: Light-induced Structural Changes in the ROS Are Due to the Interaction of Rhodopsin with Transducin

In a first series of experiments we set out to find changes in the neutron diffraction pattern of oriented ROS that can be correlated with the light-scattering signals described by Kühn et al. (1981). The largest light-induced changes turn out to be those in the intensities of orders 2 and 4.

In the experiment of Fig. 4, no GTP was added to the ROS suspension and the sample was photolyzed three times: 0.5% R* at 0 min and 10% R* at 4 and 8 min. As is clearly shown, a low level of 0.5% photolysis did not elicit a measurable change when GTP was not present. The 10% R* flash at 4 min however caused a significant increase of $\sim$5% in the intensity of order 2. This 10% level of photolysis is saturating as the second 10% photoactivation at 8 min no longer provoked a detectable increase. This diffraction change is thus very reminiscent of the "binding signal" as reported by Kühn and co-workers (1981). Their light-scattering "signal" also requires a high level of photolysis (a few percent) and saturates at $\sim$10% R*, when all the transducin molecules present have become bound to the activated rhodopsin molecules. Fig. 4 also displays the same experiment seen from the standpoint of order 4 intensity. The observations are the same: no observable change upon the 0.5% R* flash, a significant change upon the first 10% R* photolysis, and no more signal at the second 10% R* flash. The only difference is that the signal manifests itself here as a decrease in intensity. As for orders 1 and 3 they display essentially little or no intensity change in this experiment (Table I).

In the experiment of Fig. 5, we looked for changes that would correspond to the light-scattering "dissociation signal." Thus, in the presence of 600 μM GTP, the first 0.5% R* photolysis at 0 min elicited a 3% increase in the intensity of order 2. Just as observed by Kühn et al. (1981) for their light-scattering signal, saturation is also reached in our case after photolysis of a few tenths of a percent. A

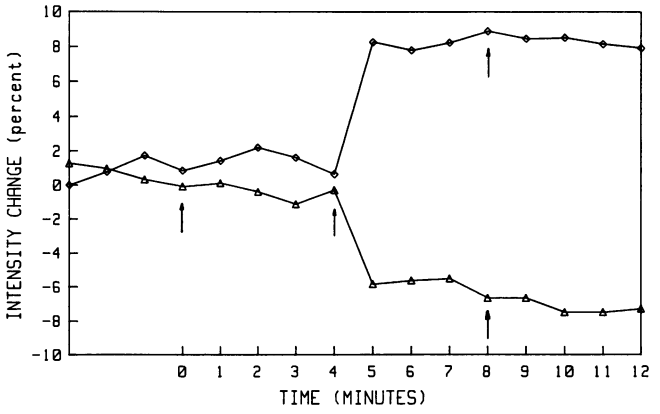

FIGURE 4 Light-induced changes of diffraction peak intensity observed for orders 2 (*diamonds*) and 4 (*triangles*) in the absence of GTP with 1-min time resolution, 20°C. Flashes photoexciting 0.5% R* at 0 min, 10% R* at 4 and 8 min (*arrows*). These data are obtained from a single sample, but this measurement has been repeated with a good reproducibility on three independent samples.

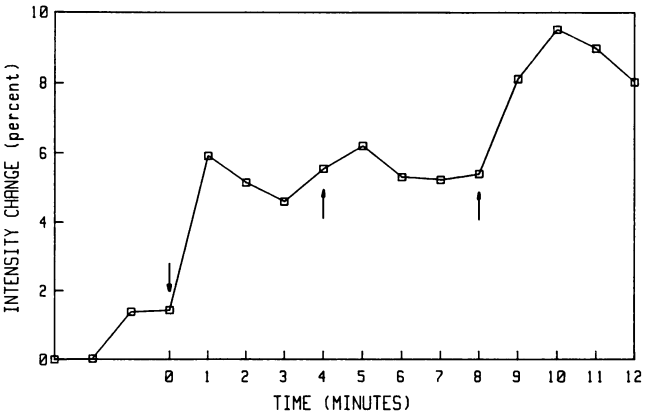

FIGURE 5 Diffraction peak intensity changes observed for order 2 in the presence of 600 μM GTP; 1-min time resolution, 20°C. Flashes: 0.5% R* at 0 min, 0.5% R* at 4 min, and 10% R* at 8 min (*arrows*). These data are obtained from a single sample, but this type of experiment has been repeated with a good reproducibility on four independent samples.

second 0.5% R* flash, triggered 4 min after the first one no longer caused any change. However a later 10% R* photolysis, 8 min after the first low flash in Fig. 5, provoked an increase in order 2 intensity of ~2%, reminiscent, but with a smaller amplitude, of the change observed upon the high intensity flashes in the absence of GTP. We therefore conjectured that this change was due to the interaction of the newly formed R* with transducin molecules that had reattached to the surface membrane. Such reattachment would require the deactivation of the previously formed R* (Wilden et al., 1986) and the hydrolysis of the bound GTP by the intrinsic GTPase activity of transducin (Fung, 1983). To test this hypothesis, in the experiment of Fig. 6 the sample was subjected to the 10% R* photolysis only 2 min after the first 0.5% R* flash. In this case, one expects transducin to reattach itself to the membrane to a much lesser extent as there is less time to perform the two deactivation operations described before. Accordingly in Fig. 6, the second 10% R* flash at 2 min elicited only a small and slow intensity increase, barely detectable after 1 min. However, if there was indeed rebinding of transducin to the disc membrane, it is puzzling that there was not even a partial recovery of the signal toward baseline (i.e., 0% change) during the 8-min delay.

TABLE I
SUMMARY OF THE RELATIVE INTENSITY CHANGES (IN PERCENT) SEEN WITH THE TIME RESOLUTION OF 1 MIN, 20°C

Order	10% R* (no GTP)	0.5% R* (600 μM GTP)
1	0	+1
2	+5	+3
3	0	+1.5
4	−3	−3

We can only speculate that a slow swelling, sometimes observed after the first flash on this time scale of minutes, could modify the peak intensity and mask the recovery. The perfect stability of the diffraction peak intensity is difficult to ascertain over these long time periods and we did not investigate further the origin of this slowly recovering sensitivity to large flashes. The important fact, however, is that this recovery was never observed, in repeated experiments, for sequences of low intensity flashes in the presence of GTP, the constant feature being that a second 0.5% R* flash applied between 1 and 10 min from the first one elicited no further step response.

Changes of −3%, +1%, and +1.5% were measured for orders 4, 1, and 3, respectively. All these results are shown in Table I. A quick perusal of the data listed reveals a simple pattern: whether GTP was present or not it was orders 2 and 4 that changed the most in intensity; as for orders 1 and 3, there was little or no change in both cases. This observation can be understood in terms of two previous results: (*a*) the nature of the "binding" and "dissocia-

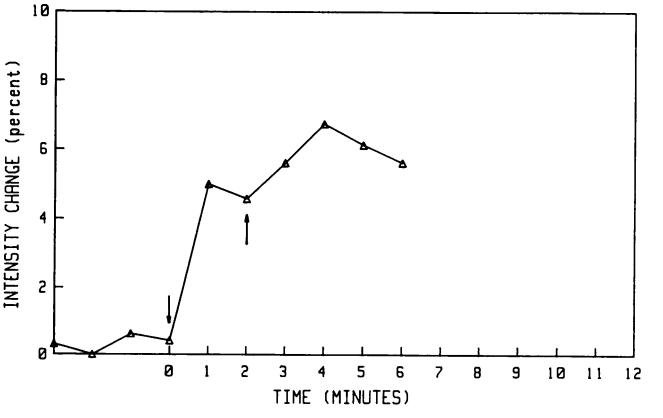

FIGURE 6 Intensity change of order 2 observed in the presence of 600 μM GTP. 1-min time resolution, 20°C. Flashes: 0.5% R* at 0 min, 10% R* at 2 min (*arrows*).

tion signals" as analyzed by light-scattering studies, and (*b*) the special crystalline arrangement of the ROS.

Both "binding" and "dissociation signals" have been shown (Kühn et al., 1981; Kühn, 1984; Vuong et al., 1984; Chabre, 1985) to be related to events concerning the disc surface. The former is a consequence of the binding of R* to transducin; this event occurs on the disc surface, involving an integral protein (rhodopsin) and a peripheral protein (transducin). We emphasize again that transducin was already bound to the membrane in the dark, and other experiments have shown (Emeis et al., 1982) that the light-scattering transient may be delayed with respect to the light-induced molecular interaction between R* and transducin. The underlying structural change is but a secondary consequence of this molecular interaction. It probably results, at least in parts, from a change in the membrane ordering in the ROS fragment, due to a change of electrostatic interactions between the membranes (Chabre, 1985).

The "dissociation signal" directly monitors two processes: the release of Tα-GTP from the disc surface into the interdiscal space and its subsequent loss from the ROS fragment (Vuong et al., 1984; Chabre, 1985). One expects this loss of mass to have an important effect on the scattering density at the disc surface as transducin accounts for a significant portion of the protein content in this location. In a nutshell then, these events, whether studied by light scattering or by fast neutron diffraction, are membrane surface phenomena.

In a fortuitous manner, the disc surface happens to occupy a very special position in the ROS structure: while the basic repeat distance of the ROS is 295 Å (distance between two adjacent discs), the thickness of each disc is ~140 Å. This arrangement places the disc surface at about the half-way point in each unit cell. The spatial frequency of the disc surface is thus twice the fundamental frequency of the overall array. Therefore, the second (and fourth) harmonics (or diffraction peaks) in Fourier space are expected to be particularly sensitive to any structural changes these disc surfaces may undergo. This structural consideration when combined with the "disc surface" nature of the "binding" and "dissociation signals" accounts very well for the observation that orders 2 and 4 exhibited the largest intensity changes. To better illustrate this point we reconstructed the disc membrane's density profile using the seven structure factors and their respective phases as given by Saibil et al. (1976). To compute the light-induced change in this profile, we considered that orders 5 through 7 remained constant, that the first 4 orders underwent the percent changes listed in Table I, and that these intensity changes are not due to lattice modifications (see below). As shown in Fig. 7, most of the change is seen to occur in the vicinity of the disc surface (from ~60 to 100 Å).

As for the lattice spacing, we observed changes in the range of a few 10^{-3}. In this resolution of 1 min/spectrum,

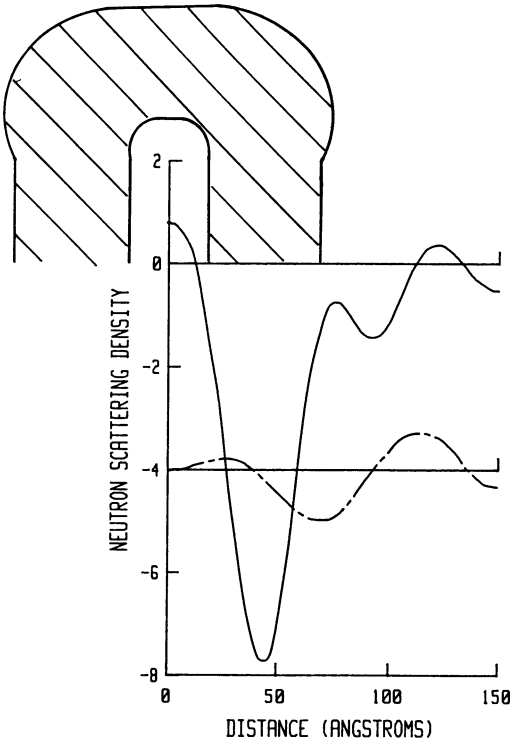

FIGURE 7 Reconstructed density profile (*solid line*) of the ROS disc membrane with the seven structural factors and phases of Saibil et al. (1976). Using the intensity changes observed in the presence of GTP for the first 4 orders (and considering the last 3 orders as unchanged), the difference (after flash – before flash) is computed and displayed expanded 20 times (*dashed line*). It is assumed in computing the difference that the lattice shift was not responsible for these intensity changes but that they were only due to an object change of the disc membrane.

these lattice changes occurred with the same time course as the intensity changes. Thus, we could not rule out the possibility that the intensity changes of orders 2 and 4 were due to a modification of the lattice. To resolve this question, a faster time resolution was needed.

Experiments with 4-s Time Resolution: The Change in Order 2 Intensity Seen in the Presence of GTP Is Not Due to a Lattice Modification but Is an Object Change

The intensity changes of orders 2 and 4 as described above conform to the criteria laid down by the light-scattering work insofar as saturation characteristics, photolysis levels, and GTP requirements are concerned. These neutron experiments however provided almost no kinetic information. Indeed, both "binding" and "dissociation signals" as described by Kühn et al. (1981) have 100-ms rise times for the cattle ROS fragment preparations. In the much larger frog rods, Vuong et al. (1984) resolved the latter signal into two components: a release of Tα-GTP from the disc surface (200-ms rise time) and its subsequent loss from the

ROS fragment (2–10-s rise time, depending on the ROS fragmentation). In frogs, cooling the sample down to 4°C can bring the kinetics of the "release component" into the 1-s range (Vuong, T. M., and M. Chabre, unpublished data). With these considerations in mind, we slightly modified the data acquisition electronics of the D11 instrument to be able to collect a diffraction pattern in 4 s. Experiments at this improved time resolution are shown in Figs. 8 and 9. The results presented below will only deal with the first three orders of diffraction as the counting statistics for order 4 were inadequate in these 4-s experiments.

In Fig. 8 is displayed the result obtained at 6°C in the presence of 1 mM GTP with a light flash that photolyzes 0.3% of the rhodopsin. Comparing top and bottom panels one notes that orders 1 and 3 show no detectable changes, in contrast to the order 2 intensity increase of ~2.5%. This result is not exactly identical to the one obtained with the time resolution of 1 min. The intensity changes were larger before: 5, 1, and 1.5% for orders 2, 1, and 3, respectively. This reduction in the observed relative intensity changes is due most likely to the increase in uniform, diffuse background as we switched from the *X-Y* mode to the *X-t* mode of acquisition (see Fig. 3 for a comparison).

The time course of the lattice spacing change (computed as the shift in the centers of gravity of the two symmetrical second order peaks) is compared with that of the second order intensity change. This comparison is facilitated by fitting an exponential of the form $A\ [1 - \exp(-t/\tau)]$ to both curves. As can be clearly seen in Fig. 9, while the change in intensity occurs in <4 s, the lattice shift takes ~20 s. The change in order 2 intensity thus precedes by a safe margin the slower lattice shrinkage. This indicates that the intensity effect is truly an object change reflecting the release of Tα-GTP from the disc surface.

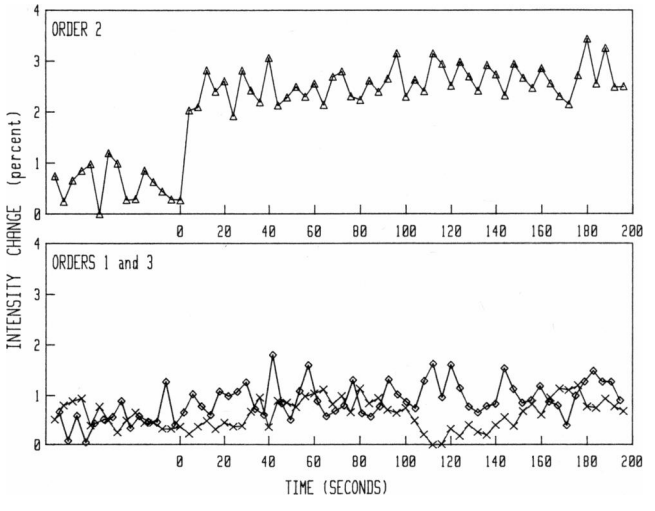

FIGURE 8 Experiments with 4-s time resolution, 6°C. 1 mM GTP, 0.3% R* at 0 s. (*Top*) Intensity change for order 2. (*Bottom*) Intensity change for orders 1 (*crosses*) and 3 (*diamonds*).

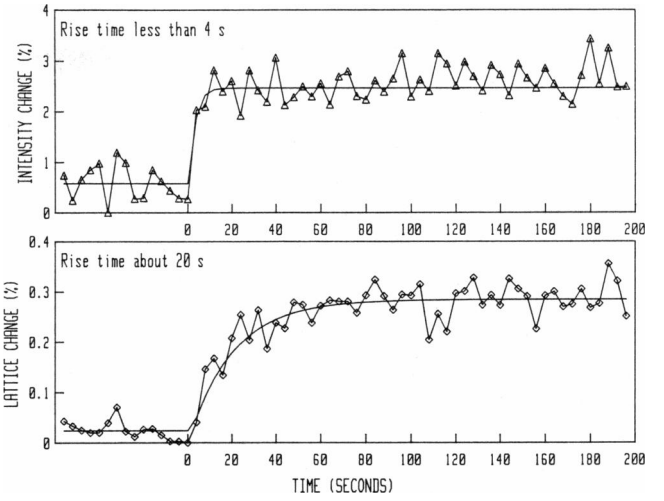

FIGURE 9 Experiments with 4-s time resolution, 6°C. Time courses of the intensity and lattice changes. Same experimental conditions as in Fig. 8. A fit of the form $A[1 - \exp(-t/\tau)]$ is shown superimposed on the two experimental curve. (*Top*) The rise time of order 2 change is seen as occurring in one time step of 4 s. (*Bottom*) The lattice shrinkage of 0.25% rises in ~20 s.

As for diffraction changes seen in the absence of GTP, order 2 show an increase of 4.5% and there is a lattice spacing shrinkage of 0.30% (data not shown). Within this time resolution of 4 s/spectrum, both lattice and intensity changes still show the same rise time (<4 s). That this intensity change is a consequence of the lattice shrinkage is a possibility one cannot currently exclude. Such lattice change might result from some sort of overall modification of the interdiscal electrostatic interaction over the whole disc stack.

DISCUSSION AND CONCLUSION

We have identified changes in the diffraction pattern of oriented frog ROS fragments that correspond to the light-scattering "signals" described and characterized by Kühn et al. (1981) and Vuong et al. (1984). In the absence of GTP, the diffraction changes appear to indirectly reflect the one-to-one binding of photoactived rhodopsin to transducin. On a structural level, this binding seems to affect the surface of the disc membrane. Presently, however, one cannot distinguish between the following three possible origins for these intensity changes. (*a*) A modification of the disc membrane's density profile, i.e., an event independent of the 0.3% shift in lattice spacing itself. (*b*) Conversely a direct consequence of the 0.3% shift in lattice spacing itself. (*c*) A combination of both processes. This ambiguity comes from the observation that both intensity and lattice changes display identical kinetics within the available time resolution of 4 s. Given the large extent of photolysis (10%) required to elicit these changes, one could conjecture that the dominant effect is an alteration of the membrane surface charge resulting in an overall change in

the electrostatic interaction between disc membranes. One might expect from such a change displacements of the disc membranes within the lattice as well as a slight "rapprochement" of the disc resulting in the observed lattice compression. Thus, these intensity changes might be caused in large part by displacement of the disk membranes and to a much smaller extent by a change in the disc membrane's density profile.

With GTP added into the suspension, the conditions are closer to those found in vivo: the light-triggered cascade can proceed beyond the R*–transducin stage. Amplification is thus possible and consequently the photolysis level required to observe the diffraction changes is much lower (0.3–0.5%). In the first series of experiments with 1-min time resolution we established that these diffraction changes exhibited the same kind of characteristics as the "dissociation signal" (Kühn et al., 1981): the absolute requirement for GTP and saturation at a low level of photolysis (~0.5% R*). Interpretation of these preliminary data encountered the same kind of ambiguity as outlined above. Within this time resolution of 1 min, the intensity changes cannot be unequivocally attributed to an object change as they follow the exact same time course as the lattice change. With the better time resolution of 4 s, the intensity changes can now be observed to have a much faster time course (<4 s) than the lattice change (~20 s). This kinetic discrimination thus characterizes the intensity changes in the presence of GTP as an object change preceding the lattice shift. This object change pertains to the disc surface as it shows up as intensity changes of orders 2 and 4, the Fourier harmonics corresponding to the spatial frequency of the disc surfaces. Such a result is consistent with the observation that one consequence of rhodopsin photolysis is the solubilization of the α-subunit of transducin: there is displacement of protein mass away from the disc surface as Tα-GTP becomes soluble (Kühn, 1980).

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REFERENCES

Blaurock, A. E., and M. H. F. Wilkins. 1972. Structure of retinal photoreceptor membrane. *Nature (Lond.).* 236:313–314.

Chabre, M. 1975. X-ray diffraction studies of retinal rods. I. Structure of the disc membrane, effect of illumination. *Biochim. Biophys. Acta.* 382:322–335.

Chabre, M. 1985. Trigger and amplification mechanisms in visual phototransduction. *Annu. Rev. Biophys. Biophys. Chem.* 14:331–360.

Chabre, M., and A. Cavaggioni. 1973. Light induced changes of ionic fluxes in the retinal rods. *Nature New Biol.* 24:118–120.

Chabre, M., and A. Cavaggioni. 1975. X-ray diffraction studies on retinal rods. II. Light effects on the osmotic properties. *Biochim. Biophys. Acta.* 382:336–343.

Corless, J. M. 1972. Lamellar structure of bleached and unbleached photoreceptor membranes. *Nature (Lond.).* 237:229–231.

Emeis, D., H. Kühn, J. Reichert, and P. Hofmann. 1982. Complex formation between Metarhodopsin II and GTP-binding protein in bovine photoreceptor membrane leads to a shift of the photoproduct equilibrium. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 136:201–207.

Fung, B. K. K. 1983. Characterization of transducin from bovine rod outer segments. I. Separation and reconstitution of the subunits. *J. Biol. Chem.* 258:10495–10502.

Fung, B. K. K., J. B. Hurley, and L. Stryer. 1981. Flow of information in the light triggered cyclic nucleotide cascade of vision. *Proc. Natl. Acad. Sci. USA.* 78:152–156.

Gras, W. J., and C. R. Worthington. 1969. X-ray analysis of retinal photoreceptors. *Proc. Natl. Acad. Sci. USA.* 63:233–237.

Kühn, H. 1980. Light and GTP-regulated interaction of GTPase and other proteins with bovine photoreceptor membranes. *Nature (Lond.).* 283:587–589.

Kühn, H. 1981. Interaction of the rod cell proteins with the disc membrane: influence of light, ionic strength and nucleotides. *Curr. Top. Membr. Transp.* 15:171–201.

Kühn, H. 1984. Interactions between photoexcited rhodopsin and light activated enzymes in rods. Progress in Retinal Research. Vol. 3. Osborne and Chader, editors. Pergamon Press, New York. 123–126.

Kühn, H., N. Bennett, M. Michel-Villaz, and M. Chabre. 1981. Interaction between photoexcited rhodopsin and GTP-binding protein: kinetic and stoichiometric analysis from light scattering changes. *Proc. Natl. Acad. Sci. USA.* 78:6873–6877.

Pfister, C., H. Kühn, and M. Chabre. 1983. Interaction between photoexcited rhodopsin and peripheral enzymes in frog retinal rods. *Eur. J. Biochem.* 136:489–499.

Saibil, H., M. Chabre, and D. L. Worcester. 1976. Neutron diffraction studies of retinal rod outer segment membranes. *Nature (Lond.).* 262:266–270.

Schwartz, S., J. E. Cain, E. Dratz, and J. K. Blasie. 1975. An analysis of the lamellar X ray diffraction from disordered membrane multilayers with an application to data from retinal rod outer segments. *Biophys. J.* 15:1201–1232.

Stryer, L. 1983. Transducin and the cyclic-GMP phosphodiesterase: amplifier proteins in vision. *Cold Spring Harbor Symp. Quant. Biol.* 48:841–852.

Stryer, L. 1986. Cyclic-GMP cascade of vision. *Annu. Rev. Neurosci.* 9:87–119.

Vuong, T. M. 1984. Kinetic studies of the activation of transducin by photoexcited rhodopsin. Ph.D. thesis. Stanford University, Stanford, CA.

Vuong, T. M., M. Chabre, and L. Stryer. 1984. Millisecond activation of transducin in the cyclic nucleotide cascade of vision. *Nature (Lond.).* 311:659–661.

Wilden, U., W. Hall, and H. Kühn. 1986. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc. Natl. Acad. Sci. USA.* 83:1174–1178.

Woodruff, M. L., and D. Bownds. 1978. Amplitude, kinetics and reversibility of a light-induced decrease in guanosine 3'-5'-cyclic monophosphate in isolated frog retinal rod outer segments. *J. Gen. Physiol.* 73:629–653.

Yeager, M., B. Schoenborn, D. Engelman, and L. Stryer. 1980. Neutron diffraction analysis of rod photoreceptor membranes in intact retinas. *J. Mol. Biol.* 137:315–348.

Yoshikami, S., W. I. Robinson, and W. A. Hagins. 1974. Topological connectivity and intactness of rod and cone outer segment membranes determined with a fluorescent probe. *Fed. Proc.* 33:1575.