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Topography of the Rhodopsin Molecule

IDENTIFICATION OF THE DOMAIN PHOSPHORYLATED

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Studies on the light-stimulated phosphorylation of rod outer segments by [γ-^{32}P]ATP showed that although nearly 1 mol of [^{32}P]phosphate was incorporated/mol of total opsin, only a small fraction of the molecules were phosphorylated, and these contained at least 2–3 mol of phosphate/mol. Rod outer segments containing the phosphorylated opsin were incubated with 11-*cis*-retinal to generate phosphorylated rhodopsin and then digested with papain to produce a cleaved complex comprising three fragments, heavy (H), medium (M) and light (L). It was shown that the L-fragment of apparent mol.wt. 6000 contained all the phosphorylation sites. This suggests that one specific domain of rhodopsin is susceptible to multiple phosphorylation.

Rhodopsin, the photoreceptor substance found in the rod outer segments of the retina, consists of a glycoprotein, opsin, linked to 11-*cis*-retinal via a Schiff base (Akhtar *et al.*, 1965, 1967, 1968; Bownds, 1967). The original observations of Bownds *et al.* (1972) and Kühn & Dreyer (1972) led to the recognition that there is present, in rod outer segments of several species of animals, a kinase that phosphorylates the bleached pigment, opsin, but not rhodopsin. The physiological significance of the phosphorylation reaction has been the subject of extensive investigations in the last 5 years. The most compelling hypothesis, first adumbrated by Kühn (1974) and subsequently supported by Weller *et al.* (1975*a*,*b*) assumes that the phosphorylation reaction is intimately associated with the mechanism of light/dark adaptation. It was found that phosphorylation of opsin in rod outer segments returned the light-induced increase in Ca^{2+} permeability of the disc membrane to its 'dark' value.

An understanding of how photochemically induced conformational changes in rhodopsin alter the membrane conductance requires correlating the structure of various parts of the molecule to their physiological functions. The present paper is concerned with the identification of the domain of opsin whose phosphorylation regulates permeability to Ca^{2+}.

Experimental

Materials

[γ-^{32}P]ATP was from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals and materials were obtained from the sources previously stated (Sale *et al.*, 1977).

Preparation of rod outer segments containing phosphorylated opsin

Rod outer segments were prepared from fresh retinae and processed up to step 2 as previously described (Sale *et al.*, 1977). They were then suspended in 50mM-Tris/HCl, pH 7.4, at 2 A_{500} units/ml, prewarmed at 37°C for 5min and incubated in the presence of 2mM-[γ-^{32}P]ATP and 1mM-$MgCl_2$, either in darkness or in the light, for up to 30min. The incorporation of ^{32}P was determined at various times by washing the rod outer segments (1ml portions) three times with 10% (w/v) trichloroacetic acid (10ml), followed by solubilization in 5% (w/v) sodium dodecyl sulphate (1ml) and counting for radioactivity 0.2ml of the resulting solution in NE260 scintillant as described under 'Radioactivity measurement'. It was found (see Fig. 1) that maximal incorporation of ^{32}P from [γ-^{32}P]ATP was obtained by incubating bleached rod outer segments (i.e. those containing opsin) in the light for 10min.

Preparation of rod outer segments containing phosphorylated rhodopsin

11-*cis*-[15-^{3}H]Retinal of specific radioactivity 24000d.p.m./nmol or unlabelled 11-*cis*-retinal were synthesized and purified as described previously (Akhtar *et al.*, 1968; Hirtenstein & Akhtar, 1970). Rod outer segments containing phosphorylated opsin (prepared by incubation with [γ-^{32}P]ATP in the light for 10min as described above) were regenerated

by adding a 2-fold molar excess of 11-*cis*-[15-^{3}H]-retinal or 11-*cis*-retinal in a small amount of Tween 80 (Hirtenstein & Akhtar, 1970) and incubating in the dark at 37°C for 15min. The regenerated rod outer segments were successively washed and centrifuged (100000*g* for 30min) with water, 2% (w/v) hydroxylamine, 1% (v/v) formaldehyde and water. In the experiments of Figs. 3, 4 and 5, the rod outer segments were then directly analysed, whereas in the experiment of Fig. 2 they were first washed with 10% (v/v) Tween 80 [(Z)-sorbitan mono-9-octadecenoate poly(oxy-1,2-ethanediyl) derivatives] as described previously (Sale *et al.*, 1977). At the concentration used, this detergent selectively removes some non-rhodopsin protein from the rod outer segments and helps remove excess retinal oxime.

Treatment of rod outer segments containing phosphorylated opsin with papain

Rod outer segments ($10A_{500}$ units) were suspended in 10ml of 0.067M-potassium phosphate buffer, pH7, containing 5mM-cysteine and 2mM-EDTA. Papain (1mg) was added and the mixture incubated under dim red light at 37°C for up to 5h. The digestion was terminated at the appropriate time by the addition of iodoacetamide to a concentration of 10mM; the rod outer segments containing papain-cleaved rhodopsin were collected by centrifugation at 190000*g* for 15min and washed twice by re-centrifugation. No loss in A_{500} occurred during proteolysis.

Column chromatography

Native and papain-treated rod outer segments were first washed twice with water and collected by centrifugation at 190000*g* for 15min after each washing to remove buffer if present. The pellets were solubilized by homogenization in 2% (w/v) Ammonyx LO containing 0.01M-imidazole buffer, pH7 ($3–5A_{500}$ units/ml), followed by centrifugation at 100000*g* for 20min to give a clear extract. The solubilized rhodopsin or papain-cleaved rhodopsin was purified by column chromatography under dim red light at 20°C. Calcium phosphate was freshly prepared by the method of Mathews *et al.* (1964) and columns (10cm × 1cm) were equilibrated with 25ml of 2% Ammonyx LO containing 0.01M-imidazole buffer, pH7. Solubilized visual pigment ($5–10A_{500}$ units in 2–4ml) was applied to the column which was then washed with 25ml of 2% Ammonyx LO containing 0.01M-imidazole buffer, pH7, to remove unbound radioactive label. Columns containing the rhodopsin sample were eluted with a 60ml gradient of 5–300mM-potassium phosphate, pH7, containing 2% Ammonyx LO, whereas those of papain-cleaved rhodopsin were eluted with a 60ml gradient of 5–500mM-potassium phosphate, pH7, containing 2% Ammonyx LO. The fractions were analysed spectrophotometrically and the radioactivity was determined in 0.2ml portions as described under 'Radioactivity measurement' by using NE260 scintillant. In all columns the A_{278}/A_{500} ratio of the purified visual pigment was 1.6–1.7 and the A_{278} always followed the A_{500}.

Polyacrylamide-gel electrophoresis

Native and papain-treated rod outer segments were solubilized at 20°C in 5% (w/v) sodium dodecyl sulphate containing 50mM-Na_2CO_3 to give a final protein concentration of 50nmol/ml. About 3nmol of protein was applied to gels (6.3mm × 70mm) containing 12.5% (w/v) acrylamide, 0.416% (w/v) bisacrylamide, 0.1% (w/v) sodium dodecyl sulphate and 6M-urea (Swank & Munkres, 1971). Electrophoresis was performed at 5mA/gel for 6h. The gels were stained with Coomassie Blue and destained by the method of Weber & Osborn (1969) and scanned at 265nm in a Joyce-Loebl densitometer (Joyce, Loebl and Co., Gateshead, Tyne and Wear, U.K.). Apparent molecular weights were estimated as previously described (Sale *et al.*, 1977).

Measurement of visual pigment

This was determined in A_{500} units from the difference spectrum of a bleached and unbleached sample at 500nm ($1A_{500}$ unit/ml = difference-spectrum absorbance of 1 unit at 500nm in 1cm-path-length cell). Where appropriate an $\varepsilon_{500} = 40000$ litre·mol^{-1}·cm^{-1} (Applebury *et al.*, 1974) was used to calculate the molarity of rhodopsin. Thus $1A_{500}$ unit/ml = 25nmol of rhodopsin/ml. As no change in A_{500} occurred during papain digestion of rod outer segments this relationship was used for the determination of papain-cleaved rhodopsin.

Radioactivity measurement

Aqueous samples (0.2ml) containing detergent were counted in 15ml of NE260 scintillant (Nuclear Enterprises, Sighthill, Edinburgh, Scotland, U.K.). Polyacrylamide gels were sliced into 2mm discs. The discs were swollen with 0.8ml of 90% (v/v) NCS tissue solubilizer by incubating overnight at 50°C in tightly capped vials. Scintillation fluid (10ml) containing 8g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4 diazole/litre of toluene was then added. The vials were then assayed for radioactivity in an Intertechnique SL40 liquid-scintillation counter programmed for automatic quench correction.

Results

Preparation of rod outer segments containing phosphorylated rhodopsin

The procedure adopted for identifying the region(s) of the visual protein phosphorylated involved the

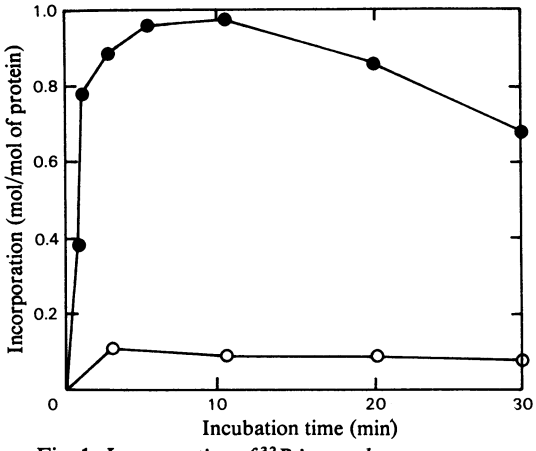
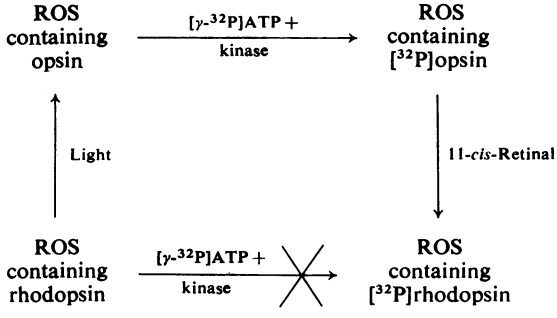


Fig. 1. *Incorporation of ^{32}P into rod outer segments*
Rod outer segments were incubated in the light or in darkness with [γ-^{32}P]ATP and the incorporation of ^{32}P determined at various times by washing the rod outer segments with trichloroacetic acid as described in the Experimental section. Incubation in the dark (○, i.e. phosphorylation of rhodopsin); incubation in the light (●, i.e. phosphorylation of opsin).

incubation of opsin containing rod outer segments with [γ-^{32}P]ATP followed by regeneration and then treatment of the rod outer segments now containing phosphorylated rhodopsin with papain. We showed previously (Sale *et al.*, 1977) that papain cleaves rhodopsin in rod outer segments into a non-covalent complex comprising three fragments, H, M and L



Scheme 1. *Protocol for the preparation of rod outer segments (ROS) containing ^{32}P-labelled rhodopsin*

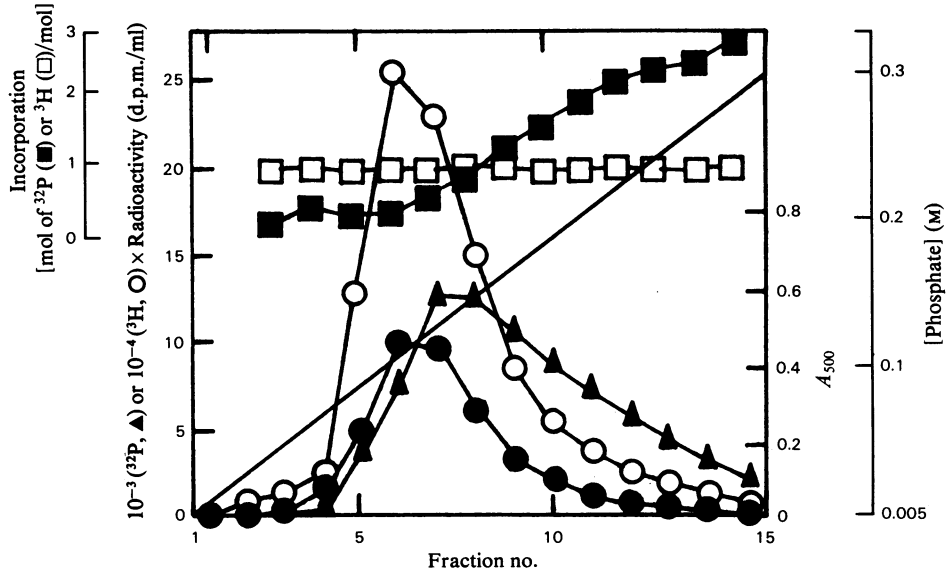


Fig. 2. *Column chromatography of solubilized rod outer segments doubly labelled with ^{32}P and* 11-cis-[15-^{3}H]*retinal*
Rod outer segments were bleached and incubated in the light for 10min with [γ-^{32}P]ATP (sp. radioactivity 2292d.p.m./nmol) under the conditions described in the Experimental section. They were then regenerated with 11-*cis*-[15-^{3}H]-retinal (sp. radioactivity 24000d.p.m./nmol; see the Experimental section) and 8.7A_{500} units subjected to Ammonyx LO/calcium phosphate chromatography. Fractions of 4ml were collected; 8A_{500} units of rhodopsin of $A_{278}/A_{500} = 1.6$ were recovered. Considering all the rhodopsin and ^{32}P eluting in fractions 4–15, 0.72mol of ^{32}P was present/mol of rhodopsin. For individual fractions the mol of ^{32}P and 11-*cis*-[15-^{3}H]retinal present/mol of rhodopsin is indicated by ■ and □ respectively. ●, A_{500}; ——, phosphate concn.

(heavy, medium and light respectively), representing different domains of the molecule. We report here which of the fragments present in the complex contains the phosphorylation site(s).

A preliminary investigation was first conducted to ascertain the conditions that favoured high phosphorylation. It was found that aged, frozen or extensively washed rod outer segments all gave a low incorporation of ^{32}P on incubation with [γ-^{32}P]-ATP. Consequently, rod outer segments were carefully prepared from fresh retinae (see the Experimental section). These rod outer segments were incubated in darkness or in the light with [γ-^{32}P]-ATP, and the incorporation of ^{32}P into protein determined at various times (Fig. 1). Very little ^{32}P was incorporated when the incubation of rod outer segments with [γ-^{32}P]ATP was performed in darkness, but in the light the incorporation was much higher, reaching a maximal value after 10min and was followed by a relatively slower dephosphorylation. The maximum incorporation was about 1mol of ^{32}P/mol of opsin. These results are in accord with previous reports and show that only bleached rod outer segments are phosphorylated, thus implying that it is opsin and not rhodopsin that is the true substrate for the kinase.

The localization of the site(s) of phosphorylation required the preparation of rod outer segments containing phosphorylated rhodopsin. As mentioned above, this was achieved by incubating phosphorylated opsin containing rod outer segments with 11-*cis*-retinal. The regeneration yield was always greater than 85%. The salient features emerging from the observations described so far are summarized in Scheme 1.

Table 1. *Treatment of column-purified phosphorylated rhodopsin with methanol*

Portions (0.2ml) from fractions 5–12 of the column in Fig. 2 were directly counted to determine the specific radioactivity of rhodopsin. The remaining samples were freeze-dried and the residues washed with methanol (2×20ml) then water (20ml) with collection each time by centrifugation at 12000*g* for 15min. The precipitate was then solubilized in 2% (w/v) sodium dodecyl sulphate and its molarity determined from the A_{278} by using $\varepsilon_{278} = 64000$ litre·mol^{-1}·cm^{-1}. The radioactivity content was determined by counting 0.2ml in NE 260 scintillant. The specific radioactivity of the opsin precipitate was then calculated.

Fraction no.	Sp. radioactivity of rhodopsin (d.p.m./nmol)	Sp. radioactivity of opsin precipitate (d.p.m./nmol)
5	642	627
6	687	695
7	1170	1200
8	1860	1825
9	2750	2760
10	3280	3300
11	4170	4150
12	4730	4795

Column chromatography of phosphorylated rhodopsin and its papain-cleaved derivative

A sample of rod outer segments containing phosphorylated rhodopsin that had been regenerated with 11-*cis*-[15-^{3}H]retinal was solubilized in the detergent, Ammonyx LO, and the rhodopsin purified by chromatography on calcium phosphate (Fig. 2). Although the ^{3}H peak exactly followed the A_{500} peak of rhodopsin, the ^{32}P peak was separated, although not completely, from the visual-pigment fractions. Treatment of individual fractions with methanol showed that in each case all the ^{32}P remained associated with the precipitated protein (Table 1). Thus the ^{32}P peak observed in the column eluates represented protein-bound radioactivity and was not due to label incorporated into phospholipid. The amount of ^{32}P associated with the rhodopsin eluted from the column corresponded to a stoicheiometry of between 0.15 and nearly 3mol/mol of rhodopsin, with the most highly phosphorylated rhodopsin being the most strongly adsorbed to the

Table 2. *Digestion of rod outer segments containing ^{32}P-labelled rhodopsin with papain*

Various batches (a–e) of ^{32}P-labelled rod outer segments prepared as in Fig. 2, but regenerated with non-radioactive 11-*cis*-retinal were incubated with papain for various times and the reactions terminated with iodoacetamide. Portions of rod outer segments were removed, and the ^{32}P content determined after washing with trichloroacetic acid as described in the Experimental section.

Time of incubation with papain	Batch ...	^{32}P content of rod outer segments (d.p.m.)				
		a	b	c	d	e
0		3500	7200	4100	8600	8400
10min		1500	3400	—	9100	—
20min		1100	2650	900	8900	—
60min		550	2500	850	8500	6300
5h		—	1900	870	8800	—

column. However, when all the fractions under the rhodopsin peak were pooled, a value of 0.72mol of ^{32}P bound/mol of rhodopsin was obtained. This average stoicheiometry is similar to that obtained by several other workers using the less critical approach of the type used in the experiments of Fig. 1, and has been generally accepted to mean that one specific site of opsin is phosphorylated. However, the more

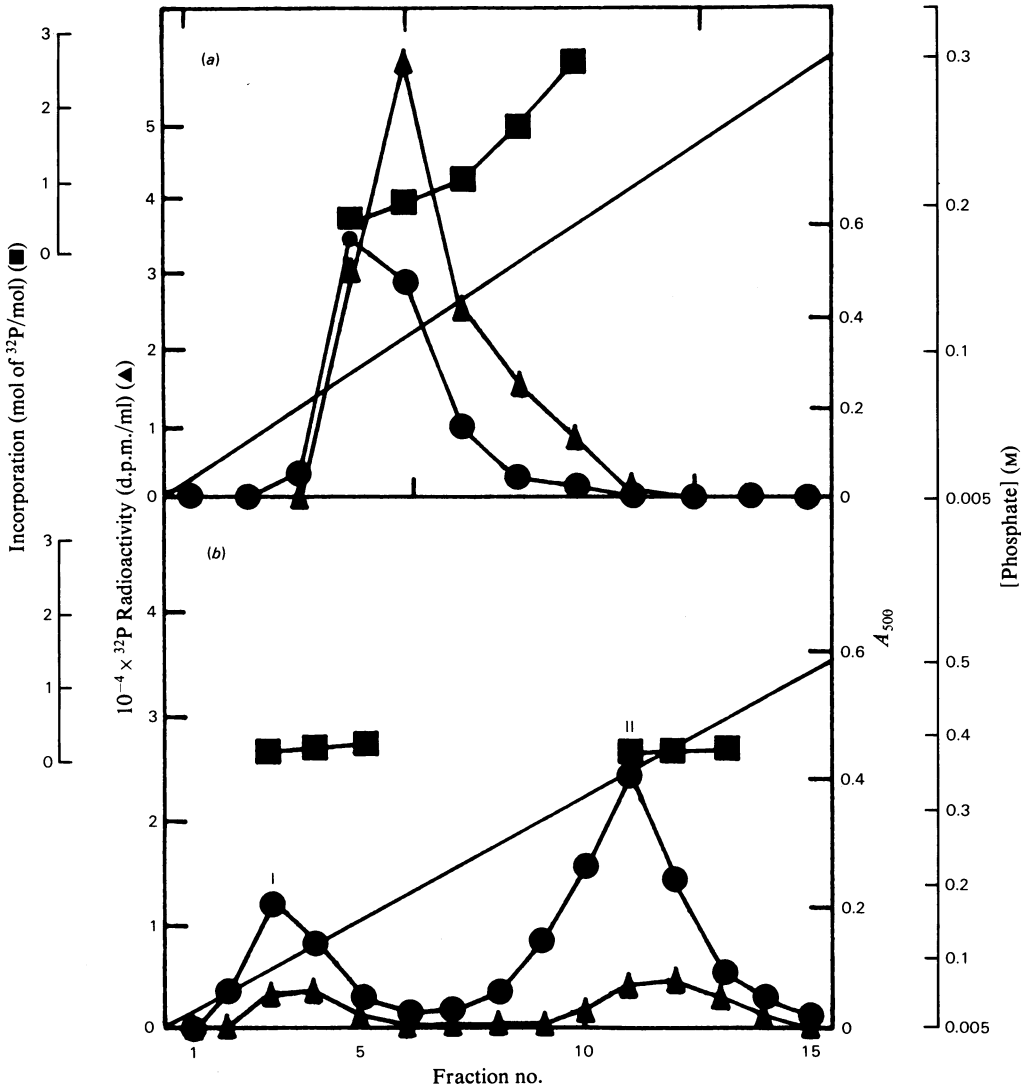


Fig. 3. *Column chromatography of solubilized rod outer segments (batch 1) containing rhodopsin or papain-cleaved rhodopsin labelled with ^{32}P*

The rod outer segments were phosphorylated by incubation in the light with [γ-^{32}P]ATP (sp. radioactivity 6900d.p.m./nmol) for 10min, then regenerated with 11-*cis*-retinal and a portion incubated with papain for 1h; (*a*) and (*b*) show the analysis of the undigested and digested rod outer segments respectively. The recovery of visual pigment was about 90% in both columns. Fractions of 5ml and 4ml were collected in (*a*) and (*b*) respectively. In (*a*) the specific radioactivity of the rhodopsin peak (fractions 3–8 combined) = 4243d.p.m./nmol, which corresponds to 0.61mol of ^{32}P/mol of rhodopsin. In (*b*) the specific radioactivity of peak I (fractions 1–6 combined) was 889d.p.m./nmol, which corresponds to 0.13mol of ^{32}P/mol of visual pigment, and peak II (fractions 7–15 combined) was 608d.p.m./nmol, which corresponds to 0.09mol of ^{32}P/mol of visual pigment. ●, A_{500}; ——, phosphate concn.

rigorous analysis by column chromatography suggests that this interpretation is incorrect. Also, Shichi *et al.* (1974) found that on chromatography of ^{32}P-labelled isorhodopsin (the pigment regenerated using 9-*cis*-retinal is called isorhodopsin) on agarose columns the ^{32}P and visual-pigment peaks did not co-elute and therefore inferred that only a minor fraction of rhodopsin molecules in rod outer segments were in fact phosphorylated. The results presented above are in agreement with this hitherto ignored proposal and suggest that the phosphorylated molecules contain at least two to three phosphorylation sites.

Table 2 shows that incubation of rod outer seg-

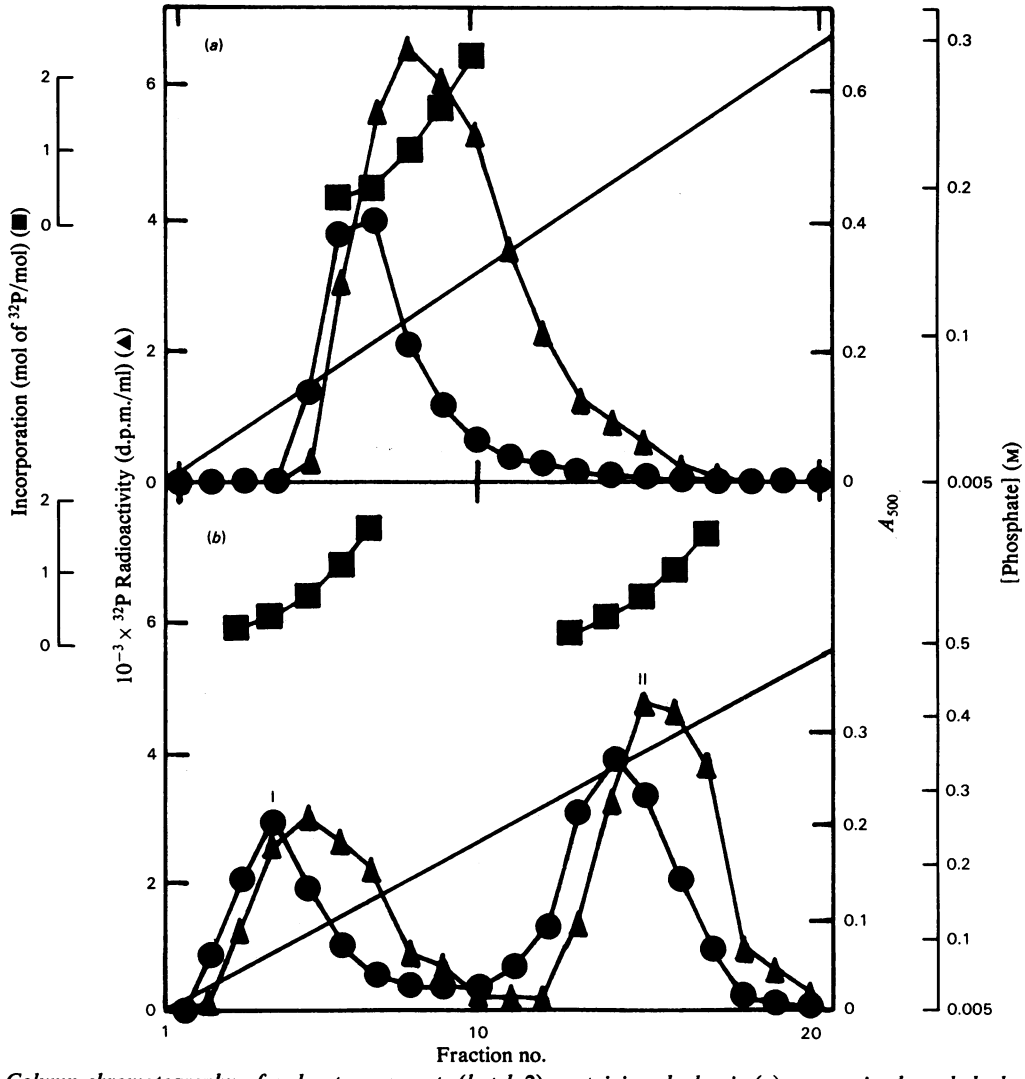


Fig. 4. *Column chromatography of rod outer segments (batch 2) containing rhodopsin (a) or papain-cleaved rhodopsin (b) labelled with ^{32}P*

The rod outer segments were phosphorylated, regenerated and analysed as described in the legend to Fig. 3. Fractions of 3ml were collected and the recovery of visual pigment was about 90% in both columns. In (*a*) the specific radioactivity of the rhodopsin peak (fractions 4–15 combined) was 1018d.p.m./nmol, which corresponds to 0.75mol of ^{32}P/mol of rhodopsin. In (*b*) the specific radioactivity of peak I (fractions 1–10 combined) was 800d.p.m./nmol, which corresponds to 0.6mol of ^{32}P/mol of visual pigment, and that of peak II (fractions 11–20 combined) was 770d.p.m./nmol, which corresponds to 0.57mol of ^{32}P/mol of visual pigment. The specific radioactivity of the [γ-^{32}P]ATP initially used in this experiment was 1350d.p.m./nmol. ●, A_{500}; ——, phosphate concn.

ments containing phosphorylated rhodopsin with papain resulted in loss of protein-bound ^{32}P, but the rate of loss was extremely variable and dependent on the batch of rod outer segments used. As rod outer segments are known to undergo a phosphorylation–dephosphorylation cycle (for example, see the loss of ^{32}P after 10min in the experiment of Fig. 1) they must contain both a kinase and a phosphatase. The loss of ^{32}P from ^{32}P-labelled rod outer segments could be due to the variable activity and/or amount of this phosphatase. To critically define the fate of ^{32}P during papain digestion, it was important to decrease the extent of the dephosphorylation reaction. In strategic experiments, therefore, the period of digestion with papain was decreased to 1h. This experimental design had the added advantage of producing rod outer segments that contained not only the papain-cleaved complex, but also the parent rhodopsin. It was thus possible to compare the ^{32}P content of the two species that had been exposed to identical biochemical treatments.

Two types of papain-treated rod outer segments were processed, one in which only 20% of the original protein-bound ^{32}P was retained after a 1h digestion period, and the other where the retention was as high as 70%. Portions of these digested rod outer segments were solubilized in Ammonyx LO and subjected to calcium phosphate column chromatography (Figs. 3 and 4). With each sample it was found that the specific radioactivities of the visual pigment eluting in the position of rhodopsin (peak I) was similar to that of the cleaved complex in peak II. This similarity in specific radioactivities indicates that the loss of ^{32}P during papain treatment, when it occurs, is not due to the removal of a phosphopeptide, but a consequence of the activity of a phosphatase.

In passing, it is noteworthy that phosphorylated cleaved rhodopsin is more strongly adsorbed to the column than is its non-phosphorylated counterpart. This behaviour parallels that already observed for rhodopsin and phosphorhodopsin.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The fate of ^{32}P during the conversion of rhodopsin into cleaved rhodopsin was also studied electrophoretically. The rod outer segments containing ^{32}P-labelled rhodopsin (as analysed by column chromatography in Fig. 4*a*) when dissolved in sodium dodecyl sulphate and electrophoresed gave most of the ^{32}P radioactivity on the gel in the opsin band (Fig. 5*a*). Solubilization of papain-treated, ^{32}P-labelled rod outer segments (from the batch analysed by column chromatography in Fig. 4*b*) in sodium dodecyl sulphate, followed by electrophoresis, gave 32% of the radioactivity on the gel in the ospin band, whereas 51% co-electrophoresed with the L-fragment and less than 5% corresponded with either the H- or M-fragments (Fig. 5*b*). Thus it appears that the L-fragment of papain-cleaved rhodopsin contains the phosphorylation sites of rhodopsin. It is noteworthy that the ratio (^{32}P in opsin)/(^{32}P in L-fragment) is similar to the ratio (^{32}P in peak I)/(^{32}P in peak II) of the column shown in Fig. 4*b*.

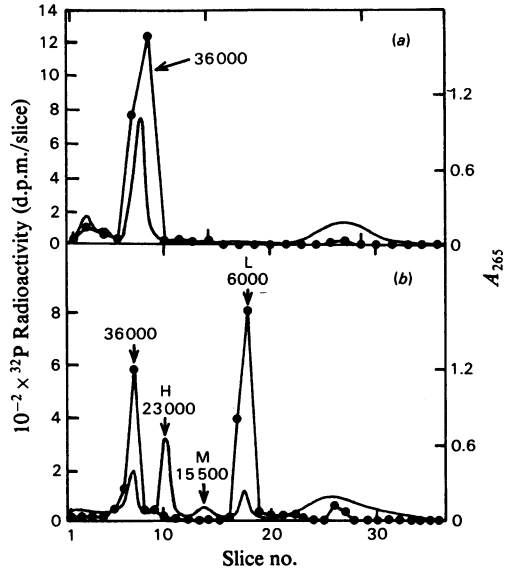


Fig. 5. *Polyacrylamide-gel electrophoresis of native and papain-treated ^{32}P-labelled rod outer segments*

^{32}P-labelled rod outer segments and those treated with papain were prepared as in Fig. 4 and contained 0.8 and 0.6mol of ^{32}P/mol of visual pigment respectively. Undigested (*a*) and digested (*b*) rod outer segments containing 3 and 3.5nmol of visual pigment respectively, were then solubilized in sodium dodecyl sulphate and electrophoresed. Of the visual-pigment-bound radioactivity originally applied, 87 and 86% was recovered in the protein bands in (*a*) and (*b*) respectively. ●, Radioactivity; ——, A_{265}

Discussion

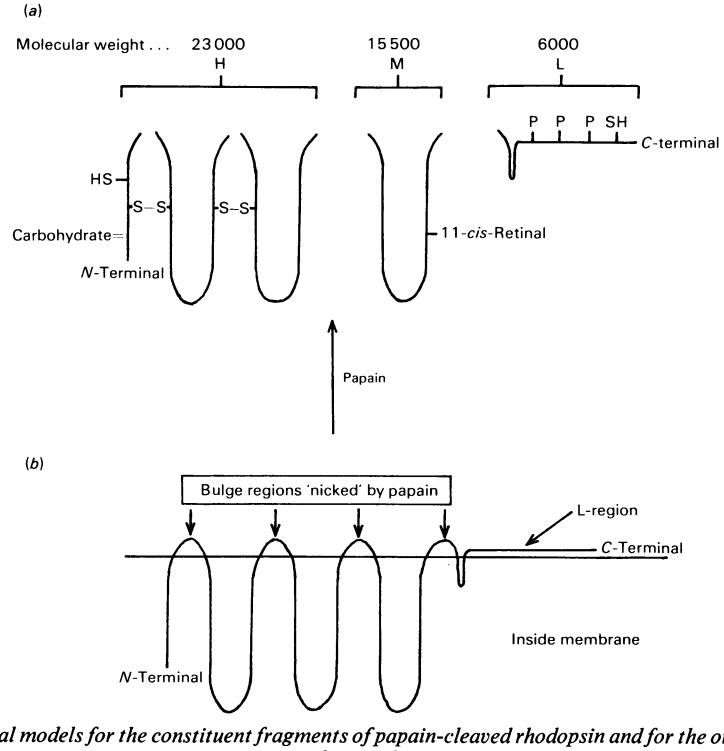
The light-induced phosphorylation of rod outer segments has been widely investigated because of its probable physiological role. It has been shown that although the bleached pigment, opsin, may be phosphorylated, rhodopsin itself is a poor substrate for the kinase (Kühn & Dreyer, 1972; Bownds *et al.*, 1972; Kühn *et al.*, 1973; Weller *et al.*, 1975*c*; Frank & Buzney, 1975; Miller & Paulsen, 1975; McDowell & Kühn, 1977). Weller *et al.* (1975*a*) have observed that phosphorylation of opsin lowers the Ca^{2+} permeability of the disc membrane back to its 'dark'

value. These results are usually rationalized by implicating the phosphorylation–dephosphorylation sequence in the mechanism of light/dark adaptation (Kühn, 1974; Miller & Paulsen, 1975; Weller *et al.*, 1975*b*).

It is thus important to localize the site(s) of light-stimulated phosphorylation in rhodopsin. Virtually the only attempt in this direction is that of Virmaux *et al.* (1975) who treated rod outer segments containing [^{32}P]phosphorylated opsin with papain, and observed the loss of ^{32}P. However, it now appears that that work was complicated by the action of a phosphatase and an inadequate knowledge of the way the protein was cleaved by papain. We have now found that when ^{32}P rhodopsin was eluted from calcium phosphate columns the peak of ^{32}P trailed behind the A_{500} of rhodopsin (see Fig. 2). Based on the specific radioactivity of the label and using the A_{500} to calculate the molarity of rhodopsin, the rhodopsin eluting in fractions 12–14 of the column shown in Fig. 2 contains 2–3 mol of ^{32}P/mol, yet that eluting in fractions 4–7 (which represents well over half the total rhodopsin eluted from the column) contains a negligible amount of ^{32}P. This suggests that during the phosphorylation reaction only a small fraction of the opsin molecules are phosphorylated and that these contain at least two to three sites of phosphorylation.

Since in papain-cleaved rhodopsin all the phosphate was found to be associated with the L-fragment (Fig. 5), the L-fragment must contain a cluster of at least two to three phosphorylation sites. This implies that one specific region of opsin is susceptible to multiple phosphorylation.

In the experiments *in vitro* described above, the region of rhodopsin phosphorylated must be accessible to both the enzyme responsible for the reaction and to exogenously added ATP. ATP is presumably membrane-impermeable. The opsin kinase is soluble in water and can easily be extracted from rod outer



Scheme 2. *Hypothetical models for the constituent fragments of papain-cleaved rhodopsin and for the organization of rhodopsin in the membrane*

(*a*) The location of the chemical features of rhodopsin known to date, namely two thiol groups, two disulphide bonds, 11-*cis*-retinal and phosphorylation sites, in papain-cleaved rhodopsin. (*b*) The arrangement of rhodopsin in the membrane as deduced from the mode of cleavage by papain. As the L-fragment contains a multiplicity of phosphorylation sites and an easily accessible thiol group, this would suggest it may be largely located in the aqueous phase outside the membrane, in contrast with the remaining bulk of the rhodopsin molecule, which is probably embedded in the membrane. For further discussion of model see text.

segments (Kühn *et al.*, 1973; Weller *et al.*, 1975*c*), which suggests that the enzyme occurs in an aqueous region or is very loosely associated with the membrane. Thus it would be expected that the phosphorylation sites reside on a region of the molecule in an aqueous phase, indicating that the L-fragment, or at least part of it, has a surface location (most likely on the interdiscal surface) as opposed to being completely buried in the hydrophobic interior of the membrane. Furthermore the L-fragment contains a thiol group that in membrane-bound rhodopsin is accessible to the relatively hydrophylic alkylating agent, 5-iodoacetamidosalicylate. This is in contrast with a thiol group located in the H-fragment, which in membrane-bound rhodopsin cannot be modified by 5-iodoacetamidosalicylate (Sale *et al.*, 1977).

When the results described in the present paper are taken in conjunction with our previous results (Towner *et al.*, 1977; Sale *et al.*, 1977), it is apparent that six strategic chemical features of the rhodopsin molecule have so far been highlighted. The retinal-binding site resides in the M-fragment, and the H- and L-fragments each contain a different thiol group. In addition the L-fragment contains a multiplicity of phosphorylation sites. Furthermore, the H-fragment consists of at least three polypeptides (each of apparent mol.wts. in the range 9000–4000) joined by two disulphide bonds, whereas both the M- and L-fragments are single polypeptide chains. Thus the production of papain-cleaved rhodopsin involves the cleavage of at least four sites of rhodopsin. Assuming that papain cannot penetrate into the membrane then this mode of cleavage favours a model in which the rhodopsin polypeptide chain alternately folds in and out the membrane forming a series of 'bulge' regions that just extend outside the membrane. As the L-fragment contains several phosphorylation sites and an easily accessible thiol group, this indicates that this represents a domain of rhodopsin that is largely located outside the membrane, in contrast with the remaining bulk of the rhodopsin molecule. A hypothetical model incorporating these structural features is presented in Scheme 2.

Note Added in Proof (Received 7 July 1978)

After acceptance of this paper for publication we learnt that Professor Hargrave (private communication) has proposed a similar model for the skeletal outline of rhodopsin. Furthermore, his group has shown that the thiol group modified by iodoacetylamidosalicylate resides in a polypeptide arising from the *C*-terminal region which interestingly contains an abundance of serine and threonine residues that might be potential phosphorylation sites (McDowell & Griffith, 1978). Thus it may be deduced that the L-fragment represents the *C*-terminal of rhodopsin.

The H-fragment reacts positively with performic acid/Schiff reagent (Sale, 1978) suggesting it contains the carbohydrate of rhodopsin. As available evidence indicates that rhodopsin only contains two sites of carbohydrate attachment both located within 15 amino acid residues of the block *N*-terminal amino acid (Hargrave, 1977) it is likely that the two oligosaccharides and the blocked *N*-terminal reside in the H-fragment. Furthermore carbohydrate has been cytochemically localized only at the inner disc surface (Röhlich, 1976). As the blocked *N*-terminal region is highly hydrophylic (Hargrave, 1977) it is reasonable to propose that this region of rhodopsin (which is present in the H-fragment) is located outside the inner surface of the disc membrane and not buried in the lipid bilayer. Because the H-fragment probably contains the blocked *N*-terminal and the L-fragment the *C*-terminal it follows that the M-fragment fits in between the H- and L-fragments.

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