

Function of the farnesyl moiety in visual signalling

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The aim of this work was to search for the biological function of protein isoprenylation. For this purpose, peptides were synthesized and, by using a convenient protocol, were farnesylated or geranylated at the thiol group of the C-terminal cysteine. The interaction of these peptides with photoactivated rhodopsin (Rho*, which is functionally equivalent to metarhodopsin II) was studied with the use of sheep rod outer segments. The sheep rod outer segments, although chosen because of the unavailability of bovine material in the U.K., had favourable optical properties for the direct determination of spectral changes in membrane suspensions. At 20 °C and pH 8.0, the $t_{\frac{1}{2}}$ of the conversion of metarhodopsin II (Meta II) (λ_{\max} 389 nm) into Meta III (λ_{\max} 463 nm) was 3.2 min (less than 1.5 min at 37 °C). The $t_{\frac{1}{2}}$ was unaltered in the presence of non-farnesyl peptides but increased by approx. 20 % with farnesyl-*N*-acetylcysteine, by approx. 60 % with farnesyl peptide containing residues 544–558 of rhodopsin kinase and by approx. 140 % with farnesyl peptide corresponding to residues 60–71 of the γ -subunit of visual transducin. The effect

of various peptides on the activities of bovine and sheep rhodopsin kinase was also studied. In this assay the non-farnesyl peptides and common detergents were found to be inactive; however, all the farnesyl peptides inhibited the activity to various extents. Cumulatively, the results show that, whereas the farnesyl peptides as well as a number of membrane-disrupting detergents affected the conversion from Meta II into Meta III, the inhibition of the activity of rhodopsin kinase was achieved only by the farnesyl peptides. The results are interpreted as showing that Meta II possesses a binding site for the recognition of the farnesyl group that can be used either by the farnesyl moiety of rhodopsin kinase or transducin to make the initial encounter, which can then develop into multivalent interactions characterized by the structure, and the desired function, of each protein.

Key words: farnesyl peptides, rhodopsin kinase, rhodopsin phosphorylation, transducin.

INTRODUCTION

Since the discovery that certain fungal mating factors contain an isoprenyl group [1], a large number of mammalian proteins have been found to be similarly modified at the C-terminal cysteine residue [2,3]. Because of the hydrophobic nature of the isoprenyl group it was originally assumed to facilitate the membrane localization of the farnesylated and geranylgeranylated proteins [4]. This view is now being modified and evidence is accumulating to suggest that the isoprenyl moiety might also promote specific protein–protein interactions [5].

Many protein components in the visual cycle have been found to possess an isoprenyl group. Rhodopsin kinase (RK) [6] and the γ subunit of transducin (T γ) [7,8] are farnesylated, whereas the α and β subunits of phosphodiesterase are farnesylated and geranylgeranylated respectively [9]. RK and transducin are of particular interest because both interact with the light-activated form of rhodopsin (Rho*) [10,11]. Transducin initiates the visual cascade, giving rise to signal transmission, whereas RK phosphorylates Rho* at its C-terminal domain, instigating the desensitization of the receptor (reviewed in [12]). The fact that both proteins contain a farnesyl group and interact with the same receptor, Rho*, but produce diametrically opposite effects provides an interesting system in which to evaluate the farnesyl moiety's role in protein–protein interactions.

Here we describe the synthesis of farnesyl peptides corresponding to the C-terminal sequences of RK and T γ and show

that these interact with metarhodopsin II (Meta II; considered to be equivalent to Rho*) and act as inhibitors of RK activity.

MATERIALS AND METHODS

Materials

[γ -³²P]ATP was from Amersham, fluoren-9-ylmethoxycarbonyl (Fmoc) amino acids were obtained from Nova Biochem, *N*-acetyl-*S*-farnesyl-*L*-cysteine was from Sigma and Sasrin resin was from Bachem; all other chemicals were purchased either from Sigma or Aldrich.

The buffers used in this work were as follows: buffer A, 20 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane ('Bis-Tris propane')/2 mM MgCl₂/0.1 mM EDTA/1 mM dithiothreitol/1 mM benzamidine/0.2 mM PMSF (pH 7.4); kinase extract, as buffer A but also containing 280 mM NaCl; buffer B, 100 mM Tris/HCl/2 mM MgCl₂/1 mM EDTA/1 mM dithiothreitol (pH 8.0) at 20 °C.

Synthesis of C-terminal cysteinyl peptides

These were assembled on Fmoc-*L*-Cys(Trityl)[4-(hydroxymethyl)phenoxy]methyl copoly(styrene/1 % divinylbenzene) resin (Wang) by using traditional Fmoc methodology [13].

Abbreviations used: Far, farnesyl; Fmoc, fluoren-9-ylmethoxycarbonyl; Ger, geranyl; 12-mer T γ , residues 60–71 of the γ -subunit of visual transducin; 15-mer RK, residues 544–558 of rhodopsin kinase; Meta I, metarhodopsin I; Meta III, metarhodopsin III; Rho*, bleached rhodopsin [considered to be equivalent to metarhodopsin II (Meta II)]; RK, rhodopsin kinase; ROS, rod outer segment; TFA, trifluoroacetic acid.

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Table 1 Synthesized peptides whose sequences correspond to the C-terminus of bovine RK and T γ

The numbers in each sequence correspond to the amino acid position in the corresponding protein. Peptides were synthesized as described in the Materials and methods section and were purified by reverse-phase HPLC with the use of a C₁₈ column with a linear gradient of 0.1% (v/v) TFA and acetonitrile containing 0.1% TFA. The percentage of acetonitrile required to elute each peptide is shown.

Peptide	Sequence	Elution [acetonitrile] (%)	[M + 1] ⁺	
			Expected	Observed
Rhodopsin				
10-mer RK	⁵⁴⁹ AAPSSKSGMC ⁵⁵⁸ (<i>S-Farnesyl</i>) 	36	939	938
Far-10-mer RK	⁵⁴⁹ AAPSSKSGMC ⁵⁵⁸	63	1143	1143
15-mer RK	⁵⁴⁴ VSGQEAPSSKSGMC ⁵⁵⁸ (<i>S-Farnesyl</i>) 	38	1438	1439
Far-15-mer RK	⁵⁴⁴ VSGQEAPSSKSGMC ⁵⁵⁸	63	1642	1643
15-mer(OMe) RK	⁵⁴⁴ VSGQEAPSSKSGMC ⁵⁵⁸ -OMe (<i>S-Farnesyl</i>) 	40	1452	1453
Far-15-mer(OMe) RK	⁵⁴⁴ VSGQEAPSSKSGMC ⁵⁵⁸ -OMe (<i>S-Geranyl</i>) 	69	1657	1658
Ger-15-mer RK	⁵⁴⁴ VSGQEAPSSKSGMC ⁵⁵⁸	56	1574	1576
20-mer RK	⁵³⁹ DDMKGVSGQEAPSSKSGMC ⁵⁵⁸ (<i>S-Farnesyl</i>) 	41	1984	1985
Far-20-mer RK	⁵³⁹ DDMKGVSGQEAPSSKSGMC ⁵⁵⁸	66	2188	2188
24-mer RK	⁵³⁵ GQMPDDMKGVSGQEAPSSKSGMC ⁵⁵⁸ (<i>S-Farnesyl</i>) 	42	2397	2397
Far-24-mer RK	⁵³⁵ GQMPDDMKGVSGQEAPSSKSGMC ⁵⁵⁸	65	2601	2601
(Cys)-14-mer RK	cys- ⁵⁴⁴ VSGQEAPSSKSGM ⁵⁵⁷ (<i>S-Farnesyl</i>) 	38	1438	1439
Far-(Cys)-14-mer RK	cys- ⁵⁴⁴ VSGQEAPSSKSGM ⁵⁵⁷	63	1642	1642
Transducin				
12-mer T γ	⁶⁰ DKNPFKELKGGC ⁷¹ (<i>S-Farnesyl</i>) 	44	1335	1335
Far-12-mer T γ	⁶⁰ DKNPFKELKGGC ⁷¹	65	1540	1540

Peptides were synthesized on a 0.25 mM scale (resin substitution 0.5–0.65 mmol/g Fmoc-amino acid) and processed as described for the peptide methyl ester below.

Synthesis of peptide methyl ester

The peptide methyl ester, farnesyl peptide containing residues 544–558 of RK [Far-15-mer(OMe) RK], was prepared by using the more acid-labile Fmoc-Cys(trityl)-Sasrin resin (400 mg containing 0.4 mmol/g Fmoc-cysteine). After assembly, the resin support was washed with dichloromethane (twice with 10 ml, each for 5 min) to remove traces of dimethylformamide. The labile peptide-linker bond was then cleaved with 1% (v/v) trifluoroacetic acid (TFA) in dichloromethane (four times with

5 ml, each for 15 min); the extracts were combined and after dilution with an equal volume of acetonitrile the solvent was removed immediately under vacuum. The resulting peptide, containing all the side-chain protections except the C-terminal carboxy group (120 mg), was dissolved in 25 ml of chloroform/methanol (1:1, v/v); the mixture, after cooling to 4 °C, was treated with a solution of diazomethane in diethyl ether until a yellow colour persisted. After 1 h, acetic acid was added to quench unreacted diazomethane and the solvent was removed under vacuum. The remaining side-chain protecting groups were removed by treatment with 10 ml of TFA/anisole/trifluoroethanol/ethanedithiol (94:2:2:2, by vol.) at room temperature for 2 h and the solvent was removed under vacuum. Scavengers were removed by filtration through a sintered funnel and washed

thoroughly with diethyl ether. The peptide methyl ester was taken up into 0.1% TFA in AnalaR water and freeze-dried overnight. Purification was by a two-step procedure initially involving gel filtration on a prepacked Sephadex G25 (PD10) column in 0.1% TFA in AnalaR water, followed by reverse-phase HPLC (C_{18} column) with a linear gradient of 0.1% aqueous TFA and acetonitrile containing 0.1% TFA. Analytical data on the peptides are presented in Table 1.

Peptide isoprenylation

Peptide farnesylation

To a foil-wrapped screw-capped tube containing peptide (10 mg, 7 μ mol) were added methanol (1 ml) and 5% (w/v) KOH in methanol (118 μ l, 105 μ mol) and the tube was flushed with argon. The reaction was started by the addition of *trans,trans*-farnesyl bromide (3.8 μ l, 14 μ mol) and the reaction was monitored for free thiol by using 5,5'-dithiobis-(2-nitrobenzoic acid) ('DTNB') [14]. At room temperature the reaction was complete in less than 5 min. The mixture was neutralized with acetic acid and the solvent was removed immediately with a stream of N_2 . The residue was dissolved in 20% (v/v) acetonitrile in AnalaR water (500 μ l) and the mixture was centrifuged (3 min at 10000 g). The colourless supernatant, containing the farnesyl peptide, was carefully removed from the excess *trans,trans*-farnesyl bromide and purified by reverse-phase HPLC (C_{18} column) as described above.

Peptide geranylation

The above method was extended to the synthesis of geranyl 15-mer RK (Ger-15-mer RK) by using geranyl chloride (3 μ l, 16 μ mol), 15-mer RK (10 mg, 7 μ mol) and 5% (w/v) KOH in methanol (64 μ l, 57 μ mol). At room temperature the reaction was complete in 1½ h.

Yields of the isoprenylated peptides after purification were 30–45%. All the peptides were obtained as white solids. Analytical data for all synthesized peptides are shown in Table 1.

Preparation of sheep rod outer segments (ROSs) and RK

During the course of this work, owing to the high incidence of bovine spongiform encephalopathy ('BSE') in the U.K., the removal of eyes from cattle was stopped by the Specified Bovine Offal Order 1995. Sheep retinae were therefore used as a substitute. The preparation of sheep ROS membranes, urea-washed ROSs and the purification of RK were performed as described previously for the bovine system [15,16]. With the use of 600 sheep retinae, ROS membranes containing up to 120 mg of rhodopsin were obtained and had an A_{280}/A_{500} of between 2.1 and 3.5. All preparations of ROSs were performed under red light at 4 °C and the material was stored at -20 °C. The sequence of sheep rhodopsin and its similarity to bovine rhodopsin has already been established [17]. All spectroscopic results obtained in this paper were obtained with sheep ROSs only with $A_{280}/A_{500} < 3.0$.

For the purification of RK, sheep ROS membranes containing 100–120 mg of rhodopsin were extracted six times by alternating between low-salt and high-salt buffers containing 0.04% (v/v) Tween 80. The combined extracts, containing up to 120 units of RK activity (see below) underwent chromatography on heparin-agarose and up to 60 units (see below) were recovered in six fractions of 1.5 ml each.

The sheep RK was similar to its bovine counterpart in terms of its molecular mass, its ability to undergo autophosphorylation

and its immunoreactivity with antisera raised against two peptides containing the C- and N-terminal sequences of bovine RK (results not shown). The phosphorylation assays below were performed with both the sheep and the bovine RK preparations; identical results were obtained. The kinase used in the work generally had an activity of more than 6 units/ml (1 unit represents the incorporation of 1 nmol of phosphate from ATP per min with the use of the assay system described below).

Standard rhodopsin phosphorylation assay

The standard rhodopsin phosphorylation assay employed in these studies consisted of a 100 μ l incubation and consisted of 10 μ l of urea-washed ROSs (2.5 nmol), 10 μ l of [γ - 32 P]ATP (3 mM final concentration; 80000 to 120000 c.p.m./nmol), 70 μ l of kinase extract and, when present, 10 μ l of farnesyl peptide (various final concentrations from 0 μ M to 3 mM in buffer A). Incubations were vortex-mixed, sonicated and incubated in the dark at 32 °C before the introduction of continuous light. Aliquots of 30 μ l were removed at 10 min and subjected to precipitation with trichloroacetic acid and washing before solubilization of the pellets and scintillation counting to determine the incorporation of phosphate into rhodopsin.

It should be noted that the stock farnesyl peptide solutions were made afresh before each experiment because storage overnight at 4 °C or freezing significantly decreased the activity of the peptides.

Phosphorylation assay with solubilized rhodopsin

Urea-washed rhodopsin was solubilized in 2% (w/v) dodecyl maltoside in buffer A. Vigorous vortex-mixing, homogenization and subsequent pelleting of unsolubilized material (240000 g for 20 min) typically generated rhodopsin concentrations in the range 2.1–2.6 mg/ml. The rhodopsin phosphorylation assay was then performed as above, except that solubilized rhodopsin replaced the urea-washed ROS membranes.

Spectroscopic analysis

Sheep ROS membranes free of soluble proteins (containing 12.5 μ M rhodopsin in buffer B) were homogenized (by using ten passes through a hand-held homogenizer) and suspended by being passed twice through a fine (25-gauge) needle. Into two cuvettes (plastic, 10 mm path-length) was added the ROS suspension (1 ml). One of these cuvettes was left unbleached and acted as a scattering blank, whereas the experimental cuvette was flash-bleached (30–40%) and wavelength spectra (350–600 nm) were measured after 1 min, at 1 min intervals for 10 min (20 °C).

Half-life data were obtained by measuring the absorbances at 389 nm [λ_{max} for metarhodopsin II (Meta II)], 463 nm (λ_{max} for Meta III) and 417 nm (isosbestic point for Meta II \leftrightarrow Meta III) every 0.5 min for 40 min. When present, peptides were added to both experimental and reference samples of ROS suspensions before being bleached to give a final concentration of 500 μ M (10 μ l of a 50 mM stock solution in buffer B). Control experiments had buffer added (10 μ l) instead of peptide. For Far-(NAc)Cys, 8.3 μ l of 60 mM stock solution in DMSO was added, whereas control experiments had DMSO added, which had no effect on $t_{1/2}$. All the absorption readings (80 in each case) at 389 and 463 nm were divided by the absorption at 417 nm to give normalized values. The ΔA_{389} values were obtained by subtracting the normalized absorptions at different time intervals from that at the notional zero time, which was approx. 1 min after the initiation of photolysis. The ΔA_{463} values were obtained by subtracting the normalized zero-time value from those at various

time intervals. The plots were constructed by using the ΔA at 40 min as 100% and the half-lives were calculated by using the mono-exponential first-order decay of Meta II or growth of Meta III (see Figure 2). The cross-over of Meta II decay and Meta III rise also corresponded to the calculated half-life.

RESULTS

Synthesis

Ever since the description of the first chemical method [18–20], farnesyl peptides have been prepared by using a range of strategies [21–23]. In the present study a convenient procedure was developed in which unprotected cysteinyl peptides were alkylated with farnesyl bromide with the use of KOH in methanol, with quantitative conversion in less than 5 min. The method was used for the preparation of a number of farnesyl peptides, as well as peptides containing a C-terminal methyl ester, and was also extended to the preparation of geranyl peptides with geranyl chloride. All the peptides were purified to homogeneity on reverse-phase HPLC and gave the expected m/z ions with electrospray and matrix-assisted laser desorption ionization–time-of-flight MS (Table 1).

Interaction of peptides with Meta II

The interaction of various ligands with bleached rhodopsin has previously been studied by monitoring their effects on the equilibrium between Meta I and Meta II at 4 °C [11,24]. An unforeseen advantage of sheep ROS membranes was their favourable optical properties, which allowed primary spectroscopic measurements to be made, without the need for deconvolution, with a high degree of reproducibility. In this work the conversion of Meta II into Meta III was selected to evaluate the interaction of the peptides because this reaction can be followed conveniently at ambient temperature and quantified accurately in terms of the $t_{1/2}$ of the conversion. Figure 1(A) shows a family of spectral curves, following the photolysis of rhodopsin in sheep ROS membranes at 20 °C, in which the decay of absorption at 389 nm and the increase in absorption at 463 nm is attended by a sharp isosbestic point. The latter facet can be used as evidence that the two species contributing the absorption maxima are in direct equilibrium without the participation of either a light-absorbing intermediate or a side product. The time course of the decay of Meta II at 389 nm and the formation of Meta III at 463 nm is plotted in Figure 2(A). Three independent measurements involving the first-order decay of Meta II, the first-order formation of Meta III and the point of intersection of

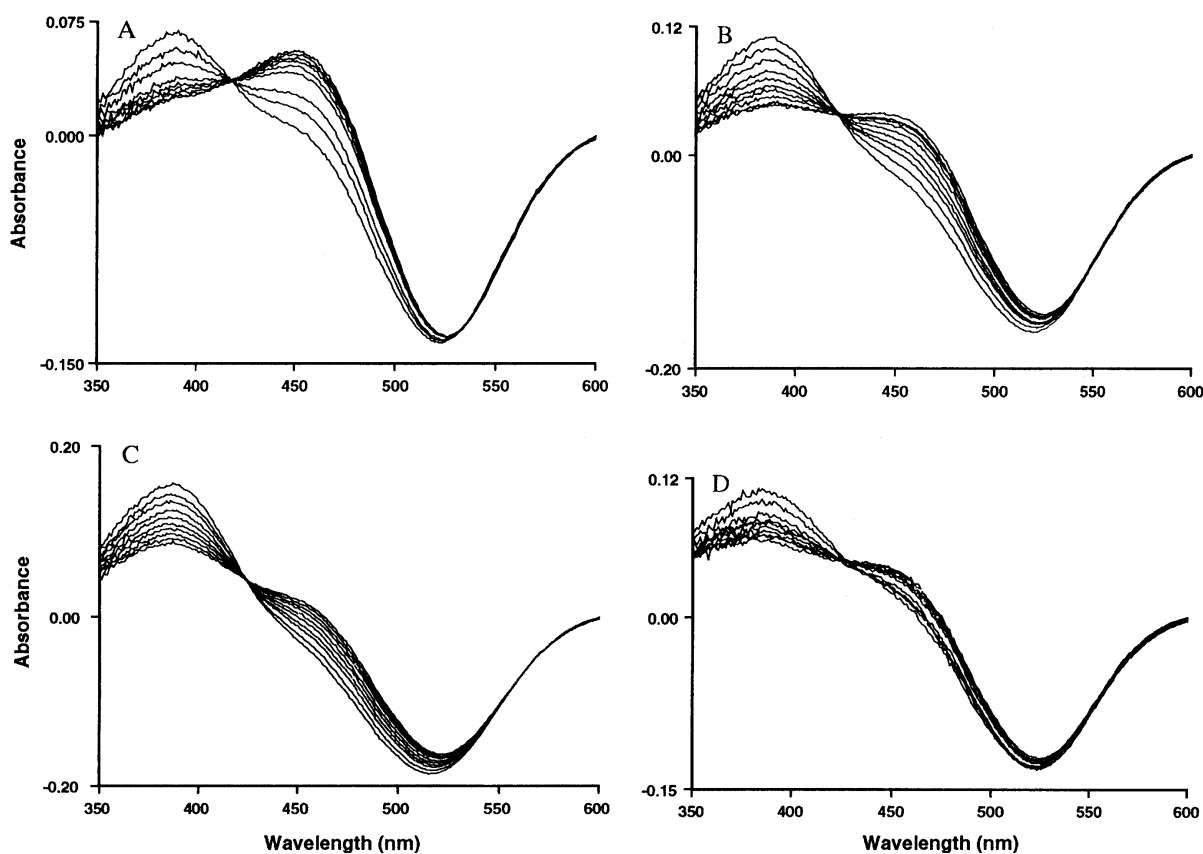


Figure 1 Spectroscopic measurement of the stabilization of Meta II by farnesyl peptides

Wavelength scans (350–600 nm) for ROS suspensions (12.5 μM) that had been exposed to known flashes of light to generate 30–40% bleaching are shown; the reference cell contained 12.5 μM unbleached ROS suspension. The scans were performed at 1 min intervals for 10 min at 20 °C. In each case the initial spectrum is that containing the maximal absorption at 389 nm. (A) No addition; (B) 500 μM Far-15-mer RK; (C) 500 μM Far-12-mer T γ ; (D) 500 μM Far-(Cys)-14-mer RK. The profile in (D) is illustrative of spectra that did not contain a well-defined isosbestic point.

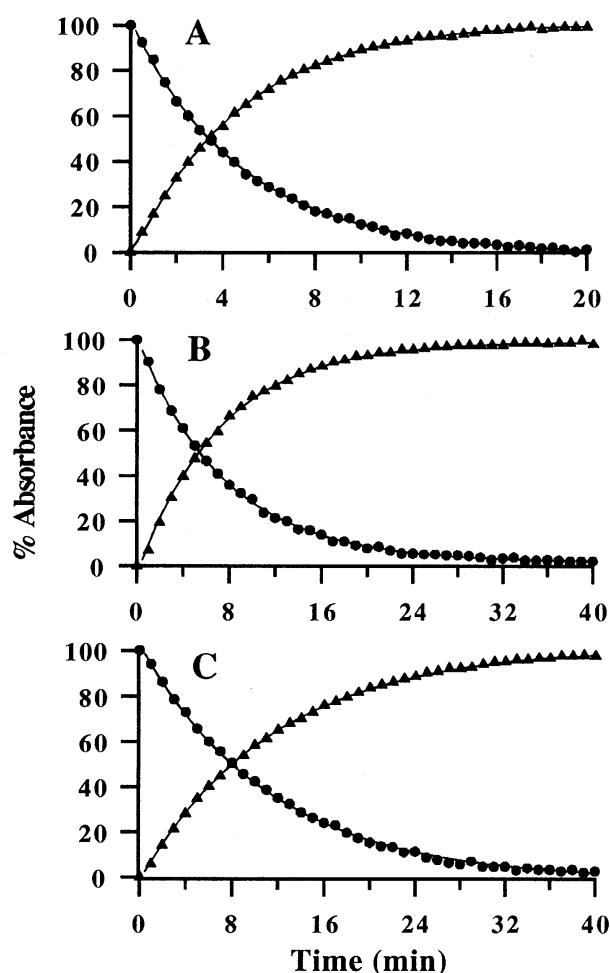


Figure 2 Half-lives of Meta II decay and Meta III formation

Data of the type in Figure 1 but recorded at intervals of 0.5 min are plotted, showing changes in absorbance at 389 nm (●) (λ_{\max} for Meta II) and 463 nm (▲) (λ_{\max} for Meta III) as described in the Materials and methods section. (A) No addition; (B) 500 μ M Far-15-mer RK; (C) 500 μ M Far-12-mer T γ . The point at which these two plots intersect represents $t_{1/2}$. In all cases in which the spectra showed isobestic points, the $t_{1/2}$ values from the intercept were similar to those obtained from the first-order mono-exponential decay or rise of the two types of curve; these are listed in Table 2.

Table 2 Effect of various additives on the $t_{1/2}$ of decay of Meta II and the formation of Meta III

Data are from experiments of the type shown in Figure 2. Values are means \pm S.D. for three experiments.

Additions	Apparent $t_{1/2}$ for decay of Meta II (min)	Apparent $t_{1/2}$ for rise of Meta III (min)	Well-defined isobestic point at 417 nm
None (control)	3.32 \pm 0.04	3.24 \pm 0.04	+
15-mer RK	3.10 \pm 0.04	3.16 \pm 0.03	+
12-mer T γ	3.21 \pm 0.05	3.18 \pm 0.03	+
Ger-15-mer RK	4.10 \pm 0.09	3.78 \pm 0.09	+
Far-15-mer RK	5.21 \pm 0.05	4.96 \pm 0.05	+
Far-12-mer T γ	8.02 \pm 0.12	7.92 \pm 0.05	+
Far-(NAc)Cys	3.77 \pm 0.08	3.85 \pm 0.12	+
Far-(Cys)-14-mer RK	6.35 \pm 0.06	4.25 \pm 0.07	–
Oleic acid	4.99 \pm 0.04	4.60 \pm 0.04	+
Dodecyl maltoside	3.41 \pm 0.11	2.67 \pm 0.09	–

the two rate curves gave similar $t_{1/2}$ values of 3.2–3.3 min at 20 °C (less than 1.5 min at 37 °C). The preceding Meta II to Meta III reaction was performed routinely, as a control, in all the spectroscopic experiments described here, for which more than 24 independently prepared samples of sheep ROSs were used over a 3-year period. In all these cases, patterns identical with those in Figures 1(A) and 2(A) were obtained, establishing the reproducibility of the approach. Furthermore, the $t_{1/2}$ found in the present work is in the same range as that reported previously [25,26], and is close to the latest reported value (4.7 min) [26].

The study was then extended to the evaluation of the effect of various peptides listed in Table 1; it was found that the non-farnesyl peptides, 15-mer RK and 12-mer T γ , at concentrations of 500 μ M were without effect on the conversion of Meta II into Meta III, whereas the simplest farnesyl derivative, Far-(NAc)-Cys, increased the $t_{1/2}$ by 20% (Tables 2 and 3; primary spectra not shown). The farnesyl peptides corresponding to the C-terminal sequences of RK and T γ increased the $t_{1/2}$ by 60% and 140% respectively (spectra in Figures 1B and 1C; absorption plots in Figures 2B and 2C; Table 2 gives the $t_{1/2}$ values and Table 3 converts these into percentages of the control). In the preceding cases the rearrangement occurred involving a sharp isobestic point (as in Figures 1B and 1C). This provided the assurance that the interconversion of Meta II and Meta III was affected selectively, and in particular no significant contribution to absorption at 389 nm was made by all-*trans*-retinal formed from Schiff-base hydrolysis. The reliability of the method was further emphasized by the fact that the three related measurements (decay of Meta II, formation of Meta III and the point of intersection of the two processes; see Figure 2) gave similar $t_{1/2}$ values. Another important point to emerge from a closer examination of the spectral curves in Figure 1 is that the farnesyl peptides not only affected the decay rate of Meta II but also shifted the position of the Meta II/Meta III equilibrium. If we assume that the absorption coefficients of Meta II and Meta III are similar, the ratio of Meta III to Meta II without any addition is the highest and decreases progressively in the presence of Far-15-mer RK and Far-12-mer T γ (compare Figures 1A, 1B and 1C). An important consequence of the alteration of the $t_{1/2}$ of decay of the reaction, and also the position of the equilibrium, is that the starting A_{389} is different from experiment to experiment, being related to $t_{1/2}$. That is, the decay process is slower for higher initial A_{389} values recorded at approx. 1 min after the initiation of the photolysis.

Isoprenyl peptides are amphipathic compounds; the possibility exists that their spectroscopic effects are mediated not by specific ligand–protein interactions but indirectly through the alteration of the properties of the membrane. To evaluate this aspect, the effect of a range of common detergents on the fate of Meta II was studied. Oleic acid and farnesylthioacetic acid slowed the decay of Meta II but only the former affected the interconversion of Meta II and Meta III via a sharp isobestic point (results not shown). The spectra obtained with all the other compounds were rather complex, with broad peaks and relatively fast changes in the 389 and 463 nm regions. Furthermore, the spectra lacked the isobestic point, indicating the presence of more than two light-absorbing species. In this connection, we note that Far-(Cys)-14-mer RK, which contains the farnesylcysteine moiety in the non-native orientation, also gave a spectral profile without a sharp isobestic point, as shown in Figure 1(D). When no isobestic point was observed, the rates for the decay of Meta II and the formation of Meta III differed from each other (see the eighth row in Table 2). In such cases the results cannot be used to draw conclusions on the type of intermediates of rhodopsin that were present at any moment. To emphasize this fact, the sharpness of

Table 3 Effect of various compounds on Meta II decay and on the activity of RK

The decay data are from plots of the type in Figure 2. The sharpness of the isosbestic point as observed for the plots in Figures 1(A)–1(C) is denoted by (+) and its absence by (–). Abbreviation: n.d., not determined. The inhibitory data are from experiments of the type shown in Figure 3.

Type of compound	Condition	Relative $t_{1/2}$ for Meta II decay (%)	Sharpness of isosbestic point	Relative rhodopsin kinase activity (%)
Non-isoprenyl peptides	(I) Control	100	+	100
	(II) 10-mer RK	n.d.	n.d.	94
	(III) 15-mer RK	94	+	100
	(IV) 20-mer RK	n.d.	n.d.	102
	(V) 24-mer RK	n.d.	n.d.	n.d.
	(VI) 12-mer T γ	97	+	103
	(VII) (Cys)-14-mer RK	n.d.	n.d.	108
Isoprenyl peptides	(VIII) Ger-15-mer RK	124	+	108
	(IX) Far-10-mer RK	n.d.	n.d.	77
	(X) Far-15-mer RK	158	+	53
	(XI) Far-20-mer RK	n.d.	n.d.	53
	(XII) Far-24-mer RK	n.d.	n.d.	68
	(XIII) Far-(NAc)-Cys	115	(+)	75
	(XIV) Far-12-mer T γ	242	(+)	61
	(XV) Far-(Cys)-14-mer RK	194	(Broad)	35
	Detergents and other compounds	(XVI) Oleic acid	151	(+)
(XVII) Farnesylthioacetic acid		136	(–)	102
(XVIII) Dodecyl maltoside		103	(–)	109
(XIX) <i>N,N</i> -dimethyl-dodecylamine- <i>N</i> -oxide		Data unobtainable	(–)	100
(XX) Dodecyltrimethylammonium bromide		Data unobtainable	(–)	98

the isosbestic point has been given special prominence in Tables 2 and 3.

Effect of peptides on RK activity

The effect of various peptides on the phosphorylation of bleached rhodopsin, catalysed by RK, was also studied. The results in Figure 3 show that at up to 1 mM the non-farnesyl peptides were without any effect on the phosphorylation reaction. Far-15-mer RK corresponds to the C-terminal sequence of RK and was found to be a moderate inhibitor of the activity of RK, having an IC_{50} of approx. 500 μ M. The IC_{50} remained unchanged when the C-terminal carboxy group was methylated, as in Far-15-mer(OMe) RK. An attempt was made to improve the inhibitory potency of Far-15-mer RK by altering the peptide chain length. An increase of five residues, to give a 20-mer (Far-20-mer RK) had no effect but an additional increase of four residues, to give a 24-mer (Far-24-mer RK), increased the IC_{50} from 500 to 700 μ M. Shortening the length of the peptide also failed to increase the inhibitory potency: a 10-mer (Far-10-mer RK) showed the least inhibition of the farnesyl peptides studied, with an IC_{50} of 1 mM. The geranyl peptide Ger-15-mer RK was completely inactive, whereas the single amino acid mimetic, Far-(NAc)-Cys, and a peptide containing the C-terminal amino acid sequence of T γ (Far-12-mer T γ) showed the same modest inhibition, with an IC_{50} of 800 μ M. However, surprisingly, when the C-terminal farnesylcysteine residue of Far-15-mer RK was transferred to the N-terminus the resulting peptide, Far-(Cys)-14-mer RK, was found to be the most potent inhibitor in this work, with an IC_{50} of 200 μ M.

Particularly important from a mechanistic viewpoint was the finding that the farnesyl peptide also inhibited the RK activity in a completely solubilized system. Detailed studies were performed with Far-15-mer RK, which in 0.4% dodecyl maltoside inhibited the RK activity, although with a decreased potency compared with the membrane system (Figure 3C, ■, □). The reason for

this decrease in inhibition is not known but the possibility exists that the effect is due to the sequestration of the inhibitory peptide by two types of micelle, with or without the substrate Rho*. The overall consequence of such a partitioning would be that a portion of the inhibitor would be unable to gain access to the RK–Rho* complex, thus decreasing its effective concentration.

DISCUSSION

Here we have studied the interaction of farnesyl peptides with the proteins of the visual system spectroscopically and also by measuring their effects on the activity of RK. For the spectral work, advantage was taken of the characteristic absorption maxima of various derivatives of rhodopsin. The 11-*cis*-retinal moiety of rhodopsin is linked to the ϵ -amino group of a lysine residue via a Schiff-base linkage [27–31], later shown to be Lys-296 [17,32,33]. It is the protonation of the Schiff base that causes the red shift of approx. 120 nm, giving rise to an absorption maximum at 498 nm for mammalian rhodopsin. The latter, on photolysis, is converted via a number of transient species into a relatively longer-lived intermediate Meta II, having a maximum absorption at 380–389 nm [34] characteristic of neutral Schiff bases. The latter intermediate, which has been implicated in visual signalling, initially interacts with transducin, promoting signal transmission, and is then deactivated through phosphorylation catalysed by RK (12). *In vitro*, in the absence of signalling proteins, Meta II slowly rearranges to Meta III, having an absorption maximum at 463–465 nm assignable to a protonated Schiff base. The latter species is then hydrolysed to all-*trans*-retinal and opsin [35], although such a reaction has been demonstrated only in detergent solutions [35,36]:



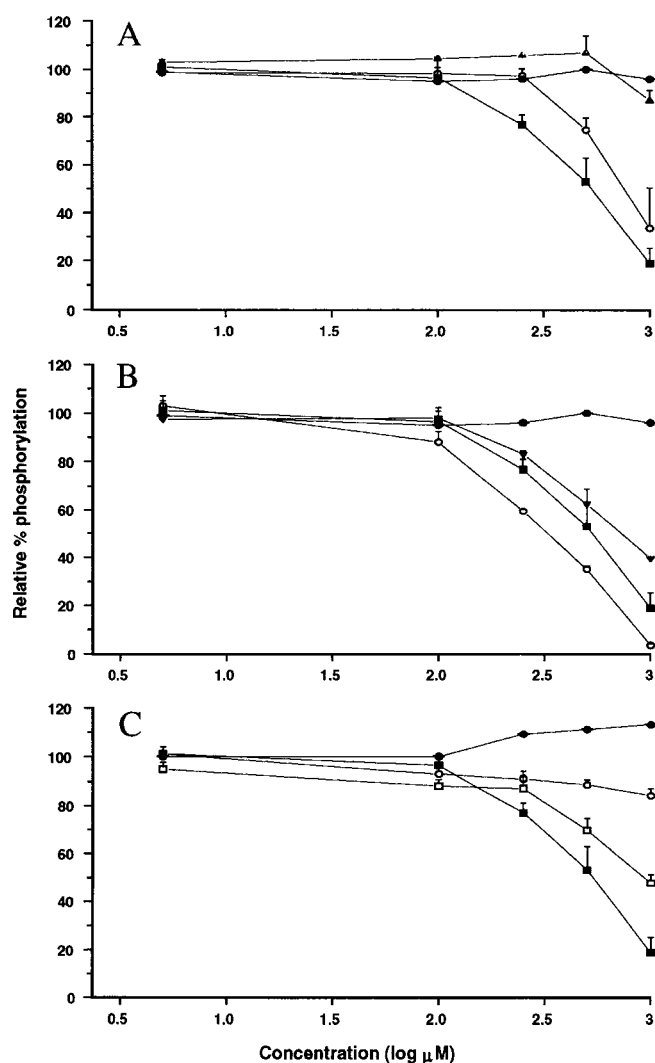


Figure 3 Effect of various peptides on the phosphorylation of Rho* catalysed by RK

Various concentrations of peptides were incubated with urea-washed rhodopsin (2.5 nmol), [γ - 32 P]ATP (3 mM, 80 000 c.p.m./nmol) and RK extract in a 100 μ l standard rhodopsin phosphorylation assay (see the Materials and methods section). Rhodopsin was pelleted, washed and scintillation-counted to determine [32 P]phosphate incorporation, which is expressed as a percentage of the phosphorylation achieved for a standard phosphorylation assay in which there was no peptide. Results are means \pm S.E.M. for three separate experiments. (A) In the presence of 5 μ M to 1 mM 15-mer RK (●), Far-(NAc)-Cys (○), Ger-15-mer RK (▲) and Far-15-mer RK (■). (B) In the presence of 5 μ M to 1 mM 15-mer RK (●), Far-(Cys)-14-mer RK (○), Far-12-mer T γ (▼) and Far-15-mer RK (■). (C) The effect of Far-15-mer RK on the phosphorylation of Rho* in ROS membranes or solubilized in dodecyl maltoside (see the Materials and methods section): phosphorylation of Rho* in ROS membranes in the presence of Far-15-mer RK (■) or dodecyl maltoside (●); phosphorylation of Rho* in the solubilized system in the presence of Far-15-mer RK (□) or additional dodecyl maltoside (○).

The stabilization of Meta II by monitoring the shift of equilibrium from Meta I towards Meta II has previously been observed with transducin in the presence of GDP (but not GTP), a peptide corresponding to the C-terminal sequence of the α -subunit of transducin, and also Far-12-mer T γ [11,24,37,38]. The present study extended the work on Far-12-mer T γ and in addition used farnesyl peptides corresponding to the C-terminal sequence of RK to show that these peptides stabilize Meta II in the

interconversion of Meta II and Meta III. Pfister et al. [39] found that the phosphorylation of bleached rhodopsin by RK was impaired in the presence of the transducin-GDP complex, which indicated that these two proteins might bind to Meta II at overlapping sites. In broad terms, the results with Far-12-mer T γ and Far-15-mer RK lend support to the hypothesis of an overlapping site.

Farnesyl peptides are amphipathic compounds and could exert their effect on bleached rhodopsin by non-specifically altering the physical properties of the surrounding membrane lipids. The effect of detergents and lipids on the interconversion of Meta I and Meta II has been studied extensively [40–42] and it is known that the nature of membrane lipids can profoundly influence the energetics of the conformational states of the species, thus altering the rate at which the conversion of Meta I into Meta II occurs and also the position of the equilibrium [43,44]. From the cumulative spectroscopic data in Table 2, we cannot totally exclude the possibility that the effects shown by the two key farnesyl peptides, Far-12-mer T γ and Far-15-mer RK, were also the consequence of non-specific alterations in membrane properties. However, the coincidence that the farnesyl group, when present in peptides corresponding to the sequence of transducin and RK, clearly affected the interconversion of Meta II and Meta III suggests that in the 500 μ M range their interaction with Meta II or Meta III has a specific component. In qualitative terms, the single amino acid mimetic, Far-(NAc)-Cys, behaved similarly to the two farnesyl peptides, indicating that the farnesylcysteine motif might be a key component of the interaction. Two observations shed further light on this aspect. First, Far-(Cys)-14-mer RK, containing the farnesylcysteine moiety in a non-native arrangement, also retarded the decay of Meta II, although in this case, perhaps, the general membrane effects characteristic of detergents contributed to the absence of a sharp isobestic point. Secondly, the detergent-like behaviour of farnesylthioacetic acid highlights the point that, for a specific effect, the farnesyl moiety alone is not sufficient: it needs to be attached to a suitable peptide vector.

In summarizing the discussion above it needs to be acknowledged that the two farnesyl peptides, Far-12-mer T γ and Far-15-mer RK, that show the most significant spectroscopic effects are by no means the perfect analogues of the protein domains that they represent. For this to be so, the analogues would need to be effective at concentrations one-fifth to one-tenth of those found in this work, so that their non-specific membrane-disruption properties are minimized.

Notwithstanding the reservation above, a stronger case for the fact that the analogue peptides might participate in specific interactions is provided by monitoring their effects on the activity of RK. For an appreciation of the properties of the enzyme, RK does not use the dark-adapted form of rhodopsin or the apoprotein, opsin, as the substrate, but acts with equal facility on Meta II, Meta III and presumably on other Schiff-base derivatives in which all-*trans*-retinal is still linked to the active-site lysine residue of opsin [45]. It therefore follows that factors that might disturb the equilibrium concentration of Meta II, Meta III and other isomerized Schiff-base species would, on their own, not affect the phosphorylation reaction unless these interfered somehow with the formation of catalytically competent complex between Rho* and RK. In the light of the preamble we examine the results in Table 3, in which the compounds used in the spectroscopic and inhibitory studies are assembled into three groups and their effects on the two parameters, Meta II decay and RK activity, are compared, keeping the concentration constant at 500 μ M.

First, the non-farnesyl peptides corresponding to the C-terminal sequence of T γ and RK did not affect the Meta II decay; neither did they inhibit the phosphorylation of rhodopsin.

In the second group the geranyl peptide, Ger-15-mer RK, retarded the Meta II decay slightly but was without any effect on the activity of RK. Of the four farnesylated C-terminal peptides of RK, detailed spectroscopic measurements were performed only on Far-15-mer RK. The latter increased the $t_{1/2}$ of Meta II decay by approx. 60% and was the second most effective inhibitor of the activity of RK. The single amino acid mimetic Far-(NAc)-Cys and the C-terminal peptide corresponding to T γ were at two extremes as promoters of the decay of Meta II (115% and 242% of control respectively), yet they had similar inhibitory effects on the activity of RK. Far-(Cys)-14-mer RK, containing the non-native orientation of the farnesylcysteine moiety, was the most potent inhibitor of the activity of RK and had the second largest $t_{1/2}$ of Meta II decay with a somewhat broad isosbestic point.

In the third group are common amphipathic compounds, which displayed widely different spectroscopic effects: oleic acid clearly slowed the decay of Meta II, whereas dodecyltrimethylammonium bromide accelerated the process, leading to the formation of a complex mixture. However, none of these amphipathic, 'potentially membrane-affecting', compounds at 500 μ M inhibited the activity of RK.

Cumulatively, the findings reported in this paper show that a range of general and specially designed agents can influence the interconversion between various photointermediates of rhodopsin. However, the inhibition of the activity of RK is mediated, in the concentration range up to 500 μ M, only by peptidomimetics that contain the farnesylcysteine motif. It might therefore be argued that in the impairment of phosphorylation the farnesylcysteine group does not act merely as a hydrophobic agent but as a more specific ligand whose delivery to its target site is facilitated when it is appended to sequences characteristic of proteins (RK and transducin) that are known to interact with rhodopsin. That Far-15-mer RK inhibited the activity of RK not only in membranes but also in a fully solubilized system further supports the involvement of specific ligand-protein interactions. However, the contribution that the peptide sequences used in the present study make to the interaction is relatively small, because the farnesyl peptides corresponding to RK and transducin were not markedly more effective as inhibitors of RK than was the single amino acid derivative, Far-(NAc)-Cys. Therefore, although the data point to the role of the farnesylcysteine motif in the interaction, beyond that no firm conclusion can be drawn. A problem often encountered when peptides are used to mimic protein domains is also highlighted here. This concerns the observation that the domain mimetic properties of peptides are not always improved by increasing the chain length, as was found with the C-terminal peptides of RK containing 15, 20 and 24 residues (compare entries X, XI and XII in Table 3). This counter-intuitive behaviour has been attributed to the formation of inappropriate secondary structures by medium-sized peptides that are not, or are only poorly, recognized by the target protein [46].

There are two broad mechanisms to explain the inhibitory properties of the farnesyl moiety. The first assumes that it directly interacts with RK. The classical enzyme inhibitors are generally analogues of the substrate and inhibit by binding to the enzyme's active site. This cannot be so in the present context because here the optimal inhibitory motif is related to the C-terminal domain of the enzyme itself. One way in which this might interact with RK requires the assumption that, as predicted by the consensus sequence, the N-terminus of the enzyme contains

a fatty acyl modification [47] that is suitably positioned close to the farnesyl group to establish lipid-lipid interactions [48], as has been deduced to occur in the structure of transducin [49,50]. The farnesyl motif could disrupt such an arrangement by establishing alternative liaisons and impair the activity of the enzyme. The second mechanism envisages that farnesyl peptides compete with RK for binding to its substrate, Meta II. The spectroscopic data showing the stabilization of Meta II by farnesyl peptides do provide support for the fact that Meta II contains a complementary motif for interaction with the farnesyl moiety. The complementary motif might be the palmitoyl groups attached to Cys-321 and Cys-322 or a site that becomes available after the *cis* \rightarrow *trans* isomerization accompanying the conversion of rhodopsin into Meta II [31].

Of the two possible mechanisms considered here, the second seems more likely because of the spectroscopic evidence suggesting the interaction between farnesyl peptides and Meta II; however, further evidence will need to be provided to establish that the interaction is indeed the primary cause of impairment of the activity of RK.

Irrespective of which of the two mechanisms operate, the results reported in this paper together with those by Kisselev et al. [37,38] show that farnesyl peptides affect the lifetime of Meta II and also impair the processes that depend on the interaction of the latter with the two farnesylated proteins, transducin and RK. This suggests that the recognition of the farnesyl group by its cognate binding site might have an important functional role.

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