Kinetics, Binding Constant, and Activation Energy of the 48-kDa Protein–Rhodopsin Complex by Extra-Metarhodopsin II[†]

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ABSTRACT: We have found that the 48-kDa protein (or S-antigen 48k) of the rod photoreceptor enhances the light-induced formation of the photoproduct metarhodopsin II (MII) from prephosphorylated rhodopsin. The effect is analogous to the known enhancement of MII (extra-MII) that results from selective interaction of MII with G-protein. We have determined some parameters of the MII–48k interaction by measuring the extra-MII absorption change induced by the 48-kDa protein. The amplitude saturation yields a dissociation constant for the MII–48k complex on the order of 50 nM. At the technical limit of these measurements, 13.7 °C and 12 μM 48-kDa protein, we find a rate of 2.3 s^{-1} for formation of the 48k–MII complex. Extrapolation of these values to cellular conditions yields an occupation time of phosphorylated MII by 48k less than 200 ms. This is short compared to estimated rates of phosphorylation. The temperature dependence of the MII–48k formation rate is very high (Q_{10} for 5 °C/15 °C = 9–10). The related Arrhenius activation energy (165 kJ mol^{-1}) is correspondingly high and indicates a considerable transient chemical change during the binding process.

Afterabsorption of a photon and the very fast isomerization of its chromophore, the visual pigment rhodopsin relaxes in milliseconds via intermediates into an equilibrium between two forms, metarhodopsin I (MI) and metarhodopsin II (MII)[1] (Matthews et al., 1963; Emeis & Hofmann, 1981; Parkes & Liebman, 1984; Hofmann, 1986). In MII, rhodopsin adopts a conformation that is recognized by several other proteins, including a GTP-binding protein (G-protein or transducin, G) (Kühn, 1980), a protein kinase that phosphorylates photoactivated rhodopsin (Kühn, 1978; Wilden & Kühn, 1982), and a 48-kDa protein (48k) (Kühn et al., 1984) also known as S-antigen (Pfister et al., 1985).

Interaction of G-protein with MII leads to its well-documented GTP-dependent activation as a transmitter to a cGMP phosphodiesterase (PDE) (Stryer, 1986). Both kinase and 48-kDa protein are currently discussed as antagonists to the G-activating property of MII. An ATP-dependent quench of light-induced PDE activity, first seen in native membrane preparations (Liebman & Pugh, 1980), was later described in partly purified kinase preparations (Sitaramayya, 1986). Available data support the idea that phosphorylation of photoactivated rhodopsin inhibits its capacity to induce signal transduction (Kühn et al., 1973) and, in particular, to interact with G-protein (Liebman & Pugh, 1980). Effective inhibition may additionally depend on a spectrally silent conformation change occurring during the lifetime of the photoproduct MII (Hofmann, 1986). Different inhibitory mechanisms involving 48-kDa protein have been proposed. Zuckerman and Cheasty (1986) proposed a model in which 48-kDa protein acts as an inhibitor of PDE, while Wilden et al. (1986a) saw its role as inhibitor of the active receptor. The results of Wilden et al. are consistent with a competition of 48-kDa protein for pho-

toactivated rhodopsin which blocks the interaction between MII and G-protein. The main points are that light-induced binding of 48-kDa protein to disk membranes is greatly enhanced by phosphorylation of rhodopsin (Kühn et al., 1984) and that in prephosphorylated membranes 48-kDa protein is required to effectively quench light-induced PDE activity (Wilden et al., 1986a). Miller et al. (1986), using purified phosphorylated rhodopsin, found a graded inhibiting effect of increasing levels of phosphorylation. Consistent with the proposal by Wilden et al. (1986a), phosphorylated rhodopsin still supported PDE activation to some degree.

The experimental evidence from these studies is consistent with an inhibitory role of 48-kDa protein at the stage of the activated receptor protein. However, direct evidence concerning the strength, kinetics, and activation parameters of the interaction between 48-kDa protein and MII is still lacking. Such information could help to answer the question whether the interaction is fast enough to serve as, or to induce, a mechanism for the rapid quench under native conditions. In addition, it could provide a direct approach to investigate the interaction between the proteins and the mechanism of inhibition of the light-activated receptor.

In this paper we report specific properties of the MII–48k interaction using a monitor (extra-MII; Emeis & Hofmann, 1981; Emeis et al., 1982; Hofmann, 1986) similar to that previously used to study MII–G interaction: 48-kDa protein, like G-protein, stabilizes MII, and the large difference in absorption between MII and its tautomeric form MI provides a spectroscopic monitor. Extrapolation of our data to conditions prevailing in vivo yields values for the MII–48k interaction time (<200 ms) and K_D (50 nM) that are relevant in view of the expected properties of a quench mechanism.

[1] Abbreviations: MII, metarhodopsin II; extra-MII, amount of MII that is due to stabilization of this species; 48k, 48-kDa protein; G, G-protein, transducin; PDE, cGMP phosphodiesterase; R*, photoactivated rhodopsin; P-Rh membranes, disk membranes containing phosphorylated rhodopsin.

EXPERIMENTAL METHODS

Preparation. Rod outer segments (ROS) were prepared from bovine eyes as previously described (Wilden et al., 1986a) and stored under argon at −70 °C. Phosphorylated disk membranes were prepared as described by Kühn et al. (1984). Briefly, ROS in 100 mM phosphate buffer were illuminated in 3 mM [γ-^{32}P]ATP from New England Nuclear and 1.5 mM $MgCl_2$ for 3 h at 30 °C and then regenerated in the dark with excess 11-*cis*-retinal and washed with isotonic and hypotonic buffers. The resulting phosphorylated rhodopsin membranes were stored at −70 °C and for the measurements resuspended in a buffer containing 100 mM piperazine-1,4-diethanesulfonic acid (PIPES), pH 7.5.

Average phosphorylation was 5.5–6.0 phosphates/rhodopsin, and regeneration yield was 98–102%. Analysis on cellulose columns showed that 2% of the rhodopsin remained unphosphorylated; the rest was phosphorylated to various levels. The control measurement in Figure 1 was done with normal disk membranes washed in hypotonic saline.

48k was purified from whole retina extracts as described by Wilden et al. (1986b) and stored at −70 °C. Protein concentration was determined as described by Wilden et al. (1986a).

Measuring Techniques. Formation of metarhodopsin II (MII) was assayed according to the two-wavelength technique described by Hofmann and Emeis (1981). This method reduces scattering artifacts by measuring the difference of the absorbances at 380 nm (maximal absorption of MII) and 417 nm (MI/MII isosbestic point). The measurements were made by employing a Shimadzu UV 300 two-wavelength spectrophotometer equipped with thermostated cuvette, temperature regulation, and flash gun as described by Kohl and Hofmann (1987). This instrument satisfies the requirements of the present assay for stability and low intensity of measuring light.

Control measurements in the near infrared have shown the absence of any flash-induced scattering change when the differential 2-wavelength method is used (cf. Figure 1, record e, panel A); the two wavelength scattering artifact in the 400-nm range is therefore negligible.

The near-infrared (800 nm) light-scattering measurements were carried out as described by Schleicher and Hofmann (1987). The angular range of scattering detection was adjusted to 10–30°.

Evaluation of the Absorption Data. In the following, we derive a relation between the measurable quantity M_{380} (amount of 380-nm absorbing species), the total amount of photolyzed rhodopsin, R_L, and the amount of complexes MIIX. X denotes a protein that binds specifically to MII, as G-protein or 48k.

Extra-MII is described by the reaction

$$\text{MI} + \text{X} \rightleftharpoons \text{MII} + \text{X} \rightleftharpoons \text{MIIX} \qquad (1)$$
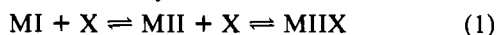
i.e., coupled equilibria of MII formation and the concatenated binding to MII of a protein X (48k in this study). The equilibria obey the equations

$$[\text{MII}] = K_1[\text{MI}] \qquad (2)$$

$$[\text{MIIX}] = (1/K_D)[\text{MII}][\text{X}] \qquad (3)$$

where [MI], [MII], and [X] represent the molar concentrations of MI, MII, and X, respectively, K_1 is the equilibrium constant of the MI/MII equilibrium, and K_D is the dissociation constant of the MIIX complex.

In addition, the following sum equations are given:

$$[R_L] = [\text{MI}] + [\text{MII}] + [\text{MIIX}] \qquad (4)$$

$$[\text{X}]_{\text{tot}} = [\text{X}] + [\text{MIIX}] \qquad (5)$$

$[R_L]$ and $[X]_{\text{tot}}$ are the total concentration of photolyzed rhodopsin and X-protein, respectively.

The saturation of X binding at stepwise photolysis of rhodopsin can be calculated starting from eq 3. The unknown concentrations of free X-protein [X] and free MII [MII] have to be expressed by the known total concentrations of X-protein $[X]_{\text{tot}}$ and photolyzed rhodopsin $[R_L]$. By use of eq 2 and 4, [MII] can be expressed by

$$[\text{MII}] = ([R_L] - [\text{MIIX}])/(1 + 1/K_1) \qquad (6)$$

With relations 5 and 6, eq 3 can now be transformed to

$$[\text{MIIX}] = (1/A)[R_L]([\text{X}]_{\text{tot}} - [\text{MIIX}]) - \\ (1/A)[\text{MIIX}]([\text{X}]_{\text{tot}} - [\text{MIIX}]) \qquad (7)$$

where $A = K_D[(K_1 + 1)/K_1]$. This gives a quadratic equation for [MIIX] with the solution

$$[\text{MIIX}] = -p/2 \pm \sqrt{(p/2)^2 - q} \qquad (8)$$

where $p = -(A + [R_L] + [X]_{\text{tot}})$ and $q = [R_L][X]_{\text{tot}}$. On the basis of eq 4, the solution of eq 8 involving the positive sign can be excluded. This is obvious when the asymtotic behavior of [MIIX] for $K_D \rightarrow 0$ and $K_D \rightarrow \infty$ is considered. Equation 8 is the desired expression for the saturation of complex formation as a function of total photolysed rhodopsin.[2]

[MIIX] is related to the measurable quantity $[M_{380}]$ by

$$[M_{380}] = [\text{MII}] + [\text{MIIX}] \qquad (9)$$

which can be transformed by using eq 2 to

$$\begin{aligned}[M_{380}] &= K_1[\text{MI}] + [\text{MIIX}] \\ &= K_1[R_L] - K_1[\text{M380}] + [\text{MIIX}] \\ &= [K_1/(1 + K_1)][R_L] + [1/(1 + K_1)][\text{MIIX}]\end{aligned}$$
$$(10)$$

where [MIIX] is given by eq 8. The term $[K_1/(1 + K_1)][R_L]$ is the amount of MII that would be measured in the absence of X-protein. The enhancement of $[M_{380}]$ observed in the presence of X-protein corresponds therefore to the term $[1/(1 + K_1)][\text{MIIX}]$ and is proportional to the concentration of complexes MIIX.

Experimentally, evaluation of the second term in eq 10 is accessible by the difference between MII formation in the presence and absence of X-protein (Figure 1B). A fit of eq 8 to a plot of this term against R_L (Figure 2) yields values for $[X]_{\text{tot}}$ and K_D. An independent determination of $[X]_{\text{tot}}$ provides a control of the quality of the fit.

RESULTS AND DISCUSSION

48-kDa Protein Enhances MII Formation. The spectrophotometric data of Figure 1 show the flash-induced formation of metarhodopsin II (MII) for different concentrations of the reaction partners R* and 48k. Each record was obtained from a new sample. Record a, panel A, shows MII formation in phosphorylated membranes and in the absence of 48k. The absorption change rises with the kinetics known from unphosphorylated membranes and with an amplitude that indicates at most only a small shift of the MI/MII equilibrium toward MII (Parkes & Liebman, 1984; Emeis & Hofmann, 1981). The presence of 48k (records b and c) preserves the initial phase of the signal. However, by contrast to record a, records b and c show an additional, slower absorbance change. This effect is due to the enhanced formation of 380-nm ab-

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[2] Equation 8 is equivalent to the expression given by Bennett and Dupont (1985) for G-protein. However, they considered photolyzed rhodopsin, R_L, rather than MII, as the ligand. The dissociation constant used in their study has therefore to be corrected and corresponds to the term A used in our study.
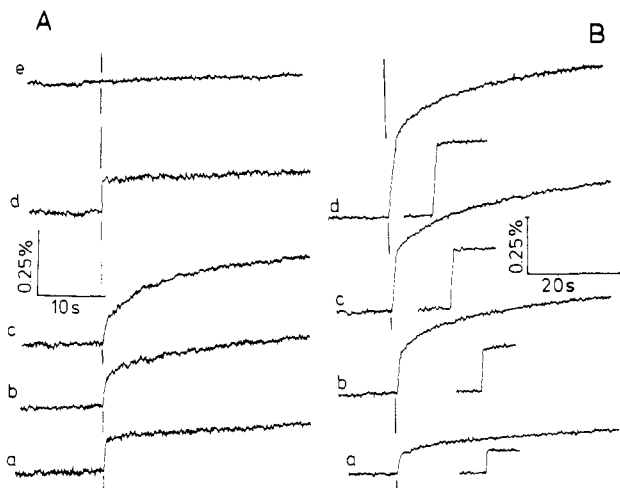
FIGURE 1: Flash-induced formation of metarhodopsin II (MII) in a suspension of bovine rod photoreceptor disk membranes. Signals are the absorbance change at 380 nm minus the absorbance change at 417 nm (except record A-e, see below). Phosphorylated (P-Rh) and normal washed membranes were isolated, regenerated, and phosphorylated as described under Experimental Methods. 48-kDa protein (48k) was added to the suspension prior to the measurement. In all measurements, total rhodopsin concentration in the cuvette was 60 μM, path length was 0.5 mm, rhodopsin photolyzed per flash was 3.9% or 2.4 μM, T was 10 °C, and pH was 7.5. (A) (a–c) MII formation in P-Rh membranes in the presence of 0 (a), 3.3 μM (b), and 6.6 μM (c) 48k; (d) normal membranes, 6.6 μM 48k; (e) P-Rh membranes, dual wavelength set at 690/720 nm. (B) (a–d) P-Rh membranes, 6.6 μM 48k, application of 1 (a), 2 (b), 3 (c), and 4 (d) flashes at 0.5-s intervals. Traces shifted to the right represent MII formation in the absence of 48k.

sorbing species (scattering under our conditions does not contribute to the absorption signal; see record e and Experimental Methods). We identify this species as MII because this is the only photoproduct that can produce such a large 380/417 absorption difference. Comparison of traces b and c shows an increase in the amplitude of the slow component with increasing concentration of 48k. This leads us to conclude that, by analogy to the G-protein-enhanced formation of MII (extra-MII), 48k also stabilizes MII. The effect is not seen when normal washed membranes are illuminated in the presence of 48k (trace d), indicating that phosphorylated membranes are required. This is in agreement with the previous finding that light-induced 48k binding is greatly enhanced by phosphorylation (Kühn et al., 1984).

Panel B of Figure 1 shows the results obtained when the amount of flash-induced metarhodopsin was varied. As in panel A, every record is from a new sample. For records a–d, one to four flashes were applied at intervals of 0.5 s to achieve different levels of photoactivation. This causes a slight apparent prolongation of the rapid initial phase of the signals recorded both in the presence and absence of 48k (traces shifted to the right). With the relatively small rhodopsin turnover (3.9%/flash), the amplitude of this initial rise increases practically linearly with the number of flashes applied. By contrast, the slow component monitoring binding of 48k saturates with increasing amounts of R*.

Amplitude Saturation. When the control without 48k is subtracted from the total absorption change (see Experimental Methods) and plotted as a function of the rhodopsin turnover, a saturation curve is obtained. The curve in Figure 2 was fitted according to eq 8. The fit yields both the concentration of active 48k and the value of the MII-related dissociation constant K_D. A value of 7.0 μM was thereby obtained for [48k]; this agrees well with the value from the protein determination of the sample (6.6 μM). For A (eq 7) one obtains 0.12 μM
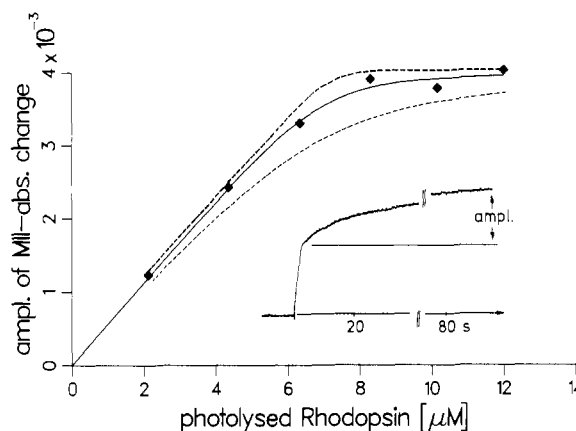

FIGURE 2: Saturation of 48k-dependent metarhodopsin II formation with increasing rhodopsin turnover. The amplitude of the slow, 48k-dependent component of the difference absorption signals (see Figure 1B) is plotted against the molarity of total photolyzed rhodopsin. The slow component was obtained by subtracting the fast component, which is measured in the absence of 48k, from the total absorption change. Conditions: P-Rh membranes, total rhodopsin concentration 60 μM, 0.5-mm path length, 6.6 μM 48k, rhodopsin photolyzed by up to six flashes at 0.5-s intervals, T = 10 °C, pH 7.5. Upper and lower dashed lines represent curves for K_D's of 5 and 500 nM, respectively (with [48k] = 7 μM).
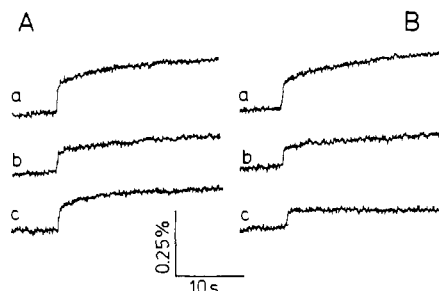

FIGURE 3: Recovery of the 48k-dependent part of metarhodopsin II (MII) formation for conditions of fast and slow decay of preformed MII. (A) 1.25 mM hydroxylamine present; (B) no hydroxylamine. Both panels show signals from a series of flashes, each photolyzing 3.5% of the rhodopsin in a suspension of P-Rh membranes (60 μM, 0.5-mm path) with 3.3 μM 48k present. In each panel, record a is from the first and record b from the last of a series of five flashes, applied at time intervals <1 min. A sixth flash (record e in each panel) was applied after 6 min; note the difference in the recovery of the signals in (A) vs (B).

and for K_D 0.046 μM (dissociation constant related to [MII], based on MI/MII = 0.73; see Experimental Methods).

In order to obtain a rough margin for the error of this evaluation, we calculated a number of curves for higher and lower values of K_D. The upper and lower dashed lines in Figure 2 represent the curves for K_D's of 5 and 500 nM, respectively (with [48k] = 7 μM). It is seen that these curves deviate systematically from the data. We estimate the K_D in the order of 50 nM with upper and lower limits of ca. 200 and 20 nM.

Fast Recovery by Hydroxylamine. Hydroxylamine is an agent that terminates the lifetime of MII by forming retinal oxime with the chromophore. It has previously been applied to study the effect of the MII to opsin transition on the binding of G-protein (Hofmann et al., 1983). Figure 3 shows a test of the recovery of extra-MII in the presence and absence of hydroxylamine. The figure shows that exposure to flashes (four flashes, each bleaching 3.5% of rhodopsin, time interval <1 min) eliminates the slow component of MII in response to a subsequent flash. We interpret this to indicate the absence of excess 48k, due to its stable binding to the metarhodopsin already formed. Further stimulation after a 6-min period
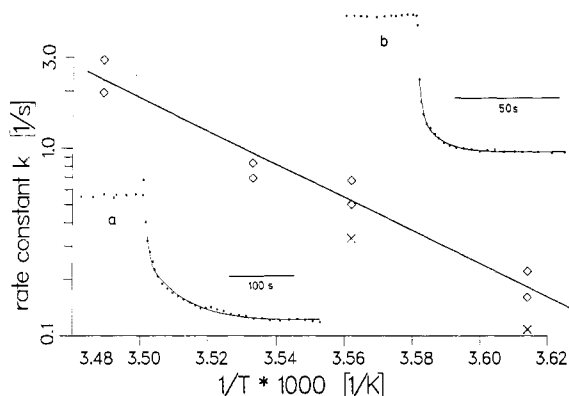
FIGURE 4: Temperature dependence and Arrhenius plot of the rate of formation of metarhodopsin II (48k-dependent part). MII formation by the first flash on a fresh sample was measured between 3.7 and 13.7 °C on P-Rh membranes at pH 7.5; 48k was added prior to the measurement to a final concentration of 12 μM. Total rhodopsin concentration in the cuvette was 60 μM, path length was 0.5 mm, and rhodopsin photolyzed per flash was 3.9% (2.4 μM). The signals were digitized and analyzed by two first-order rate constants (pseudo-first-order approximation, see text); two examples for 3.7 °C (a) and 13.7 °C (b) are shown as insets. Absorption increases are displayed as downward deflections in this figure. For the Arrhenius representation, the rate of the slow component is plotted as a function of the inverse absolute temperature. Different symbols (diamonds and crosses) show results from different preparations. From one preparation (diamonds), two samples were measured at each temperature.

yields a response that is greatly influenced by hydroxylamine: no slow component is seen in the absence, but substantial recovery in the presence, of hydroxylamine. Thus, the hydroxylamine-induced decay of MII has led to dissociation of MII–48k complex; free 48k made available by this dissociation binds to MII generated by the sixth flash. The result demonstrates that 48k does not bind to free opsin with an affinity comparable to MII.

Arrhenius Plot. Exact description of the bimolecular reaction between MII and 48k requires an analysis by second-order kinetics. However, a sufficiently high excess of 48k affords evaluation by a pseudo-first-order approach. The data for the Arrhenius plot in Figure 4 are from samples with a 6-fold excess of 48k to R* and were fitted with a sum of two exponentials. The rate of the slow component is the entry for the Arrhenius plot. Plotted rates are corrected for the temperature-dependent MI/MII equilibrium; this means that the Arrhenius activation energy does not incorporate the enthalpy of MII formation. Evaluation of the plot yields an Arrhenius activation energy of 165 kJ/mol.

Light-Scattering Signal. Flash-induced binding of 48k to phosphorylated rhodopsin expresses itself not only in formation of extra-MII but also in a light-scattering signal. As shown in Figure 5, the scattering change increases with the concentration of 48k. By analogy with the "P_D signal" arising from the transition of soluble G-protein to disk membranes (Schleicher & Hofmann, 1987), the effect is interpreted as the gain of mass of the membranes when 48k is bound. This scattering change can be understood as a "binding signal" for 48k analogous to that described by Kühn et al. (1981) for binding of G-protein.

The time course of the 48k-induced signal is comparable to those of the P_D signal, and its amplitude fits roughly to the amount of 48k that binds to MII (as evaluated from the extra-MII data). However, the instability of the records precluded their quantitative evaluation.

Dissociation Constants for Binding of 48k and G-Protein. Competitive inhibition of G-protein binding by 48k depends
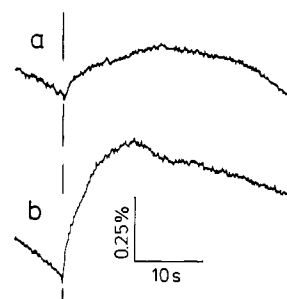

FIGURE 5: Flash-induced light-scattering signals in a suspension of phosphorylated bovine rod photoreceptor disk membranes. Total rhodopsin concentration in the samples was 5 μM, path length was 10 mm, rhodopsin photolyzed per flash was 3.9%, and temperature was 30 °C. The concentration of 48k was 1 μM (a) and 2 μM (b).

on the relationship between the K_D's of binding of the proteins to phosphorylated membranes. The value for the K_D of 48k determined above is 50 nM.

The binding of G-protein to unphosphorylated membranes has been estimated from the saturation of the light-scattering binding signal (Bennett & Dupont, 1985) and from MII data (Hofmann et al., 1984). When related to MII (once formed), the respective values are 150 and 120 nM. From the efficiency of PDE activation, one estimates that G-protein binds ca. 5 times weaker to rhodopsin when phosphorylated as in our preparation [Wilden et al., 1986a). The resulting K_D is about 15 times larger than the one for 48k. However, this consideration neglects the membrane binding of G-protein, which enhances the effective concentration of G-protein and favors its light-induced binding. Therefore, the volumetric K_D must be corrected for the concentration-dependent dark binding. Equation 11 describes the concatenation of dark and light

$$G_f + A + R^* \rightleftharpoons G_m + R^* \rightleftharpoons R^*G \qquad (11)$$
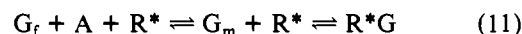
binding, where G_f and G_m denote free and membrane-bound G-protein, respectively. A denotes the anchor site at the membrane to which G is bound in the dark (Schleicher & Hofmann, 1987). Both binding processes are assumed as equilibria and are described in terms of their respective dissociation constants.

The dark binding

$$G_f + A \rightleftharpoons G_m \qquad (12)$$

is described by K_m, with

$$K_m = ([G_f][A])/[G_m] \qquad (13)$$

and the light binding

$$R^* + G_m \rightleftharpoons R^*G \qquad (14)$$

by

$$K_{RG} = ([R^*][G_m])/[R^*G] \qquad (15)$$

The volumetrically determined constant K_{app} of light-induced binding does not recognize the preequilibrium of membrane binding. This means, by definition

$$K_{app} = \{[R^*]([G_m] + [G_f])\}/[R^*G] \qquad (16)$$

Considering eq 13

$$[G_f] = K_m[G_m]/[A]$$

and

$$[G_m] + [G_f] = [G_m](1 + K_m/[A])$$

eq 15 and 16 lead to

$$K_{app} = K_{RG}(1 + K_m/[A]) \qquad (17)$$

which shows how the measured K_{app} depends on the dissociation constants of light-binding K_{RG} and of dark binding K_m and on the membrane concentration [A].

According to eq 17, determination of K_{RG} from a measurement of K_{app} requires knowledge of the values of K_m and [A]. The concentration of dark binding sites [A] is conventionally approximated by the rhodopsin concentration (Schleicher & Hofmann, 1987). The following calculation assumes a value of 5 μM for [A]. K_m depends on the type of preparation (Liebman & Sitaramayya, 1984). For example, isolated and purified disks (Smith et al., 1975) exhibit a K_m value of 5–10 μM (Schleicher & Hofmann, 1987), while native and reconstituted membranes as studied by Liebman and Sitaramayya (1984) exhibited K_m values of 0.1 and 1 μM, respectively. This yields correction factors $(1 + K_m/[A])$ (eq 17) between 3 and 1.02. This yields K_D ratios for 48k and G between 5 and 14.7 instead of 15, as derived above on a volumetric basis.

Thus, we can only say that the affinities of 48k and G to phosphorylated metarhodopsin II differ by about 1 order of magnitude.

Extrapolation of 48k Kinetics to Cellular Conditions. A critical issue for any mechanism involving 48k in the shut-off of the PDE cascade is the rate of its binding to photoactivated rhodopsin. Such binding must occur on a time scale that is comparable to or shorter than the preceding phosphorylation of R*, which is its necessary condition. The evident lifetime of R* in situ (at 20–22 °C) of less than 3 s (Pepperberg et al., 1988) establishes a time frame within which these reactions must operate.

For an estimation we may extrapolate the measured rates to cellular conditions. A simple approach to the rate of binding is based on the following expression for a bimolecular reaction:

$$d[R^*\cdot48k]/dt = k[48k][R^*]$$

Under the conditions of the measurement (flash 1, [R*] = 0.035 × 60 μM = 2.1 μM, [48k] = 12 μM) as well as under cellular conditions, it holds that [R*] $\ll$ [48k]. Therefore, the concentration of free 48k is approximately constant during formation of MII–48k after the first flash, and the reaction kinetics can be described by a (pseudo-)first-order law:

$$[R^*\cdot48k] = const[1 - \exp(k[48k]t)]$$

$$[R^*] = const \exp(-k[48k]t)$$

Thus, the observed time constant of the reaction is

$$k_{obs} = k[48k]$$

The equation states that the effective rate of occupation of R* by 48k is proportional to [48k]. The concentration of 48k recovered with isolated rod outer segments is high, with a molar ratio to rhodopsin of several percent in bovine (Wilden et al., 1986a) and in frog material (Hamm & Bownds, 1986). 48k immunolabeling in toad photoreceptors shows 48k present in the outer segments at a molar ratio to opsin of 1:16 in the dark and 1:6 after strong illumination (Mangini & Pepperberg, 1988). By such techniques, 48k also appears to be an abundant protein of mammalian photoreceptors (Broekhuyse et al., 1985, 1987). In view of the abundance of 48k in the intact cell, a conservative estimate of 48k in bovine rod outer segmants is 1% or 30 μM [see also Bennett and Sitaramayya (1988)].

The highest rate measured in this study (for $T = 13.7$ °C, [48k] = 12 μM, see Figure 4) is $k_{obs} = 2.3$ s^{-1}. On this basis, one calculates $k = 2.3(30/12) = 5.75$ s^{-1} for this temperature. Since the increase of the rate at higher temperatures cannot be determined, we take the value at 13.7 °C, 1/5.75 = 0.175 s, as an upper limit for the time to bind 48k to phosphorylated

R*. Even this time is shorter than the shortest estimated reaction times for phosphorylation (Liebman & Sitaramayya, 1984), and 48k binding would not be rate limiting.

CONCLUSION

This study demonstrates that 48-kDa protein stabilizes the biochemically active, spectroscopically distinct form of photolyzed rhodopsin, metarhodopsin II (MII). The only other protein that is known to form extra-MII is the G-protein (Emeis & Hofmann, 1981; Emeis et al., 1982). Certain other proteins, for example, various monoclonal antibodies against epitopes at the rhodopsin surface, are ineffective (König, Hargrave, and Hofmann, unpublished results). The 48k-induced extra-MII provides a kinetic and stoichiometric (eq 8) monitor of 48-kDa protein binding in which the chromophore of rhodopsin itself, retinal, serves as the reporter molecule.

The saturation data (Figures 1 and 2) are consistent with a 1:1 complex between MII and 48k. MII is distinguished with respect to both the preceding and the subsequent form of the light–activated receptor: the preceding MI cannot bind to a degree comparable to MII because otherwise the metarhodopsin equilibrium would not be shifted to MII, and the same is true for the subsequent opsin because 48k becomes free when MII has decayed (Figure 3).

As a very similar behavior has been found in earlier investigations for the G-protein, one might assume that G-protein and 48k recognize the same conformation of photoactivated rhodopsin. However, although both proteins interact with MII, the phosphorylation of MII imposes an additional constraint that distinguishes between G and 48k: only phosphorylated MII binds 48k effectively (this study; Kühn et al., 1984), while G-protein prefers unphosphorylated MII (Wilden et al., 1986a; Miller et al., 1986). Thus, it is expected that G-protein is bound with preference prior to phosphorylation and 48k after a certain degree of phosphorylation. At which degree of phosphorylation binding of 48k is optimal could not be determined with our randomly phosphorylated preparation. However, even conservative extrapolation to cellular conditions of the measured rate of 48k binding led to reaction times <200 ms. This appears to be fast enough to allow its interference at any stage of the phosphorylation reaction, which is estimated to take at least 1 s, until it is effective in PDE deactivation (Sitaramayya & Liebman, 1983).

In the preparation used in this study, phosphorylation yields were 5.5–6.0 phosphates bound per average rhodopsin. It will be of interest to entend the present approach to studies of purified, phosphorylated rhodopsin (Miller et al., 1986). Separation into fractions containing 1–2, 2–4, 4–6, etc. phosphates/rhodopsin should give an insight as to how many phosphates are required for effective extra-MII formation by 48-kDa protein. It also appears possible to obtain relative values for the dissociation constants of G and 48k for mono-, di-, and triphosphorylated rhodopsin. There is a putative phosphoryl binding site on 48k (Shinohara et al., 1987) that may correspond to a site of interaction at the C-terminal tail of rhodopsin. Saturation of 48k binding affinity at low phosphorylation levels has been inferred from observations of 48k-dependent shut-off of PDE and of shortening of the period for G deactivation. It is noteworthy that the concentration of 48-kDa protein which led to G-protein inactivation with half-maximal rate is identical with the dissociation constant of 48k binding determined above (50 nM, Figure 2) (Bennett & Sitaramayya, 1988).

Concerning the mechanism of action, our results are generally consistent with a block of the activated receptor induced by 48k. The original proposal by Wilden et al. (1986a) has

been that the competition of 48-kDa protein with G-protein (Kühn et al., 1984) as such terminates the light-induced activity of rhodopsin. In this study, we have compared the measured dissociation constants of 48-kDa protein and G-protein. Available data indicate that 48k binds by about 1 order of magnitude stronger than G-protein to phosphorylated rhodopsin. The resulting total quench capacity of phosphorylation (Wilden et al., 1986a; Miller et al., 1986) plus 48k binding would be about 2 orders of magnitude. However, one has to be aware that the competition between 48k and G for metarhodopsin II in vivo is complicated because it depends on the instantaneous affinities and concentrations of both proteins. For example, G-protein no longer competes once it is in the activated, GTP-binding state. Any estimation of the effectiveness of a competitive mechanism has to be compared to the PDE shut-off in the intact retina, for which a light-scattering assay is now available (Pepperberg et al., 1988). Such comparison may help to decide whether additional mechanisms have to be assumed that would occur under more native conditions.

The bimolecular interaction between rhodopsin and the 48-kDa protein is preceded by phosphorylation and MII formation. When the reaction enthalpies of both these reactions are subtracted, an activation energy remains that must be due to events that are simultaneous to complex formation. The Arrhenius energy is very high, comparable to that of G-protein activation (Kohl & Hofmann, 1987). This indicates the presence of major chemical changes during formation of the MII–48k complex. Specification of the mechanism will require a combination of the binding assay with molecular probing by synthetic peptides corresponding to the rhodopsin binding domain(s) of 48k (Shinohara et al., 1986), as recently applied to the rhodopsin–G-protein interaction (Hamm et al., 1988).

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