Inactivation of Photoexcited Rhodopsin in Retinal Rods: The Roles of Rhodopsin Kinase and 48-kDa Protein (Arrestin)†

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Received September 17, 1987; Revised Manuscript Received October 22, 1987

ABSTRACT: The inactivation of excited rhodopsin in the presence of ATP, rhodopsin kinase, and/or arrestin has been studied from its effect on the two subsequent steps in the light-induced enzymatic cascade: metarhodopsin II catalyzed activation of G-protein and G-protein-dependent activation of cGMP phosphodiesterase. The inactivation of G-protein (from light-scattering measurements) and that of phosphodiesterase (from measurements of cGMP hydrolysis) have been studied and compared in reconstituted systems containing various combinations of the proteins involved (rhodopsin, G-protein, phosphodiesterase, kinase, and arrestin). Our results show that rhodopsin kinase alone can terminate the activation of G-protein and that arrestin speeds up the process at a relative concentration similar to that reported in the rod (half-maximal effect at 50 nM for 4.4 μM rhodopsin). Measurements of rhodopsin phosphorylation under identical conditions show that in the presence of arrestin total metarhodopsin II inactivation is achieved when only 0.5–1.4 phosphates are bound per bleached rhodopsin, whereas in the absence of arrestin it requires binding of 12–16 phosphates per bleached rhodopsin. Phosphodiesterase activity can similarly be turned off by kinase, and the process is similarly accelerated by arrestin.

Absorption of a photon by a molecule of rhodopsin in the disk membrane induces a transient reduction of the sodium conductance of the plasma membrane of the rod, which results in hyperpolarization of the cell. The mechanism of visual transduction has been well studied: photoexcitation of rhodopsin leads to the formation of an active intermediate (metarhodopsin II), which catalyzes the binding of GTP to the GTP-binding protein (G-protein or transducin). The activated G-proteins (G_{GTP}) then activate a cGMP phosphodiesterase (PDE),[1] and the reduction of cGMP concentration in the cytoplasm results in the closing of sodium channels in the plasma membrane [see review by Pugh and Cobbs (1986)]. Fast recovery from light activation, allowing distinct perception of repeated stimuli, implies the existence of a rapid turnoff mechanism: the termination of cGMP hydrolysis requires inactivation of the PDE either independently or as a consequence of inactivation of G_{GTP} and metarhodopsin II (MII). It was first hypothesized that MII is inactivated by phosphorylation by rhodopsin kinase (Liebman & Pugh, 1980) and that this results in inactivation of G_{GTP} due to hydrolysis of GTP by endogenous GTPase activity of the G-protein; in the absence of G_{GTP} the PDE reverts to its inhibited state. More recently, a role for a 48-kDa protein has been proposed in the recovery process. According to Wilden et al. (1986), phosphorylation of MII only reduces its ability to activate G-proteins, total inactivation of MII being achieved by binding of the 48-kDa protein to phosphorylated rhodopsin. Miller et al. (1986) propose that increasing levels of phosphorylation produce a graded reduction of the binding affinity of G-protein for bleached rhodopsin and that the inactivation is accelerated by addition of a 48-kDa protein. On the other hand, Zuckerman and Cheasty (1986) suggest that the PDE is inactivated by direct binding of a 48-kDa protein, which is itself activated by MII-induced ATP binding. The 48-kDa protein was for this reason termed "arrestin" by these authors. In this latter scheme, PDE is thus inactivated independently of rhodopsin phosphorylation and of inactivation of G_{GTP}. It was shown, however, in a recent report (Sitaramayya, 1986a) that in contradiction both with Wilden et al. and with Zuckerman and Cheasty, the light-induced PDE activity in a reconstituted system can be fully terminated in the absence of arrestin.

We report here a study of the inactivation of excited rhodopsin in the presence of kinase and/or arrestin not only from the turnoff of PDE activity as previously reported by Wilden et al. (1986), Miller et al. (1986), and Zuckerman and Cheasty (1986) but also from the preceding step, the inactivation of G_{GTP}, which can be monitored by light scattering (Bennett, 1986). Moreover, while the above-cited studies used phosphorylated regenerated opsin, we have separated all the constituents of the system, including the kinase. Experiments were carried out using reconstituted systems containing various combinations of purified PDE, G-protein, and arrestin and of a kinase preparation (Sitaramayya, 1986a).

Materials and Methods

Protein Preparations. Dark washed membranes were obtained from frozen dark ROS pellets. The membranes were washed 3 times with hypotonic buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and once in the same buffer without

† A.S. thanks the National Science Foundation (BNS 84 19341) and the National Eye Institute (EY07158) for support and the Pennsylvania College of Optometry and the Commissariat a l'Energie Atomique for supporting a visit to Grenoble.

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[1] Abbreviations: G, G-protein; G_{GTP} (G_{GDP}), G with GTP (GDP) bound; PDE, cGMP phosphodiesterase; MII, metarhodopsin II; ROS, rod outer segment; DTT, dithiothreitol; TCA, trichloroacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography.

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EDTA. Urea-washed membranes were obtained by incubating washed membranes for 30 min in 5 M urea and 10 mM Tris-HCl, pH 7.5, followed by three hypotonic washes. Membranes washed without urea had a low kinase activity; urea-washed membranes had no measurable kinase activity.

PDE and G-protein were prepared in room light from fresh rods according to the method of Baehr et al. (1979, 1982). PDE was purified on a DE 52/Sephadex G-100 column. The proteins were supplemented with 1 mM DTT and stored at 4 °C.

Arrestin was prepared according to the method of Wilden and Kühn (1986), except that FPLC purification was omitted. The pellets were rinsed with extraction buffer before extraction of the 48-kDa protein in order to remove most of the remaining impurities as described. Aliquots were kept in liquid nitrogen.

Kinase preparation was carried out according to the method of Sitaramayya (1986a). The extraction buffer contained protease inhibitors as described (benzamidin, leupeptin, aprotinin, and PMSF). The kinase preparation was stored at 4 °C. The kinase itself represented about 10% of the total proteins, and its activity was 0.02 nmol of P_i transferred min^{-1} (μg of kinase)$^{-1}$ (room light assay, see below). As previously described (Sitaramayya, 1986a), the major impurity was G_β, but the amount of other contaminant proteins was slightly different: much less 56-kDa protein was present, while another protein of about 37 kDa was more abundant. Although the gel shows a faint band migrating as a 48-kDa protein (Figure 2), this protein was not stained with antibody raised against arrestin.

Protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as standard.

Gel electrophoresis (10% acrylamide) in the presence of sodium dodecyl sulfate was carried out according to the method of Laemmli (1970).

Reconstitution. In all experiments described, the concentrations of proteins were (unless otherwise specified) 4.4 μM rhodopsin, 0.5 μM G-protein, 0.1 μM PDE when added, 16 μg/mL kinase preparation (corresponding to 1.6 μg of the 65-kDa kinase, i.e., approximately 25 nM kinase), and 25–500 nM arrestin. Experiments were carried out at 20 °C in 120 mM KCl, 1 mM $MgCl_2$, 5 mM Tris, and 10 mM MOPS, pH 7; ATP and GTP concentrations were 500 and 250 μM, respectively.

Ligh-scattering changes were measured in transmittance at 708 nm as described (Kühn et al., 1981) by using a modified Durrum D117 spectrophotometer.

PDE activity was measured according to the method of Liebman and Evanczuk (1982) from the proton release associated with cGMP hydrolysis by using a Radiometer PHM 64 pH meter. The concentration of cGMP added for the assay was 5 mM.

Bleaching was performed with an electronic flash (Sunpak autozoom 3400) of 1-ms duration through a Balzers K3 filter (500 nm) attenuated with neutral density filters as required. The bleaching extent was calibrated as previously described (Bennett, 1982).

Rhodopsin Phosphorylation. (a) Room light kinase assays were performed at 24 °C in 25 mM potassium phosphate, 2 mM $MgCl_2$, and 2 mM DTT, pH 7.5. [γ-^{32}P]ATP (1.7×10^5 cpm/nmol) concentration was 50 μM (rhodopsin 250 μg/mL). The reaction was stopped by TCA.

(b) Flash-activated kinase activity was assayed under conditions (buffer, ATP, GTP, and protein concentration, flash intensity) identical with the conditions used for light-scattering measurements shown in Figure 3, except that [γ-^{32}P]ATP (7×10^5 cpm/nmol) was used. The membrane suspension was placed in a plastic cuvette in the thermostated cuvette holder of the spectrophotometer; aliquots of the suspension were taken at various time intervals after the flash and immediately injected into cold TCA.

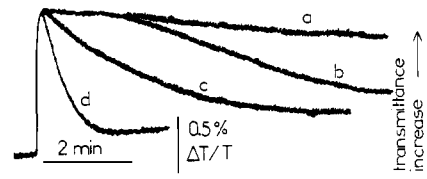

FIGURE 1: Dissociation signals measured in the presence or absence of kinase and/or arrestin. Rhodopsin concentration 2.5 μM (urea-washed membranes), G-protein 0.5 μM, ATP 500 μM, GTP 250 μM, no PDE. Flash intensity 5×10^{-4} bleach. (a) No addition, or addition of 0.5 μM arrestin in the absence of ATP. A second flash of the same intensity applied 5–10 min after the first one induces a very small signal, indicating that only slow recovery due to the thermal decay of MII occurs. (b) Addition of arrestin (0.5 μM) and ATP. (c) Addition of 25 nM kinase and ATP. (d) Addition of kinase (25 nM), arrestin (0.5 μM), and ATP. In (b–d), a second flash after return of the scattering to the initial level induces a second signal of the same amplitude, indicating that all the MII produced by the first flash have been inactivated at that point.

Results and Discussion

G-Protein and PDE Are Inactivated in the Presence of Rhodopsin Kinase and ATP; Arrestin Speeds Up the Effect. (*a*) *Inactivation of G-Protein.* It has been shown previously that the light-induced activation of the G-protein (binding of GTP) in rod suspensions or in reconstituted systems containing only washed disk membranes and purified G-protein is specifically associated with a light-scattering decrease, which was called a "dissociation signal" (Kühn et al., 1981). This signal has been shown to precede the onset of PDE activity, and its amplitude is proportional to the amount of bound GTP and to PDE activity (Bennett, 1982; Bennett & Dupont, 1985). The level of scattering remains stable as long as GTP is present in the cuvette since the G_{GDP} formed upon hydrolysis of GTP are immediately reactivated by MII molecules. When all the free GTP has been exhausted, the scattering returns to the initial level as the bound GTP is hydrolyzed and G returns to inactive G_{GDP} (Bennett, 1982, 1986). Addition of the soluble proteins of the rod outer segment (including kinase and arrestin) and ATP to a reconstituted system containing rhodopsin and purified G-protein induces immediate reversal of the dissociation signal (Bennett, 1986), as expected if the ability of excited rhodopsins to reactivate the G_{GDP} is rapidly suppressed.

Figure 1 shows the recordings of dissociation signals in reconstituted systems in the absence of PDE, with addition of kinase alone, with addition of arrestin alone, or with addition of both kinase and arrestin in the presence of ATP. The intensity of the flash (5×10^{-4} bleach) was almost saturating. The proteins used for reconstitution are shown in the gel of Figure 2. The membranes used in this experiment (urea washed) had no measurable kinase activity, and the amount of arrestin used had 0.6% the activity of the amount of kinase used. Arrestin alone in the presence of ATP has a slight effect, which could be due to this kinase contamination. Kinase alone clearly induces full inactivation of G_{GTP} since a second flash applied after complete reversal of the dissociation signal induces a second signal of the same amplitude. This result is consistent with the finding that light-induced PDE activity can be turned off in the absence of arrestin (Sitaramayya, 1986a). It should be noted that the reversal of the second signal is reproducibly slower by approximately a factor 2 than that of

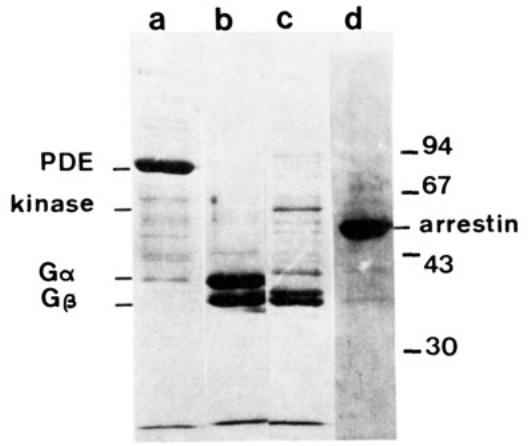

FIGURE 2: Protein fractions used for the reconstitution experiments: (a) PDE; (b) G-protein; (c) kinase; (d) arrestin. The amount of total protein loaded on the gel was 3 μg in lanes a and c and 6 μg in lanes b and d.

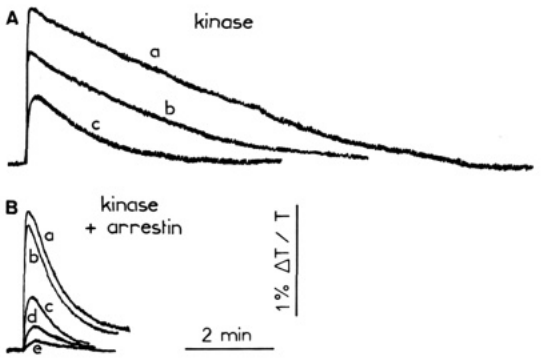

FIGURE 3: Influence of the bleaching extent on the rate of reversal of the dissociation signal in the presence of kinase (A) or kinase and arrestin (B). Bleaching extent: (a) 10^{-3}; (b) 5×10^{-4}; (c) 10^{-4}; (d) 2.5×10^{-5}; (e) 10^{-5}. Other conditions as in Figure 1.

the first one (see Figure 5C below). If the membranes are submitted to further flashes of the same intensity, however, the kinetics of the reversal remains identical with that of the second flash: up to six flashes (5×10^{-4} bleach) have been applied to the same suspension without modification of the kinetics of inactivation.

In the presence of both kinase and arrestin, the reversal of the dissociation signal is unambiguously faster. Identical responses are observed upon a succession of flashes of the same intensity. A possible explanation for the fact that the reversal of the first signal in the absence of added arrestin is faster than that of subsequent flashes is that the other protein fractions may contain small amounts of contaminating arrestin: once all the arrestin present bound to phosphorylated MII produced by the first flash, the inactivation of MII produced by subsequent flashes is achieved by kinase alone. While the kinase fraction seems to be free of arrestin (see Materials and Methods), the contamination level of the G-protein and of the disk membranes used for the experiments has not been tested.

(*b*) *Influence of the Bleaching Level on the Inactivation of G_{GTP} in the Presence of Kinase Alone or with Added Arrestin.* The kinetics of the reversal of the dissociation signal by kinase alone depends on the flash intensity (Figure 3A), the reversal being slower at higher bleach, as expected if inactivation is related to phosphorylation of MII.

In the presence of both kinase and arrestin (Figure 3B), on the other hand, the kinetics of recovery of the signal is almost independent of the flash intensity from 10^{-5} up to at least 10^{-3} bleach. This suggests that the inactivation of MII is much faster under these conditions and that the reversal of the

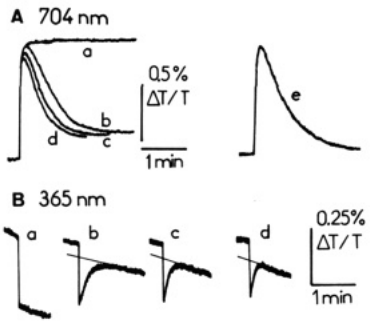

FIGURE 4: Hydroxylamine-induced inactivation of G-protein monitored by reversal of the dissociation signal (A) and corresponding decay of metarhodopsin II (B): (a) no hydroxylamine; (b) 5 mM hydroxylamine; (c) 10 mM hydroxylamine; (d) 15 mM hydroxylamine. The inactivation of G-protein in the presence of kinase (25 nM) and arrestin (0.5 μM) is shown for comparison in [A(e)]. Bleaching extent 5×10^{-4} (A) and 10^{-2} (B). Other conditions given under Materials and Methods.

dissociation signal is mainly limited by a reaction that is slower than inactivation of MII and independent of the amount of MII formed, such as the hydrolysis of bound GTP or a subsequent process. The accelerating effect of arrestin on G_{GTP} inactivation is less obvious at dim flashes, in agreement with Wilden et al. (1986), since the inactivation of MII by kinase alone is already very rapid.

(*c*) *Estimation of the Kinetics of MII Inactivation by Kinase and Arrestin from Comparison with Experiments in the Presence of Hydroxylamine.* Hydroxylamine is known to degrade MII (Liebman & Pugh, 1982; Hofmann et al., 1983) and is therefore expected to induce reversal of the dissociation signal. The concentration of hydroxylamine that produces a reversal whose kinetics are similar to those induced by kinase and arrestin for the same flash intensity was found to be between 5 and 15 mM (Figure 4A). The transient formation of MII in the absence or in the presence of 5, 10, and 15 mM hydroxylamine is shown in Figure 4B: MII decay is complete after 20 (15 mM) to 50 s (5 mM). It can therefore be concluded that the total inactivation of MII by rhodopsin kinase and arrestin also takes between 20 and 50 s under the experimental conditions used (20 °C, 5×10^{-4} bleach).

(*d*) *Inactivation of PDE.* PDE activity is measured under exactly the same conditions as the dissociation signal except that PDE is also added. Recordings of the pH drop associated with cGMP hydrolysis are shown in Figure 5A. In agreement with Sitaramayya (1986a) and with the results shown in Figure 1, we find that kinase alone is capable of terminating PDE activation and that addition of arrestin markedly speeds up the effect. The variation of PDE activity as a function of the time after the flash can be represented by the derivative of the pH trace as a function of time (Figure 5B). Transient dissociation signals observed with the same protein preparations are shown for comparison in Figure 5C: The inactivation of PDE is found to be faster than that of G_{GTP}. It is possible that the reversal of the dissociation signal in the presence of kinase and arrestin is in fact slower than GTP hydrolysis and is associated with a slower process such as rebinding of G-protein to the disk membrane following GTP hydrolysis. Indeed, from the rate of GTP hydrolysis measured with [γ-^{32}P]GTP (Sitaramayya & Casadevall, 1987) under similar conditions, 0.5 μM GTP (corresponding to 1 GTP bound/G-protein) would be hydrolyzed in approximately 10 s, which is about 30 s less than the delay that we observe between MII inactivation and reversal of the dissociation signal in Figure 4. The dissociation signals shown in Figure 5C were, however, measured in the absence of PDE.

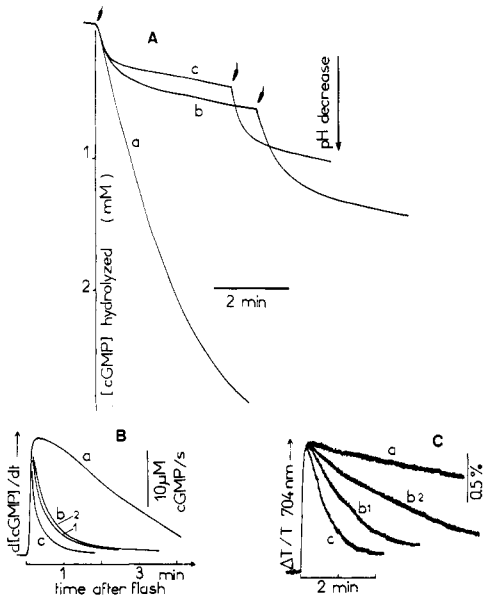

FIGURE 5: Inactivation of PDE by kinase in the presence or absence of arrestin. (A) Proton release associated with flash-induced (5×10^{-4} bleach, arrows) cGMP hydrolysis (a) in the absence of kinase and arrestin; (b) in the presence of kinase alone; (c) in the presence of both kinase and arrestin. ATP was present in all three measurements. The slow inactivation in (a) is not due to lowering of cGMP concentration, which is well above the K_m (total cGMP concentration before the flash 5 mM), but to the presence of contaminant kinase in the membranes (the membranes used for the experiments shown in (A–C) have not been washed with urea). Protein and nucleotide concentrations given under Materials and Methods. (B) Mathematical derivative of the pH trace as a function of time, representing the variation of PDE activity (concentration of cGMP hydrolyzed per second) as a function of the time after the flash. (a–c): as in (A); (b1) was obtained from cGMP hydrolysis induced by the first flash and (b2) from the second flash. (C) Dissociation signals observed under identical conditions as PDE activity measurement except that no PDE is added. (a–c) as in (A) and (B); (b1) and (b2) as in (B). Reversal of the dissociation signal corresponds to inactivation of G-protein upon GTP hydrolysis. The reversal of the dissociation signal in (a) (no addition) is slightly faster than in the experiment shown in Figure 1, in which urea-washed membranes (having no kinase activity) were used.

We have indeed reported that the activation of PDE by G_{GTP} is associated with a specific light-scattering signal (Bennett, 1986; unpublished results), which was termed "PAS" (for "PDE activation signal"). This signal requires, in addition, swelling of the disks by the dark Mg-ATPase described by Uhl et al. (1979). In the presence of ATP and of the soluble proteins of the rod outer segments, the PAS is a transient signal. We show in Figure 6 that the reversal of the PAS, as the reversal of the dissociation signal and the inactivation of the PDE, can be induced by kinase alone and is accelerated by arrestin. Considering the total amplitude of the PAS measured in the absence of kinase and arrestin (trace a), it is possible that the half-time of its reversal is shorter than that of the dissociation signal shown in Figure 5C. The fact that the PAS is a slow process (Figure 6a) as compared to the dissociation signal (Figure 5C) and to the activation of the PDE (visualized by the derivative of the pH variation, Figure 5B), however, makes it difficult to measure the kinetics of its reversal and to correlate it precisely with the inactivation of PDE. The reversal of the PAS might, moreover, be limited by another process subsequent to inactivation of PDE.

In order to test whether the unexpected difference between PDE turnoff and reversal of the dissociation signal exists when measurements are carried out under identical conditions, we have compared these two processes for varying flash intensities, using rod fragments that contain all the proteins involved and

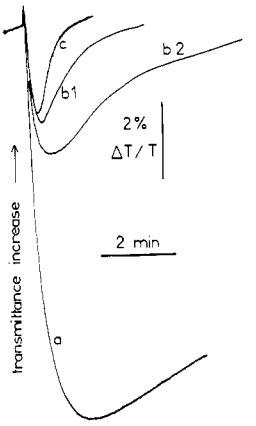

FIGURE 6: Light-scattering signal associated with the activation of PDE in the presence of ATP ["PAS" described by Bennett (1986)] in the presence or absence of kinase and arrestin. (a) No addition; (b1, b2) addition of kinase (first and second flash, respectively); (c) addition of kinase and arrestin. Bleaching extent per flash 5×10^{-4}.

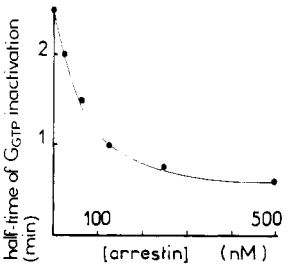

FIGURE 7: Dependence of the rate of G-protein inactivation on the concentration of arrestin. The half-time of G_{GTP} inactivation is measured from the reversal of the dissociation signal. Bleaching extent 5×10^{-4}.

that consist of stacks of flattened disks. Vanadate was added in order to inhibit the ATP-induced swelling, which would result in replacing the dissociation signal by a PAS. The rate of reversal of the dissociation signal was found to be identical with the rate of PDE turnoff in all experiments. Since the GTPase activity is not modified in the presence of PDE (Sitaramayya, unpublished results), it can be suggested that the reversal of the dissociation signal in the absence of PDE in reconstituted systems is probably slower than GTP hydrolysis itself. The reversal of the dissociation signal may be related to reassociation of α and $\beta\gamma$ subunits of G, which may indeed be facilitated for PDE-bound (i.e., membrane-bound) α subunits.

Concentration Dependence of the Accelerating Effect of Arrestin on G_{GTP} Inactivation. The half-time of the reversal of the dissociation signal is plotted as a function of the concentration of added arrestin in Figure 7 (5×10^{-4} bleach). The half-time in the absence of arrestin (which corresponds to inactivation of MII by phosphorylation) was measured from the dissociation signal induced by the second flash in order to eliminate contaminant arrestin (see above). The effect of arrestin is half-maximal at a concentration of 50 nM and fully saturated at 500 nM, i.e., at the same concentration as G-protein in the experiment (about 12% that of total rhodopsin). It should be stressed that this proportion is very close to that reported in the rod cell (Pfister et al., 1985). These results therefore strongly suggest that arrestin can indeed influence the fast inactivation of MII. It has been recently found, however, by N. Philip (personal communication) that the concentration of arrestin in dark-adapted rat rod outer segments prepared according to the method of Schnetkamp et al. (1979) is only 1.6–2.4% that of rhodopsin and that it in-

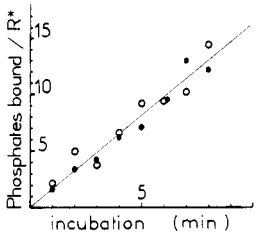

FIGURE 8: Phosphorylation of excited rhodopsin by the rhodopsin kinase in the presence or absence of arrestin: (○) kinase alone; (●) kinase and arrestin. Urea-washed membranes (2.5 μM rhodopsin), bleaching extent 10^{-3}. See Figure 3 for the rate of G-protein inactivation at the same flash intensity.

creases to 9.6% after 2 h in room light. Similar results (0.1 arrestin per G-protein in the dark, rising to 2 per G-protein after light exposure) have been found by N. Ryba, R. Wagner, and R. Uhl (personal communication). It is therefore necessary to discuss whether arrestin plays a role in the termination of the cGMP cascade in vivo. The effect of arrestin on the inactivation of G_{GTP} is shown to be greater at higher flash intensities (Figure 3) under our conditions (25 nM kinase) and appears very small below 5×10^{-4} bleach. In the rod outer segment, where the kinase concentration is as high as 8 μM (1 kinase for 360 rhodopsins; Sitaramayya et al., 1985), i.e., 320 times higher than in our experiments reported here, phosphorylation of excited rhodopsin is expected to be much faster. It seems therefore unlikely that the intervention of arrestin will be needed even at the highest flash intensities used in the experiment shown (10^{-3} bleach). The fact that reversal of the dissociation signal is in fact slower than PDE turnoff (Figure 5) makes it even less likely. It appears therefore that arrestin may only have a role in the turnoff mechanism at a high level of bleaching.

Although Wilden et al. (1986) and Wilden and Kühn (1986) report that binding of arrestin to phosphorylated rhodopsin is necessary for turning off PDE activity, they need about 10 times more arrestin than we do for the same effect. The reason for such a discrepancy is unclear. Our arrestin was prepared according to the simplified method of Wilden and Kühn (1986), without FPLC purification. It is not known whether FPLC could denature the protein to such an extent. Another possible explanation is that arrestin may bind to regenerated phosphorylated rhodopsin. Although the results reported by Kühn et al. (1984) show that binding of arrestin to phosphorylated regenerated rhodopsin is certainly increased by light, they do not show that there is no binding in the dark. Since 98% of the rhodopsins are phosphorylated in the experiments described by Wilden et al. (1986) and Wilden and Kühn (1986), the concentration of free arrestin might be much less than the total concentration.

Rhodopsin Phosphorylation in the Presence or Absence of Arrestin. The results reported above clearly demonstrate that arrestin accelerates the inactivation of MII by the rhodopsin kinase. In order to understand the mechanism of action of arrestin, we have measured kinase activity in the presence or absence of arrestin. The experiment was carried out under exactly the same conditions as the light-scattering measurement. Results are shown in Figure 8: The kinase activity is identical whether or not arrestin is added to the suspension. However, we have shown that in the presence of arrestin MII is fully inactivated between 20 and 50 s after the flash (Figures 3B and 4), whereas it takes 8–10 min for the dissociation signal to reverse in the absence of arrestin (10^{-3} bleach, Figure 3A). From Figure 8, 0.5–1.4 phosphates are bound per bleached rhodopsin between 20 and 50 s after the flash and 12–16 phosphates between 8 and 10 min.

It can therefore be deduced from the experiments described in Figures 3, 4, and 8 that (i) arrestin binds to MII at low phosphorylation levels, probably after binding of only one phosphate; (ii) phosphorylated MII with bound arrestin is not capable of activating G-protein; (iii) in the absence of arrestin phosphorylation alone can inactivate MII, but much more phosphate per bleached rhodopsin is needed to reach total inactivation; and (iv) binding of arrestin to MII less than 1 min after the flash does not hinder access of kinase to phosphorylation sites on MII since phosphorylation continues at the same rate as in the absence of arrestin. This reveals that the binding sites for kinase and arrestin on the rhodopsin molecule are distinct.

The large extent of phosphorylation required to prevent interaction with G-protein in the absence of arrestin may explain why Wilden et al. (1986) find that phosphorylation alone is not capable of turning off PDE activity. Indeed, they used phosphorylated membranes with 5.5–6 phosphates bound per rhodopsin, which in our experiments is not sufficient to fully inactivate MII (even though the value of 12–16 phosphates per rhodopsin is probably an overestimation since MII is likely to be inactivated before total reversion of the dissociation signal). It should be noted that although the number of serines and threonines at the cytoplasmic side of rhodopsin is only 16 (Hargrave et al., 1983), very high levels of phosphorylation have been observed under low bleach conditions [19 phosphates in Sitaramayya and Liebman (1983) and up to 30 in Miller et al. (1977)], suggesting some sort of cooperativity in rhodopsin inactivation (Aton, 1986). Finally, in agreement with the present results, a low level of phosphorylation (1 or 2 phosphates) was previously reported to inactivate rhodopsin in rod preparations containing a high amount of kinase (Sitaramayya, 1986b): Although the reason for this was not understood at that time, it is clear from the gels shown (Sitaramayya, 1986b) that this preparation had a high content of arrestin as well as of kinase.

Although phosphorylation of rhodopsin is often compared to that of β-adrenergic receptors, since both only occur upon activation (Benovic et al., 1986), the results presented here underline the difference between the two systems. Phosphorylation of β-adrenergic receptors upon agonist binding (Sibley et al., 1984) leads to partial desensitization of the receptors [by about 40% (Stadel et al., 1981)] to further stimulation by the agonist. On the other hand, we show that rhodopsin is totally inactivated by multiple phosphorylation and does not stabilize at an intermediate partially active state. Such partially active states do exist, as shown by Miller et al. (1986), but are only transient intermediates in the process of multiple phosphorylation of excited rhodopsin.

Registry No. PDE, 9068-52-4; rhodopsin kinase, 54004-64-7.

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Molecular Cloning, Sequencing, and Characterization of cDNA for Sarcotoxin IIA, an Inducible Antibacterial Protein of *Sarcophaga peregrina* (Flesh Fly)†

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Received June 1, 1987; Revised Manuscript Received October 5, 1987

ABSTRACT: A cDNA clone for sarcotoxin IIA, an antibacterial protein of *Sarcophaga peregrina* (flesh fly) larvae [Ando, K., Okada, M., & Natori, S. (1987) *Biochemistry 26*, 226–230], was isolated and characterized. Sarcotoxin IIA was found to consist of 270 amino acid residues. Northern blot analysis showed that the sarcotoxin IIA gene was activated in response to injury of the body wall of the larvae. The gene was activated for much longer after injection of *Escherichia coli* into the abdominal cavity of larvae than after injection of saline alone. A common nucleotide sequence for mammalian inflammatory mediator protein cDNAs, TTATTTAT, was found in the 3′-untranslated region of sarcotoxin IIA cDNA, suggesting that this protein plays a role in the inflammatory response of this insect.

We are all familiar with the immune system in vertebrates. However, there are many unknown defense mechanisms that kill bacteria and viruses nonspecifically. The prototype of such nonspecific defense mechanisms can be found in invertebrates like insects. Therefore, it is important to investigate the defense mechanisms of insects from the viewpoint of comparative immunology.

Holometabolous insects have both humoral and cellular defense systems to protect themselves from various pathogens and to scavenge own-tissue fragments produced during metamorphosis (Boman et al., 1974; Pye & Boman, 1977; Scott, 1971; Gagen & Ratcliffe, 1976; Schmit & Ratcliffe, 1977). Of the humoral antibacterial proteins of *Lepidoptera*, cecropins and attacins, a series of antibacterial proteins of *Hyalophora cecropia*, have been purified (Steiner et al., 1981; Hultmark et al., 1982, 1983; Lee et al., 1983). Their primary structures were determined both by chemical sequencing of the proteins and from their cDNAs (engstrom et al., 1984; Kockum et al., 1984; von Hofsten et al., 1985). Cecropins were also isolated from *Antheraea pernyi*, and their primary structures were found to be almost identical with those of cecropins from *H. cecropia* (Qu et al., 1982).

In *Sarcophaga peregrina* (flesh fly), humoral antibacterial proteins are induced when the body wall of third instar larve is injured by a hypodermic needle (Natori, 1977). Previously we reported purification of antibacterial proteins termed sarcotoxin I and II (Okada & Natori, 1983; Ando et al., 1987). Sarcotoxin I consists of three structurally related proteins termed sarcotoxin IA, IB, and IC. Each protein has been purified to homogeneity and sequenced. These proteins each consist of 39 amino acid residues and differ only in 2 or 3 amino acid residues. From the sequences of sarcotoxin I and

† This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.
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0006-2960/88/0427-1715$01.50/0