CHEMICAL MODIFICATION OF BACTERIORHODOPSIN WITH *N*-BROMOSUCCINIMIDE

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1. Introduction

Bacteriorhodopsin in the purple membrane of halophilic bacteria functions as a light-driven proton pump. Proton translocation is driven by light absorption by the retinal chromophore [1], but the way in which the chromophore excitation interacts with protein, and the process of vectorial proton translocation which involve the apo-protein are not yet well characterized. There is evidence that deprotonation and reprotonation of the Schiff base at the site of attachment of retinal to the protein is a primary step in proton translocation. The deprotonated intermediate state is detected by an absorption band at 412 nm during the photoreaction cycle [2–5]. The amino acid residues involved in this reaction and in the movement of the proton through the protein molecule are unclarified.

Chemical modification of bacteriorhodopsin in purple membrane preparations is a good approach to study such questions because this protein is the only one in the preparation, and because it exhibits considerable stability [1,6]. In a previous study, we demonstrated that modification of amino groups using certain lysine specific reagents (imidoesters) and glutaraldehyde inhibited proton pump activity concommitant with a loss of intrinsic tryptophan fluorescence [7]. Oesterhelt and Hess [9] found a decrease of tryptophan fluorescence during illumination of purple membranes in ether saturated salt solutions. Furthermore Bensasson et al. [8] have proposed that in mammalian rhodopsin, tryptophan residues interact with the retinal Schiff base to form a charge transfer complex resulting in a large red shift of chromophore absorption. A similar situation may occur in the case of bacteriorhodopsin.

In the present study the effect of a tryptophan specific reagent, *N*-bromosuccinimide, on bacteriorhodopsin has been investigated. Correlation of chemical modification of tryptophan residues by this reagent with measurements of the formation of the 412 nm photoreaction cycle intermediate and chromophore absorption, reveal that tryptophan residues are essential for proton pump activity.

2. Materials and methods

Purple membrane preparations kindly provided by Dr J. Lanyi (NASA-Ames Research Center) containing 4–8 μM of bacteriorhodopsin, as calculated from 570 nm absorption using the molar extinction coefficient of 63 000 M^{-1} cm^{-1}, [9] were employed in experiments. Absorption spectra were recorded in a Cary 14 spectrophotometer and difference spectra were measured in the Aminco Chance DW2 dual wavelength spectrophotometer.

The height of the 412 nm peak induced by a single flash is a direct indication of the number of bacteriorhodopsin molecules photocycling, and therefore provides an assessment of proton pump activity. The rate of decay of the 412 nm intermediate is proportional to the kinetics of proton pump activity. Flash photometry was carried out in an apparatus designed for these measurements in our laboratory which contained a photoflash unit (Vivitar 283) 1 ms maximum flashes from a xenon lamp. A pair of narrow band interference filters with a peak transmission of 410 nm (Baird Atomic series) were used to isolate the

measuring tungsten light source and the photomultiplier tube. Xenon flashes were filtered through a broad band filter, 490–610 nm transmission. This arrangement permitted accurate measurements of the height of the 412 nm species and its subsequent decay. Since the lower limit of the flash duration is 30 μs, it is also possible to make estimates of the rise time of the 412 nm species.

Chemical modification of bacteriorhodopsin was carried out in the pH range between 4.0 and 7.1 by titrating purple membrane preparations with aliquots of a 10 mM *N*-bromosuccinimide (NBS) aqueous solution to give various molar ratios of NBS : bacteriorhodopsin. The reaction mixture was buffered with 20 mM phosphate for pH above 5.0 and 20 mM acetate for pH less than 5.0. Recordings of absorption and difference spectra were commenced 40–45 s after mixing NBS solutions with purple membrane preparations. The degree of modification by NBS was estimated from difference spectra showing decreases in absorbancy at 280 nm at different NBS : bacteriorhodopsin molar ratios in native and sodium dodecylsulphate (SDS) solubilized purple membrane preparations.

3. Results and discussion

The specificity of chemical modification by NBS is pH-dependent; at lower pH it reacts exclusively with tryptophan and at neutral or alkaline pH reacts mainly with tryptophan, but also somewhat with tyrosine [11]. NBS also avidly reacts with cysteine, but this amino acid is not present in bacteriorhodopsin [10]. Figure 1 shows a loss of ultraviolet absorbance in the region where tryptophane and tyrosine absorb as the molar ratio of NBS to bacteriorhodopsin increased; complete loss of absorbance occurred at a molar ratio of about 30 at pH 4.7. NBS is specific for reaction with tryptophan at pH 4.7 and therefore this represents approximately a 7.5-fold molar excess of NBS to tryptophan residues. NBS treatment of SDS solubilized bacteriorhodopsin yielded the same changes (molar extinction coefficient) as with native purple membrane preparations using a 10–12 molar ratio of NBS. There are 4 tryptophan residues in bacteriorhodopsin [10] and since the total loss of absorbance in difference spectra is the same in these two preparations, we presume that all tryptophan residues undergo reaction with NBS.

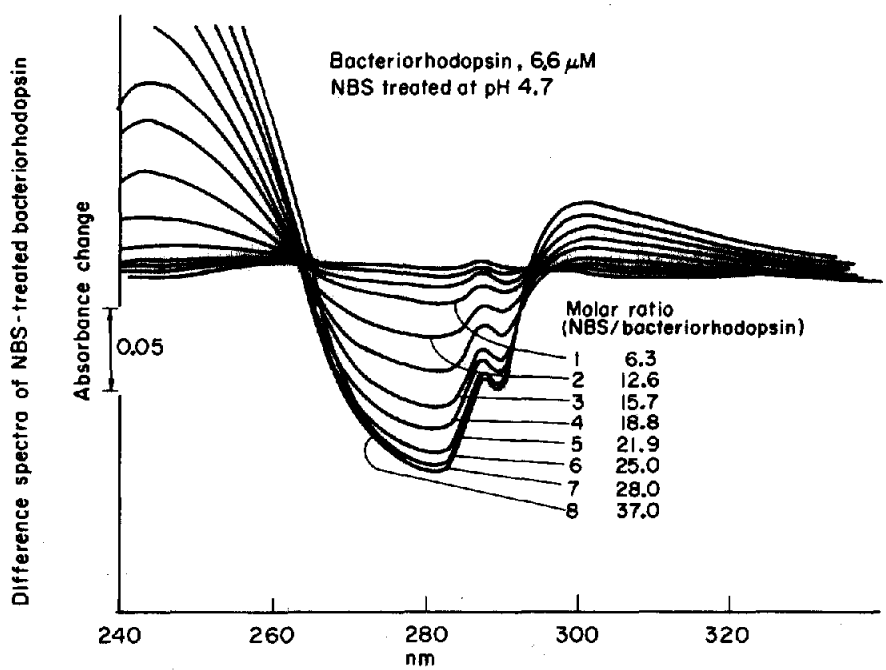

Fig.1. Difference spectra of *N*-bromosuccinimide-treated purple membrane preparations at pH 4.7.

Under conditions which caused NBS modification of tryptophan, a loss of the 570 nm chromophore was observed. This involved a progressive broadening and shift of the absorption peak to around 505 nm (fig.2). By adding SDS to solubilize the treated and untreated bacteriorhodopsin, a spectrum corresponding to the free protonated retinal Schiff base at 435 nm appeared (cf. [1]). Hence, reaction of tryptophan residues with NBS does not modify the retinal Schiff base in the purple membrane.

To evaluate the effect of tryptophan modification on the proton pump activity, the formation and decay of 412 nm intermediate were measured at various stages of NBS titration. Figure 3 summarizes results for experiments performed at pH 4.7 and pH 7.1. At low pH the loss of tryptophan residues correlated with loss of the light-induced formation of the 412 nm intermediate, and occurred before bleaching of the chromophore. At higher pH, loss of the 412 nm intermediate and chromophore bleaching both showed a biphasic curve. However, after complete disappearance of tryptophan absorption, 20–25% of 570 nm absorbance still remains; this indicates a fraction of the red-shifted chromophore absorption depends upon other amino acid residues. An enhancement of the 412 nm intermediate formation is also observed in the initial stage of NBS titration; this finding and the initial lag of the tryptophan modification cannot exclude a possibility of modification of other residues in the early stage of titration, but further experiments are needed to explain this finding. Figure 4 shows that tryptophan modification and the amount of the 412 nm intermediate formed by a single flash showed a good correlation in the pH range between 4.0 and 6.3.

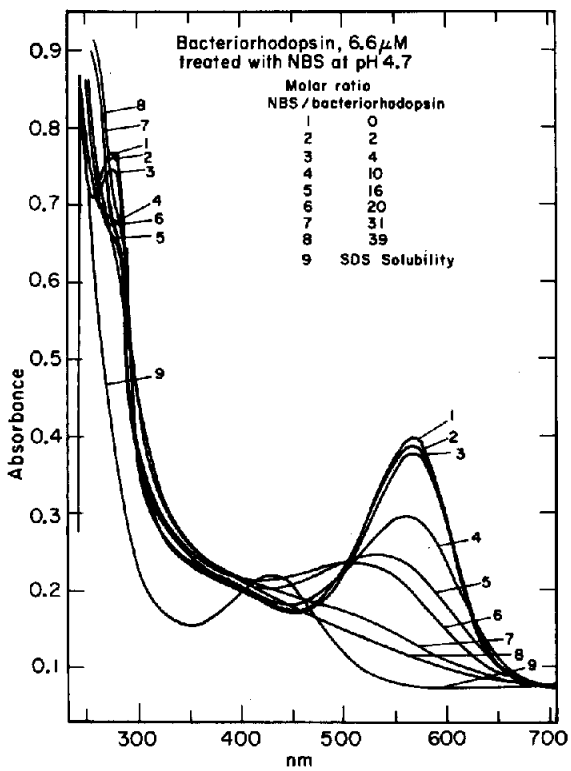

Fig.2. Changes in the absorbance spectrum of bacteriorhodopsin in purple membrane preparations upon titration with *N*-bromosuccinimide, at pH 4.7. Note that curve 8 includes the turbidity of the sample and the maximum absorbance change by NBS titration. After SDS treatment, curve 9, the turbidity of the sample is decreased.

These results indicate that tryptophan residues have an essential role in maintaining the environment of the chromophore and in formation of the photoreaction cycle intermediates of bacteriorhodopsin. Hence, tryptophan residues are involved in the process of proton translocation. It is not, however, known which step(s) in the process involves these residues; to determine this it would be necessary to analyze each photoreaction cycle intermediate with respect to a correlation with tryptophan loss. Our studies are consistent with one, perhaps two of the total tryptophan residues being essential for formation of the 412 nm intermediate, as judged by the analysis of the formation and rate of decay of the 412 nm species with progressive chemical modification. The other tryptophan residues may have structural significance in maintaining proper interaction of the chromophore with the protein. Thus at low and high pH a large discontinuity in the decay of the 412 nm intermediate was observed when 50% of the total tryptophan residues had been modified by NBS (fig.3, bottom). Recently R. Bogomolni and J. Lanyi [12] have observed tryptophan fluorescence changes during operation of the photochemical cycle of bacteriorhodopsin and Lubert et al. [13] have reported a light-induced, very fast depolarization of tryptophan fluorescence in bovine rhodopsin. Thus, tryptophan may have a similar role in both types of visual pigment.

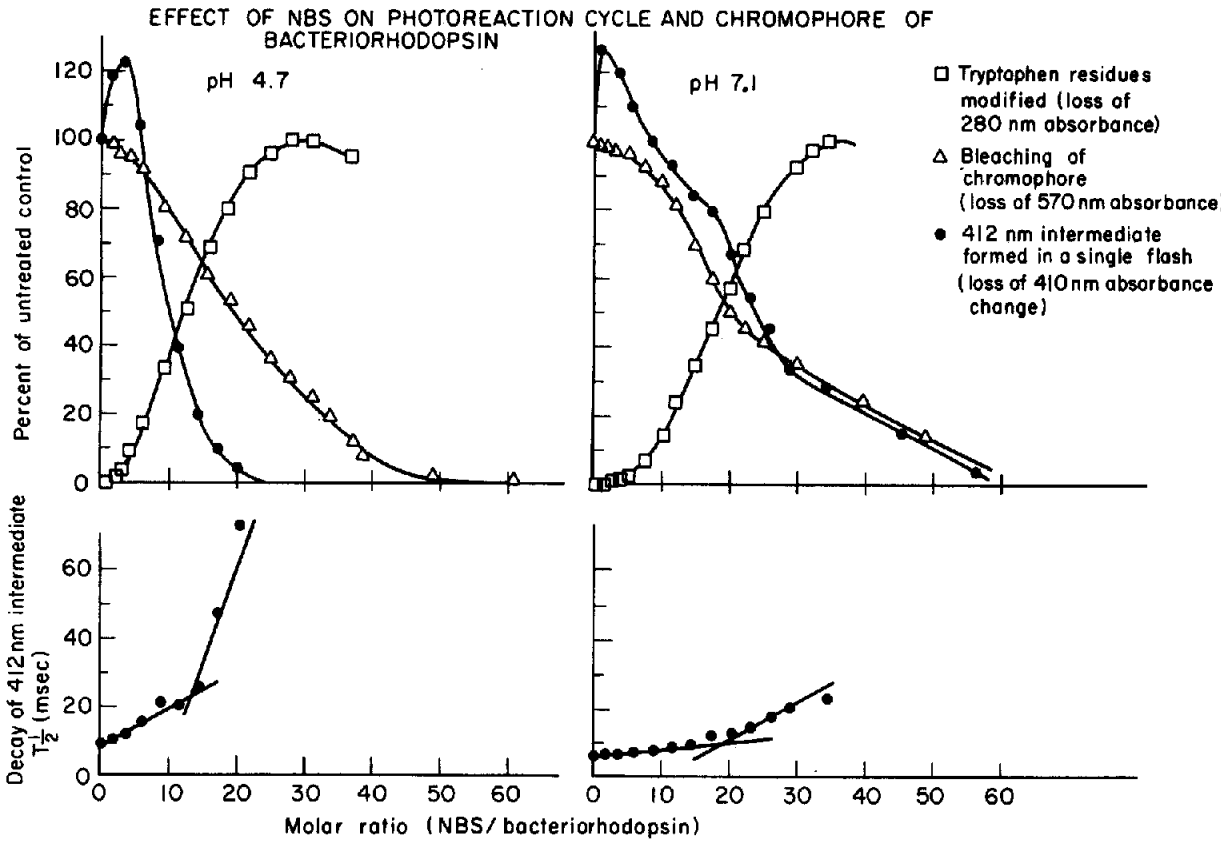

Fig.3. Effect of *N*-bromosuccinimide titration on the photoreaction cycle and chromophore absorbance of bacteriorhodopsin at pH 4.7 and pH 7.1. Bacteriorhodopsin, 4–6.6 μM; results shown are the average of two experiments.

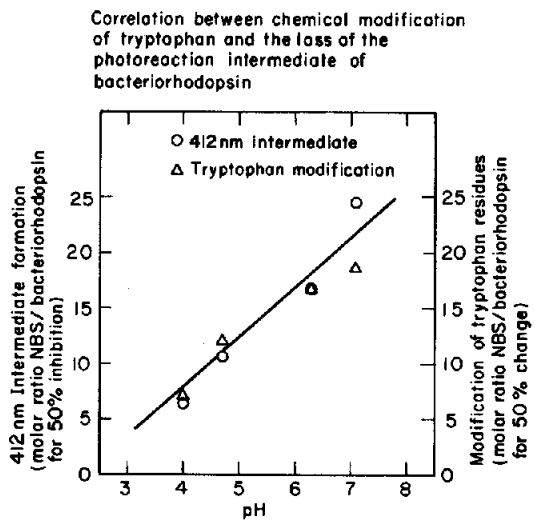

Fig.4. Correlation between chemical modification of tryptophan and loss of the 412 nm photoreaction intermediate of bacteriorhodopsin.

Acknowledgements

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References

[1] Oesterhelt, D. and Stoeckenius, W. (1971) Nature New Biol. 233 149–152.
[2] Lewis, A., Spoonhower, J., Bogomolni, R. A., Lozier, R. H. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. USA 71, 4462–4466.

[3] Lozier, R. H., Bogomolni, R. A. and Stoeckenius, W. (1975) Biophys. J. 15, 955–962.
[4] Lozier, R. H., Niederberger, W., Bogomolni, R. A., Hwang, S. B. and Stoeckenius, W. (1976) Biochim. Biophys. Acta 440, 545–556.
[5] Eisenbach, M., Bakker, E. P., Lorenstein, K. and Caplan, S. R. (1977) Biochim. Biophys. Acta in press.
[6] Blaurock, A. E. and Stoeckenius, W. (1971) Nature New Biol. 233, 152–155.
[7] Konishi, T. and Packer, L. (1976) Biochem. Biophys. Res. Commun. 72, 1437–1442.
[8] Bensasson, R., Land, E. J. and Truscott, T. T. (1975) Nature 258, 768–770.
[9] Oesterhelt, D. and Hess, B. (1973) Eur. J. Biochem. 37, 316–326.
[10] Bridgen, J. and Walker, I. (1976) Biochemistry 15, 792–798.
[11] Spande, T. F. and Witkop, B. (1967) in: Methods in Enzymology, Vol. XI (Hirs, C. H. W. ed) pp. 506–522, Academic Press, New York.
[12] Bogomolni, R. A. and Lanyi, J. (1977) Abst. Int. Symp. Membrane Bioenergetics, July 10–15, Spetsai, Greece.
[13] Lubert, G. P., Cox, J. and Georghiou, S. (1977) Abst. 5th Ann. Meet. Am. Soc. Photobiology, p. 52, May 11–15. San Juan, Puerto Rico.