Phosphorylation of ovine rhodopsin

Identification of the phosphorylated sites

Paul THOMPSON and John B. C. FINDLAY

Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

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Light-dependent phosphorylation of sheep opsin was obtained in purified discs to which was added a partially purified preparation of rhodopsin kinase. A maximum ratio of 1.8 mol of phosphate/mol of rhodopsin bleached was obtained. Perturbing the lipid bilayer did not alter the phosphorylation ratio. Dephosphorylation in both segments and discs was only achieved when the supernatant fraction from a retina homogenate was added. Complete dephosphorylation required the presence of the detergent dodecyltrimethylammonium bromide in the incubation medium. Treatment of phosphorylated disc membranes with Staphylococcal aureus V8 proteinase generated two membrane-bound fragments, only one of which (V8-S, Mr 12000) was labelled, together with a soluble seven-residue peptide that contained $^{32}$P phosphoserine. Peptide sequencing, together with subdigestion procedures, localized the phosphorylation sites to serine residues at positions 334, 338 and 343 in the whole sequence and threonine residues at positions 335 and 336.

On photon capture the chromophore of rhodopsin isomerizes from the 11-cis to the all-trans configuration, and a series of conformational changes in the protein moiety is initiated. At one of these stages [probably the lumirhodopsin–meta-rhodopsin transition (Paulsen & Bentrop, 1983)], opsin becomes a temporary substrate for a specific cyclic nucleotide-independent kinase present in the outer segment (McDowell & Kuhn, 1977; Shichi & Somers, 1978). This light-activated phosphorylation has been observed in vitro by using $^{32}$P ATP and isolated bovine ROS (Bownds et al., 1972) and in vivo by injecting $^{32}$P phosphate into living frogs (Kuhn, 1974). In both cases the rate of phosphorylation was slow relative to the events of transduction, thereby effectively eliminating any primary involvement in the generation of the rod-cell hyperpolarization.

As part of a study into the structure of rhodopsin, the amino acid sequence of the ovine protein has been determined (Findlay et al., 1981; Brett & Findlay, 1983) and within that sequence the attachment sites of chemical probes have been identified (Barclay & Findlay, 1984). In this respect, light-dependent phosphorylation can be regarded as a physiological hydrophilic probe that can reveal information on the structure of the protein and on some of the complex conformational changes that accompany bleaching.

The present work describes some of the characteristics of the phosphorylation of ovine rhodopsin and presents the sequence of the phosphorylated region, identifying those residues to which the phosphate groups become attached.

Experimental

Materials

All chemicals were obtained from BDH (Poole, Dorset, U.K.), unless listed below. AnalR-grade chemicals were used when available. $^{32}$P Orthophosphate was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.) and Dowex 1 (X4) and Bio-Gel P resins from Bio-Rad Laboratories (Bromley, Kent, U.K.). For other reagents see Abu Salah et al. (1982), Brett & Findlay (1983) and Pappin & Findlay (1984).
Methods

Extraction of rhodopsin kinase. About 70 dark-adapted sheep retinas were illuminated for 5 min with a 100 W lamp and immediately gently homogenized manually in a total volume of 90 ml of ice-cold 0.1 M-Tris/HCl buffer, pH 7.4, made 50% (w/v) with respect to sucrose. All subsequent steps, excluding the dialysis, were performed in the dark or under dim red light. The homogenate was centrifuged for 15 min at 65 000 g, and floating material, consisting of ROS, carefully removed. The segment suspension was adjusted to 50 ml with 1 M-NH₄Cl, 50 mM-Tris/HCl, pH 7.4, and incubated for 45 min at room temperature. After thorough homogenization, the suspension was centrifuged at 45 000 g for 15 min. The supernatant, containing rhodopsin kinase, was decanted and extensively dialysed against ice-cold 10 mM-ammonium bicarbonate (5 x 5 litres). The preparation was then freeze-dried and stored at -20°C until required.

Preparation of [32P]ATP. [γ-32P]ATP was synthesized by a modification of the method of Glynn & Chapell (1964). The enzymic exchange between [32P]phosphate and unlabelled ATP was allowed to proceed for 2 h (Thatcher, 1978). [32P]ATP was purified by anion-exchange chromatography on Dowex 1 (X4) resin with a linear gradient of 500 ml of 10 mM-NH₄HCO₃ and 500 ml of 1 M-NH₄HCO₃. After removal of residual NH₄HCO₃ by repeated freeze-drying and resuspension in distilled water, the product was checked for purity by PEI-cellulose t.l.c. in 0.85 M-LiCl and stored at -20°C in 40 mM Tris/HCl buffer, pH 7.4, adjusted to 0.05% (v/v) with respect to ethanol.

Phosphorylation. Phosphorylation was performed in disc membranes with added kinase extract. Phosphorylation conditions were, unless otherwise stated, 2 mM [32P]ATP, 3 mM MgCl₂, and 40 mM-Tris/HCl, pH 7.4. The specific radioactivity of [32P]ATP, determined for each experiment, was usually between 10⁴ and 10⁵ d.p.m./nmol. Phosphorylation ratios were expressed as mol of phosphate incorporated per mol of rhodopsin bleached. Rhodopsin assays based on A₅₀₀ were performed as described by Pappin & Findlay (1984).

Disc membranes, purified as described by Brett & Findlay (1979), and kinase extract, were added to prewarmed buffer/ATP solution under dim red light such that the disc membrane protein concentration was 3 mg/ml and that of the kinase extract was up to 11 mg/ml (dry weight of freeze-dried material). The reaction was allowed to proceed either in the light or in the dark at 37°C for up to 60 min. Phosphorylation was assayed at intervals by subjecting samples to polyacrylamide-gel electrophoresis in SDS (Weber & Osborn, 1969) or SDS/urea (Swank & Munkres, 1971) and measuring the 32P content of gel slices or alternatively, since opsin was the only significant species phosphorylated, by sampling the mixture (50–100 μl) into 600 μl of ice-cold 10% (w/v) trichloroacetic acid/1 M-H₃PO₄. Precipitated material was recovered by centrifugation, washed eight times with 1 ml portions of the trichloroacetic acid/phosphoric acid solution and solubilized in NCS tissue solubilizer. Portions of the solubilized precipitates were removed, neutralized with acetic acid and mixed with 4 ml of scintillation cocktail.

Dephosphorylation. After phosphorylation, excess [32P]ATP was removed by diluting the reaction mixture with 10 vol. of 40 mM-Tris/HCl, pH 7.4, and centrifuging for 15 min at 45 000 g. The pellets were resuspended to a protein concentration of 1 mg/ml in 40 mM-Tris/HCl (pH 7.4)/3 mM-MgCl₂ and incubated at 37°C. Portions were periodically removed into 600 μl of 10% (w/v) trichloroacetic acid/1 M-H₃PO₄ and the precipitated material was washed, solubilized and assayed for 32P as described for phosphorylation in disc membranes.

Supernatants of retina homogenates were prepared by diluting ten fresh retinas to 10 ml with 40 mM-Tris/HCl (pH 7.4)/3 mM-MgCl₂ and homogenizing with a Potter homogenizer for 2 min at 4°C. The suspension was centrifuged for 20 min at 45 000 g and the supernatant removed. These supernatants were added to phosphorylated opsin preparations to give protein concentrations of 8 and 1 mg/ml respectively, and the mixtures were incubated at 37°C as described above.

Protein methods. Phosphorylated discs were subjected to digestion with S. aureus V8 proteinase (Brett & Findlay, 1983) and the supernatant freeze-dried and subjected to gel filtration with Sephadex G-50 and Bio-Gel P resin in 20% (v/v) formic acid or 5% (v/v) acetic acid. The membrane-bound fraction was reduced, carboxymethylated and the V8-L (Mr, 27 000) and V8-S (Mr, 12 000) fragments separated by using Sephadex LH-60 as described previously (Brett & Findlay, 1983).

Subdigestion procedures. Cleavage with CNBr was performed in 70% (v/v) formic acid as previously described (Pappin & Findlay, 1984). Cleavage at lysine residues was achieved in 0.1 M-NH₄HCO₃ with Tos-Phe-Ch₂Cl-treated trypsin (5%, w/w, with peptide). The mixture was incubated at 37°C with stirring, for 6 h, after which time a further portion of fresh trypsin was added and the material was freeze-dried after a second 6 h incubation. Digestion of peptides with S. aureus V8 proteinase was performed in a similar manner, except the digestion times were increased to 18 h.

Peptide analysis. 32P-labelled peptides were partially hydrolysed with 6 M-HCl at 110°C for 2 h in sealed evacuated tubes. Phosphorylated amino acids were separated by electrophoresis at 3 kV for
90 min on Whatman 3MM chromatography paper with acetic acid/90% (v/v) formic acid/water (2:1:25, by vol.) as solvent, and identified by autoradiography and staining with fluorescamine. Amino acid analysis and peptide sequencing was carried out as described by Brett & Findlay (1983). The coupling of peptides to resins with EDC was performed as described by Laursen (1977). Protein, in the 10–50 μg range, was determined by the manual ninhydrin method after alkaline hydrolysis of the sample (Hirs, 1967).

Samples were counted for radioactivity in a Beckman LS230 liquid-scintillation counter. The scintillation cocktail used was Triton X-100/toluene (1:2, v/v), containing 0.4% (w/v) PPO (2,5-diphenyloxazole) and 0.02% (w/v) POPOP [1,4-bis-(5-phenyloxazo-2-yl)benzene]. Before mixing with this scintillation cocktail, gel slices were solubilized by incubation at 37°C in 30% (v/v) H2O2 containing 1% (v/v) 0.88-sp.g. NH3 (150 μl/3 mm gel slice). The 32P radioactivity in column eluates was assayed by Čerenkov counting.

**Results**

**Characterization of phosphorylation**

Irradiation of discs, in contrast with ROS, with light in the presence of [32P]ATP resulted in no significant phosphorylation of opsin. Reconstitution of the phosphorylating system was possible by the addition of kinase previously extracted from ovine ROS using the 'in situ' affinity method (Fig. 1). The reaction in discs, as in ROS, was slow relative to the events of transduction, a result similar to that obtained for bovine and frog rhodopsin (Miller et al., 1977; Shichi & Somers, 1978). Phosphorylation ratios for disc membranes were 1.42 ± 0.36 (10) mol of phosphate incorporated/mol of rhodopsin bleached.

After brief illumination, the ability to phosphorylate rhodopsin with kinase extract decayed with time. This decay was not due to kinase inactivation during the course of the experiment, since incubation of kinase at the end of the time course with freshly bleached rhodopsin gave 88% of the phosphorylation observed at the beginning of the experiment.

Illumination of rhodopsin in the presence of hydroxylamine results in very rapid bleaching with premature formation of opsin. The speed of this process suggested that it could exclude, or at least reduce, the lifetime of later conformational changes associated with the bleaching sequence. Inclusion of 10 mM-hydroxylamine in the phosphorylation assay decreased light-stimulated phosphorylation to 14.8 ± 0.5% of the control value. Both sets of experiments, therefore, lead to the conclusion that freshly bleached rhodopsin is only a temporary substrate for the kinase.

**Phosphorylation in altered systems**

Certain hydrophobic agents are known to perturb the activity of membrane-transport proteins. In particular, the hydrophobic anaesthetics althesin and propanidid inhibit transport of glucose and phosphate across the membrane of the human erythrocyte (Abu Salah et al., 1982). The effect of these agents and cholesterol on light-induced phosphorylation of rhodopsin in disc membranes was studied. No significant inhibitory effects were observed (Table 1).

**Dephosphorylation**

After removal of excess [32P]ATP, incubation of phosphorylated discs did not reveal significant

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**Fig. 1. Effect of kinase extract on light-stimulated disc phosphorylation**

Discs and [32P]ATP were incubated in continuous light or in the dark in the presence (○) or absence (●) of kinase extract. Each point represents the difference between the degree of phosphorylation in the light and in the dark. Phosphorylation was assayed by precipitation with trichloroacetic acid.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative phosphorylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Althesin (1 mM)</td>
<td>102</td>
</tr>
<tr>
<td>Propanidid (1 mM)</td>
<td>113</td>
</tr>
<tr>
<td>Cholesterol (1 mM)</td>
<td>94</td>
</tr>
</tbody>
</table>

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dephosphorylation. If, however, phosphorylated discs were incubated with the supernatant from a whole retina homogenate, partial dephosphorylation occurred. The addition of a further portion of the supernatant during the course of the experiment or sonication of the disc preparation during the incubation did not increase phosphate removal. However, almost total dephosphorylation resulted if the discs were dissolved in the ionic detergent DTAB before the addition of supernatant. The detergent alone did not induce any dephosphorylation. No such increases in dephosphorylation were observed, however, when digitonin was used as the detergent in place of DTAB.

Gel filtration of the radioactivity released in such experiments [with Sephadex LH-20 resin equilibrated with 90% (v/v) formic acid/ethanol (3:7, v/v)] demonstrated it to be free of associated peptide material. This excludes proteolysis as a possible explanation for the observed loss of phosphate, a conclusion supported by the inability of proteinase inhibitors, such as phenylmethanesulphonyl fluoride, to inhibit dephosphorylation.

Structural studies

(a) Soluble 32P radioactivity. S. aureus-V8-proteinase treatment of phosphorylated disc membranes released 29% of the membrane-bound radioactivity into the supernatant. Gel filtration of this soluble material on Sephadex G-50 in 5% (v/v) acetic acid revealed two radioactive peaks, one being at the column volume. The included and column-volume pools were re-chromatographed on Bio-Gel P4 and P2 respectively. Amino acid analysis showed the Bio-Gel-P4-purified radioactivity to be associated with a heptapeptide (denoted as peptide C, Fig. 4 below) of composition:

$$\text{Thr}_{0.9}\text{Ser}_{1.04}\text{Glu}_{1.03}\text{Pro}_{0.92}\text{ Ala}_{2.06}\text{Val}_{1.03}$$

[32P]Phosphoserine was detected when a partial acid hydrolysate of this pool was subjected to high-voltage paper electrophoresis. A similar investigation of the Bio-Gel-P2-purified material, which again was eluted at the column volume, revealed the absence of amino acids, suggesting that it was free phosphate.

The 32P-labelled heptapeptide was sequenced automatically after coupling to aminopolystyrene, via its C-terminal carboxy group, with EDC. The radioactive content of the various rounds revealed the presence of [32P]phosphoserine at round 2 (Table 3 below). This peptide represented the C-terminus of the intact protein. Manual sequencing confirmed its structure.

![Fig. 2. Fractionation of CNBr-1 tryptic fragments on Sephadex LH-20](image)

Digested material was solubilized in 90% (v/v) formic acid/ethanol (3:7, v/v) (Gerber et al., 1979) and applied to a column (135 cm x 2 cm) of Sephadex LH-20 equilibrated with this solvent. The column eluate was pooled as indicated. Ninhydrin-positive material eluted in the column volume represents NH$_3$HCO$_3$ remaining after freeze-drying of the digest. O, $A_{570}$ (manual ninhydrin); ○, radioactivity.
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Table 2. Amino acid composition of tryptic and S. aureus-V8-proteinase (V8) peptides of CNBr-1

<table>
<thead>
<tr>
<th>Composition (residue/molecule)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>CM-Cys</td>
</tr>
<tr>
<td>Asx</td>
</tr>
<tr>
<td>Thr</td>
</tr>
<tr>
<td>Ser</td>
</tr>
<tr>
<td>Gly</td>
</tr>
<tr>
<td>Ala</td>
</tr>
<tr>
<td>Val</td>
</tr>
<tr>
<td>Leu</td>
</tr>
<tr>
<td>Lys</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N-Terminus</th>
<th>Leu</th>
<th>Asx</th>
<th>Thr</th>
<th>Leu</th>
<th>Ala</th>
</tr>
</thead>
</table>

(b) Membrane-bound $^{32}P$ radioactivity. S. aureus-V8-proteinase treatment of disc membranes also cleaved phosphorylated opsin into two membrane-bound fragments of apparent $M_2$, 27000 and 12000, designated 'V8-L' and 'V8-S' respectively (Findlay et al., 1981). The $^{32}P$ radioactivity migrated with V8-S on SDS/urea/polyacrylamide-gel electrophoresis. V8-L contained no significant radioactivity.

Purified $[^{32}P]V8-S$ was then treated with CNBr and the resulting six peptides resolved by gel filtration on Sephadexes LH-60 and LH-20 (Findlay et al., 1981; Pappin & Findlay, 1984). All the $^{32}P$ radioactivity was associated with a 24-residue fragment (CNBr-1) in which both $[^{32}P]$phosphoserine and $[^{32}P]$phosphothreonine were detected when a partial acid hydrolysate of this peptide was analysed by high-voltage paper electrophoresis and autoradiography.

Table 3. Release of radioactivity on solid-phase sequencing

<table>
<thead>
<tr>
<th>Residue</th>
<th>$^{32}P$ released (d.p.m.)</th>
</tr>
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<tbody>
<tr>
<td>Glu-15</td>
<td>190</td>
</tr>
<tr>
<td>Ala-16</td>
<td>105</td>
</tr>
<tr>
<td>Ser-17</td>
<td>1220</td>
</tr>
<tr>
<td>Thr-18</td>
<td>1290</td>
</tr>
<tr>
<td>Thr-19</td>
<td>2300</td>
</tr>
<tr>
<td>Val-20</td>
<td>1305</td>
</tr>
<tr>
<td>Ser-21</td>
<td>3760</td>
</tr>
<tr>
<td>Lys-22</td>
<td>3860*</td>
</tr>
<tr>
<td>Thr-23</td>
<td>†</td>
</tr>
<tr>
<td>Glu-24</td>
<td>—</td>
</tr>
<tr>
<td>Thr-25</td>
<td>960‡</td>
</tr>
<tr>
<td>Ser-26</td>
<td>4560</td>
</tr>
<tr>
<td>Gln-27</td>
<td>930</td>
</tr>
<tr>
<td>Val-28</td>
<td>720</td>
</tr>
<tr>
<td>Ala-29</td>
<td>550</td>
</tr>
<tr>
<td>Pro-30</td>
<td>440</td>
</tr>
<tr>
<td>Ala-31</td>
<td>510</td>
</tr>
</tbody>
</table>

* Peptide (100nmol; 152000d.p.m.) activated with DITC and coupled to aminoethylaminopropyl-glass (yield 58%). The sequencing yield was 32%. Lys-22 remained bound to the resin. Radioactivity released at this step may therefore be carry-over and $^{32}P$ in the C-terminal part released from support.
† Peptide T3, not radioactive and sequenced manually (see Fig. 2).
‡ Peptide 56nmol; 27920d.p.m.) coupled to aminoethylaminopropyl-glass (yield 88%). The sequencing yield was 25%.
which yielded two peptides, A and B (Figs. 3 and 4). Their compositions and N-terminal amino acids identified them as representing residues 1–15 and 16–24 respectively of CNBr-1 (Table 2). Only the latter was radioactive (Fig. 3).

In summary, serine residues 21 and 26 were most heavily phosphorylated (0.5–0.6 mol/mol) and Ser-17, Thr-18 and Thr-19 less so (0.2–0.3 mol/mol).

Discussion

Reported phosphorylation ratios for bovine ROS range from 0.3 to 9 mol of phosphate/mol of rhodopsin (Kuhn et al., 1973; Hargrave et al., 1980; Wilden & Kuhn, 1982). Variable ratios also occurred for phosphorylation in ovine ROS, although the range of values was much less. Possible reasons for this include a variable loss of kinase from unsealed ROS during preparation, since the addition of kinase increased the level of phosphorylation. The presence of variable amounts of G-protein, which may restrict the access of the kinase to the C-terminal region of the protein, could also be a contributory factor. The phosphorylation of rhodopsin in disc preparations using kinase extract gave more reproducible ratios than in ROS preparations.

After photon capture, the ability to phosphorylate rhodopsin gradually decayed, with a half-time of about 20 min at 37°C. Bleached rhodopsin is, therefore, only a temporary substrate for the kinase, and the phosphorylation sites exposed on illumination are then rendered inaccessible again. The structural alterations that seques- ter the sites of phosphorylation have yet to be characterized, although they do not appear to corre- spond to the appearance or disappearance of any particular photointermediate in the bleaching sequence (Miller et al., 1977; Kuhn, 1978).

The presence of hydroxylamine during bleaching strongly inhibited light-dependent phosphorylation. Hydroxylamine is thought to react with the aldehydic group of retinal to form a stable oxime at a very early stage of the bleaching cycle. This reaction may prevent the appearance of the structural intermediate that is recognized by the kinase, or the life-time of this intermediate may be so decreased that only minimal phosphorylation is possible.

![Image of Fig. 3](image-url)  
*Fig. 3. Fractionation of peptides generated by S. aureus V8-proteinase digestion of CNBr-1 on Sephadex LH-20. Fractionation was described in Fig. 2, except that the column size was 142 cm x 2.1 cm.*

![Image of Fig. 4](image-url)  
*Fig. 4. Sequence of the phosphorylated regions of ovine rhodopsin. A, B and C represent peptides generated by treatment with S. aureus V8 proteinase; T1, T2 and T3 represent those generated with trypsin. * Denotes phosphorylated residues; numbers in parentheses show the residue position in intact rhodopsin.*
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In an effort to determine whether the bilayer exerts any marked effects on the conformational changes undergone by rhodopsin (as monitored by the phosphorylation reaction), anaesthetics were added to the disc preparation. Although althesin and propanidid enter the bilayer and can have dramatic effects on the activity of transport proteins (Abu Salah et al., 1982), they did not influence the extent of phosphorylation. This suggests that altering the fluidity of this very fluid bilayer did not markedly affect the conformational changes that are initiated at the intramembranous chromophore-binding site being transmitted to the extramembranous C-terminal domain.

Dephosphorylation

If rhodopsin phosphorylation serves some physiological role, one might expect a system of phosphate removal to return the protein to the dark-adapted dephosphorylated state. No endogenous phosphatase activity was detected in ovine segment or disc preparations, suggesting loss or inactivation of the enzyme during preparation of the material. This agrees with attempts to observe dephosphorylation in bovine ROS (Weller et al., 1975), but disagrees with the observed dephosphorylation of opsin in frog ROS (Miller & Paulsen, 1975). However, opsin phosphatase activity was detected in the supernatants of retina homogenates. This was necessarily a crude experiment, so one cannot be certain if the phosphatase(s) detected are specific to the ROS or to rhodopsin. This dephosphorylation was incomplete until DTAB was added, when almost total phosphate removal occurred. Interestingly, digitonin, which is reputed to preserve the structural integrity of membrane-bound rhodopsin, did not increase the extent of phosphate loss compared with detergent-free controls. Possible explanations for the effect of DTAB are that the structure of the membrane or of phosphorylated opsin is altered by the detergent so as to expose inaccessible phosphorylated sites to the phosphatase. Alternatively, the phosphatase itself may be activated in some manner. It is conceivable that one of the sites is preferentially dephosphorylated in the absence of detergent. Since dark-adapted protein is not phosphorylated when isolated, however, total dephosphorylation must occur in vivo.

Structural studies

Studies on isolated whole phosphorylated protein, and on protein digested with S. aureus V8 proteinase while still in the membrane, located all the phosphorylation sites to a 31-residue peptide that constitutes the extramembranous C-terminal region of the protein. No significant radioactivity was seen in fragments representing the remainder of the protein.

By using sequence information and the specific radioactivity of the various peptides, it is possible to calculate the approximate degree of phosphorylation at each of the phosphorylated residues on the basis of a phosphorylation ratio for the whole protein of 1.8 (Table 3). We did not observe phosphorylation of threonine residues 2, 3, 23 and probably not 25 (see Fig. 4).

Sale et al. (1978), using proteolysis of the bovine protein in situ, localized all the phosphorylation sites to a C-terminal fragment with an apparent Mr of 6000. These data are in agreement with studies by Hargrave et al. (1980), who restricted the bovine phosphorylation sites to the sequence from Ser-17 to Ser-26 inclusive. Until recently, the maximum phosphorylation ratio for bovine protein was reported to be 7, and since the phosphorylated region contains three serine and four threonine residues, it was inferred that each of these residues can be fully phosphorylated. We can only identify the phosphorylation of a maximum of five of these sites, no matter how exhaustive our conditions. More recently, Wilden & Kuhn (1982) have reported maximum bovine phosphorylation ratios of 9, and they suggest that there are two further sites which are separated by at least two hydrophobic membrane α-helices. In recent models for the protein, up to five such sites (three on V8-S, two on V8-L) may exist, but in the case of ovine rhodopsin were not phosphorylated.

An attempt was made to perturb the bleached-rhodopsin–kinase interaction by subjecting disc membranes to proteolysis by S. aureus V8 proteinase in situ before phosphorylation. Proteolysed protein could still be phosphorylated and the phosphorylation sites were again restricted to V8-S. It is apparent, therefore, that the removal of the seven C-terminal residues from rhodopsin does not prevent the bleached-rhodopsin–kinase interaction. Kuhn & Hargrave (1981) have found that proteolysis for 18 h in situ with thermolysin, a procedure which removes the 12 C-terminal residues and cleaves rhodopsin at an 'internal' site in the polypeptide chain, abolished the light-induced binding of the G-protein to disc membranes. Assuming that the GTPase binds to rhodopsin, these observations imply, therefore, that the binding of the G-protein and the kinase occur at different sites, although it is possible that there will be some steric hindrance to both binding at the same time.

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