TOPOGRAPHIC AND ACTIVE-SITE STUDIES ON BOVINE RHODOPSIN

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1. Introduction

Rhodopsin, the photosensitive pigment found in the rod outer segments of retina consists of a glycoprotein, opsin, linked to 11-*cis* retinal via the ϵ-amino group of a lysine [1–4]. Continuing our studies on the structural analysis of rhodopsin we now report on the location of the active-site lysine in the partial sequence of rhodopsin and tentatively identify one region of the polypeptide chain which protrudes from the membrane in the interdiscal phase.

2. Experimental

Rod outer segments containing rhodopsin were prepared from bovine retina, bleached and regenerated with a 2-fold molar excess of 11-*cis* [15-^{3}H]retinal (spec. act. 3.1×10^3 cpm/nmol) as in [5]. Tritiated rhodopsin (2.1 μmol) was cleaved with papain [5] and the label fixed with sodium borohydride to give cleaved [^{3}H]retinylopsin [3]. The SH-groups on the cleaved [^{3}H]retinylopsin were carboxymethylated by adding an aqueous suspension of the protein (5 ml, 12 mg/ml) to iodoacetic acid (60 mg), sodium dodecyl sulphate (SDS, 100 mg) and sodium hydrogen carbonate (180 mg) and leaving the mixture for 3 h under N_2 at room temperature. The protein was precipitated from the detergent solution with 80% (v/v) methanol (25 ml) as in [6]. The incorporation of [^{3}H]retinal into the protein, calculated from ϵ_{280} 68 000 litre . mol^{-1} . cm^{-1} as in [6], was 1.06 mol retinal/mol opsin. A portion of the material was analysed on SDS–polyacrylamide gel electrophoresis as in [6].

The carboxymethylated, cleaved [^{3}H]retinylopsin was dissolved in a solution of 50 mM Tris–HCl (pH 7.0) containing 2% w/v Ammonyx LO (Venture Chemicals, Reading, Berks) and 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ in 4 ml passed through a 1.5 cm × 9 cm column of con A–Sepharose 4B equilibrated in the same buffer, and washed with 50 ml buffer. The fractions containing radioactivity (10–20 ml) which corresponded to a mixture of L- and M-fragments, were pooled, freeze dried, washed twice with ice-cold 80% methanol and twice in water by suspension and centrifugation at 56 000 × *g*. In 4 such experiments 1.3 μmol (4.0×10^6 cpm) of L- plus M-fragments were obtained.

The above material (1 μmol, 3.0×10^6 cpm) was then subjected to CNBr cleavage [7], and the mixture separated by high-voltage paper electrophoresis in pyridine:acetic acid:water (25:1:225, by vol.) as in [7]. The only radioactive region, which was found at the origin, was eluted in 1% (v/v) formic acid and freeze dried. The peptide mixture was applied to a Water's μBondapack CN high-performance liquid chromatography column equilibrated with 0.1% (v/v) trifluoroacetic acid and eluted with a gradient of 0–60% of acetonitrile containing 0.07% (v/v) trifluoroacetic acid run for 25 min at 1.5 ml/min [7]. The radioactive peptide was eluted at 18% acetonitrile. A total of 284 nmol (8.8×10^5 cpm) of purified peptide was collected in this manner.

Both the amino acid composition and the sequence determination of the peptide were done as in [7].

3. Results and discussion

Bovine photoreceptor membrane in which rhodopsin was labelled at the active site with 11-*cis* [15-^{3}H]-retinal was prepared and treated with papain as in [5]. The functionally-active cleaved complex thus produced was photolysed in the presence of $NaBH_4$ to fix the chromophore to the active-site lysine. The

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resulting dihydro-derivative was carboxymethylated and a portion dissolved in SDS and subjected to polyacrylamide gel electrophoresis. Fig.1 shows the presence of 3 characteristic fragments previously designated as heavy (H), medium (M) and light (L) with apparent M_r-values of 23 000, 15 500 and 6000, respectively [5]. Fig.1 also shows that the ^3H originally associated with the retinyl moiety was found exclusively in the M-fragment. This confirms the observations in [5,8] on which doubt had been cast [9].

The mixture containing the 3 carboxymethylated fragments was dissolved in Ammonyx LO and subjected to chromatography on con A–Sepharose. The carbohydrate containing H-fragment was retained on the column, whilst the L- and M-fragments were eluted in the void volume. After the removal of the detergent the L- plus M-fragments were treated with CNBr and the mixture of peptides separated in two steps involving first high-voltage paper electrophoresis and then high-performance liquid chromatography to give in ~30% overall yield a single labelled peptide having the composition:

Asp 0.9, Thr 3.0, Ser 1.4, Pro 1.9, Ala 3.1, Val 2.5, Ile 3.0, Tyr 0.82, Lys 0.7, Homoserine 0.5

The sequence analysis of the peptide was performed by the method in [10] as described in [7]. The first 13 amino acids of the peptide were:

Thr–Ile–Pro–Ala–Phe–Phe–Ala–(Lys)–Thr–Ser–
1 2 3 4 5 6 7 8 9 10

Ala–Val–Tyr
11 12 13

and ≥50% of the radioactivity originally used in sequence analysis was released at cycle 8. The residue removed at that cycle therefore must represent the active-site lysine of rhodopsin.

The sequence of 83 amino acids from the carboxyl-terminal of bovine rhodopsin may be deduced from the structural studies in [11,12] and is shown in fig.2. The sequence information on the 13 residues in the active-site region of rhodopsin obtained here is fully in accord with the results in [12] and allows the retinal-binding lysine to be identified as the 53rd resi-

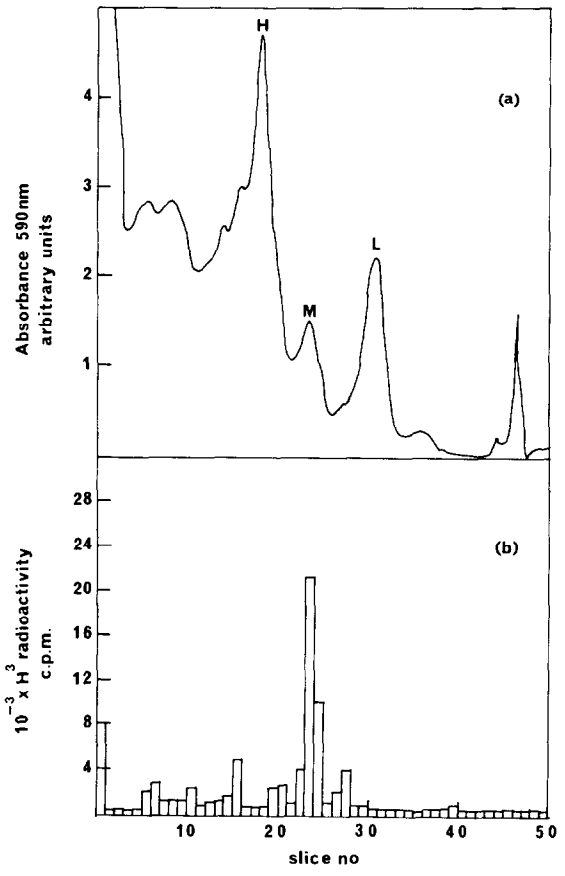

Fig.1. Polyacrylamide gel electrophoresis of papain-cleaved [^{3}H]retinylopsin after carboxymethylation. The protein was prepared as in section 2. A sample (15 nmol, 4.6 × 10^4 cpm) was electrophoresed in the presence of SDS [6]. The gels were stained, scanned at 590 nm (a) and sliced into 2 mm discs for the determination of radioactivity (b) as in [6].

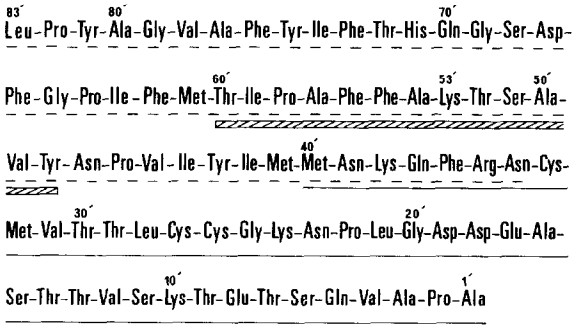

Fig.2. The partial amino acid sequence of bovine rhodopsin showing the position of the active-site lysine (53′). The peptides were sequenced by: Hargrave et al. [11] (——); Pellicone et al. [12] (– – –); and in this paper (ZZZ). The numbering system 1′,2′,3′ . . . denotes positions from the carboxyl-terminal.

Table 1
The partial amino acid sequence of the active-site peptide

Turn no.	Amino acid identified	cpm in the extracted derivative after cleavage
1	Thr	1174
2	Ile	553
3	Pro	359
4	Ala	586
5	Phe	405
6	Phe	284
7	Ala	250
8	n.d.	33 891
9	Thr	1540
10	Ser	206
11	Ala	n.d.
12	Val	n.d.
13	Tyr	n.d.
Radioactivity remaining in the peptide after turn 13 (cpm)	–	6560

The peptide (21 nmol, 6.5×10^4 cpm) was sequenced as in [7]. A portion of the coloured hydantoin derivative was used for the identification of the amino acid residue and the remainder for the determination of radioactivity. Except for residue 8 when the entire butyl acetate extract was used for the determination of radioactivity (n.d., not determined)

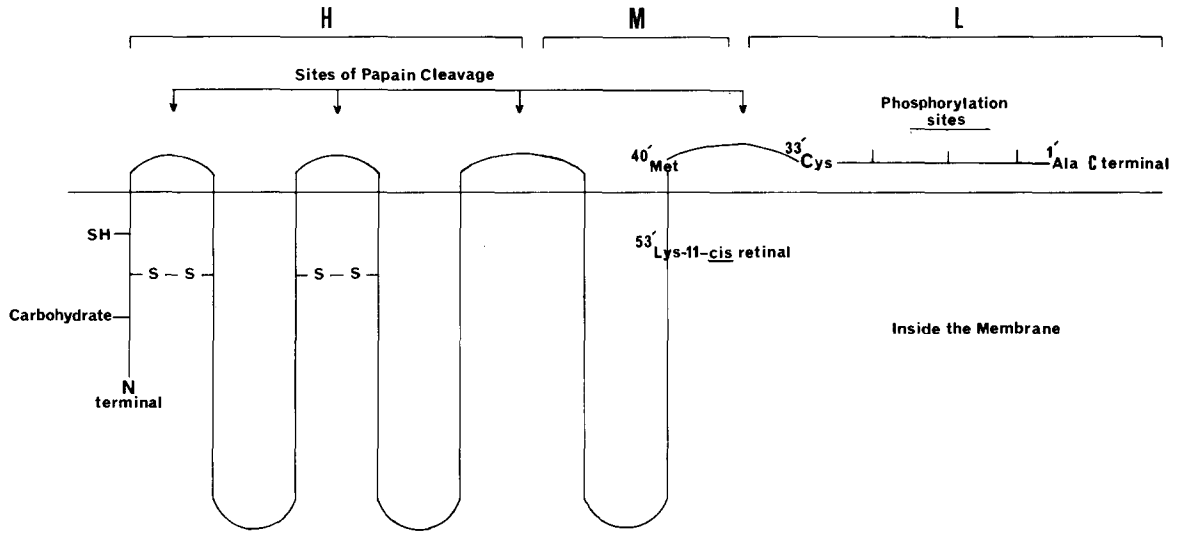

Fig.3. Further elaboration of the model of Sale et al. [16] showing the organisation of rhodopsin in the membrane. The arrangement of rhodopsin in the membrane is deduced from the mode of cleavage by papain, which produces 3 fragments, heavy (H), medium (M) and light (L). The L-fragment contains an SH group modified by 5-iodoacetamidosalicylate [5], which is assigned position 33 from the carboxyl-terminal of rhodopsin [11]. The phosphorylation sites are also shown to be localised in the L-fragment [16] and have been suggested to be between 6′–15′ [11]. The M-fragment contains the retinal-binding lysine which is now assigned position 53′. A papain cleavage site must exist between residues 40′ and 33′ (see text). Some chemical features of the H-fragment, which is shown in a highly stylised form, are discussed in [5], though neither the location of the reactive SH group in the fragment nor the positions of the disulphide linkages are known.

due from the carboxyl-terminal of rhodopsin. In [13] a dipeptide, alanyl–retinyl lysine, was isolated from a pronase digestion of $NaBH_4$-treated rhodopsin and it was suggested to correspond to the Ala–Lys residues present in the sequence in [12] and now assigned positions 52′ and 53′. We believe that our experiments provide the first direct and unambiguous identification of the retinal-binding region in the partial sequence of bovine rhodopsin. These findings, when taken in conjunction with the demonstrations [7,14, 15] that Lys_{216} is the retinal-binding residue in bacteriorhodopsin, highlight the fact that no homology exists in the sequences around the active-site regions of the two types of rhodopsins. In both cases, however, the chromophore-binding lysines are located towards the carboxyl-terminal regions of the proteins; the lysines occupying positions 53′ and 33′ in rhodopsin and bacteriorhodopsin, respectively.

This study also sheds light on a topographical feature of rhodopsin. The peptide used in the sequence analysis was one of the CNBr cleavage products of the M-fragment and was shown to contain homoserine. This fact when taken in conjunction with the sequence data in table 1 allows the peptide to be assigned the stretch of residues from position 60′–41′ in the sequence of fig.2. It therefore follows that the carboxyl-terminal of the M-fragment must extent beyond residue 41′. The L-fragment contains the SH-group of rhodopsin that is modified by 5-iodoacetamidosalicylate [5]. The same SH-group has been identified as residue 33 from the carboxyl-terminal of rhodopsin [11]. This would suggest that the rhodopsin molecule protrudes from the membrane somewhere around residues 40′–34′ providing a site for cleavage by papain between the M- and L-fragments.

The original model of the organisation of membrane-bound rhodopsin originally proposed [16] may be extended to embody the additional information as shown in fig.3.

Acknowledgements

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