Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 926-931

DANSYLATION OF BACTERIORHODOPSIN NEAR THE RETINAL ATTACHMENT SITE

G.Harris[1,b], R. Renthal[2,a,b,c], J. Tuley[a], and N. Robinson[c]

[a]Division of Earth and Physical Sciences, and [b]Division of Allied Health and Life Sciences, University of Texas at San Antonio, San Antonio, Texas 78285

[c]Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Received October 23, 1979

SUMMARY: The purple membrane of Halobacterium halobium was reacted with 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) at pH 8.0. Chromophoric and functional properties of the product appear unaltered. Approximately 2 moles of dansyl group were incorporated per mole of bacteriorhodopsin, part bound to bacteriorhodopsin and part bound to lipids. Purification and fragmentation of the protein showed most of the dansyl modification in a fragment containing residues 33 to 56. Amino acid analysis indicates that the major dansylated site is lysine 40. We conclude that, contrary to published models, 1) bacteriorhodopsin folds in a way that exposes lysine 40 at the membrane surface, and 2) this side chain is not involved in the proton pump mechanism.

The purple membrane of Halobacterium halobium acts as a light driven proton pump (1). The membrane occurs naturally as a two dimensional crystal (2) and contains only a single retinyl protein, bacteriorhodopsin (3). The crystal structure (4) and amino acid sequence analyses (5,6) have provided a framework for understanding the molecular mechanism of the purple membrane proton pump. The 7 Å resolution crystal structure showed that bacteriorhodopsin is largely folded into seven regions of α-helix (4). However, the connection scheme of the helices is unknown. The amino acid sequence has suggested several possible folding patterns (5,7). While a detailed structural model is not yet available, much attention has focused on spectroscopic methods to elucidate the proton pump mechanism. In particular, resonance Raman (8) spectroscopy points to the involvement of certain amino acid side chains in pump activity. A proposed mechanism is based on this evidence (8).

We have been examining various chemical derivatives of the purple membrane as a means of studying the proton pump mechanism (9). We now report the reaction of the purple membrane with dansyl chloride[3]. The major site of modifica-

1. This work is part of the M.S. thesis of G. Harris. Present address: University of Texas Medical School at San Antonio, San Antonio, Texas 78284
2. To whom to address reprint requests.
3. Abbreviations: Dansyl: 5-dimethylaminonaphthalene-1-sulfonyl; HPLC: high performance liquid chromatography

0006-291X/79/230926-06$01.00/0

tion, lysine 40, is adjacent to the lysine residue which bears the retinal chromophore. The high reactivity of lysine 40 with dansyl chloride is surprising and contradicts recent ideas about bacteriorhodopsin structure (5) and mechanism (8).

MATERIALS AND METHODS

Purple membrane was isolated from Halobacterium halobium S9 by the method of Oesterhelt and Stoeckenius (10). Dansyl chloride, α-chymotrypsin and LH-60 (Pharmacia) were obtained from Sigma. All other materials used were reagent grade, available from standard suppliers.

In a typical experiment, the reaction mixture consisted of 12 ml of purple membrane suspension in water (10^{-4} M bacteriorhodopsin), 161 ml of water, and 21 ml of 0.2 M phosphate buffer, pH 8.0. To start the reaction, 4.8 ml of 10 mM dansyl chloride in ethanol was added. The reaction was allowed to proceed for 24 hours at 5^{0} C in the dark. The product was washed by centrifugation and resuspension in 0.05 M NaCl. The washing was repeated twice with 0.015 M NaCl. The final pellet was used for further studies.

Cleavage of dansyl bacterio-opsin with chymotrypsin, separation of the chymotryptic fragments, and cleavage of the small chymotryptic fragment with CNBr were done according to the procedures of Gerber et al (6). The CNBr peptides were isolated on a Laboratory Data Control HPLC with a 0.4 X 25 cm Biosil ODS-10 column (Biorad) using the solvent system developed by Gerber et al (6).

Light-induced pH changes of the dansyl purple membrane were measured as previously described (11).

RESULTS

The purple membrane was reacted with dansyl chloride at pH 8 in the dark for 24 hours at 5^{0} C. The membrane incorporated apporximately two moles of dansyl group per mole of bacteriorhodopsin, as measured by ultraviolet difference spectroscopy, using a molar extinction coefficient (330 nm) of 5000 (12). Polyacrylamide gel electrophoresis of dansyl purple membrane in dodecyl sulfate showed that most of the fluorescence was incorporated into bacteriorhodopsin, although some migrated with the tracking dye. (This may be due to reaction with lipids or to tight non-covalent binding of dansic acid to the membrane.) The dansyl purple membrane was treated with chymotrypsin and chromatographed on LH-60 as described by Gerber et al (6) (fig. 1). Two bacteriorhodopsin peptides, containing residues 1-71 and 72-247, were obtained. The smaller fragment contained 91% of the fluorescence of cleaved protein. The fluorescence migrating with the larger fragment was mostly due to the presence of uncleaved material, as shown by polyacrylamide gel electrophoresis in dodecyl sulfate.

The 1-71 fragment was reacted with CNBr, and the products were chromatographed on LH-20 according to the procedure of Gerber et al (6). The fluorescence overlapped the region of the peptides containing residues 1-20 and 33-56.

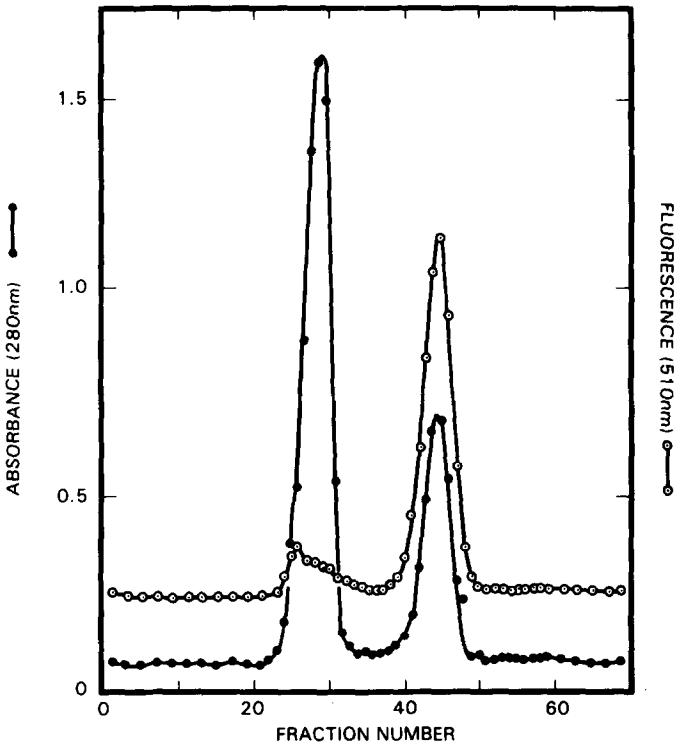

Figure 1. Separation of chymotrypsin-cleaved dansyl bacterio-opsin by LH-60 chromatography. First peak: amino acid residues 72-247. Second peak: amino acid residues 1-71. Absorbance (filled circles) was measured at 280 nm and fluorescence (open circles) was measured at 510 nm (excitation wavelength: 340 nm). The sample of lyophilized dansyl bacterio-opsin (1.2 μmol) was dissolved in 1.8 ml of 88% formic acid and 4.2 ml ethanol and applied to a 2.5 X 90 cm column of LH-60, which was then eluted with 88% formic acid : ethanol 30 : 70. Fraction size: 2.0 ml.

However, when this mixture, or the entire CNBr cleavage product, was chromatographed by reverse phase HPLC, a clean separation of the fluorescent product was obtained (fig. 2).

The major fluorescent fraction obtained by HPLC (71% of the fluorescence) was analyzed for amino acid content. The result (Table I) matches the amino acid composition of peptide 33-56, with one significant difference: the low lysine content (expected: 2.0; found: 1.1). Since the sulfonamide linkage is resistant to the acid hydrolysis conditions (12), this result is consistent with the dansylation of one of the two lysines in the peptide, 40 or 41. Lysine 41 has been shown to form the Schiff base with retinal in bacteriorhodopsin (13, 5). Dansyl purple membrane has a visible absorbance spectrum indistinguishable from unmodified purple membrane. It is unlikely that lysine 41 could be converted to a sulfonamide without loss of the 570 nm absorbance band. O-dansyl tyrosine is also resistant to acid hydrolysis (14). The tyrosine

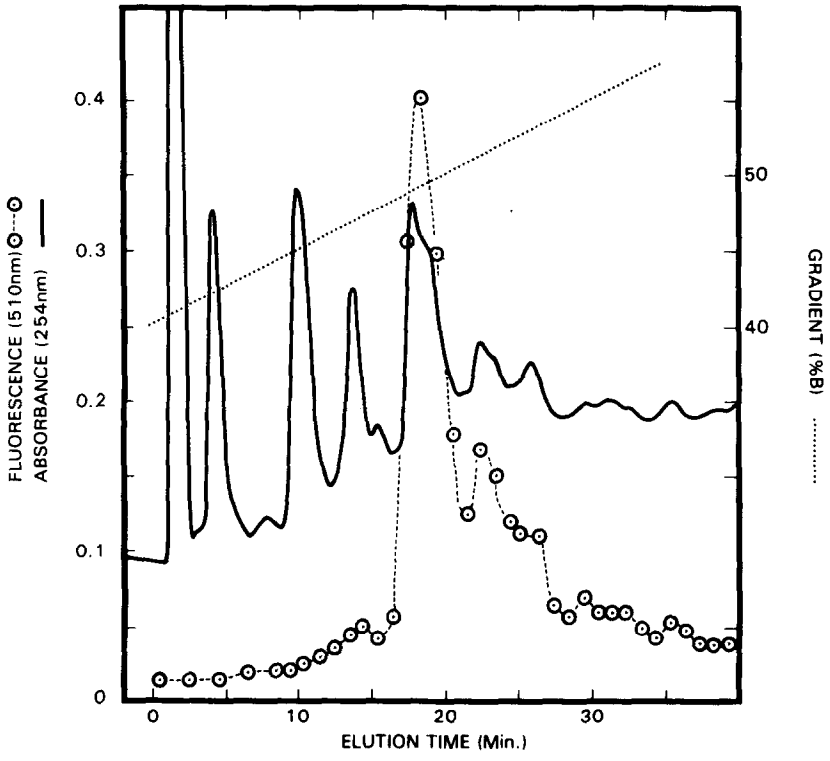

Figure 2. Separation of dansyl bacterio-opsin CNBr fragments on HPLC. Peptide containing residues 1-71 of dansyl bacteriorhodopsin (approx. 1 µmol) was cleaved with CNBr. Product was dissolved in 0.4 ml 88% formic acid and injected into 0.4 X 25 cm Biosil ODS-10 column. Gradient elution: solvent system of Gerber et al (6). Solvent A: 5% formic acid in water. Solvent B: 5% formic acid in ethanol. Flow rate: 1 ml/min. Fraction size: 1 ml. Absorbance (solid line) monitored at 254 nm. Fluorescence (open circles): excitation wavelength, 340 nm; emission, 510 nm. Major fragment: 17 - 19 minutes (see Table I for amino acid analysis).

content of the fluorescent peptide is close to the expected value (expected: 1.0; found: 0.8), so it is unlikely that the peptide contains significant amounts of O-dansyl tyrosine. Thus, we conclude that dansylation of the purple membrane occurs predominantly at lysine 40 of bacteriorhodopsin.

The minor fluorescent fragments (29% of the fluorescence) have not yet been identified due to poor chromatographic separation from the major peak.

Upon continuous illumination, dansyl purple membrane releases protons at alkaline pH in a manner indistinguishable from unmodified membrane. Light-induced release and uptake of protons by the purple membrane is due at least in part to proton pumping activity (11, 9). Therefore, it appears that the dansyl purple membrane has not undergone any drastic alteration in proton pumping activity.

TABLE I

Amino acid composition of major dansyl bacteriorhodopsin CNBr peptide[a]

amino acid	found[b]	expected, peptide 33-56[c]
Lysine	1.2, 1.0	2
Histidine	0.0, 0.0	0
Arginine	0.2, 0.0	0
Aspartate	1.8, 1.9	2
Threonine	2.9, 2.9	3
Serine	0.9, 1.2	1
Glutamate	0.0, 0.5	0
Proline	2.2, 2.0	2
Glycine	1.6, 1.7	1
Alanine	4.0, 4.2	4
Cysteine	0.0, 0.0	0
Valine	1.8, 1.9	2
Methionine	0.0, 0.0	0
Isoleucine	1.9, 2.1	2
Leucine	1.6, 1.6	1
Tyrosine	0.8, 0.9	1
Phenylalanine	1.7, 1.8	2
Homoserine + Hse lactone	0.8[d]	1

[a]Hydrolysis in 6 N HCl at 108° for 20 hours

[b]Mole ratios, two samples: first sample purified on LH-20 and HPLC; second, HPLC only

[c]From refs. 5 and 6

[d]One determination only

DISCUSSION

The results indicate that the amino acid residue in bacteriorhodopsin most reactive with dansyl chloride is lysine 40. This finding is surprising in two respects. The helix connection scheme proposed by Ovchinnikov et al (5) places both lysines 40 and 41 within the second helical segment. This arrangement suggests that lysine 30 should be as exposed to modification as lysine 40. Our results are more consistent with the model of Walker et al (7) which places both lysines 40 and 41 in a connecting peptide (between transmembrane helices 1 and 2). The proton pump mechanism proposed by Lewis (8) has the Schiff base lysine hydrogen bonded to a second lysine side chain. Since the proton release and uptake rates for dansyl purple membrane (as measured by light-induced changes in proton binding) appear identical to unmodified membrane, we can exclude lysine 40 as the source of the H-bond acceptor nitrogen.

Several previous reactions of lysine in bacteriorhodopsin have been reported (15,16,17). These sites have not been identified in the sequence. Henderson et al found biotin N-hydroxy succinimide ester reacts specifically at the extracellular surface of the membrane (17) and concluded that no accessible lysine residues are found on the cytoplasmic side. However, two proposed folding patterns for bacteriorhodopsin have placed the retinyl sequence (and hence the dansyl reaction site at lysine 40) near the cytoplasmic surface (5,7). Further chemical modification and electron microscopy studies will be necessary to clarify this discrepancy.

ACKNOWLEDGEMENTS

We are grateful to Dr. Austen Riggs for help with the amino acid analysis shown in Table I. We thank the Robert A. Welch Foundation (grant AX-736 to R.R.), NIH (grant GM 25483 to R.R. and GM 25795 to N.R.) and NSF (grant PCM 7822732 to R.R.) for supporting parts of this work. G.H. was a Robert A. Welch Predoctoral Fellow, 1978-79.

REFERENCES

1. Stoeckenius, W., Lozier, R., and Bogomolni, R. (1979) Biochim. Biophys. Acta 505, 218-278
2. Blaurock, A. and W. Stoeckenius (1971), Nature N.B. 233, 152-155
3. Oesterhelt, D. and W. Stoeckenius (1971), Nature N.B. 233, 149-152
4. Henderson, R. and N. Unwin (1975), Nature 257, 28-32
5. Ovchinnikov, Yu., N. Abdulaev, M. Feigina, A. Kiselev, and N. Lobanov (1979), FEBS Letters 100, 219-224
6. Gerber, G., R. Anderegg, W. Herlihy, C. Gray, K. Biemann, and H.G. Khorana (1979), Proc. Nat. Acad. Sci. 76, 227-231
7. Walker, J., A. Carne, and H Schmitt (1979),Nature 278, 653-654
8. Lewis, A., M. Marcus, B. Ehrenberg and H. Crespi (1978),Proc. Nat. Acad. Sci. 75, 4642-4646
9. Renthal, R., G. Harris and R. Parrish (1979), Biochim. Biophys. Acta 547, 258-269
10. Oesterhelt, D. and W. Stoeckenius (1974), Methods Enzymol. 31, 316-326
11. Renthal, R. (1977), Biochem. Biophys. Res. Comm. 77, 155-161
12. Seiler, N. (1970), Methods Biochem. Anal. 18, 259-337
13. Bridgen, J. and I. Walker (1976), Biochem. 15, 792-796
14. Veatch, W. and E. Blout (1976), Biochem. 15, 3026-3030
15. Konishi, T. and L. Packer (1976), Biochem. Biophys. Res. Comm. 72, 1437-1442
16. Dellweg, H.-G. and M. Sumper (1978), FEBS Lett. 90, 123-126
17. Henderson, R., J. Jubb and S. Whytock (1978), J. Mol. Biol. 123, 259-274