Modification of ovine opsin with the photosensitive hydrophobic probe 1-azido-4-[¹²⁵I]iodobenzene

Labelling of the chromophore-attachment domain

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The hydrophobic photosensitive probe 1-azido-4-[¹²⁵I]iodobenzene (AIB) partitioned preferentially into photoreceptor disc membranes and, upon u.v. irradiation, became covalently bound to opsin and phospholipid. The labelling of both protein and phospholipid was linearly related to AIB concentration. The amount of probe incorporated into protein was not significantly different when membranes were irradiated at -100° , 4° or 25°C , but irreversible aggregation of monomeric opsin was dramatically reduced by performing the photolysis at -100°C . Labelling of opsin after irradiation at -100° or 4° was not significantly reduced by the presence of lysine in the aqueous buffer, indicating that significant amounts of reactive species did not enter the aqueous phase. The incorporation into phospholipid, unlike that into opsin, decreased as the temperature of irradiation increased. Some labelling of opsin occurred on incubation with pre-photoactivated AIB, indicating that reaction may also occur with reactive species of longer lifetimes than the nitrene. Proteolysis of labelled opsin with *Staphylococcus aureus* V8 proteinase yielded two radiolabelled membrane-bound fragments. The location of the modified sites (cysteine, tryptophan, tyrosine, lysine and histidine residues: all nucleophiles) in the smaller fragment was entirely consistent with putative models for the protein derived from other studies.

INTRODUCTION

Several techniques have been used to investigate the disposition of the polypeptide chain of integral membrane proteins with reference to the phospholipid bilayer. The location of proteolytic cleavage sites *in situ* and of residues modified by water-soluble reagents have helped to identify those regions exposed to the aqueous environment (Eytan & Schatz, 1975; Mas *et al.*, 1980; Ovchinnikov, 1982; Barclay & Findlay, 1984). The identity of the intramembranous regions, however, has usually been inferred from a knowledge of those parts of the structure available at the two aqueous surfaces or from hydrophobicity profiles (Spatz & Strittmatter 1971; Steffen & Buse, 1979; Ovchinnikov, 1982; Hoppe *et al.*, 1983). The use of hydrophobic photosensitive probes capable of modifying residues located within the bilayer provides one potential method for directly identifying these intramembranous segments.

In recent years several hydrophobic reagents which, upon u.v. irradiation, generate very reactive nitrene or carbene species have been synthesized (Klip & Gitler, 1974; Karlsh *et al.*, 1977; Bayley & Knowles, 1978*b*; Bayley *et al.*, 1979; Brunner & Semenza, 1981) and been shown to attach covalently to integral membrane proteins and lipids (Kahane & Gitler, 1978; Wells & Findlay, 1979*a,b*; Bayley & Knowles, 1980; Brunner & Semenza, 1981).

In these studies, we have used azido[¹²⁵I]iodobenzene with the aim of identifying the intramembranous regions of ovine opsin, a protein whose amino acid sequence is

known and whose surface regions have been mapped [for a review, see Findlay *et al.* (1984)].

EXPERIMENTAL

Materials

All chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. unless listed below. Carrier-free Na¹²⁵I was obtained from Amersham International, Amersham, Bucks., U.K.; TFA anhydride was from Sigma Chemical Co. Ltd., Poole, Dorset, U.K., and *o*-phthalaldehyde and *n*-propylamine from Fluka distributed by Fluorochem Ltd., Glossop, Derbyshire, U.K. For sources of all other reagents and solvents, see Brett & Findlay (1983) and Pappin & Findlay (1984).

Methods

Preparation of AIB. AIB (20 Ci/mol) was prepared from purified 4-iodoaniline by isotope exchange with carrier-free Na¹²⁵I and iodine monochloride (ICl) reagent (Helmkamp & Sears, 1970) followed by diazotization and subsequent addition of NaN₃ (Wells & Findlay, 1979*a*; Booth *et al.*, 1979).

Labelling of photoreceptor disc membranes. Intact bleached photoreceptor disc membranes were prepared at 4°C from ovine eyes by the method of Brett & Findlay (1979) with 0.1 M-Tris/HCl buffer, pH 7.5. These membranes (1 mg of protein/ml) were incubated with AIB at 20°C or 4°C and in the dark. After 10 min, the

Abbreviations used: AEAP, *N*-(2-aminoethyl)-3-aminopropyl; AIB, 1-azido-4-[¹²⁵I]iodobenzene; DMF, dimethylformamide; DTAB, dodecyl trimethylammonium bromide; FACE, 90% (v/v) formic acid/acetate/chloroform/ethanol (1:1:2:1, by vol.); FE, 90% formic acid/ethanol (3:7, v/v); Hse>, homoserine lactone; PTH, phenylthiohydantoin; ROS, rod outer segments; TFA, trifluoroacetic acid.

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mixture, where indicated, was very rapidly frozen as a thin film by dropwise addition of the suspension to a wide-necked vessel equilibrated in liquid N₂. All these procedures were carried out under dim red light. The various membrane suspensions were irradiated for up to 90 min at -100 °C, 4 °C and 25 °C using a Philips Ultraphil 300 W u.v. lamp placed 15 cm from the reaction mixture.

Determination of covalent incorporation into opsin. Incorporation of radioactivity into opsin was determined using two assay systems.

(1) Gel chromatography. Membranes (0.5–1 mg of protein) were pelleted by centrifugation (50000 *g* for 5 min), solubilized by the sequential addition of the components of FACE, and chromatographed on Sephadex LH-60 equilibrated with the same solvent. The eluate was sampled for radioactivity, protein and phosphate content. Lipid labelling was determined after rechromatography on Sephadex LH-20.

(2) SDS polyacrylamide-gel electrophoresis. Samples of labelled photoreceptor membranes were solubilized in a mixture containing 1% (w/v) SDS, 5% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 0.25% (w/v) Bromophenol Blue tracking dye in 0.5 M-Tris/HCl, pH 6.8. Samples containing 10–50 µg of protein were loaded on gradient slab gels (10–20 or 10–30% acrylamide) and subjected to electrophoresis at 60 V for 18 h, using 0.025 M-Tris/0.192 M-glycine/0.1% (w/v) SDS, pH 8.3, as running buffer (Laemmli & Favre, 1973). Gels were fixed, stained and scanned as described previously (Brett & Findlay, 1979) and cut into 2 mm slices for the determination of radioactivity with a γ -radiation counter.

Proteolysis, carboxymethylation and delipidation of opsin. Photoreceptor membranes were incubated with 0.65 mM-AIB at 4 °C for 10 min in 100 mM-Tris/HCl/100 mM-lysine, pH 7.5, very rapidly frozen to a thin film in liquid N₂ and subsequently irradiated at -100 °C for 60 min. The suspension was slowly thawed in the dark and the washed membranes then digested with 2% (w/w) *Staphylococcus aureus* V8 proteinase, solubilized, reduced, carboxymethylated and the V8L and V8S fragments isolated by the procedures described in Findlay *et al.* (1981) and Brett & Findlay (1983). The cleavage of V8S with 100-fold molar excess of CNBr and the purification and sequence of the various peptides have been reported previously (Pappin & Findlay, 1984).

Amino-acid-sequence analysis. The following coupling procedures were used.

(i) Peptides were coupled to AEAP-glass by the C-terminal Hse> method (Horn & Laursen, 1973). The coupling solvent used was DMF/propanol (3:1, v/v).

(ii) An alternative coupling method involving acidic residues was developed for certain samples. These latter peptides were chromatographed on Sephadex LH-20 in FE, dried *in vacuo*, then solubilized in 0.5 ml of 10% (v/v) TFA anhydride in anhydrous TFA and incubated at room temperature for 1 h. The peptide mixture was then dried and incubated with AEAP-glass using DMF as the coupling solvent.

Solid-phase sequencing. Amino acid and sequence analyses were performed as described previously (Brett & Findlay, 1983). The buffer used in the solid phase was that

of Pappin & Findlay (1984). The TFA fraction collected during each sequencer round was counted for radioactivity with a γ -radiation counter, before conversion and PTH-derivative analysis, by an adaptation of the method of Zimmerman *et al.* (1977).

Determination of organic phosphate. The method of Chen *et al.* (1956) was used, after digestion of samples with 4% (v/v) HClO₄/30% (v/v) H₂SO₄ to release P_i.

RESULTS

Incorporation into membrane components

The percentage of AIB that partitioned into the membranes remained constant at 86–90% over the concentration range 0.1–1.0 mM. Partitioning was reversible and rapid, reaching equilibrium in less than 5 min, and remaining stable for at least 2 h. Above 2 mM-AIB, the appearance of the pellets changed noticeably, suggesting that gross changes in membrane structure had occurred. Partitioning at 4 °C was a little lower (82–87%), but followed the same trend as obtained at room temperature. This initially surprising observation is consistent with the very fluid characteristics of this membrane brought about by the high proportion of C_{22:6} fatty acid species.

Covalent incorporation of ¹²⁵I into opsin was measured by solubilizing pelleted membranes in FACE and subjecting them to chromatography on Sephadex LH-60 (in the dark). With irradiated preparations, the excluded peak of radiolabel contained delipidated opsin. The second major included peak of radiolabel consisted of membrane phospholipid, as shown by the detection of phosphate. The radiolabel at the column volume comprised AIB photolysis products not covalently associated with protein or phospholipid. With unirradiated samples, the excluded opsin peak contained no radioactivity and only an extremely low level, less than 2% of that obtained for the equivalent irradiated system, was associated with the phospholipid peak.

The molar ratio of binding to opsin, using membranes incubated with 0.5 mM-AIB and then irradiated at 4 °C, increased with the duration of u.v. irradiation, reaching a maximum of nearly 2 after 30 min. The incorporation of ¹²⁵I into opsin at 4 °C increased in an approximately linear fashion over an AIB concentration range of 0.1–4 mM. However, since structural changes to the membrane may occur above 2 mM, all subsequent labelling was restricted to an upper limit of 0.8 mM-AIB. Omitting lysine from the buffer had little effect on the molar ratio of binding.

In order to examine the effects of diffusion of reactive species within the bilayer, photoreceptor membranes, pre-incubated with AIB, were instantaneously frozen at -100 °C as a thin film and then irradiated. Under these conditions, incorporation of ¹²⁵I into opsin increased to a maximum after about 60 min irradiation and was linear over the range 0–0.8 mM-AIB. The molar ratio of binding was only very slightly reduced from that obtained on irradiation at 4 ° and 25 °C.

Interestingly, incorporation into membrane phospholipids, which also showed a linear relationship with AIB concentration (0–0.9 mM), increased with decreasing temperature [i.e. 100% (-100°), 70% (4°), 50% (25°)].

In order to test for long-lived reactive species,

Fig. 1. Separation of the CNBr fragments of [¹²⁵I]V8S

[¹²⁵I]V8S (300 nmol) was treated with a 50-fold molar excess of CNBr for 24 h at 20 °C. The resultant peptides were dissolved in FE and applied to a column (128 cm × 1.5 cm) of Sephadex LH-60. The eluate was monitored at 280 nm and 0.15 ml portions were taken for determination of radioactivity (●) and protein (○). Fractions (2.15 ml) were pooled as shown.

Fig. 2. Sequencing of V8S-I

The material in Pool B1 (53 nmol, 175000 c.p.m.) was coupled to AEAP-glass by the Hse > method in 63% yield. The initial sequencing yield was 60% and the repetitive yield 92%. The one-letter amino acid notation is used.

40 mM-AIB was irradiated in ethanol at 4 °C; samples were withdrawn at various time intervals and added immediately (0.5 mM) to photoreceptor membranes in the dark at 4 °C. The membranes were incubated in the dark for 3 h, then chromatographed on Sephadex LH-60, also in the dark. The amount of radioactivity associated with opsin under these conditions increased with the length of pre-irradiation, reaching a maximum after 120 min of about 25% of that obtained using the normal labelling procedure. Similarly, the labelling of phospholipid reached nearly 40% of that obtained under the normal labelling procedures. It seems likely, therefore, that at least part of the radiolabelling of membrane components may be due to reaction with very-long-lived reactive species generated after irradiation.

Membranes that had reacted were also examined by SDS/polyacrylamide-gel electrophoresis, a procedure that totally removed unirradiated AIB from opsin. Photoactivation at -100 °C gave rise to no further irreversible aggregation of opsin compared with the untreated sample. Irradiation at 4 °C and 25 °C, however, resulted in increasing degrees of SDS-resistant aggregation. At all three temperatures, both monomeric opsin (*M_r* 38000) and its oligomers contained radioactivity. The incorporation of radiolabel into the opsin monomer, as calculated from the radioactive profiles and gel scans of each sample, was roughly similar for all three irradiation temperatures and agreed to within 25% with the ratios obtained from gel chromatography of the same samples.

Characterization of the intramembranous regions

After modification with photoactivated AIB as indicated above, opsin was subjected to proteolytic cleavage with *S. aureus* V8 proteinase. The seven-residue peptide released into the supernatant by this procedure was purified and found to contain no detectable radioactivity. The two membrane-bound fragments (V8L and V8S) were solubilized, carboxymethylated and separated by Sephadex LH-60 gel filtration (Findlay *et al.*, 1981). Cleavage with CNBr of labelled V8S and subsequent chromatography on Sephadex LH-60 gave the profile shown in Fig. 1. A portion of the material in Pool A was sequenced by using the spinning-cup method (results not shown) and was found to contain a mixture of V8S and partial cleavage products.

Pool B. The material in Pool B was subjected to acid cleavage in aq. 70% (v/v) TFA at room temperature for 8 h and re-chromatographed on Sephadex LH-60. This treatment specifically cleaves the resistant Met-31–Thr-32 bond in V8S-I (51 residues), producing the V8S-IA (31 residues) and V8S-IB (20 residues) peptides (Pappin & Findlay, 1984).

The radiolabel and A_{570} (ninhydrin) column elution profiles for the three pools obtained were not exactly coincident, suggesting that incorporation of photolysed AIB molecules slightly affected the chromatographic behaviour of peptides in this chromatography system. In order to obtain accurate specific radioactivities it was necessary to amalgamate fractions comparatively widely, thereby running the risk of slight contamination. It is important to stress that any resort to other forms of purification procedure in order to obtain absolute purity will result, particularly with the small peptides, in the separation of the labelled and unlabelled versions, thereby complicating both identification and quantification.

Fig. 3. Sequencing of V8S-IB

The peptide material in Pool B3 (58 nmol, 76000 c.p.m.) was coupled to AEAP-glass by the Hse > method in 56% yield. The initial sequencing yield was 61% and the repetitive yield 95%. The one-letter amino acid notation is used.

Fig. 4. Identification of radioactive residue Lys-296

Radioactive PTH-amino acid samples were solubilized in 100% acetonitrile and applied to a μ Bondapak C_{18} h.p.l.c. column, developed with a linear gradient from 20% (w/v) acetonitrile in aq. 0.05% (v/v) TFA to 100% acetonitrile over 50 min, then held at this level for 10 min. The flow rate was 1 ml/min and 1 ml fractions were measured for radioactivity. (a) Round 8 from Fig. 3; (b) a derivatized tryptophan residue.

Sequencing of the material in Pool B1 confirmed it as V8S-I (Fig. 2). The residues Cys-7 and Trp-8 were clearly identified as major modification sites, with Tyr-11 and Tyr-44 as more minor sites of modification. This result was confirmed when sequencing the material in Pool B2 (V8S-IA). Pool B3 was found to contain V8S-IB, together with V8S-IA, in the molar ratio 5:1 (Fig. 3). The radiolabel released at round 7 was totally accounted for by Cys-7 in V8S-IA (31 residues). By contrast, most of that recovered at round 8 could not be due to Trp-8 in the same peptide. H.p.l.c. analysis of the radioactive material suggested that it represented a derivative of lysine rather than tryptophan (Fig. 4). It is probable, therefore, that Lys-8 in V8S-IB is a site of modification. The radiolabel released at rounds 13 and 18 was assigned to tyrosine residues at these positions.

Fig. 5. Fractionation of Pool C (Fig. 1) on a column (145 cm × 1.2 cm) of Sephadex LH-20

Portions (0.2 ml) were taken for the determination of radioactivity (●) and protein (○). Fractions (2.2 ml) were pooled as shown.

Fig. 6. Sequencing of V8S-II

The material in Pool C from Fig. 5 (150 nmol, 50000 c.p.m.) was first coupled to AEAP-glass by using method (i) then the non-coupling fraction treated with 15% n-propylamine, before being bound to AEAP-glass via the TFA anhydride method (18% overall yield). The initial sequencing yield of the latter peptide was 59% and the repetitive yield 95%.

Pool C. The material in Pool C was first rechromatographed on Sephadex LH-20 (Fig. 5) and that in the resultant Pool C1 treated to eliminate future coupling of any traces of V8S-IB. This procedure consisted of incubation in anhydrous TFA for 1 h at 20 °C, drying and treatment with 15% (v/v) n-propylamine in DMF for 1 h at room temperature. The mixture was then repurified by using Sephadex LH-20. The peptide V8S-II (24 residues) was then covalently bound to AEAP-glass by the TFA

Table 1. Specific radioactivities of ¹²⁵I-modified V8S residues

Residue	Specific radioactivity (d.p.m./nmol)
Cys-264	1627
Trp-265	900
Tyr-268	231
Lys-296	211
Tyr-301	1205
Tyr-306	345
Cys-316	2327
Cys-322	44
Cys-323	40
Lys-325	78

anhydride method, and sequenced (Fig. 6). The small amounts of radioactivity released at rounds 5, 6 and 8 represented modified Cys-5, Cys-6 and Lys-8 residues, but their specific labelling was very low (see Table 1). Major decreases in sequencing yield occurred at Asp-13 and Glu-15, consistent with coupling of V8S-II (24 residues) via carboxylic acid side chains. Pool C2, on repurification, contained V8S-III (14 residues), which was not radioactive (see also Fig. 5).

After rechromatography of Pool C3 on Sephadex LH-20, the material was coupled and sequenced (Fig. 7). This showed the presence of V8S-III, V8S-IV (eight residues) and the partial cleavage product Met-V8S-IV (nine residues) in a molar ratio 6:3:10. The radiolabel released at round 7 was assigned to Cys-7 in V8S-IV, and that at round 8 to the equivalent cysteine residue in the extended V8S-IV peptide. This assignment was confirmed by the close agreement between the specific radioactivities calculated for these residues (Table 1).

Pool C4 was rechromatographed on Sephadex LH-20, coupled by using the Hse > method and sequenced. This

Fig. 7. Sequencing of Pool C3

Pool C3 from Fig. 5 (45 nmol, 50000 c.p.m.) was coupled by the Hse > method to AEAP-glass in 46% yield. The initial sequencing yield was 60% and the repetitive yield 93%.

revealed the presence of three peptides [V8S-V (four residues), V8S-IV and Met-V8S-IV] in the molar ratio 10:2:1. Radiolabel was released only at rounds 7, 8 and 9, consistent with the derivatized cysteine residues in the latter two peptides. V8S-V therefore contained no modified residues.

In order to confirm residue assignment, particularly where mixtures were present, the radioactive PTH derivatives were subjected to h.p.l.c. analysis as indicated in Fig. 4. The elution position of the radioactivity was always consistent with the assumed identity of the modified PTH-amino acid and differed from that of the unmodified version.

The specific radioactivities of the modified residues in V8S are shown in Table 1.

DISCUSSION

These studies demonstrated that AIB rapidly partitioned into photoreceptor membranes at both 4 °C and room temperature to reach concentrations approx. 1000-fold higher than in the aqueous phase.

The amount of photolysed AIB incorporated into opsin was directly proportional to the initial AIB concentration and, as judged by gel chromatography and electrophoresis, did not vary greatly when irradiation was carried out at -100°, 4° or 20 °C. At first sight this could indicate that the labelling of opsin was independent of the freedom of the activated reagent to diffuse, but it is important to recognize some contribution by long-lived

reactive species of the probe. The observations from experiments involving the exposure of membranes to pre-irradiated AIB suggested that reaction with long-lived reactive species other than the nitrene may have occurred. Others have also reported that proteins can be modified by reactive intermediates, generated from photolysed arylazides, with lifetimes considerably longer than those possessed by nitrenes (Mas *et al.*, 1980; Vandest *et al.*, 1980). The isolation at cryogenic temperatures of strongly electrophilic benzazirines and azocycloheptatrienes formed by internal rearrangement reactions of aryl nitrenes (Chapman & Leroux, 1970; Nielsen & Buchardt, 1982) raises the possibility that intermediates such as these may have some role in the labelling of opsin, particularly during the period when the membranes are thawing to 4 °C.

Between 70 and 80% of the total radioactivity incorporated at 4 °C was associated with phospholipid, probably via addition reactions to unsaturated-fatty-acid side chains (Reiser & Leyshon, 1971; Gupta *et al.*, 1979a; Bayley & Knowles, 1978a). About half of the fatty-acyl content of photoreceptor membranes consists of polyunsaturated species, with a 30% contribution by docosahexaenoic (C_{22:6}) acid (Daemen, 1973; Papermaster & Schneider, 1982). The incorporation of photolysed AIB into phospholipid, unlike that into opsin, decreased with increasing temperature of irradiation. Possible explanations for this include exclusion of the probe from gel-phase lipid or, less likely, the formation of covalent bonds, which are less probable at temperatures that permit diffusion.

The conditions of labelling induced the irreversible aggregation of opsin in a temperature-dependent fashion. Opsin is particularly prone to aggregation under normal handling procedures, and the protection afforded by carrying out the irradiation at -100 °C was important for the subsequent purification of labelled peptides in high yield.

Previous experiments had indicated that the addition of lysine to the Tris buffer was effective in reducing the labelling of extrinsic proteins of erythrocyte membranes by photolysed azido[³H]benzene (Wells & Findlay, 1979a). The identification of lysine residues, amongst other amino acids, as sites within opsin modified by photoactivated AIB (M. D. Davison & J. B. C. Findlay, unpublished work) further demonstrated the suitability of lysine as a scavenger of photolysed AIB. The failure of exogenously added lysine to reduce significantly the incorporation of photolysed AIB into opsin irradiated at 4° or -100 °C suggests that significant amounts of reactive AIB photolysis products did not diffuse into the aqueous phase.

On the other hand, reduced glutathione, which has been widely used as an aqueous scavenger of hydrophobic photogenerated nitrenes and carbenes (Bayley & Knowles, 1978a,b), decreased labelling of opsin and phospholipids in this study by between 30 and 50%. Previous reports have also demonstrated that the labelling of glycophorin by photolysed adamantane diazirine was reduced by half in the presence of glutathione (Bayley & Knowles, 1980). In contrast, it is suggested that the extent of labelling of the (Na⁺ + K⁺)-transporting ATPase and of membrane proteins in sarcoplasmic-reticulum membranes by photolysed [¹²⁵I]iodonaphthylazide was not greatly depressed by the peptide, although lipid labelling was reduced in both systems (Jorgensen *et al.*, 1982; Gitler & Bercovici,

Fig. 8. Diagrammatic representation of the chromophore-attachment domain (V8S)

The CNBr peptides are shown, arranged with respect to the bilayer surface. Modified residues are indicated by stippled circles, unmodified but potential sites are shown by plain circles.

1980). From the identification of the residues in opsin modified by photolysed AIB when irradiated at -100°C in the presence of lysine (M. D. Davison & J. B. C. Findlay, unpublished work), and assuming that glutathione does not penetrate into the membrane, it would appear probable that the peptide very effectively competes for activated AIB that otherwise would react with amino acid residues or other moieties in the vicinity of the membrane interface. Paradoxically, therefore, its use in these circumstances would restrict the totality of information that could be obtained from membrane-labelling studies using such hydrophobic reagents.

The region of opsin sequenced here is thought to represent the sixth and seventh transmembrane segments of the protein, together with the cytoplasmic C-terminal portion that possesses, amongst other things, the phosphorylation sites (Fig. 8). The sixth transmembrane helix contains the labelled Cys-264, Trp-265 and Tyr-268 residues, but Tyr-274 and His-278, predicted to lie near the membrane surface, were not modified to any significant extent. In the seventh transmembrane segment, cycles corresponding to tyrosine residues at positions 301 and 306 contained radioactive material, Tyr-301 being particularly heavily labelled. It is interesting that Lys-311 was not modified, suggesting a location outside the confines of the bilayer. On the other hand, Cys-316 was heavily labelled by the hydrophobic probe, by hydrophilic alkylating reagents (Findlay *et al.*, 1984) and by 3,5-di-iodo-4-diazobenzenesulphonate (Barclay & Findlay, 1984), all pointing to an accessible location at the bilayer/aqueous-phase interface. Cysteine residues at positions 322 and 323 and Lys-325 were essentially

unmodified, supporting their putative extramembranous disposition. The results presented here are consistent with those obtained previously with hydrophilic probes (Barclay & Findlay, 1984) and structure-prediction algorithms (Eliopoulos *et al.*, 1982; Pappin *et al.*, 1984). The absence of label associated with lysine residues 245 and 248 supports the suggestion that the hydrophilic 5,6-interhelical loop projects some way for the membrane surface.

Further noteworthy points emerge from these data. If the retinal chromophore lies within the structure of opsin, then Cys-264 and Trp-265 are probably not directly involved in the binding site, since their extensive modification suggests that they are available from the intramembranous surface of the protein. Moreover, Cys-264 cannot be involved in any inter-helix disulphide-bonding, an inference that can also be gleaned from the ability to separate the N- and C-terminal fragments after proteolysis with thermolysin (Pober & Stryer, 1975) or *S. aureus* V8 proteinase (Findlay *et al.*, 1981).

Finally, it is interesting that Lys-296, which in the dark-adapted state forms the protonated Schiff base with the aldehydic group of retinal, reacts with the activated probe. This suggests that, after bleaching, the side chain of Lys-296 is situated in an environment to which the probe has access via either an aqueous or an intramembranous route.

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