A PROBABLE LINKING SEQUENCE BETWEEN TWO TRANSMEMBRANE COMPONENTS OF BACTERIORHODOPSIN

Nandini V. KATRE and Robert M. STROUD
Department of Biochemistry and Biophysics, University of California, School of Medicine, San Francisco, 94143, USA

Received 19 October 1981

1. Introduction

Purple membrane from *Halobacterium halobium* contains predominantly one protein, bacteriorhodopsin, which acts as a light-driven proton pump [1], and which consists of 7 transmembrane rods of electron scattering density [2]. X-Ray diffraction analysis [3,4] suggests that the rods correspond predominantly to α-helical regions, oriented roughly perpendicular to the membrane plane. However, which parts of the sequence are contained in the rods within the bilayer and which correspond to the linking regions between helices is not known. Enzymatic cleavage [5,6] or chemical modification of the protein, using reagents which preferentially position either into the aqueous, or the lipid phase identify linking or transmembrane regions.

We describe specific labelling of tyrosines accessible to lactoperoxidase iodination of bacteriorhodopsin from the aqueous phase. The site of modification is located by separation of peptides using Sephadex LH-60 chromatography and amino acid analysis.

2. Methods

2.1. *Isolation of purple membrane*

Halobacterium halobium strain R_1 was grown and purple membrane was isolated as in [7].

2.2. *Iodination of tyrosines in purple membrane*

The tyrosine residues of bacteriorhodopsin were modified using sodium [^{125}I]iodide and the lactoperoxidase–glucose oxidase enzyme system [8] immobilized on hydrophilic polyacrylamide beads (Enzymobeads), obtained from BioRad. Purple membrane (2 mg) was suspended in 1 ml 0.2 M phosphate buffer (pH 7.2). The Enzymobead reagent (1 mg) dissolved in 0.5 ml distilled water was added to the membrane suspension. A 10-fold excess of Na^{125}I over purple membrane was then added and the reaction started by addition of 0.25 ml 2% glucose. After 15 min at room temperature, the enzymobeads were removed by centrifugation 1000 × *g* for 5 min. The purple membrane was washed several times with phosphate buffer, and concentration assayed using extinction coefficient $\epsilon_{570} = 63 \times 10^3$ cm^{-1} for the light-adapted form [9]. The same procedure was used to iodinate 20 mg protein, using non-radioactive NaI.

2.3. *Identification of labeled residues*

The radioactive and non-radioactive iodinated samples were mixed and initial cleavage of labeled protein was performed by treatment of apomembrane with α-chymotrypsin (Sigma Chemical Co.) at a ratio of 1.5×10^{-2} mg/mg protein, for 5 h, as described [5]. Apomembrane was generated by bleaching in 1 M hydroxylamine hydrochloride, pH 7.0 at 37°C for 2–3 hours [10], and collected by centrifugation at 25 000 × *g*. The two chymotryptic fragments generated were separated by Sephadex LH-60 chromatography (column size 2.0 cm diam. × 60 cm length), using 96% formic acid and ethanol (2.5:7) as elution buffer and 2 ml fractions were collected. The larger of the 2 chains (residues 72–248), named (CTI) and the smaller chain (CTII residues 1–71) were separated. CTI was lyophilized, dissolved in 70% HCOOH and treated with cyanogen bromide (CNBr) in 70% HCOOH. A 100-fold molar excess of CNBr over methionine residues was used. The reaction mixture was incubated at 37°C for 24 h, after which it was diluted 10-times with distilled water and lyophilized.

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

The lyophilized CNBr fragments of CTI were separated on Sephadex LH-60 as above.

The peak containing radioactivity was lyophilized and subjected to amino acid analysis and polyacrylamide (15%)–SDS gel electrophoresis [11].

Amino acid analysis was carried out on a Beckman 121 M amino acid analyzer following complete acid hydrolysis in 12 N HCl for 22 h. Radioactivity was counted on a Beckman model 300 gamma-counter.

3. Results

Chymotryptic cleavage of bacteriorhodopsin gave 2 fragments migrating with app. M_r 19 000 (CTI) and 7000 (CTII) on SDS–polyacrylamide gels. Separation of these 2 fragments on Sephadex LH-60 is shown in fig.1. The difference in absorbance at 280 nm for the 2 fractions was primarily due to the different numbers of tryptophan and tyrosine residues in the sequences (6 Trp, 7 Tyr in CTI; 2 Trp, 4 Tyr in CTII). 0.2 ± 0.04 mol ^{125}I was bound/mol purple membrane. ^{125}I was associated mainly with the CTI fragment. The separation of the CNBr fragments of labeled CTI gave primarily one radioactive peptide (fig.2). The radioactivity with the void volume was due to aggregated material.

CNBr cleavage of CTI results in 5 fragments. Starting from the N-terminus, I has residues 72–118, II (119–145), III (146–163) IV (164–209) and V (210–248). V contains no tyrosine or tryptophan, hence no absorbance at 280 nm. The amino acid analysis of the main radioactive peptide, containing the modified tyrosine(s) is shown in table 1. This peptide best

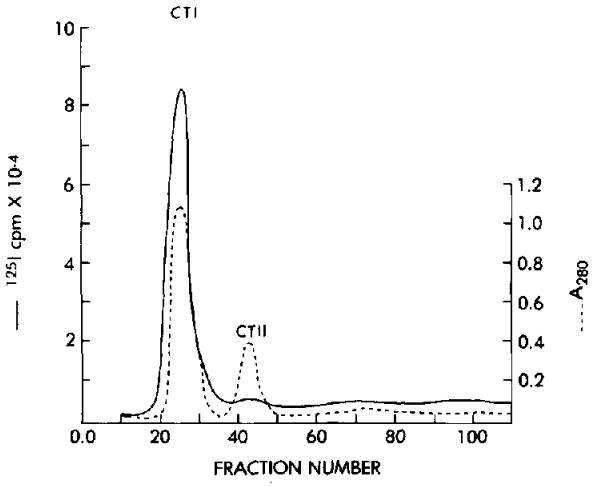


Fig.1. Fractionation of iodinated purple membrane after cleavage, on Sephadex LH-60: (– – –) absorbance at 280 nm of the fractions; (——) cpm of the iodinated purple membrane; CTI contained residues 72–248; CTII contained residues 1–71.

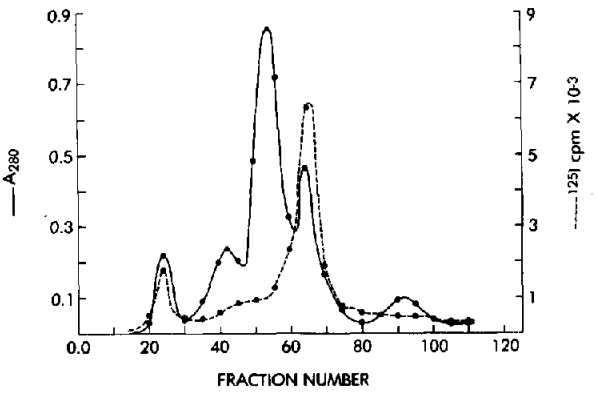
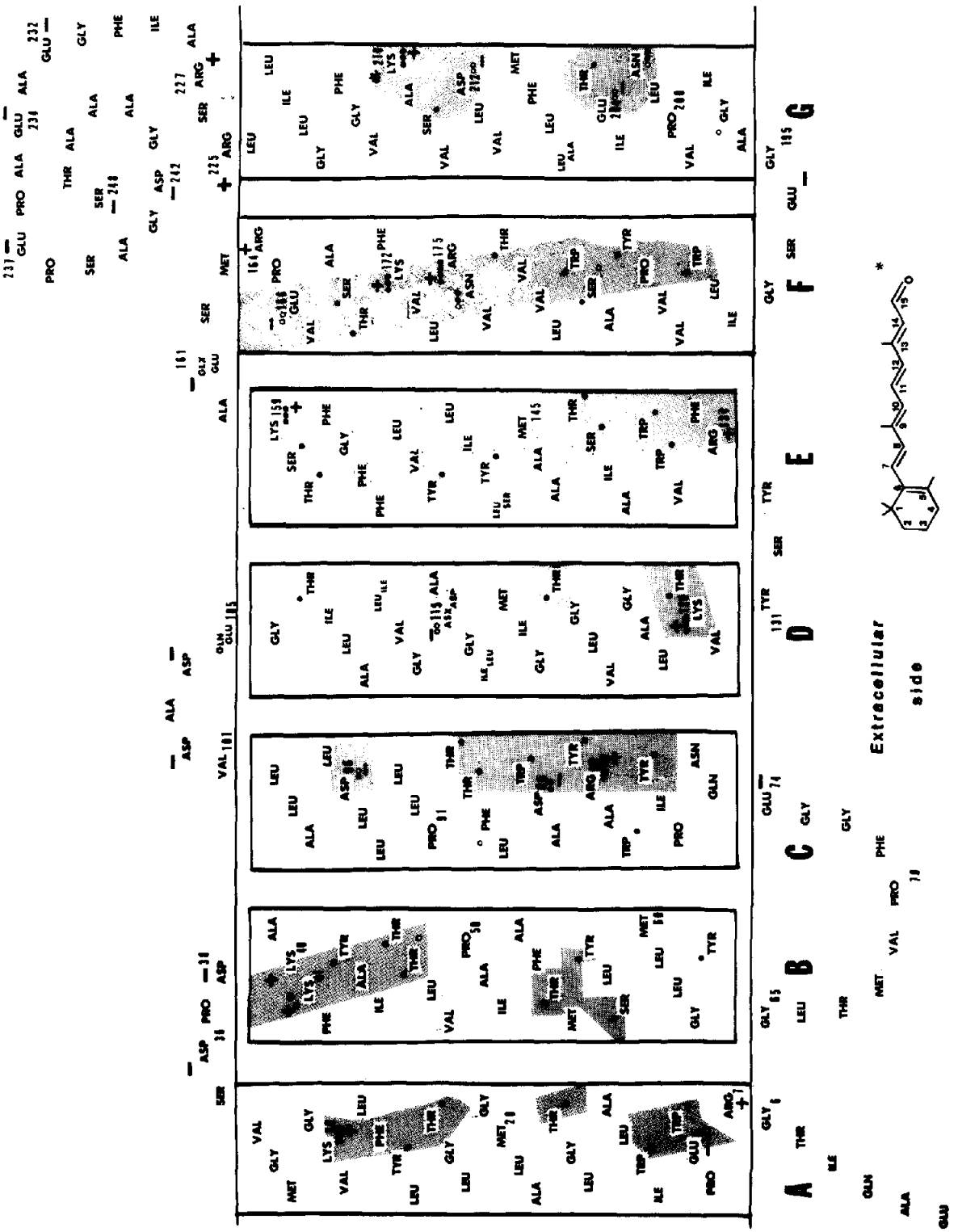


Fig.2. Fractionation of CTI (the larger chymotryptic fragment) of iodinated bacteriorhodopsin after CNBr cleavage, on Sephadex LH-60: (——) represents absorbance of fractions at 280 nm; (– – –) represents cpm of the iodinated peptide.

Table 1
Amino acid analysis: CNBr fragment containing modified tyrosine

Found	Expected for the 5 CNBr fragments of CTI				
	I	II	III	IV	V
Lys 1.1	0	1	1	1	1
Arg 0.8	1	1	0	2	2
Asp 0.8	5	0	0	2	2
Thr 2.8	3	3	1	3	1
Ser 1.6	0	2	2	3	4
Glu 0.6	3	0	1	3	3
Pro 0.7	2	0	0	3	2
Gly 2.3	5	3	1	3	5
Ala 4.0	6	4	1	3	8
Val 2.4	2	3	1	8	3
Ile 1.8	3	2	1	3	2
Leu 2.8	10	2	3	7	4
Tyr 1.5[a]	2	2	2	1	0
Phe 1.5	1	1	0	2	2
RMS deviation $\sqrt{\chi^2/12}$	±2.8	±0.5	±1.3	±2.4	±1.9

[a] Tyr was not used to calculate χ^2


A
B
C
D
E
F
G
Extracellular side

matched the CNBr peptide II (amino acids 119–145), which contains tyrosines 131 and 133.

The iodination reaction did not result in any shift in λ_{max} at 570 nm of the light-adapted modified purple membrane, indicating no major structural changes involving the retinyl moiety.

4. Discussion

The conditions we utilized for iodination of tyrosines were optimized to label the residues most accessible from the aqueous phase, which can constitute the linking regions between the transmembrane components of bacteriorhodopsin. The tyrosines involved in the transmembrane rod-like structures, buried in the hydrophobic region of the membrane are not likely to be modified. The stoichiometry of labeling was low, 0.2 ± 0.04 mol ^{125}I/mol protein under these conditions. The labeling pattern indicates that the modified tyrosine(s) are contained in the peptide, amino acids 119–145. Therefore one or both of the only tyrosines 131 and 133 are likely to be in the linking region.

The scheme shown in fig.3 shows an arrangement of the amino acid sequence organized into 7 transmembrane stretches and arranged in an α-helical pattern. Slightly different arrangements have been published in [6,12].

Experimental evidence, mainly enzymatic cleavage using α-chymotrypsin, papain and trypsin, has determined the amino acid residues which constitute the N-terminus, the C-terminus tail and the linking region between helix B and helix C [5,6,13]. The iodination of tyrosines 131 and/or 133, establishes the likely linking region between helix D and helix E. There are 3.4 residues/α-helical turn, thus ~27 residues contained in 8 turns would be sufficient to just span the distance between lipid head groups across the bilayer [14,15]. Using this, the remaining amino acid residues have been arranged in the scheme shown in fig.3.

There is some minor radioactivity associated with CTII, shown in fig.1. This may be modification of tyrosine 64 which is close to the external surface of the protein. Longer reaction times leads to labeling of both CTI and CTII. This is likely to be due to the modification of less accessible tyrosines, and tyrosines in the membrane region. Earlier modifications of tyrosines in purple membrane showed 1–2 tyrosines modified by free iodine [16], or more (up to 8–9 total [17]) or (up to 4 [18]) by tetranitromethane. In the latter cases, the reagents may be sufficiently hydrophobic to enter the lipid phase.

These data help establish the linking sequence of amino acids between helices D and E, that is between the helices 4 and 5, from the N-terminus.

Acknowledgements

This work was carried out under support from the National Institutes of Health (GM 27057) and from the National Science Foundation (PCM77-25407). We thank Dr John Kane of the Cardiovascular Research Institute for the amino acid analyses.

References

[1] Lozier, R. H., Niederberger, W., Bogomolni, R. A., Hwang, S. B. and Stoeckenius, W. (1976) Biochim. Biophys. Acta 440, 545–556.
[2] Henderson, R. and Unwin, P. N. T. (1975) Nature 257, 28–32.
[3] Henderson, R. (1975) J. Mol. Biol. 93, 123–138.
[4] Blaurock, A. E. (1975) J. Mol. Biol. 93, 139–158.
[5] Gerber, G. E., Anderegg, R. J., Herlihy, W. C., Gray, C. P., Biemann, K. and Khorana, H. G. (1979) Proc. Natl. Acad. Sci. USA 76, 227–231.
[6] Ovchinnikov, Yu. A., Abdulaev, N. G., Feigina, M. Yu., Kiselev, A. V., and Lobanov, N. A. (1979) FEBS Lett. 100, 219–224.
[7] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–678.
[8] Schenkein, I., Levy, M. and Uhr, J. W. (1972) Cell. Immunol. 5, 490–493.
[9] Rehorc, K. and Heyn, M. P. (1979) Biochemistry 18, 4977–4983.

Fig.3. The primary sequence of bacteriorhodopsin is depicted as arranged into 7 α-helical segments spanning the lipid bilayer. Residues are indicated as if on an unfolded cylinder for each helical region (pitch ~5.4 Å). The sequence is from [19], with differences from [6], noted below. Retinal, shown in all *trans* forms on the lower right is attached as a Schiff's base to lysine 41/lysine 216 [20–23]: (•) proton donors; (○) proton acceptors associated with side chains or main chain amides. The shaded areas include almost all donors and acceptors and are thought to indicate possible areas involved in intra- (or possibly inter-) molecular protein–protein contacts.

[10] Bauer, P. J., Dencher, N. A. and Heyn, M. P. (1976) Biophys. Struct. Mech. 2, 79–92.
[11] Laemmli, U. K. (1970) Nature 227, 580–585.
[12] Engelman, D. M., Henderson, R., McLachlin, A. D. and Wallace, B. A. (1980) Proc. Natl. Acad. Sci. USA 77, 2023–2027.
[13] Gerber, G. E., Gray, C. P., Wildenauer, D. and Khorana, H. G. (1977) Proc. Natl. Acad. Sci. USA 74, 5426–5430.
[14] Blaurock, A. E. and King, G. (1977) Science 196, 1101–1104.
[15] Stroud, R. M. and Agard, D. A. (1979) Biophys. J. 25, 495–512.
[16] Konishi, T. and Packer, L. (1978) FEBS Lett. 92, 1–4.
[17] Campos-Cavieres, M., Moore, T. A. and Perham, R. N. (1979) Biochem. J. 179, 233–238.
[18] Lemke, H.-D. and Oesterhelt, D. (1981) Eur. J. Biochem. 115, 595–604.
[19] Khorana, H. G., Gerber, G. E., Herlihy, W. C., Gray, C. P., Anderegg, R. J., Nihei, K. and Biemann, K. (1979) Proc. Natl. Acad. Sci. USA 76, 5046–5050.
[20] Lemke, H.-D. and Oesterhelt, D. (1981) FEBS Lett. 128, 255–260.
[21] Bayley, H., Huang, K.-S., Radhakrishnan, R., Ross, A. H., Takagaki, Y. and Khorana, H. G. (1981) Proc. Natl. Acad. Sci. USA 78, 2225–2229.
[22] Mullen, E., Johnson, A. H. and Akhtar, M. (1981) FEBS Lett. 130, 187–193.
[23] Katre, N. V., Wolber, P. K., Stoeckenius, W. and Stroud, R. M. (1981) Proc. Natl. Acad. Sci. USA 78, 4068–4072.