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Phosphorylation of ovine rhodopsin

Identification of the phosphorylated sites

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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, M_r 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–metarhodopsin 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.

Abbreviations used: ROS, rod outer segments; SDS, sodium dodecyl sulphate; DITC, *p*-phenylene di-isothiocyanate; EDC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodi-imide; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane; DTAB, (sodium) dodecyltrimethylammonium bromide; PEI-, poly(ethyleneimine)-.

Experimental

Materials

All chemicals were obtained from BDH (Poole, Dorset, U.K.), unless listed below. AnalaR-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 5min with a 100W lamp and immediately gently homogenized manually in a total volume of 90ml of ice-cold 0.1M-Tris/HCl buffer, pH7.4, 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 15min at 65000*g*, and floating material, consisting of ROS, carefully removed. The segment suspension was adjusted to 50ml with 1M-NH_4Cl, 50mM-Tris/HCl, pH7.4, and incubated for 45min at room temperature. After thorough homogenization, the suspension was centrifuged at 45000*g* for 15min. The supernatant, containing rhodopsin kinase, was decanted and extensively dialysed against ice-cold 10mM-ammonium bicarbonate (5×5 litres). The preparation was then freeze-dried and stored at −20°C until required.

Preparation of [^{32}P]*ATP.* [γ-^{32}P]ATP was synthesized by a modification of the method of Glynn & Chapell (1964). The enzymic exchange between [^{32}P]phosphate and unlabelled ATP was allowed to proceed for 2h (Thatcher, 1978). [^{32}P]ATP was purified by anion-exchange chromatography on Dowex 1 (X4) resin with a linear gradient of 500ml of 10mM-NH_4HCO_3 and 500ml of 1M-NH_4HCO_3. After removal of residual NH_4HCO_3 by repeated freeze-drying and resuspension in distilled water, the product was checked for purity by PEI-cellulose t.l.c. in 0.85M-LiCl and stored at −20°C in 40mM Tris/HCl buffer, pH7.4, 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 state, 2mM-[^{32}P]ATP, 3mM-$MgCl_2$ and 40mM-Tris/HCl, pH7.4. The specific radioactivity of [^{32}P]ATP, determined for each experiment, was usually between 10^4 and 10^5 d.p.m./nmol. Phosphorylation ratios were expressed as mol of phosphate incorporated per mol of rhodopsin bleached. Rhodopsin assays based on A_{500} 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 3mg/ml and that of the kinase extract was up to 11mg/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 60min. 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 ^{32}P content of gel slices or alternatively, since opsin was the only significant species phosphorylated, by sampling the mixture (50–100μl) into 600μl of ice-cold 10% (w/v) trichloroacetic acid/1M-H_3PO_4. Precipitated material was recovered by centrifugation, washed eight times with 1ml portions of the trichoroacetic acid/phosphoric acid solution and solubilized in NCS tissue solubilizer. Portions of the solubilized precipitates were removed, neutralized with acetic acid and mixed with 4ml of scintillation cocktail.

Dephosphorylation. After phosphorylation, excess [^{32}P]ATP was removed by diluting the reaction mixture with 10vol. of 40mM-Tris/HCl, pH7.4, and centrifuging for 15min at 45000*g*. The pellets were resuspended to a protein concentration of 1mg/ml in 40mM-Tris/HCl (pH7.4)/3mM-$MgCl_2$ and incubated at 37°C. Portions were periodically removed into 600μl of 10% (w/v) trichloroacetic acid/1M-H_3PO_4 and the precipitated material was washed, solubilized and assayed for ^{32}P as described for phosphorylation in disc membranes.

Supernatants of retina homogenates were prepared by diluting ten fresh retinas to 10ml with 40mM-Tris/HCl (pH7.4)/3mM-$MgCl_2$ and homogenizing with a Potter homogenizer for 2min at 4°C. The suspension was centrifuged for 20min at 45000*g* and the supernatant removed. These supernatants were added to phosphorylated opsin preparations to give protein concentrations of 8 and 1mg/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 resins in 20% (v/v) formic acid or 5% (v/v) acetic acid. The membrane-bound fraction was reduced, carboxymethylated and the V8-L (M_r 27000) and V8-S (M_r 12000) 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 0.1M-NH_4HCO_3 with Tos-Phe-CH_2Cl-treated trypsin (5%, w/w, with peptide). The mixture was incubated at 37°C with stirring, for 6h, after which time a further portion of fresh trypsin was added and the material was freeze-dried after a second 6h incubation. Digestion of peptides with *S. aureus* V8 proteinase was performed in a similar manner, except the digestion times were increased to 18h.

Peptide analysis. ^{32}P-labelled peptides were partially hydrolysed with 6M-HCl at 110°C for 2h in sealed evacuated tubes. Phosphorylated amino acids were separated by electrophoresis at 3kV for

90min 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 10–50μg 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-phenyloxazol-2-yl)benzene]. Before mixing with this scintillation cocktail, gel slices were solubilized by incubation at 37°C in 30% (v/v) H_2O_2 containing 1% (v/v) 0.88-sp.gr. NH_3 (150μl/3mm gel slice). The ^{32}P 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 [^{32}P]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 10mM-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 [^{32}P]ATP, incubation of phosphorylated discs did not reveal significant

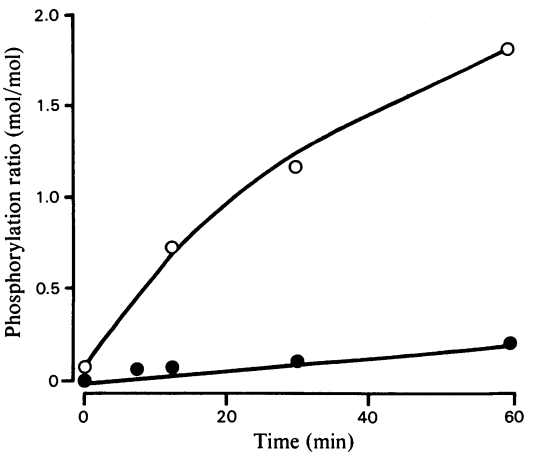

Fig. 1. *Effect of kinase extract on light-stimulated disc phosphorylation*
Discs and [^{32}P]ATP were incubated in continuous light or in the dark in the presence (○) or absence (●) of kinase extract. Each point represents the difference between the degree of phosphorylation in the light and in the dark. Phosphorylation was assayed by precipitation with trichloroacetic acid.

Table 1. *Influence of selected hydrophobic compounds on light-stimulated disc phosphorylation*
Discs were preincubated with each compound for 45min at room temperature in the dark.

	Relative phosphorylation (%)
Control	100
Althesin (1mM)	102
Propanidid (1mM)	113
Cholesterol (1mM)	94

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* ^{32}P *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:

$$Thr_{0.97}Ser_{1.04}Glu_{1.02}Pro_{0.92}Ala_{2.00}Val_{1.03}$$

[^{32}P]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 ^{32}P-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 [^{32}P]phosphoserine at round 2 (Table 3 below). This peptide represented the *C*-terminus of the intact protein. Manual sequencing confirmed its structure.

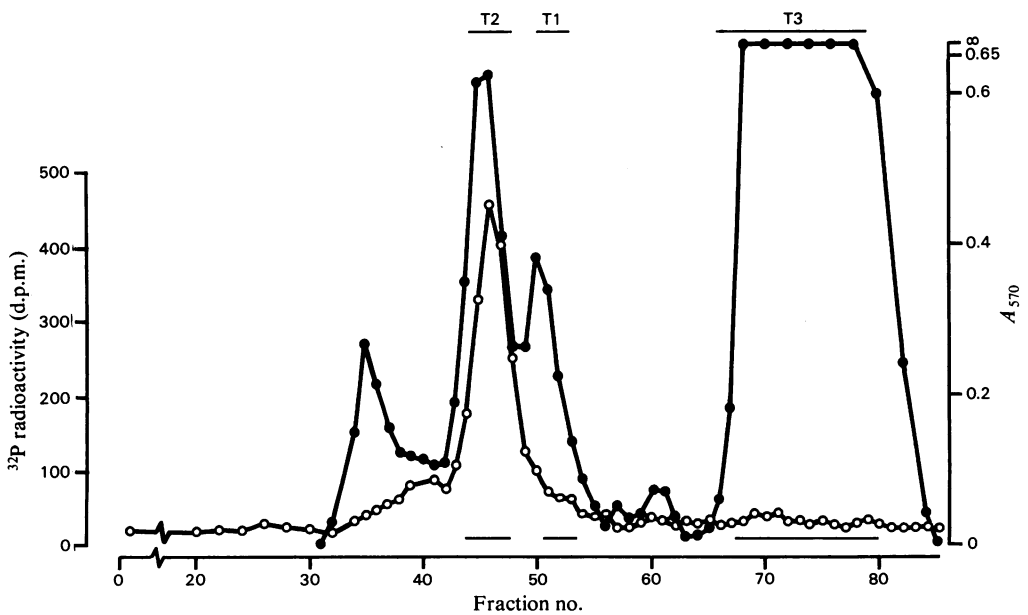

Fig. 2. *Fractionation of CNBr-1 tryptic fragments on Sephadex LH-20*
Digested material was solubilized in 90% (v/v) formic acid/ethanol (3:7, v/v) (Gerber *et al.*, 1979) and applied to a column (135cm × 2cm) of Sephadex LH-20 equilibrated with this solvent. The column eluate was pooled as indicated. Ninhydrin-positive material eluted in the column volume represents NH_3HCO_3 remaining after freeze-drying of the digest. ●, A_{570} (manual ninhydrin); ○, radioactivity.

Table 2. *Amino acid composition of tryptic and S. aureus-V8-proteinase (V8) peptides of CNBr-1*
Abbreviation used: CM-Cys, carboxymethylcysteine.

	Composition (residue/molecule)					
		Tryptic peptides			V8 peptides	
Amino acid	CNBr 1	T1	T2	T3	VA	VB
CM-Cys	2	1.3 (2)			1.1 (2)	
Asx	3		2.9 (3)		2.6 (3)	
Thr	5	2.0 (2)	1.9 (2)	1.1 (1)	2.1 (2)	3.2 (3)
Ser	2		2.0 (2)			2.0 (2)
Glx	2		1.1 (1)	0.9 (1)	1.2 (1)	1.4 (1)
Pro	1		1.2 (1)		1.1 (1)	
Gly	2	1.1 (1)	1.3 (1)		2.3 (2)	
Ala	1		1.0 (1)			0.9 (1)
Val	1		0.7 (1)			0.7 (1)
Leu	3		1.8 (2)	1.2 (1)		2.9 (3)
Lys	2	1.0 (1)	1.0 (1)		0.7 (1)	0.9 (1)
N-Terminus ...	Leu	Leu	Asx	Thr	Leu	Ala
Residue nos. ...	1–24	1–8	9–22	23–24	1–15	16–24

(*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_r 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.

Since CNBr-1 coupled poorly to aminopropyl-glass using either EDC or DITC, phosphorylation sites were identified by subdigestion with trypsin. *N*-Terminal, amino acid and solid phase automated sequence analysis revealed that trypsin digested CNBr-1 into three peptides of two, eight and fourteen residues, the last containing all the radioactivity (Fig. 2, Table 2). This eliminated threonine residues at positions 2, 3 and 23 in CNBr-1 as primary phosphorylation sites. Solid-phase sequencing of the lysine-coupled peptide identified the labelled residues as Ser-17, Thr-18, Thr-19 and Ser-21 (Table 3).

Confirmation of these results was obtained by digestion of CNBr 1 with *S. aureus* V8 proteinase,

Table 3. *Release of radioactivity on solid-phase sequencing*
See Fig. 4 for numbering of residues.

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

* 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 aminopolystyrene with EDC (yield 88%). The sequencing yield was 25%.

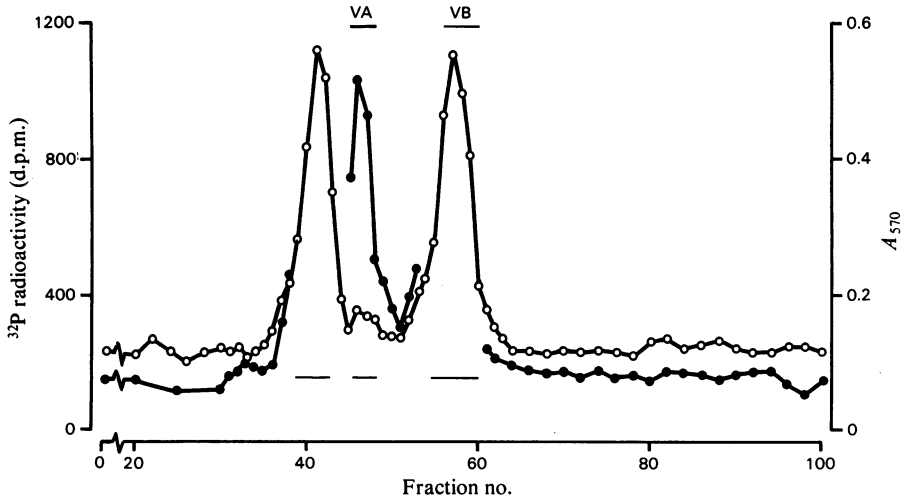

Fig. 3. *Fractionation of peptides generated by S. aureus V8-proteinase digestion of CNBr-1 on Sephadex LH-20*
Fractionation was described in Fig. 2, except that the column size was 142cm × 2.1cm.

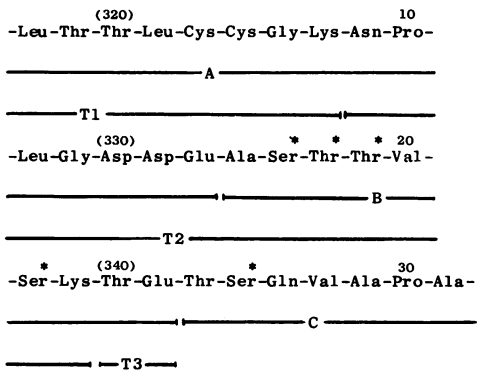

Fig. 4. *Sequence of the phosphorylated regions of ovine rhodopsin*
A, B and C represent peptides generated by treatment with *S. aureus* V8 proteinase; T1, T2 and T3 represent those generated with trypsin. * Denotes phosphorylated residues; numbers in parentheses show the residue position in intact rhodopsin.

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 (0.5–0.6mol/mol) and Ser-17, Thr-18 and Thr-19 less so (0.2–0.3mol/mol).

Discussion

Reported phosphorylation ratios for bovine ROS range from 0.3 to 9mol 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 20min 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 sequester the sites of phosphorylation have yet to be characterized, although they do not appear to correspond 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.

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 M_r 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 18h *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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