Biochimica et Biophysica Acta, 725 (1983) 60–70
Elsevier

BBA 41368

INTERPLAY BETWEEN HYDROXYLAMINE, METARHODOPSIN II AND GTP-BINDING PROTEIN IN BOVINE PHOTORECEPTOR MEMBRANES

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(Received April 21st, 1983)

Key words: Rhodopsin; GTP-binding protein; Vision; Photoreceptor membrane; (Bovine retina)

The decay reactions of metarhodopsin II and the dissociation of the complex between rhodopsin (in the metarhodopsin II state) and the GTP-binding protein (G-protein) (in its inactive, GDP-binding form) have been compared at various concentrations of hydroxylamine. The reactions of the chromophore were measured by absorption changes in the visible range, the complex dissociation by changes in the near-infrared scattering. An additional monitor of the complex was given by the G-protein-dependent equilibrium between metarhodopsin I and metarhodopsin II. For all measurements, fragments of isolated bovine rod outer segments in suspension were used. In the absence of hydroxylamine, the rhodopsin-G-protein complex dissociated within 20–30 min at room temperature. The presence of hydroxylamine greatly accelerated (e.g., 5-fold at 1 mM NH_2OH) the dissociation. Under all conditions, the free, dissociated G-protein can reassociate to metarhodopsin II produced by subsequent bleaching. Dissociation of the metarhodopsin II-G-protein complex required the decay of photoproducts with a maximal absorbance of 380 nm, but was not affected by the simultaneous presence of metarhodopsin III or metarhodopsin III – like photoproducts with a maximal absorbance between 450 and 470 nm. Despite the acceleration of metarhodopsin II-G-protein dissociation by NH_2OH, metarhodopsin II-G-protein was relatively stabilized as compared to free metarhodopsin II. The ratio of the decay rates of free metarhodopsin II and metarhodopsin III-G-protein was increased as much as 10-fold in the presence of 25 mM NH_2OH. The results indicate a mutual interdependence of retinal, opsin and G-protein.

Introduction

The absorption of a light quantum by the visual pigment rhodopsin results in a very rapid isomerization of the chromophore (picosecond range), 11-*cis*-retinal, followed in the dark by a series of thermal relaxations identified by different intermediate pigments, each with a characteristic absorption spectrum [1]. The metarhodopsin I-to-metarhodopsin II transition is the last transition, which preceeds the activation of the rod outer segment phosphodiesterase by bleached rhodopsin. The first step in the latter process is the association of bleached rhodopsin with a peripheral membrane protein called the G-protein (GTP-binding protein [2,3]). This study investigates further the link between the intramolecular photochemistry of rhodopsin and its intermolecular 'enzymatic' activity. Previous work has suggested that the photoproduct metarhodopsin II, which arises in a few milliseconds at room temperature, is the first to show a high affinity for the

Abbreviations: Pipes, piperazine-1,4-diethanesulfonic acid; Guo*PPP*[S], guanosine 5'-[γ-thio]triphosphate.

G-protein. This can be concluded from observations that the complex formation closely follows metarhodopsin II production, and is strong enough to draw metarhodopsin II from the equilibrium with metarhodopsin I [4–6]. We have investigated the dependence of the dissociation of the complex between rhodopsin and G-protein on metarhodopsin II decay at various concentrations of hydroxylamine.

In this study, two different indicators of the association and dissociation between rhodopsin and the G-protein have been used. The first one uses the fact that the complex formation draws metarhodopsin II from its equilibrium with metarhodopsin I, the second is a change in the near-infrared scattering of the rod outer segments. This so-called 'binding signal' [3] is basically the same observation [5] as the 'signal P' [7], which was previously studied in some phenomenological investigations.

Materials and Methods

All measurements were done in isotonic saline containing 130 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 10 mM Pipes (pH 7.5). Bovine rod outer segments were prepared according to a standard procedure [4] and were stored in liquid N_2. Prior to the measurements, the thawed suspensions were diluted to a measuring concentration of $3 \cdot 10^{-6}$ M rhodopsin.

The simultaneous measurement of absorption and near-infrared scattering signals was previously described in detail [8]; flash (20 μs, EG & G), cuvette configuration (effective path length 1.4 cm), optical blocking filters (dielectric filters), semiconductor detectors and amplifiers were the same as previously described. All signals were measured on randomly oriented rod outer segments, and the scattering signals were corrected for the small so-called N-signal.

Concerning the use of the monitors of the rhodopsin-G-protein complex, the metarhodopsin I-metarhodopsin II equilibrium shift is limited to low temperatures and/or high pH values ('classical' equilibrium favors metarhodopsin I); the scattering indicator (signal P), on the other hand, can be used at all pH values and temperatures but it is physically complicated and dependent on the integrity of the rod outer segments. In isolated discs recombined with the G-protein, for example, the binding reaction occurs in milliseconds while the scattering signal can take tens of seconds [5,9]. In ordinarily stacked rod outer segments, signal P consists of two highly anisotropic components which can be separated by orientation of the rod outer segments in a magnetic field. We found a fast (10 ms) change in the axial scattering of the rod outer segments and a slower change (50–500 ms) in the radial scattering [9].

The present study is restricted to rod outer segments. The signal P will be measured under isotropic conditions, i.e., as a superposition of the radial and axial effects which are similarily affected by hydroxylamine.

Results

A. Recovery measurements

a. Recovery of the scattering signal. In the experiment shown in Fig. 1, we measured the binding of G-protein to flash-induced metarhodopsin II by recording the P signals evoked by three consecutive test flashes. The first test flash (bleaching 1.5% of rhodopsin) was applied on a previously unbleached sample. After a subsequent bleach of 20% rhodopsin, a further test flash (bleaching 1.5%) applied within seconds evoked no further signal. This can be understood by the fact that most of the G-protein (total amount approx. 10% of rhodopsin) is now bound in a 1 : 1 complex [3] to metarhodopsin II [5]. The rapid dissociation of the complex by GDT-GTP exchange [3] was prevented by the absence of GTP.

The third signal in Fig. 1 indicates, however, an additional slower dissociation mechanism: after an hour, the third test flash evoked a signal of about 60% of the amplitude observed with flash No. 1. The system regains sensitivity within this time. This result suggests that within this time period the metarhodopsin II-G-protein complexes were dissociated and that newly formed metarhodopsin II (by the third test) can bind again with the free G-protein. Because it is known that metarhodopsin II undergoes a slow decay (to metarhodopsin III and retinal + opsin) it is further suggested that some of these subsequent products are no longer able to bind the G-protein, thereby resensitizing

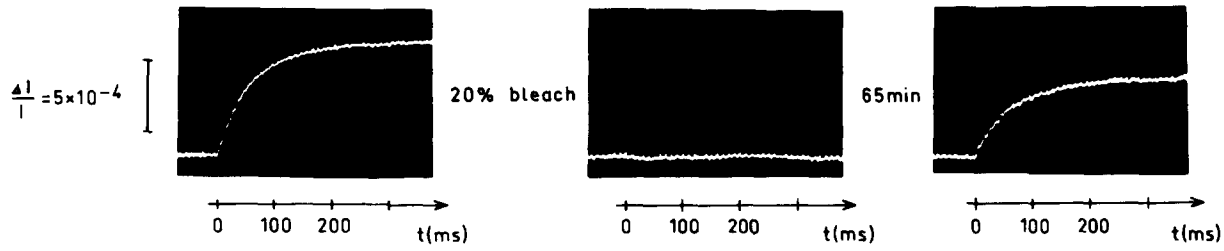

Fig. 1. Recovery of the binding of G-protein to flash-induced metarhodopsin II. The figure shows flash-induced near-infrared scattering signals (P signals) under three different conditions: (Left) Signal evoked by a flash bleaching 1.5% of rhodopsin in an unbleached sample; the signal shows the time course of G-protein binding by metarhodopsin II; the amount of metarhodopsin II is equal to the amount of bleached rhodopsin. (Middle) No signal is observed after a second 1.5% flash applied within seconds after the first one, but following a 20% bleach. 20% of rhodopsin was bleached between flash Nos. 1 and 2, the total amount of G-protein within the sample (approx. 10–12% of the rhodopsin content [3]) now being bound to metarhodopsin II molecules. (Right) 65 min later, a third 1.5% flash was again able to evoke a signal. Measuring conditions: $T = 20°C$, pH 7.5.

the membranes to a flash of light.

In the following, we have tried to establish which of the slow photoproducts is incompetent at binding the G-protein by accelerating the decay of metarhodopsin II through the addition of hydroxylamine.

b. Effect of hydroxylamine on recovery. Following the same protocol as in the experiment shown in Fig. 1, we have measured the signals from test flashes (bleaching 1%) following a 20% bleach. In Fig. 2, the amplitude of the response relative to that of the test flash preceding the 20% bleach is plotted versus time. Each curve belongs to a fresh sample. The data show that increasing concentrations of NH_2OH dramatically shortened the time required for the membranes to regain sensitivity for responding the test flashes.

All curves displayed a sigmoidal time course. Under all conditions the recovery of the sensitivity to respond to the flash is incomplete: the ampli-

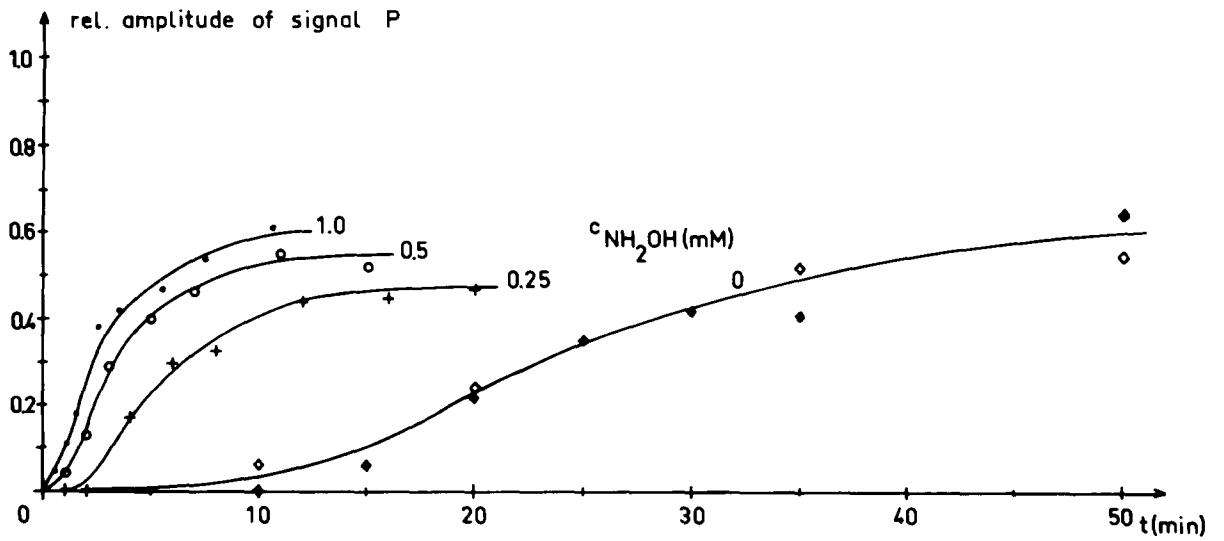

Fig. 2. Time course of the recovery shown in Fig. 1. Parameter of the curves is the concentration of hydroxylamine present in the sample suspension; NH_2OH is also present during and after flashes. After a short bleach of 20% rhodopsin, small test flashes were applied at the times indicated. The observed amplitudes of the P signal evoked by these small flashes (bleaching 1.5%) are normalized to the amplitude of the first test flash, i.e., a measure of complexable G-protein. Different symbols at the ($C = 0$) curve denote different samples. Measuring conditions: $T = 20°C$, pH 7.5.

tude of the signal after recovery from previous bleaches was 50–60% at all NH_2OH concentrations.

c. Recovery of the metarhodopsin I-metarhodopsin II equilibrium shift. In order to corroborate that the above observations on signal P are really based on binding and not on some property of the scattering mechanism, we measured the shift of the metarhodopsin I-metarhodopsin II equilibrium caused by the metarhodopsin II-G-protein complex. This effect can most readily be observed in so-called exhaustion curves. Their use was discussed in more detail in a previous study [4]. The 380 − 417 nm absorption difference is thereby measured in a series of equally intensive flashes gradually photolysing all rhodopsin. The differential measurements avoids scattering artefacts. One effectively measures production of metarhodopsin II; due to the shift of the metarhodopsin I-metarhodopsin II equilibrium by the preference of G-protein for metarhodopsin II, a pronounced deviation from the normal logarithmic photolysis curve is observed in the relevant low-bleaching range (below 10%).

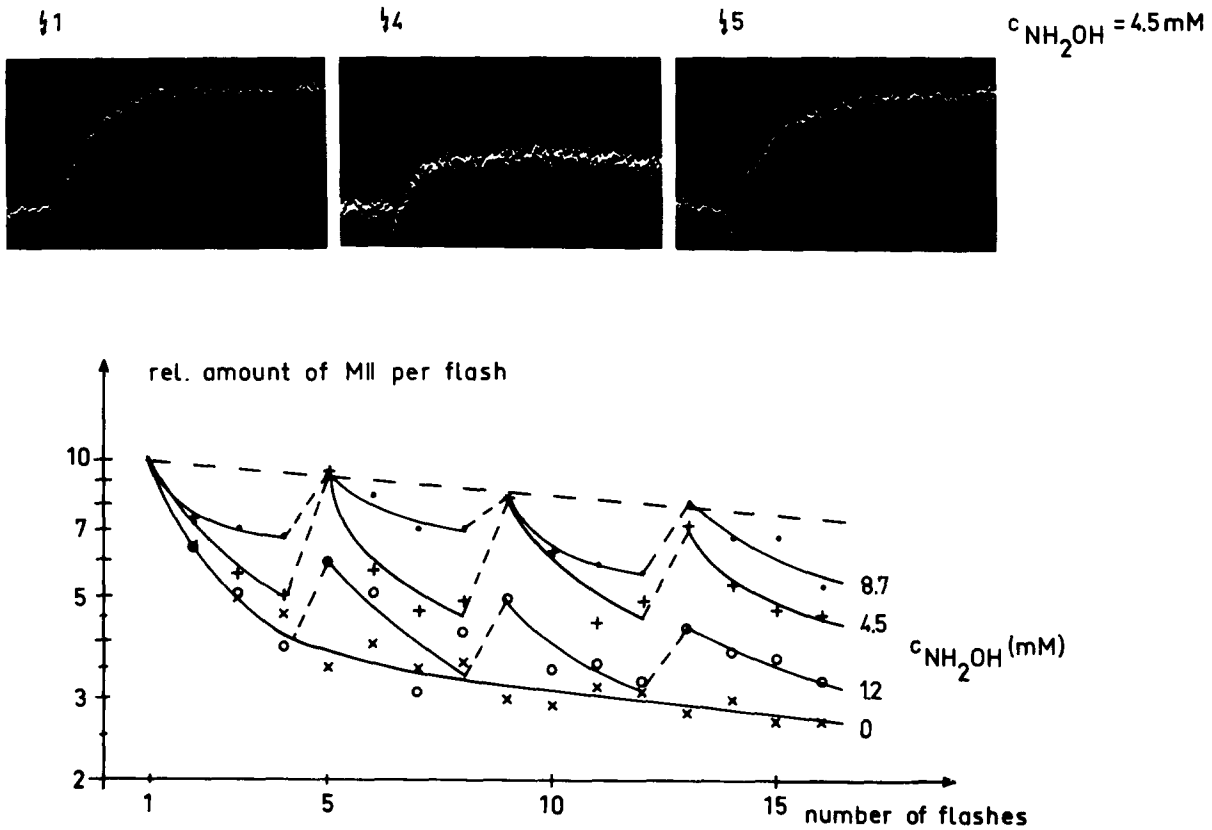

Fig. 3. Formation of metarhodopsin II (MII) upon successive bleaches of rhodopsin in series of flashes (exhaustion curves). Parameter of the curves is the concentration of hydroxylamine in the suspension. The flashes were applied in groups of four with a time interval of 1 min. After flash Nos. 4, 8 and 12, the time interval was 5 min. The metarhodopsin II production was measured by the difference in the flash-induced absorption changes at 380 and 417 nm. The amplitudes of these 380 − 417 nm difference signals (see above) are plotted as single flash recordings versus the flash number. Signals are the differences of the relative absorption changes at 380 and 417 nm [8]. Every flash evokes a signal by producing metarhodopsin II and bleaches the sample a further step. The more G-protein is free prior to a flash, the more metarhodopsin II is bound; this is observed by a shift of the signal amplitude to higher values. Limits are the lower solid line representing the equilibrium amount of metarhodopsin II under the conditions (pH 7.5 and $T = 7°C$), and the dotted line above where the total amount of rhodopsin produced by a flash is bound to free G-protein, thereby fully shifted to metarhodopsin II.

Consider in Fig. 3 first the exhaustion for the samples without NH_2OH. Under the conditions used in this experiment (pH 7.5, $T = 5°C$) the metarhodopsin I-metarhodopsin II equilibrium constant is K = [metarhodopsin II]/[metarhodopsin I] = 1/3.

As described previously [4], the equilibrium curve was only achieved after a bleaching of 10–15% rhodopsin, i.e., after the binding of all G-protein available to the metarhodopsin II produced by the first few flashes. Thus, these first flashes produced more metarhodopsin II as was expected from the metarhodopsin I-metarhodopsin II equilibrium constant under these conditions. This means the signal amplitude is a measure of the amount of G-protein which is bound to the metarhodopsin II molecules produced by the flash and therefore of the free G-protein prior to the flash.

The important point for the following is that the amplitude of the metarhodopsin II signal follows the upper dotted straight line if the metarhodopsin I-metarhodopsin II equilibrium of the rhodopsin produced by a flash is fully shifted to metarhodopsin II (i.e., when excess G-protein is available). In the experiment (at pH 7.5, $T = 7°C$) this is the case when all G-protein molecules prior to the flash are dissociated from rhodopsin, as in the unbleached sample (flash No. 1). With increasing [NH_2OH], the curves are expected to progressively shift up from the lower solid line to the upper line because of the increased dissociation rate of the metarhodopsin II-G-protein complex. This is really observed in Fig. 3. Furthermore, the flashes were applied in a certain rhythm, applying them in groups of four with intervals of 1 min between the flashes in each group and with an interval of 5 min between groups. One expects the observed saw tooth-like curves: within the 1 min interval, the production of metarhodopsin II dominated over hydrolysis of metarhodopsin II-G-protein complex, but the 5 min interval allowed the production of more free G-protein and consequently the first flash in each subsequent group could produce more metarhodopsin II-G-protein.

While the recovery time course is better estimated on the basis of the scattering signals shown in Fig. 2, the metarhodopsin II data in Fig. 3 allowed the determination of the total amount of free G-protein available after recovery. For the two highest NH_2OH concentrations, the first flash of each group (i.e., Nos. 1, 5, 9 and 13) follows the upper dotted line, indicating a complete dissociation of the preexisting metarhodopsin II-G-protein complex.

B. The inverse scattering signal

In Fig. 4, the effect of a high NH_2OH concentration on the scattering signal P is shown. The increase in light scattering is transient under these conditions, indicating the formation and subsequent dissociation of the metarhodopsin II G-protein complex.

Apparently, all kinetic components of the P signal (axial and radial shrinkage of the scattering mass and also the slow polarizability change [9] showed complete relaxation. Because of the necessity of rather high concentrations of NH_2OH, a side effect of NH_2OH cannot be excluded but it is improbable that such an effect would just cause the observed complete relaxation of the light-induced scattering change.

C. Absorption changes after metarhodopsin II in the presence of NH_2OH

In order to investigate the reactions of the chromophore which come into question as precursors of the complex dissociation, we investigated the absorption changes during the decay of metarhodopsin II, in its free form and in the metarhodopsin II-G-protein complex, and in the presence of NH_2OH. Absorption and light-scattering changes in the complex were recorded simultaneously; the scattering data will be described separately in the next section.

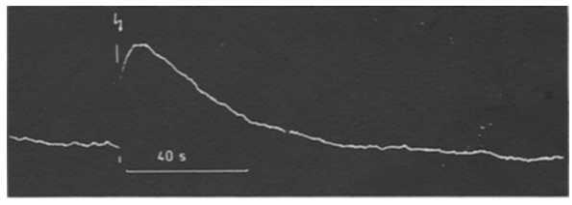

Fig. 4. Flash-induced near-infrared scattering signal in the presence of 35 mM NH_2OH. Measuring conditions: $T = 20°C$, pH 7.0. Flash bleached 3% of the rhodopsin present. Note that the positive scattering signal, indicating the formation of metarhodopsin II-G-protein complexes, is totally reversed.

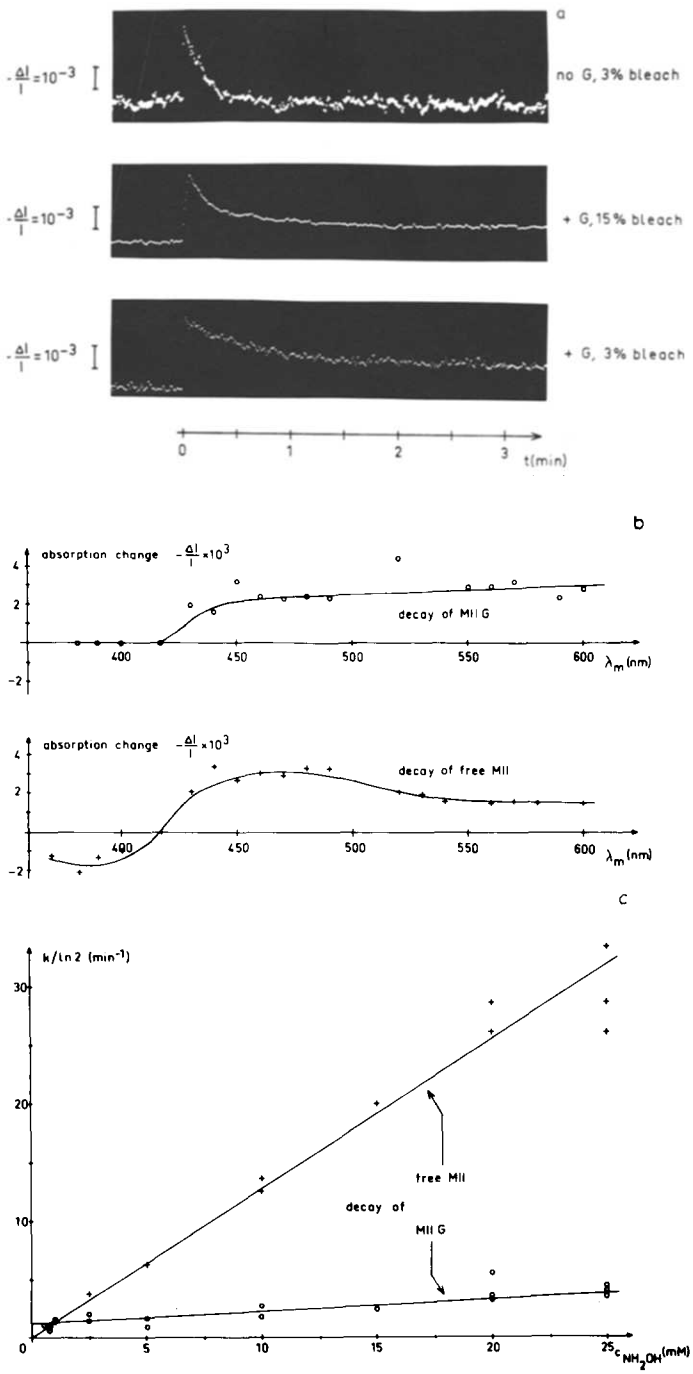

Fig. 5. Flash-induced absorption changes in the presence of NH_2OH. The initial jump is due to the formation of metarhodopsin II (MII) (cf. Fig. 3, flash No. 1). $T = 20°C$, pH 7.5. (a) Original recordings of the 450 − 417 nm absorption difference (difference in the flash-induced absorption changes at 450 and 417 nm); [NH_2OH] = 5 mM, three different conditions: no G-protein present at the membrane (by application of 20 μM Guo*PPP*[S] [5]), upper trace; G-protein present, but bleaching high enough that a mixture of free metarhodopsin II and metarhodopsin II-G-protein was formed, middle trace; G-protein present, low bleaching, the total amount of metarhodopsin II was bound to G-protein, lower trace. Note the kinetic difference between the decays in the upper and lower traces and the superposition of both kinetics in the middle trace. (b) Difference spectrum of the absorption changes shown in a [NH_2OH] = 25 mM. Reference wavelength was 417 nm. The plots represent the difference between the intensity changes at the wavelength indicated on the abscissa and at 417 nm. The upper spectrum belongs to the upper, faster trace (no G-protein present, decay of free metarhodopsin II) in a, the lower spectrum to the lower trace (G-protein present, decay of metarhodopsin II-G-protein). (c) Decay rates (inverse half-mean times) of the decay of free metarhodopsin II and metarhodopsin II-G-protein, as a function of the concentration of hydroxylamine. Meaning of the symbols as in b.

There are two ways to obtain free metarhodopsin II: either one can bind the total amount of G-protein by bleaching approx. 15% of rhodopsin prior to the test flash (cf. Fig. 1, flash No. 2) or one can remove the G-protein completely from the membrane, using Guo*PPP*[S] [5].

Fig. 5a shows original recordings of flash-induced absorption changes in the Guo*PPP*[S] treated sample (a), in the prebleached sample (b) and in the untreated sample (c), all in the presence of 5 mM NH_2OH. The measuring wavelength of these selected recordings is 450 nm where the maximal absorption difference occurs (Fig. 5b, see below). It is seen (and confirmed by a detailed kinetic analysis, not shown) that the absorption change in the absence of bound G-protein and that in the metarhodopsin II-G-protein complex approximate simple first-order kinetics, the latter being a factor of 5 faster.

The middle trace, a recording after higher bleaching by a group of five rapidly following flashes in the presence of G-protein, shows a superposition of the upper and lower traces. This is expected because there is more metarhodopsin II than G-protein after the flash group, and the signal of a mixture of free and complexed metarhodopsin II must be observed.

In addition to a faster reaction pathway in free metarhodopsin II than in metarhodopsin II-G-protein, the difference spectrum of the fast (metarhodopsin II) signal showed a maximum near 470 nm while the slow signal (metarhodopsin II-G-protein) showed a broader difference spectrum. These spectra were observed for all NH_2OH concentrations where the signals were fast enough for a reliable measurement ($[NH_2OH] \geq 2$ mM).

As a consequence of the difference technique, where one measuring beam remains adjusted to 417 nm, all signals are set to zero at this wavelength. This explains why the spectra approximate to positive values at high wavelengths. The choice of 417 nm as a reference wavelength is due to its position in the center of the range of interest and to the fact that there is an isosbestic point between metarhodopsin I and metarhodopsin II.

The difference spectra and kinetics seem to be consistent with the NH_2OH-accelerated production of a metarhodopsin III-like product (see, for example, Refs. 10 and 11) in the absence of G-protein. This product is not found in the metarhodopsin II-G-protein complex. In this situation, retinal reacts much slower to give another product which is most probably free retinal oxime, as estimated by its difference spectrum which can be understood as a shift of the absorption maximum from 380 to 365 nm.

The difference spectra do not allow a clear decision as to whether this reaction is exclusive or if it is contaminated by metarhodopsin II → metarhodopsin III or other processes. However, the uniformity of the kinetics shows that, irrespective of the contaminants, they must be produced via one and the same rate-limiting step.

In this study we shall not attempt to identify the products further, but focus on the finding that there are two kinetically and spectrally different reaction pathways with hydroxylamine in free metarhodopsin II and metarhodopsin II-G-protein. We will use the operational terms 'retinal oxime 365' and 'retinal 470'. Additional recordings (not shown) showed that retinal 470 is a transient product, which is also transformed to retinal oxime 365 (approx. 30 min at $[NH_2OH] =$ 25 mM).

Fig. 5c shows in addition that retinal must already have reacted with NH_2OH prior to the formation of the products because there is in both cases a clear dependence of the rate on $[NH_2OH]$.

For free metarhodopsin II, the rate of decay (retinal 470 formation) increased linearly with NH_2OH concentration. The decay of metarhodopsin II-G-protein may also show a linear dependence on the NH_2OH concentration but in this case the slope was more than a factor of 10 smaller than in the case of free metarhodopsin II.

The lack of a noticeable lag phase in the signals shown in Fig. 5a and their linear acceleration by NH_2OH (Fig. 5c) suggests that in both systems and in the measured concentration range, the bimolecular reaction is rate limiting. Because there is always much more hydroxylamine than retinal, the bimolecular reaction and therefore the reaction to retinal oxime 365 as well as that to retinal 470 can be treated as pseudo-first-order reactions and described by two different first-order rate constants. Thus, one obtains the following equations (for $[NH_2OH] \geq 2$ mM) (M, metarhodopsin; G, G-protein):

$$\text{MII-G} + \text{NH}_2\text{OH} \xrightarrow{k_{\text{MII-G}}} \text{retinal oxime 365} + \text{G-opsin} \quad (1)$$

$$\text{MII} + \text{NH}_2\text{OH} \xrightarrow{k_{\text{MII}}} \text{retinal opsin 470} \quad (2)$$

D. Comparison between the absorption changes of the chromophore and the metarhodopsin II-G-protein complex dissociation

In Fig. 6, the [NH_2OH] dependencies of all reactions described in the foregoing sections are compared to each other. The two upper curves are the same as those which were already discussed in the last section, with an extended ordinate. The lower curve represents the decay rate of P signals such as that shown in Fig. 4. These signals – which indicate the dissociation of the metarhodopsin II-G-protein complex (section B) – were recorded simultaneously (i.e., on the same sample and from the same flash) with the absorption changes in the presence of G-protein. As discussed in the foregoing section, the latter most probably represents the formation of free retinal oxime.

Comparing the absorption change and the light-scattering change observed in the inverse P signals, it is seen that the latter is slower at all NH_2OH concentrations. This result suggests that the retinal reaction(s) observed in the absorption changes could be involved in the induction of complex dissociation.

The correlation becomes clearer in the range of very low [NH_2OH]. In this range, the inverse P signal and the retinal absorption changes became too slow to be measured as transients at constant wavelength. However, the recovery time course, as shown in Fig. 2, could be conveniently evaluated. The results are shown in the inset of Fig. 6 (P_{invers},

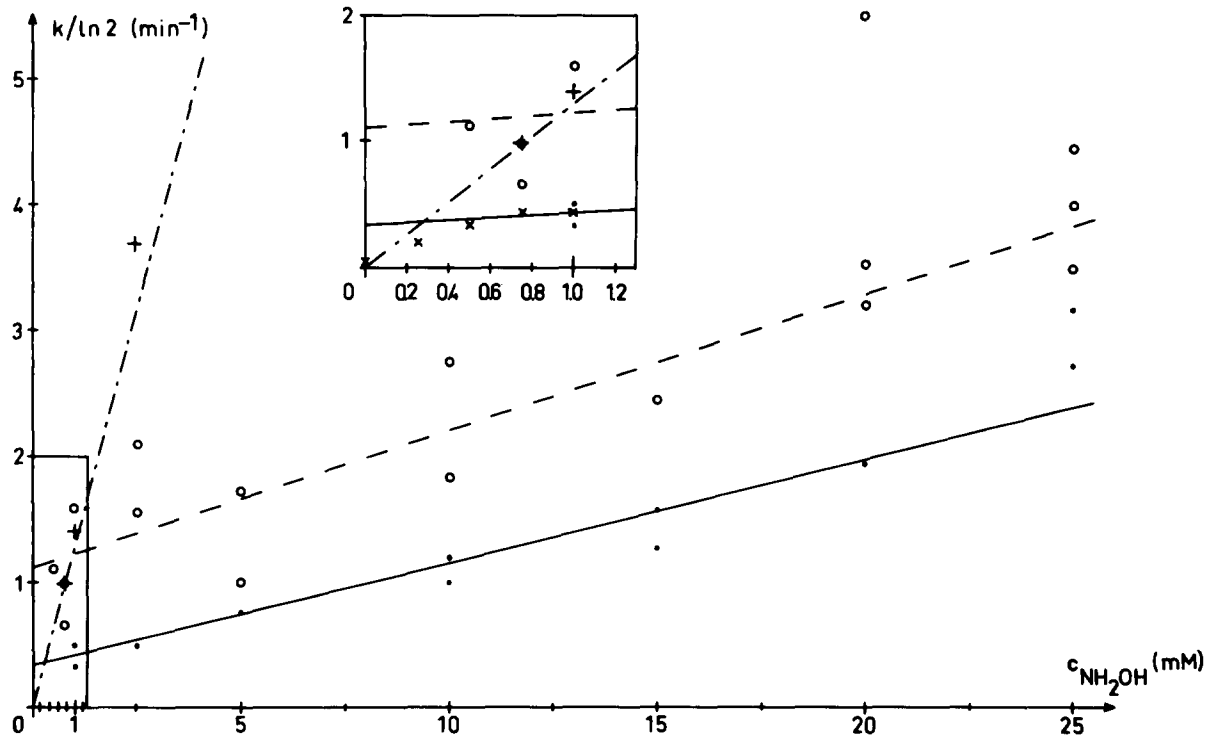

Fig. 6. Decay rates of metarhodopsin II in the absence of G-protein (+) and in the metarhodopsin II-G-protein complex (○), compared to the decay of the metarhodopsin II-G-protein complex as indicated by near-infrared signals. The two upper dotted straight lines (– · – and — —) are the same as those shown in Fig. 5c (ordinate scale expanded). The additional lower solid line (values with symbol ●) represents the decay rate taken from scattering signals as shown in Fig. 4 (signal reversal). The two lower straight lines, therefore, belong to the metarhodopsin II-G-protein complex; they were measured simultaneously from the same sample. (Inset) Symbols have the same meaning (expanded abscissa). Symbol x represents the inverse half-mean time of the recovery time course, as shown in Fig. 2.

symbol ●; recovery rate, symbol x). First of all, it is important to note the kinetic equivalence of the recovery and inverse P signal which is seen from the congruence of the rates at $[NH_2OH] = 1.0$ mM where both measurements were possible.

It is further seen that, at NH_2OH concentrations below 0.5 mM, the recovery rate (inverse half-mean times taken from Fig. 2) did not follow the solid line but became much slower, parallel to the increasing lag phase seen in Fig. 2. Apparently, the recovery becomes gradually rate limited by one or more NH_2OH-dependent precursor-retinal reaction(s).

These key reactions of retinal, which terminate the complex of rhodopsin with G-protein, certainly do not take place in the slow photoproducts metarhodopsin III and retinal 470, because neither metarhodopsin III nor retinal 470 binds the G-protein. This follows from the binding that in the presence of high concentrations of these products free G-protein is found which binds to new flash-induced metarhodopsin II. We tested this by recording spectra of the slow photoproducts after a full bleaching (not shown). Metarhodopsin III remained stable (greater than 80%) beyond 1 h after bleaching (pH 7, 20°C). After that time the binding of G-protein to metarhodopsin II had already fully recovered (Fig. 2), in spite of the high amount of metarhodopsin III. The same was found for retinal 470 (1 mM NH_2OH): also in this case, metarhodopsin II produced by new flashes bound the maximal amount of G-protein while most of the slow photoproduct was still present.

Thus, we have collected several qualitative correlations between metarhodopsin II decay and rhodopsin G-protein complex dissociation: both were similarly accelerated by NH_2OH, metarhodopsin II (or products absorbing at 380 nm) always decayed faster than the complex dissociated and the products following metarhodopsin II did not bind to G-protein.

This suggests a reaction scheme where the decay of metarhodopsin II preceeds the dissociation of the metarhodopsin II-G-protein complex:

$$\text{MII-G} + NH_2OH \overset{k_{\text{MII-G}}}{\rightarrow} \text{retinal oxime} + \text{opsin-G}$$

$$\overset{k_s}{\rightarrow} \text{retinal oxime} + \text{opsin} + \text{G} \qquad (3)$$

The simplest assumptions for the rate constants would be that $k_{\text{MII-G}}$ is the same as in Eqn. 1 and that the complex splitting rate k_s is constant and independent of $[NH_2OH]$. However, we have to note that the data did not allow an exact kinetic correlation between the first and second steps in Eqn. 3. Especially the nature of the rate-limiting processes at low NH_2OH concentration (inset of Fig. 6) is unknown.

Discussion

The results show first of all that the reaction of hydroxylamine with the chromophore (retinal) of the visual pigment rhodopsin induces the dissociation of the complex between bleached rhodopsin and the GTP-binding protein (G-protein).

This result has to be considered in relation to our previous finding that the formation of the photoproduct metarhodopsin II is rate limiting for the complex formation [4,5]. The present study shows in addition that the metarhodopsin II-G-protein complex dissociates when the metarhodopsin II conformation is terminated by the reaction of retinal with hydroxylamine, forming retinal oxime.

However, the present study demonstrates not only an effect of a retinal reaction on the interaction between rhodopsin and G-protein but also an inverse effect: the presence of complexed G-protein blocked the retinal 470 pathway for the reaction of retinal with hydroxylamine.

It can therefore be derived that there is a transient mutual interaction in the system retinal/opsin/G-protein during the lifetime of metarhodopsin II. The specific role of metarhodopsin II, already suggested in former investigations, is thereby supported.

Further conclusions will mainly depend on the identification of the unknown product which we – so far – have termed retinal 470. This product is comparable in its absorption to metarhodopsin III. Identifying this product with retinal 470, one would have to assume that NH_2OH binds transiently to metarhodopsin II, thereby catalysing the reaction metarhodopsin II → metarhodopsin III in the free rhodopsin molecule. In the complexed system, the pathway to metarhodopsin III is blocked and the decay to free retinal oxime is thereby preferred.

Appendix

A possible reaction scheme

Chabre and Breton [11] have demonstrated a pH-dependent equilibrium between metarhodopsin III and metarhodopsin II. They pointed to the similarity between metarhodopsin I and metarhodopsin III with respect to their absorption maxima as well as to the orientation of the chromophore.

In recent work, Pfister, Kühn and Chabre (personal communication, 1983) have demonstrated that metarhodopsin III is formed faster and in a much lower amount in metarhodopsin II-G-protein, compared to free metarhodopsin II, suggesting a preference of the G-protein for metarhodopsin II, with respect to not only metarhodopsin I but also metarhodopsin III.

Extending this concept on the hydroxylamine-induced metarhodopsin III-like product retinal 470 of this study and using Eqns. 1–3, the following scheme is suggested:

$$MI + NH_2OH \rightleftharpoons MII + NH_2OH \underset{k_{-1}}{\overset{C_H \cdot k_1}{\rightleftharpoons}}$$

$$MII - NH_2OH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{retinal opsin 470} + NH_2OH$$

$$\downarrow k_3$$

$$\text{opsin-retinal oxime 365}$$

$$\downarrow k_5$$

$$\text{opsin} + \text{retinal oxime 365}$$

where C_H is the concentration of NH_2OH.

The effect of G-protein on this system is to shift the equilibrium in the upper horizontal line to metarhodopsin II. An effect of G-protein on the spontaneous decays with k_3 and k_5 (cf. Eqn. 3) is not assumed. We shall now describe the overall rates of the reactions starting from free and complexed metarhodopsin II in this scheme and compare them to the experimental rates k_{MII} and $k_{MII\text{-}G}$ (Eqns. 1 and 2).

Free metarhodopsin II, no G-protein complexed

The equilibria with subscripts 1 and 2 are shifted to the right. It follows that if $k_3 \ll k_2$, branch 2 is preferred. Because step 1 is rate limiting, the overall rate for metarhodopsin II decay starting from free metarhodopsin II is given by:

$$k_{MII} = k_1 \frac{k_2}{k_{-1} + k_2} C_H \approx k_1 C_H$$

Thus, metarhodopsin III is produced with the first-order rate $k_1 C_H$, retinal oxime 365 rises very slowly with k_2, returning via the step 2 equilibrium (and/or via a separate pathway not contained in the scheme).

Rhodopsin-G-protein complex

In this situation, equilibria 1 and 2 are shifted to the left and if $k_3 \gg k_2 + k_{-2}$, branch 3 is preferred. The overall rate is now given by:

$$k_{MII\text{-}G} = K_1 k_3 = \frac{k_3}{k_{-1}} k_1 C_H$$

$K_{MII\text{-}G}$ is, therefore, like k_{MII}, proportional to C_H, but slowed down by the factor k_3/k_{-1} which depends on the assumptions for k_3 and k_{-1}. For sufficient shift of the equilibrium to the left, the experimental finding $k_{MII\text{-}G} \approx 1/10\ k_{MII}$ can be realized. The crucial point of the scheme above is the product metarhodopsin II-NH_2OH which cannot be spectrally identified as such. This postulate is based on the kinetic data in section C which are limited to the range $[NH_2OH] \geqslant 2$ mM.

Acknowledgements

We wish to thank Professor W. Kreutz, Dr. F. Siebert, D. Walter and J. Reichert for valuable discussions. The financial support of the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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