Light-induced binding of 48-kDa protein to photoreceptor membranes is highly enhanced by phosphorylation of rhodopsin

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The 48-kDa protein, a major protein of rod photoreceptor cells, is soluble in the dark but associates with the disk membranes when some (5–10%) of their rhodopsin has absorbed light and if this rhodopsin is additionally phosphorylated by ATP and rhodopsin kinase. If rhodopsin has been phosphorylated and regenerated prior to the protein binding experiment, the binding of 48-kDa protein depends on light but no longer on the presence of ATP. Another photoreceptor protein, GTP-binding protein, associates with both phosphorylated and unphosphorylated rhodopsin upon illumination. Excess GTP-binding protein thereby displaces 48-kDa protein from phosphorylated disks; this indicates competition between these two proteins for binding sites on illuminated phosphorylated rhodopsin molecules.

Rhodopsin *Phosphorylation* *Light-dependence* *48-kDa protein* *GTP-binding protein* *Photoreceptor*

1. INTRODUCTION

Absorption of light by rhodopsin in rod outer segment (ROS) disk membranes leads to conformational changes in the rhodopsin molecule [1], making it available as a substrate for a specific protein kinase. This kinase transforms the terminal phosphate group from ATP [2,3], and from GTP [4], into serine and threonine residues [2,5] of bleached rhodopsin.

Light absorption by rhodopsin also causes several ROS proteins to bind more strongly to the disk membrane than before illumination. Proteins that undergo such 'light-induced binding' include: a peripherally membrane-associated GTP-binding protein (G-protein) [6–9]; the soluble enzyme rhodopsin kinase [10]; and a soluble protein of $M_r \sim 48\,000$ [10]. The function of this 48-kDA protein is still unclear, although it is quite abundant in ROS [11].

It is known that both ATP and GTP enhance the light-induced binding of 48 kDa protein to disk membranes [6,9,11,12]. This report demonstrates that this enhancement is specifically due to phosphorylation of rhodopsin. We chose a reconstituted experimental system in which rhodopsin was phosphorylated and regenerated with 11-*cis*-retinal prior to the actual protein-binding experiments. This allowed us to study the binding of soluble and peripheral ROS proteins to the washed phosphorylated membranes in the absence of ATP, thus eliminating other possible reactions [13,14] of ATP. We find that the 48-kDa protein binds to the previously phosphorylated disk membranes in a strictly light-dependent fashion, under conditions in which it does not bind to unphosphorylated control membranes. This observation should provide a clue to elucidating the function of both the 48-kDa protein and rhodopsin phosphorylation.

Abbreviations: G-protein, GTP-binding protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); AMP-PNP, adenylyl imidodiphosphate; R*, photoexcited rhodopsin; P.-disks, phosphorylated and regenerated disk membranes; C.-disks, unphosphorylated control membranes

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2. MATERIALS AND METHODS

Rod outer segments were purified from fresh bovine eyes by a standard procedure [15]. Rhodopsin concentrations were determined as in [15]. Experiments were performed in dim red light. Dithiothreitol (1 mM) was present in all buffers.

Disk membranes with highly phosphorylated and then regenerated rhodopsin ('P.-disks') were prepared by incubating ROS suspensions (0.5 mg rhodopsin/ml) in 100 mM sodium phosphate (pH 7.4), 3 mM [γ-^{32}P]ATP and 1 mM $MgCl_2$ for 3 h at 30°C under continuous illumination [15]. The resulting ^{32}P-opsin was regenerated to ^{32}P-rhodopsin by incubation with a 3-fold molar excess of 11-*cis*-retinal in the dark for 15 h at 20°C. The membranes were then washed twice with 70 mM phosphate buffer and twice with 5 mM Hepes, pH 7.2. The resuspended membranes were stored frozen (-70°C) in aliquots. Regeneration yield was 97–102%, and phosphorylation ranged from 5.2 to 7.0 phosphates bound per rhodopsin. Unphosphorylated control membranes ('C.-disks') were prepared similarly by bleaching, regenerating and washing ROS membranes as described above, but in the absence of ATP.

Binding of soluble and peripheral proteins to these membrane preparations was assayed [16] by adding various extracts of ROS proteins with and without illumination. Three types of extracts were used, all freshly prepared by centrifuging suspensions of dark-adapted ROS (5–9 mg/ml) in different buffers: (i) Extract E_1 containing the 'soluble proteins' [12] including 48-kDa protein and rhodopsin kinase, in 120 mM KCl, 10 mM Hepes (pH 7.0), 1 mM $MgCl_2$ (buffer A). This extract was used in most experiments (except that of fig.1). (ii) Extract E_3 containing the 'peripheral proteins' [12] (mainly G-protein and phosphodiesterase), obtained by lysing ROS which had been previously washed twice with buffer A, in 2 mM Hepes, pH 7.0 (see fig.3). (iii) 'Total extract' containing both soluble and peripheral proteins, obtained by lysing previously untreated ROS in 2 mM Hepes (see fig.1). In binding assays, we mixed either phosphorylated or control disks (350 μg rhodopsin/sample) to one of the extracts in a final volume of 350 μl in transparent centrifuge tubes [16]. The amount of added extract corresponded to that obtained from ROS containing 500–900 μg rhodopsin. The final ion concentration was adjusted to that of buffer A. Samples were warmed normally to 20°C (except fig.2) for 2 min in the dark. 'Light' samples were then illuminated for 30–60 s with orange light ($\lambda \geq 530$ nm) which bleached 5–10% of the rhodopsin. All samples were then immediately cooled in ice water and centrifuged for 15 min at $48\,000 \times g_{max}$ [16]. Aliquots of the clear supernatants (normally 50 μl) were analyzed by SDS-polyacrylamide gel electrophoresis [17]. Gels were stained with Coomassie brilliant blue and destained under carefully controlled conditions [18] to allow quantitative evaluation of densitograms.

3. RESULTS

Under the conditions used here (5–10% rhodopsin bleached), virtually no light-induced binding of 48-kDa protein occurs to unphosphorylated control (C.) disks (cf. gels 3 *vs* 4, and 7 *vs* 8 in fig.1). In contrast, almost all of the 48-kDa protein binds, under the same conditions, to the previously phosphorylated disks (P.-disks), provided they have been illuminated (cf. gels 1 *vs* 2, and 5 *vs* 6 in fig.1). Note that neither phosphorylation alone (fig.1, gels 1 and 5) nor light alone (gels 4 and 8) causes the 48-kDa protein to bind (i.e., to disappear from the supernatant); both light and phosphorylation are required (gels 2 and 6).

The 48-kDa protein binds to illuminated P.-disks more quantitatively if GTP is present (fig.1, gel 6) than in its absence (gel 2). This is due to the presence of G-protein in the preparation (total extract) used. It is known that the G-protein binds to photoexcited rhodopsin (R*) and is dissociated from it by GTP [6,7,9,12]. The difference between gels 2 and 6 in fig.1 is explained by the notion that G-protein binds, in absence of GTP, to phosphorylated R* and thereby displaces some of the 48-kDa protein; GTP which solubilizes the G-protein eliminates this competing effect of G-protein (see also fig.3 for this competition).

That the G-protein in fact binds to both the phosphorylated and the control rhodopsin upon illumination is illustrated in fig.1 by the second set of supernatants (1b–4b) in which the peripherally associated proteins have been extracted from the membranous pellets by a subsequent hypo-osmotic treatment. Comparison of supernatants 1b *vs* 2b

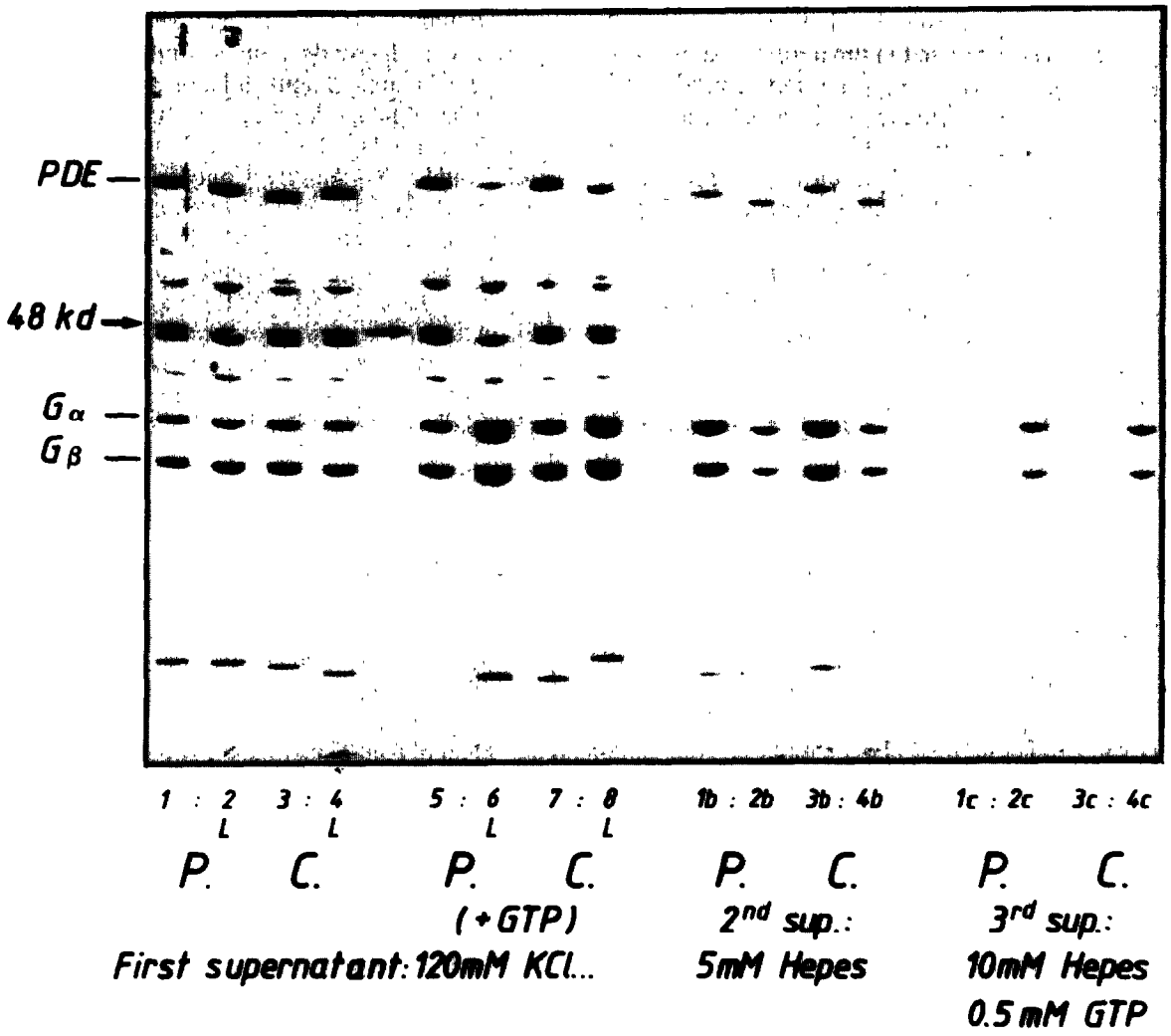

Fig.1. Polypeptide pattern [17] of supernatants obtained from a binding experiment in which previously phosphorylated disks (P.), or control disks (C.), were mixed with 'total extract' containing soluble and peripheral proteins from ROS (see section 2). Suspensions 2, 4, 6 and 8 were illuminated for 1 min, bleaching 10% of their rhodopsin, before centrifugation. Gels 1–8 show the supernatants (50 μl each) obtained directly from the binding experiment in buffer A. The 48-kDa protein is the upper band of the doublet seen in the middle of the gels (arrow). Note the absence of 48-kDa protein from supernatants 2 and 6. Samples 5–8 contained 0.5 mM GTP, which solubilized some of the G_β and most of the G subunit of G-protein upon illumination (gels 6 and 8). The membranous pellets resulting after centrifugation of samples 1–4 were treated in darkness with 5 mM Hepes, leading to the second set of supernatants (1b–4b; 25 μl/gel). The resulting pellets were finally treated with 0.5 mM GTP in 10 mM Hepes (supernatants 1c–4c; 25 μl/gel); this treatment released the G-protein bound to R [6,9]. Note that G-protein binds to illuminated P.-disks as well as to illuminated C.-disks. Similar results were obtained in 5 binding experiments with different preparations of P.-disks, C.-disks, and total extract.

and 3b *vs* 4b shows nearly the same light-dark difference of G-protein binding for the phosphorylated as for the control disks. The third set of supernatants (1c–4c) shows that portion of G-protein that had been bound to R* and released by the final treatment with GTP [6]. Again, nearly similar light-dark differences are observed for the phosphorylated as for the control disks.

Extract of soluble proteins alone, not containing G-protein, has been used in the binding experiment shown in fig.2. In this case, quantitative binding of 48-kDa protein to illuminated P.-disks occurs even in absence of GTP, since no G-protein is present to compete. Light-induced binding is quantitative at both 20 and 10°C and is still nearly quantitative at 0°C.

Competition between 48-kDa protein and G-protein for binding on phosphorylated R* is clearly demonstrated in fig.3. G-protein, added in increasing amounts, progressively displaces the 48-kDa protein from its light-induced binding to P.-disks. The competing effect of G-protein is eliminated by GTPγS (fig.3, open triangle) which dissociates G-protein from R* [9].

The influence of some adenosine nucleotides on the light-induced binding of 48-kDa protein is shown in fig.4A. The only effective nucleotide is ATP, and it has an effect only in the case of control disks: incubation of illuminated C.-disks with ATP progressively and slowly increases the portion of 48-kDa protein that binds to the membranes (open circles in fig.4A). This effect of ATP is most likely due to the slow phosphorylation of R* which takes place under these conditions (fig.4B). However, an exact correlation between phosphorylation extents of individual R* molecules [15]

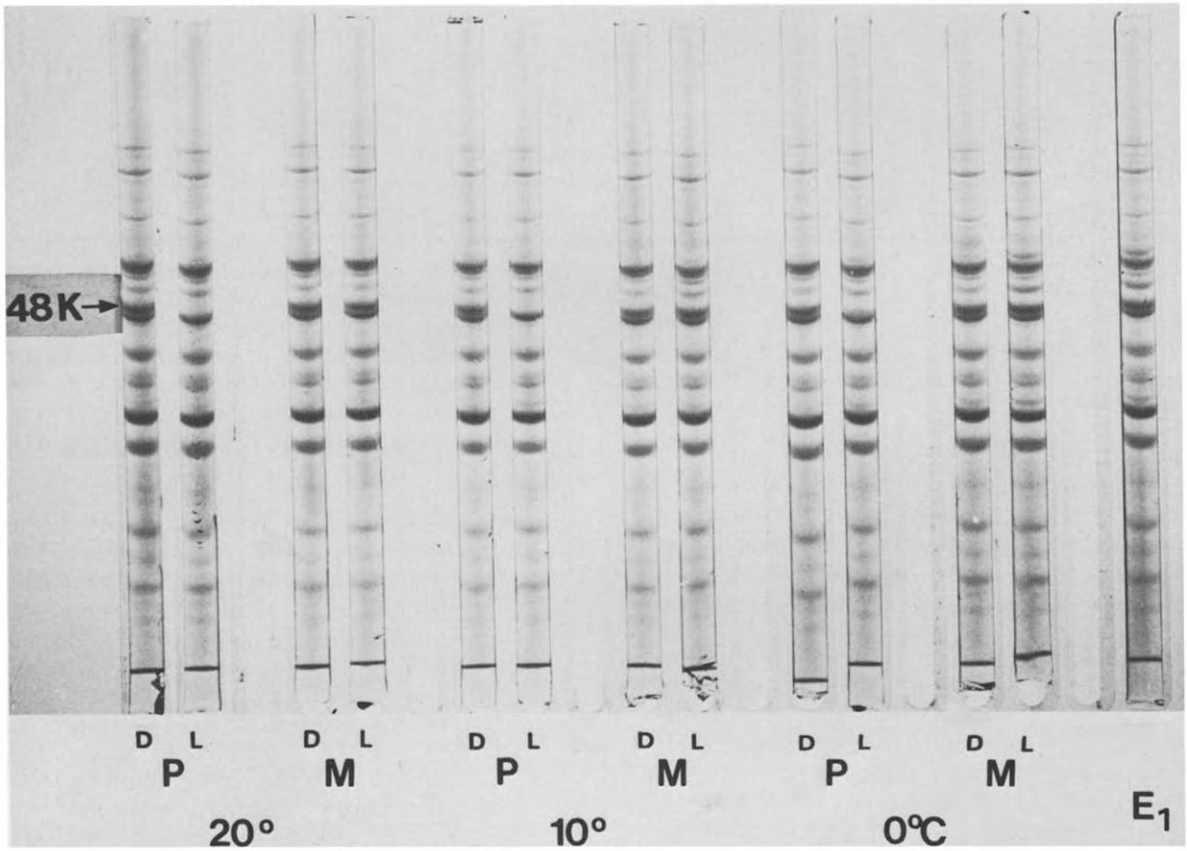

Fig.2. Supernatants from a binding experiment in which previously phosphorylated disks (P), or control disks (M) were mixed with extract of 'soluble proteins' (E_1, shown on the last gel.) In the L-samples, 10% of the rhodopsin had been bleached shortly before centrifugation. Binding experiments were performed at the temperatures (°C) indicated in the bottom line, with groups of 4 supernatants for each temperature. As in fig.1, the 48-kDa protein ('48 K') corresponds only to the upper band of the doublet.

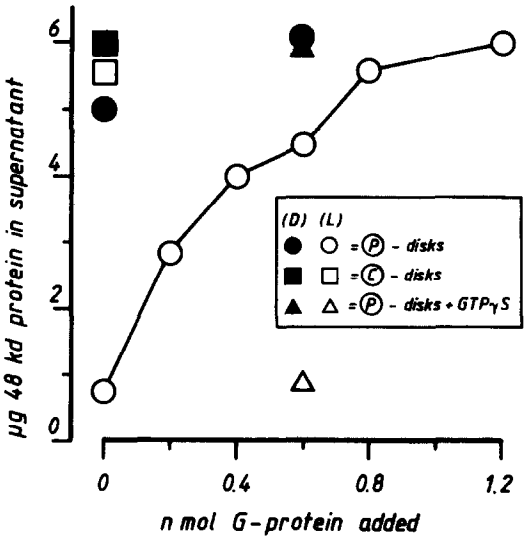

Fig.3. Excess G-protein inhibits light-induced binding of 48-kDa protein to phosphorylated disks. Each suspension in the binding assay contained the same amount of soluble proteins (extract E_1, see section 2) added to either phosphorylated disks (circles and triangles) or control disks (squares). Various amounts of G-protein, contained in an extract of 'peripheral proteins' (E_3, see section 2), were added to P.-disk suspensions as indicated on the abscissa. Two samples (▲,△) contained 20 µM GTPγS in addition to G-protein. Suspensions were either kept dark (filled symbols), or illuminated for 30 s (5% rhodopsin bleached; open symbols) before centrifugation. Supernatants were analyzed for their amounts of 48-kDa protein by densitometry of stained SDS gels, with bovine serum albumin serving as a standard. The concentration of G-protein in the extract E_3 was determined similarly. Plotted in the ordinate is the total amount of 48-kDa protein present in each supernatant. Similar results were obtained in 3 separate experiments.

and binding of 48-kDa protein cannot be drawn from these data.

The poorly hydrolysable analog AMP-PNP (fig.4A), as well as ADP (not shown), had no influence on the binding of 48-kDa protein to C.-disks. Similarly, the binding of 48-kDa protein to bleached P.-disks, as well as the absence of its binding to dark-kept P.-disks, was not changed by the addition of ATP, ADP, or AMP-PNP (0.5 mM each; not shown). This indicates that the sole binding of these nucleotides to a protein is not important for the interactions of 48-kDa protein with R* but that the phosphorylation of R* is important.

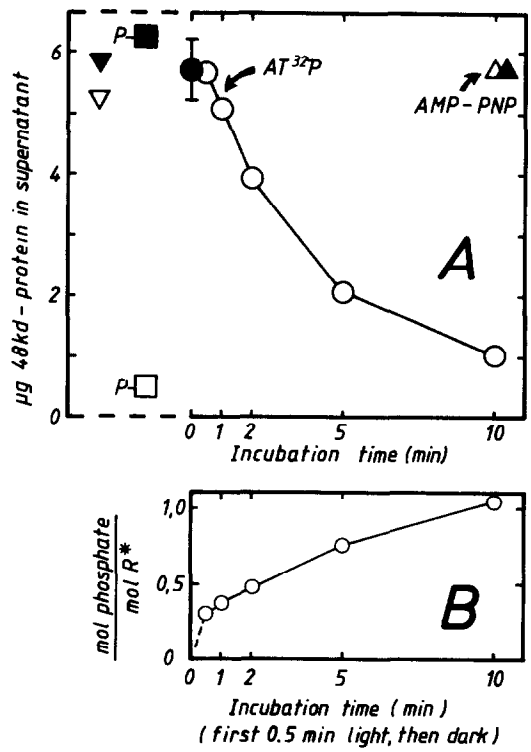

Fig.4. Time course of the light- and ATP-dependent binding of 48-kDa protein to control disks (A), and of the phosphorylation of rhodopsin in the same disks (B). Suspensions of C.-disks, each containing an equal amount of added extract E_1 ('soluble proteins', see section 2) and 0.5 mM [γ-^{32}P]ATP in buffer A, were incubated in the dark at 20°C either unbleached (●), or after an initial light exposure of 30 s bleaching 9% of the rhodopsin (○). After the times indicated on the abscissa, the tubes were cooled to 0°C and centrifuged with a delay of ~ 2 min. The amount of 48-kDa protein present in the supernatants was determined from gel densitograms and is plotted in A. Phosphorylation of rhodopsin in the corresponding pellets was determined [15] and has been plotted in B, after subtraction of the dark phosphorylation level. The following additional samples are shown in A: C.-disks incubated for 10 min with AMP-PNP either unbleached (▲) or bleached (△); C.-disks incubated for 30 s without added nucleotides, unbleached (▼) or bleached (▽); P.-disks incubated for 30 s without added nucleotides, unbleached (P-■) or bleached (P-□). Bleaching extent was 9% in all cases (open symbols).

4. DISCUSSION

The data show that 48-kDa protein binds to previously phosphorylated and regenerated disk

membranes specifically upon illumination. Rhodopsin is by far the predominant phosphorylated protein of these disk preparations [15]. The following arguments lead us to propose that the 48-kDa protein has its binding site on the photobleached, phosphorylated rhodopsin (P-R*) itself, and not just somewhere on the disk surface. (i) The binding requires light absorption by rhodopsin. (ii) It has been shown earlier [9] that G-protein binds directly to purified R* even in the absence of membranes. Our present finding, that G-protein also binds to phosphorylated disks (fig.1) and thereby displaces the 48-kDa protein (fig.3) makes it very likely that the 48-kDa protein also binds directly to the phosphorylated R*, competing with G-protein for binding sites on the same P-R* molecule. (iii) In an analogous binding experiment with a membrane-free system, 48-kDa protein was also found to bind to purified P-R* (preliminary results).

It is not clear at present if 48-kDa protein can also bind to unphosphorylated R* or if phosphorylation is absolutely required. In the earlier experiments when light-induced binding of 48-kDa protein to bovine ROS membranes was routinely observed in the absence of ATP [6,9,10,12], the ROS were always fully bleached. Since bovine material is normally not quite dark-adapted, it still contains a few percent of in vivo phosphorylated, regenerated rhodopsin (unpublished) which, at full bleaching, may account for the light-induced binding of 48-kDa protein. Interestingly, in ROS from dark-adapted frogs, ATP or GTP is absolutely required for light-induced binding of 48-kDa protein to occur [11].

The 48-kDa protein has recently gained additional interest in two respects: it has been shown [19] to be identical with the so-called 'S-antigen', a protein that causes an auto-immune disease of the eye (uveoretinitis [20]); and it has been proposed to be a light-dependent ATP-binding protein [14]. Its function in the visual process remains to be elucidated. It may well play a role, via its binding to P-R*, in the regulation of the light-triggered phosphodiesterase enzyme cascade, for instance, by competing with G-protein – but this needs to be verified experimentally.

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REFERENCES

[1] Kühn, H., Mommertz, O. and Hargrave, P.A. (1982) Biochim. Biophys. Acta 679, 95–100.
[2] Kühn, H. and Dreyer, W.J. (1972) FEBS Lett. 20, 1–6.
[3] Bownds, D., Dawes, J., Miller, J. and Stahlman, M. (1972) Nat. New Biol. 237, 125–127.
[4] Chader, G.J., Fletcher, R.T., O'Brien, P.J. and Krishna, G. (1976) Biochemistry 15, 1615–1620.
[5] Hargrave, P.A., Fong, S.-L., McDowell, J.H., Mas, M.T., Curtis, D.R., Wang, J.K., Juszczak, E. and Smith, D.P. (1980) Neurochem. Int. 1, 231–244.
[6] Kühn, H. (1980) Nature 283, 587–589.
[7] Kühn, H., Bennett, N., Michel-Villaz, M. and Chabre, M. (1981) Proc. Natl. Acad. Sci. USA 78, 6873–6877.
[8] Fung, B.K., Hurley, J.B. and Stryer, L. (1981) Proc. Natl. Acad. Sci. USA 78, 152–156.
[9] Kühn, H. (1984) Prog. Retinal Res. 3, 123–156.
[10] Kühn, H. (1978) Biochemistry 17, 4389–4395.
[11] Pfister, C., Kühn, H. and Chabre, M. (1983) Eur. J. Biochem. 136, 489–499.
[12] Kühn, H. (1981) Curr. Top. Membranes Transp. 15, 171–201.
[13] Farber, D.B., Brown, B.M. and Lolley, R.N. (1979) Biochemistry 18, 370–378.
[14] Zuckerman, R., Buzdygon, B. and Liebman, P.A. (1984) Biophys. J. 45, 292a (abstr.).
[15] Wilden, U. and Kühn, H. (1982) Biochemistry 21, 3014–3022.
[16] Kühn, H. (1982) Methods Enzymol. 81, 556–564.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Fairbanks, G., Steck, T.L. and Wallach, D.F.W. (1971) Biochemistry 10, 2606–2617.
[19] Pfister, C., Dorey, C., Vadot, E., Mirshahi, M., Deterre, P., Chabre, M. and Faure, J.P. (1984) CR Acad. Sci. Paris, in press.
[20] Dorey, C., Cozette, J. and Faure, J.P. (1982) Ophthalmic Res. 14, 249–255.