THE IDENTIFICATION OF Lys_{216} AS THE RETINAL BINDING RESIDUE IN BACTERIORHODOPSIN

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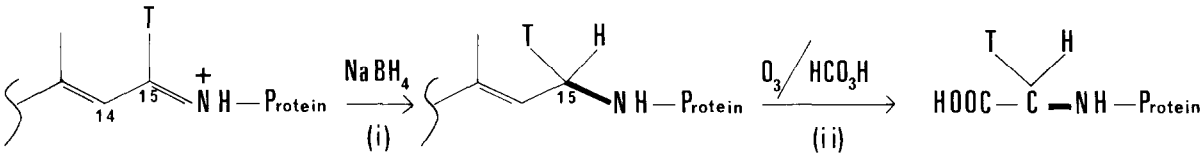
1. Introduction

Extending our studies on the development of methods for the structural analysis of the chromophore-binding sites in various rhodopsins [1–5] we now report experiments showing that in bacteriorhodopsin the retinal-moiety is attached to Lys_{216} in the primary sequence recently established by the painstaking work of the Russian [6] and American groups [7]. The two main features of our original strategy to explore the nature and location of the retinal-opsin bond involved labelling the rhodopsin active-site with [15-^{3}H]retinal and then fixing the Schiff-base in the reconstituted species with $NaBH_4$ [2,3].



(1) (2) (3)

Scheme 1

The retinyl-opsin bond (–in 2) formed in Reaction (i) is stable to alkaline hydrolysis and also to weak acids, but behaves capriciously under the varied conditions normally used for the purification and isolation of active-site peptides. In order to avoid a possible loss of the label through the cleavage of the retinyl-opsin bond (–in 2) so laboriously constructed, we have developed a protocol for the oxidative transformation of a retinyl moiety into a carboxymethyl group. The tritium label originating from C-15 of retinal now occupies a relatively stable position in (3). The incorporation of this feature in a degradative strategy coupled with some other methodological improvements have permitted the unambiguous identification of the retinal-binding site in bacteriorhodopsin.

Note: The lysine is in position 216 as in [7] but would correspond to 215 in [6]

2. Experimental

2.1. *Preparation of purple membranes*

Purple membranes from the bacterium *Halobacterium halobium* were prepared by the method of Oesterhelt and Stoeckenius [8]. The purified membranes showed a single band of approximate relative molecular mass 26 000 on sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

2.2. *Bleaching of purple membranes*

Purple membranes (300 nmol, calculated from ϵ_{568} 63 000 l. mol^{-1} . cm^{-1}), [9] were suspended in 1.0 M-hydroxylamine (10 ml), adjusted to pH 7.8, and bleached by the addition of about 400 μl of diethyl ether in 10 μl aliquots over a period of 10–15 min with constant mixing until the purple colour just disappeared. The bleached purple membranes were pooled and centrifuged at 56 000 × *g* for

30 min. The pellet was washed three times by suspension in water and centrifugation. 6.4 μmol of purple membrane were processed in 22 such operations. It was found that, whereas retinyl containing bacteriorhodopsin species are not denatured by ether, the apoprotein, bacterio-opsin, is rapidly denatured by the solvent necessitating that the concentration of ether is adjusted to allow only the exposure of the Schiff-base linkage to the external nucleophile without affecting the opsin.

2.3. *Preparation and fixation of [^{3}H]bacteriorhodopsin [4]*

The pellet of bleached purple membranes was suspended in 67 mM potassium phosphate buffer, pH 7.0, (100 ml) and divided into two parts of 47 ml and 53 ml. Ethanolic solutions of 30 mM-all-*trans*-[15-^{3}H]-retinal (3.3 μmol and 3.7 μmol; 5.5×10^6 cpm μmol^{-1}) [3] were added to the bleached material. The mixtures were left in the dark overnight. Regeneration, as measured by the appearance of the 568 nm absorption band of bacteriorhodopsin, was found to be 97% and 80% respectively (2.9 μmol) and 3.0 μmol). The samples were centrifuged at 56 000 × *g* for 30 min, resuspended in aqueous hydroxylamine solution (2% w/v) and re-centrifuged. The pellets were then washed once in the hydroxylamine solution and twice more in water by resuspension and recentrifugation. The final pellets were resuspended in water (20 ml). Sodium borohydride (200 mg) was added to each at intervals while the samples were incubated for 40 min under illumination from a 250 W light source. During fixing the temperature of the stirred suspensions was maintained at approx. 10°C. The fixed material was centrifuged at 56 000 × *g* for 30 min and the pellets washed twice in water by suspension and centrifugation. The pellets were dissolved in 5% (w/v) sodium dodecylsulphate solution (4 ml) and the protein precipitated with methanol (20 ml). The precipitates were centrifuged at 56 000 × *g* for 30 min and washed twice in water by suspension and centrifugation. From a knowledge of the specific activity of the all-*trans*-[15-^{3}H]retinal used and the amount of protein recovered (2.3 μmol and 2.5 μmol, calculated from ϵ_{280} 169 000 mol^{-1} . cm^{-1}) and the amount of radioactivity associated with that protein; 1.07 mol and 1.12 mol of retinal were found to be incorporated/mol of bacterio-opsin respectively in the two batches. Analysis of tritiated retinyl-bacterio-opsin on polyacrylamide gel electrophoresis as described in [4] showed that in both cases the only radioactive region on the gel was that corresponding to the single band of M_r 26 000.

2.4. *Ozonolysis and performic acid oxidation of tritiated retinyl-bacterioopsin*

The tritiated retinyl-bacterio-opsin obtained as described above were pooled (4.8 μmol, 2.9×10^7 cpm), dissolved in formic acid (15 ml) and ozone was passed through the solution for 20 min at room temperature at a rate of approx. 0.6 mg ozone per min. Freshly prepared performic acid (30 ml) was added and the mixture left on ice for 40 min. After dilution with water (300 ml) the solution was freeze dried.

2.5. *Preparation of peptide A*

Tritiated ozonised retinyl-bacterio-opsin (4.3 μmol, 2.36×10^7 cpm) was dissolved in 0.1 M ammonium bicarbonate solution, pH 8.0 (30 ml), trypsin (0.5 mg; EC 3.4.21.4, Sigma Chemical Co., Poole, Dorset, BH17 7NH, UK) was added and the mixture incubated by shaking at 37°C overnight. The trypsin treated material was heated in a boiling water bath for 1 min and the mixture freeze dried.

A portion of preceeding material was dissolved in pyridine:acetic acid:water (25:1:225, by vol.), pH 6.4 (1 ml) and subjected to high voltage paper electrophoresis in pyridine:acetic acid:water (25:1:225, by vol.), pH 6.4 for 45 min at 2 KV in a Gilson Medical Electronics Model D High Voltage Electrophorator. The area corresponding to the radioactivity was found by spraying a section of the electrophoretogram with ninhydrin (0.1% w/v ninhydrin in 14% v/v acetic acid in butanol) and determining the radioactivity in 1 cm strips cut from the paper. The only radioactive region, which was approx. 2 cm from the origin towards the anode, was eluted in ethanol:water:pyridine (50:50:1, by vol.). The eluants from four such procedures were pooled. The material (903 nmol, 4.97×10^6 cpm) was divided into two portions, and each subjected to overnight descending paper chromatography in butanol:acetic acid:water (4:1:5, by vol. upper layer). The zone corresponding to the radioactivity was found, as described for the electrophoresis, approx. 20 cm from the origin on a 40 cm long paper, and was eluted in ethanol:water:pyridine (50:50:1, by vol.).

The purified radioactive peptide (A) was pooled (271 nmol 1.49×10^6 cpm) and freeze dried. The material was further 'cleaned' by passing through a

Water's bondapack CN high performance liquid chromatography column equilibrated with 0.1% (v/v) trifluoroacetic acid. The peptide was eluted with a 0–60% gradient of 0.07% (v/v) trifluoroacetic acid in acetonitrile run for 25 min at 1.5 ml min^{-1} (system 1). The single peak, which eluted at 40% acetonitrile and contained the radioactivity was collected. 106 nmol (5.8×10^5 cpm) of purified peptide A were collected in this manner.

2.6. *Preparation of peptide B*

In a second experiment, 2.84 μmol of bacteriorhodopsin was regenerated with 30 mM ethanolic all-*trans*-[15-^{3}H]retinal (3.1 μmol, 1.97×10^6 cpm μmol) and fixed as described. 1.2 mol of retinal was incorporated/mol of bacterio-opsin.

Tritiated retinyl-bacterio-opsin (2.0 μmol 4.72×10^6 cpm) was dissolved in 70% aqueous formic acid (5 ml) and stirred with an excess of cyanogen bromide (20 mg) overnight in the dark. The reaction mixture was diluted with water (50 ml) and freeze dried. The residue was dissolved in formic acid (7 ml), ozonised and treated with performic acid (10 ml) as described. After freeze drying, the residue was treated with trypsin (0.25 mg) in 0.1 M ammonium bicarbonate, pH 8.0 (10 ml). The resulting peptide mixture was divided into two and each portion run on high voltage paper electrophoresis pH 6.4 as described. The radioactive zone, found 2 cm towards the cathode, was eluted. The material (637 nmol, 1.25×10^6 cpm) was run on descending paper chromatography, the radioactive area, 38 cm from the origin eluted and the eluant (162 nmol 3.2×10^5 cpm) run on high performance liquid chromatography in system 1. A single peak, containing all of the radioactivity was eluted at 35% acetonitrile. A final yield of 122 nmol (2.4×10^5 cpm) of purified peptide B was collected in this manner.

2.7. *Sequence determination*

The sequence analysis was carried out essentially as described [11]. The peptide (15–20 nmol), in a glass vial (35 mm × 6 mm internal diameter), was taken up in pyridine:water (1:1, v/v, 80 μl) and admixed with a solution of 4-*N,N*-dimethylamino-azobenzene 4′-isothiocyanate (BDH, Poole, Dorset, UK) in pyridine (40 μl; 5–6 mg of the reagent dissolved per ml of pyridine). The vial, after being flushed with argon, was stoppered and placed in a heating block at 54°C for 30 min, then further treated under argon at the same temperature with phenylisothiocyanate (10 μl) for 30 min at the same temperature to allow the modification of the amino groups remaining unreacted in the first coupling step. The excess reagents were removed by washing with two aliquots of benzene:pyridine (20:1, v/v, 300 μl each) and once with ethyl acetate:heptane (1:2, v/v; 300 μl). In the three washing steps the organic layer was separated by centrifugation at about 6000 × *g* for 1 min. The walls of the vials were freed of any remaining reagent by rinsing twice with a mixture of ethyl acetate:heptane (2:1, v/v; 150 μl). The latter operation when performed carefully, without disturbing the aqueous layer, obviated the need for centrifugation. The aqueous phase was evaporated to dryness under vacuum and the residue dissolved in trifluoroacetic acid (80 μl) and the vial flushed with argon, stoppered and placed in the heating block for 13 min. After the removal of the acid the residue was taken up in water (20 μl) and the coloured thiazolinone derivative extracted with butyl acetate (150 μl) and converted to the corresponding 4-*N,N*-dimethylazobenzene thiohydantoin derivative as described in ref. [11]. The identification of the derivative was also performed as described in the latter reference except that the solvent mixture used in the second dimension had the ratios of toluene:hexane:acetic acid, 4:2:1, (by vol.) and not 2:1:1 (by vol.) as quoted therein.

The differentiation between leucine and isoleucine was not performed in the present work.

3. Results and discussion

3.1. *The preparation and oxidation of [^{3}H]retinyl-bacterio-opsin with O_3/performic acid*

Bacteriorhodopsin prepared by a new method, described in the Experimental section was combined with all-*trans*-[15-^{3}H]retinal and the resulting pigment fixed with $NaBH_4$ under illumination. The conditions for the $NaBH_4$ reduction step were intentionally chosen to be the same as those used by Bridgen and Walker in their original attempt to locate the retinal-binding site [10], so that the results from the two studies could be directly compared.

[15-^{3}H]Retinyl-bacterio-opsin (structure of the type 2) was subjected to oxidation with O_3/performic acid under conditions which had previously been worked out for the conversion of *N*-ε-retinyl-lysine into *N*-ε-carboxymethyl-lysine. The ozonised protein

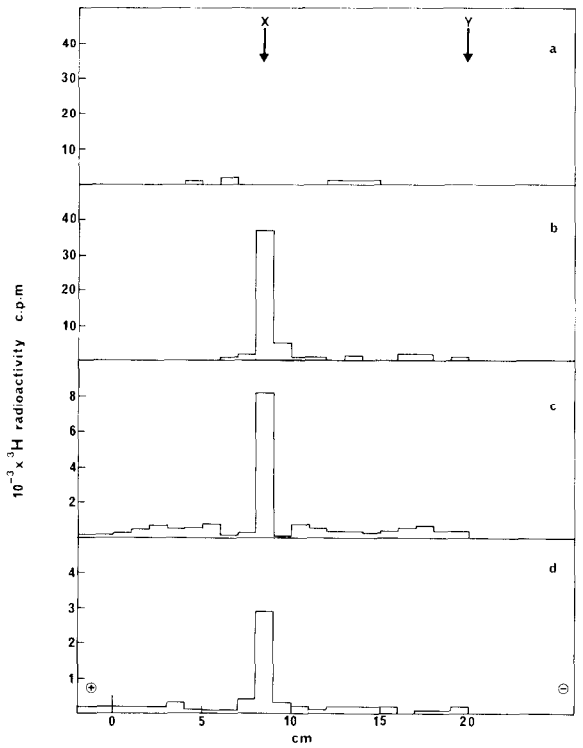


Fig.1. High voltage paper electrophoresis of hydrolysed retinyl-bacterio-opsin and ozonized retinyl bacterio-opsin. Tritiated retinyl-bacterio-opsin (20 nmol, 10.98 × 10^4 cpm); the preceding material ozonized (22 nmol, 12.1 × 10^4 cpm); the peptide A (11.3 nmol, 6.2 × 10^4 cpm) and peptide B (11.1 nmol, 2.2 × 10^4 cpm) were hydrolyzed overnight at 110°C in 6 M HCl and electrophoresed in pyridine:acetic acid:water (1:10:289, by vol.) pH 3.5 for 2.5 h at 2 kV. 1 cm strips of the electrophoretogram were cut and used for the determination of radioactivity. The results are shown in panels a,b,c and d respectively. X and Y are the positions of authentic *N*-ε-carboxymethyl lysine and lysine; + is the anode and − the cathode.

was hydrolysed and subjected to electrophoresis when the only radioactive region on the paper was found to be that corresponding to the position of authentic *N*-ε-carboxymethyl-lysine. The overall recovery of radioactivity from the oxidised protein to the derivative was 30%. Fig.1 also shows that the unozonised material on hydrolysis, as expected, gave no well defined zone of radioactivity. The amino acid analysis data in table 1 show that the ozonolysis

Table 1
The amino acid composition of bacteriorhodopsin, ozonised bacteriorhodopsin and the peptides A and B

Amino acid residue	[^{3}H]Retinyl bacterio-opsin	Ozonised [^{3}H]retinyl bacterio-opsin	Peptide A	Peptide B
Met sulphone	0	11.1	0.9	0
Asp	13.0	16.0	2.8	1.1
Thr	18.7	18.7	2.0	0.5
Ser	12.9	13.3	2.0	1.2
Glu	21.0	21.0	3.6	1.5
Pro	10.5	10.0	1.6	0
Gly	27.2	27.2	5.4	2.0
Ala	29.8	27.7	3.2	1.0
Cys	0	0	0	0
Val	21.6	21.12	6.3	0.8
Met	8.89	0	0	0
Ile	15.0	13.3	2.3	0.8
Leu	36.7	32.6	4.9	1.6
Tyr	12.0	0	0	0
Phe	14.1	11.9	3.1	0
His	0	0	0	0
Lys	6.91	8.6	0	0
Arg	13.7	9.1	1.3	0

Tritiated retinyl-bacterio-opsin (5 nmol, 2.75 × 10^4 cpm), the preceeding material ozonised (5 nmol, 2.75 × 10^4 cpm), the peptide A (3 nmol, 1.65 × 10^4 cpm) and peptide B (5 nmol, 2.75 × 10^4 cpm) were hydrolyed overnight at 110°C in 6 N HCl in the presence of a standard of norleucine and the acid hydrolysate analysed on a Rank Hilger J 180 automated amino acid analyser

treatment in addition to performing the desired conversion (2 → 3) had also resulted in the destruction of tyrosine and therefore by implication, tryptophan side chains. Methionine, as expected, was converted to Methionine sulphone. A desirable consequence of the unwanted modification of the aromatic residues being that the ozonised protein was now readily soluble in water, without the addition of any detergent, and therefore could be fragmented using conventional methods.

3.2. *Isolation of peptides A and B*

The following degradation sequence:

$$[^3H]\text{Bacteriorhodopsin} \rightarrow$$

$$[^3H]\text{Retinyl-bacterio-opsin} \xrightarrow{O_3/HCO_3H}$$

$$[^3H]\text{Carboxymethylbacterio-opsin} \xrightarrow{\text{Trypsin}}$$

$$[^3H]\text{Carboxymethylpeptide A}$$

gave a single labelled peptide, peptide A, which was purified by two-dimensional paper separation and HPLC and sequenced by the method of Chang et al. [11]. The first eight amino acid of the peptide were:

$$\underset{173}{\text{Val}}\ \text{Leu/Ile Arg Asn Val Thr Val}\ \underset{180}{\text{Val}}$$

and corresponded to residues 173 to 180 in the primary sequence of bacteriorhodopsin. The peptide remaining after the eighth residue still retained at least 72% of the radioactivity originally used in the sequence analysis. Since the only lysine following the above sequence is that at position 216, it may be inferred that Lys_{216} represents the retinal-binding site.

In order to further evaluate this assertion, the following degradative strategy was planned;

$$[^3H]\text{Retinyl-bacterio-opsin}$$

$$\xrightarrow{\text{(i) CNBr, (ii) } O_3/HCO_3H\text{, (iii) trypsin}}$$

$$[^3H]\text{Carboxymethylpeptide B}$$

to produce peptide B. It should be stressed that peptide B was the only labelled species formed in the degradation and had the sequence:

$$\underset{210}{\text{Val}}\ \text{Leu/Ile Asp Val Ser Ala} - \underset{217}{\text{Val}}$$

The data in table 2 show that all the radioactivity was clearly removed at the 7th cycle corresponding to the position of Lys_{216}. The residue at the 8th cycle was valine and the peptide material remaining after this step was devoid of any radioactivity. The sequence

Table 2
The amino acid sequence of peptides A and B

Turn no.	Peptide A		Peptide B	
	Amino acid identified	Radioactivity from butyl-acetate extract (cpm)	Amino acid identified	Radioactivity from butyl-acetate extract (cpm)
1	Val	72	Val	659
2	Leu/Ile	1250	Leu/Ile	335
3	Arg	398	Asp	130
4	Asn	448	Val	138
5	Val	192	Ser	93
6	Thr	169	Ala	4082
7	Val	28	nd	19 728
8	Val	1821	Val	672
Radioactivity remaining in peptide		81 156		1016

Peptide A (20 nmol, 1.1×10^5 cpm) and peptide B (23 nmol, 4.6×10^4 cpm) were sequenced by the method of Chang et al. [11]. 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 the 7th residue in the peptide B, when the entire butyl acetate extract was used for the determination of radioactivity. The identification of the 7th residue as modified lysine is based on the radioactive information in fig. 1(c) and (d). (nd not determined)

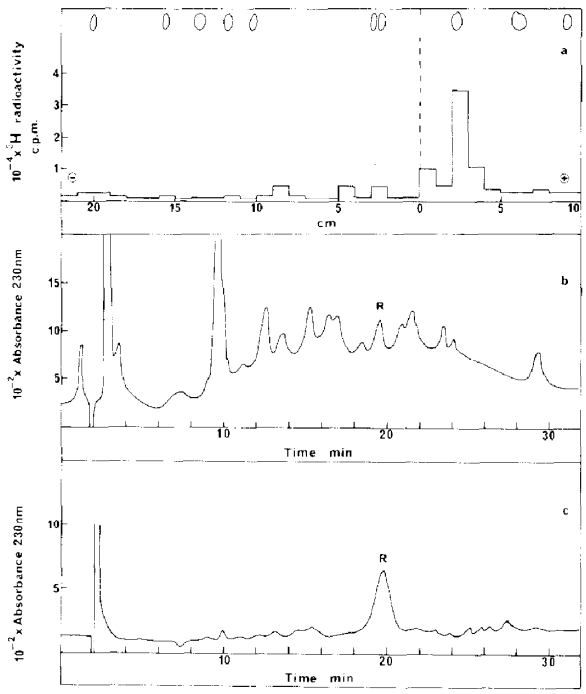


Fig.2. The purification of peptide A. (a) Ozonised tritiated retinyl-bacterio-opsin (5.5. × 10^4 cpm) was treated with trypsin and the tryptic peptides electrophoresed on high voltage paper electrophoresis pH 6.4 as described in the experimental section. 1 cm strips of paper were cut and used for determination of radioactivity. The circles represent the ninhydrin positive spots. (b) The radioactive material from the high voltage paper electrophoresis was analysed on high performance liquid chromatography as described in the experimental section. 80% of the applied radioactivity was eluted in the peak R. (c) In other experiment the radioactive material from 2(a) was subjected to descending paper chromatography as described in the experimental section. All of the radioactivity applied to the column was eluted in peak R.

and radiochemical information detailed in table 2 when taken in conjunction with the primary sequence of the protein prove beyond doubt that $NaBH_4$ under illumination had fixed the chromophore to the ε-amino group of Lys_{216} [7]. This residue therefore should be regarded as the retinal-binding site in the native protein.

This is in contrast to the currently prevailing view, based on the work of Bridgen and Walker [10] that Lys_{41} is the retinal-binding residue in bacteriorhodopsin. It should be stressed that the conditions used for the reduction of the Schiff-base linkage in the present study and in the original work were similar. The earlier identification of Lys_{41} was however based on an indirect approach in which native bacteriorhodopsin was fixed with $NaBH_4$ and the reduced species then treated with [^{14}C]succinic anhydride in an attempt to modify all the remaining lysines. The lysine deemed not to contain the radioactivity was ascribed the chromophore-binding role.

We consider the approach theoretically sound but based on two assumptions. First, that $NaBH_4$ treatment had in fact resulted in the fixation of the retinyl moiety to the protein and second, that succinic anhydride had quantitatively modified all the remaining lysine residues in the protein. Neither of these assumptions was evaluated experimentally, thus casting doubt on their validity. Notwithstanding this criticism, the possibility exists that either Lys_{41} and Lys_{216} are both involved in retinal-binding but at two different stages of the proton translocation cycle or are located sufficiently close to one another to permit an intramolecular migration of the retinyl moieity during the $NaBH_4$ reduction step. In these circumstances the trapping of the Schiff-base linkage depending on unpredictable factors would give ambiguous results. However, for such a view to deserve serious consideration, the attachment of retinal to a lysine, other than the one identified in this paper, would need to be demonstrated using a direct and reliable approach. As far as we are aware, this has not yet been achieved.

While this work was in progress we learnt that Professor H. G. Khorana had provided evidence that the binding of retinal in bacteriorhodopsin involved Lys_{216}. We acknowledge with pleasure that Professor Khorana's group had firm information on this aspect prior to the completion of our own studies. The question of priority aside, that two groups using independent approaches have come to the same conclusion would require a reinterpretation of the 3-dimensional models proposed for bacteriorhodopsin [12,13].

Acknowledgements

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