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Cause of the blue shift of the absorption spectrum of tetranitromethane-treated bacteriorhodopsin

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The reaction of tetranitromethane with bacteriorhodopsin under irradiation was separated into two stages by selecting wavelengths of the irradiation light. The first stage of the reaction was characterized by a blue shift of the absorption spectrum and the second stage was characterized by decrease of the absorption of the visible region. The blue shift at the first stage was not caused by nitration of tyrosine residues but a modification of the chromophore. This conclusion was confirmed by reconstitution experiments and chromophore analysis. Nitration of tyrosine residues progressed at the second stage of the reaction.

Bacteriorhodopsin; Purple membrane; Retinal; Tetranitromethane; Nitration; Reconstitution

1. INTRODUCTION

Bacteriorhodopsin is a unique protein in the purple membrane of *Halobacterium halobium*. This membrane protein has retinal as its chromophore and acts as a light-driven proton pump. To study its functional site, chemical modifications of amino acid residues have been applied. Some of these studies reported that nitration or iodination of tyrosine residues causes a blue shift of the absorption spectrum and effects physiological functions [1-5]. Therefore, it has been considered that some tyrosine residues play a part of proton carriers and modification of tyrosine residues causes the blue shift of the absorption spectrum. There remains, however, some uncomprehensible points regarding tetranitromethane-treated BR. For example, reduction of nitrated tyrosine residues did not change the absorption spectrum [1,3], and it is difficult to extract the chromophore from TNM-treated BR [4]. Therefore, it is possible that the chromophoric retinal is modified. Here, we investigate the reaction of TNM with BR in detail and demonstrate that a reconstituted BR with retinal and the apoprotein prepared from the TNM-treated BR did not show the spectral shift, but that with the chromophore extracted from TNM-treated BR and native apoprotein did. The results show that the chromophoric retinal was modified by TNM and the modification of retinal caused the shift of the absorption spectrum.

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Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; BO, bacterioopsin; TNM, tetranitromethane; TNM-BR, TNM-treated BR; TNM-BO, TNM-treated BO; HPLC, high performance liquid chromatography

2. MATERIALS AND METHODS

BR was isolated according to the standard method [6]. The concentration of BR was determined by using the molar extinction coefficient of light-adapted BR ($\epsilon_{568} = 63\,000\ M^{-1} \cdot cm^{-1}$).

Buffer A: 0.1 M Na phosphate, 0.025% NaN_3, pH 6.0. Buffer B: 10 mM Na phosphate, 0.2% SDS, 0.025% NaN_3, pH 6.0.

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TNM in ethanol (5% solution) was added to a BR suspension (15 μM BR) in buffer A to give a 30-fold molar excess over BR. This mixture was exposed to red light (>600 nm, Toshiba glass filter R60) from a projector lamp (1000 *W*). When the reaction reached steady state, the irradiation light was changed from red to yellow light (>500 nm Toshiba glass filter Y50). The absorption spectrum change due to this reaction was monitored by a spectrophotometer. The sample at the quasi-photo-steady state produced by the red light irradiation was used for the following experiments as TNM-BR.

Bleached BR (BO) or bleached TNM-BR (TNM-BO) were prepared according to the ordinary procedure [7].

Extraction of the chromophore from native BR or TNM-BR was performed according to Iwasa et al. [8]. As it was hard to separate the chromophore from TNM-BR, the extraction was repeated several times.

all-*trans*-Retinal or the extract from TNM-BR was dissolved in ethanol. all-*trans*-Retinal was added to the bleached TNM-BR suspension to give an equivalent molar ratio, and the extract from TNM-BR was added to the BO suspension in the same way. Regeneration of the pigments was monitored by the absorbance increase around 550 nm.

The extract from the TNM-BR was analyzed by reverse-phase HPLC (C18 column, 4.6 × 150 M&S pack) with water and acetonitrile as the solvent. The flow rate was 1 ml/min. The elution was monitored by absorption at 335 nm.

Chymotryptic fragments were prepared by the method reported by Huang et al. [9]. Nitrated tyrosine residues were estimated from absorbance changes of the solubilized chymotryptic fragments (Glu-1-Phe-71 and Gly-72-Ser-248) by a pH shift from 6 to 13.

All absorption spectra were recorded with a Shimadzu UV-300 spectrophotometer.

3. RESULTS

TNM was not reacted with BR in the dark at pH 6.0. Under this condition the absorption spectrum of BR did not change and the nitroform ion was not generated. Irradiation of visible light preceded the reaction. The reaction of TNM and BR under

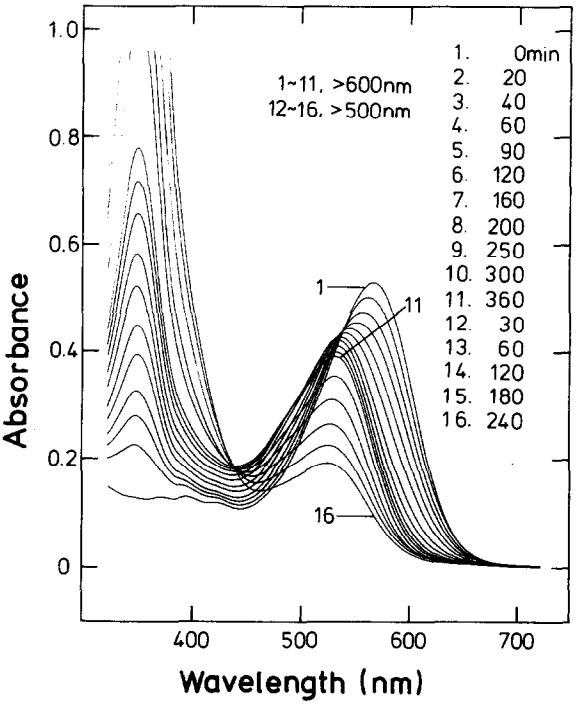

Fig.1. Spectral change of BR during the reaction with TNM. Curve 1, native PM. Curve 2–11, absorption spectra of the product of the reaction of BR and TNM under the red light (>600 nm) irradiation for 20, 40, 60, 90, 120, 160, 200, 250, 300 and 360 min, respectively. Curve 11, absorption spectrum of BR at the quasi-steady state of the reaction under the red light irradiation. Curves 12–16, absorption spectra of the product produced by reaction of BR and TNM under the yellow light (>500 nm) irradiation for 30, 60, 120, 180 and 240 min, respectively. Absorption around 350 nm was due to generation of nitroform ion.

irradiation was distinctly separated into two stages by the selection of irradiation wavelengths. The first stage is characterized by a blue shift of the absorption maximum from 568 nm to 530 nm with a concomitant decrease of the molar extinction coefficient from 63 000 to ~48 000 (fig.1, curves 1–11). Since this reaction is light-dependent and the product has only a little absorption at wavelengths longer than 600 nm, the absorption change scarcely proceeded after the irradiation of red light (>600 nm) for 360 min. When the suspension was exposed to yellow light (>500 nm), the second stage of the reaction proceeded. In the second stage, the absorption decreased in the visible

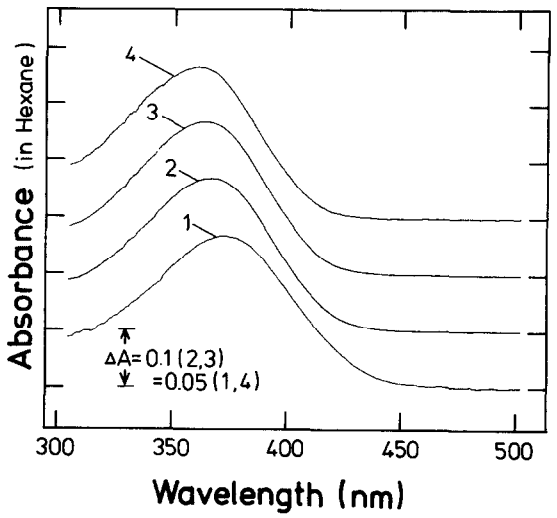

Fig.2. Absorption spectra of the chromophores extracted from BR and TNM-BR. Curve 1, chromophore extracted from native BR. The extraction of chromophore of TNM-BR was repeated three times. Curves 2–4, absorption spectra of the first, second and third extract, respectively.

region and increased around 360 nm.

Chymotryptic fragments were prepared from TNM-BR according to Huang et al. [9]. Two fragments (Glu-1–Phe-71 and Gly-72–Ser-248) contained a few nitrated tyrosine residues. This result suggests that nitration of tyrosine residues is not the cause of the blue shift of the absorption spectrum. Tyrosine residues were nitrated in the second stage of the reaction. In this stage, modification of tryptophan residues was also observed by a change in the UV absorption spectrum.

Having repeated the extraction four times, only 20% of the total chromophore was extracted from TNM-BR, in contrast, 90% of the retinal was extracted from the native BR. The absorption spectrum of the chromophore extracted from TNM-BR showed a blue shift compared to the chromophore extracted from native BR (fig.2). This blue shift was preceded by repetition of the extraction (fig.2, curves 2–4).

In order to clarify the origin of the color change of BR during the reaction with TNM, the BR was regenerated with the combination of native BO and the chromophore extracted from TNM-BR, or TNM-BO and all-*trans*-retinal. Reconstitution with native retinal and TNM-BO gave the same absorption spectrum as native BR, but the pigment reconstituted from the chromophore extracted from TNM-BR and BO showed a blue-shifted absorption spectrum similar to TNM-BR (fig.3). This observation shows that the cause of the blue shift of the absorption spectrum was not modification of the apoprotein but modification of the chromophoric retinal.

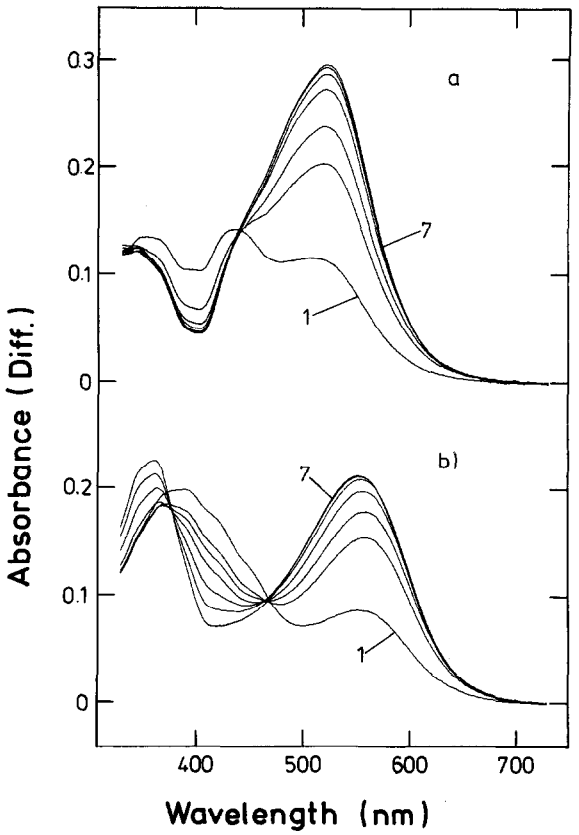

Fig.3. Regeneration of the reconstituted BRs. (a) Reconstituted BR from BO and the chromophore extracted from TNM-BR. (b) Reconstituted BR from TNM-BO and all-*trans*-retinal.

The chromophore extracted from TNM-BR was not eluted under the usual conditions for isomer separation analysis on silica gel column, where mixtures of diethyl ether and petroleum ether are used as elution solvents. In order to analyze the chromophore, we used a reverse-phase HPLC with a solvent of acetonitrile and water. The chromophore extracted from TNM-BR was eluted much earlier than the retinal (fig.4). The elution profile has several peaks. These peaks might correspond

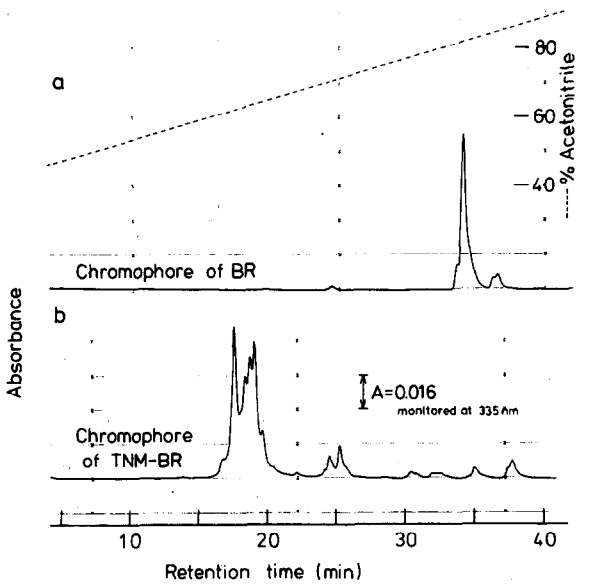

Fig.4. Elution profiles of the chromophores extracted from BR (a) and TNM-BR (b) obtained by using reverse-phase HPLC. The column had been pre-equilibrated with 60% water and 40% acetonitrile and eluted at 1 ml/min with a gradient as indicated.

to the isomers and/or multi-products which may be produced during the extraction. Anyway, fig.4 indicates that the chromophore of TNM-BR was much more hydrophilic than the native retinal, though it is unknown how retinal was modified. Now we are analyzing the structure of the chromophore in TNM-BR by mass spectroscopy, laser Raman scattering and so on.

4. DISCUSSION

It has already been reported in several papers [1-3] that the blue shift of the absorption spectrum of BR is caused by the reaction with TNM. It has been believed that the blue shift was caused by nitration of tyrosine residues or monomerization of BR. Our experimental results, however, clearly showed that the cause of the light-induced blue shift was modification of retinal, and this conclusion contradicts the previous reports. The modification product prepared by Sherrer and Stoeckenius may have been much the same as our product, judging from their reacting condition, but we think that the product of Lemke and Oesterhelt was not similar to our product, because their concentrations of the reactants were about 10-times higher than ours. Therefore, it is possible that their observation is a rare or nonspecific phenomenon, the rate of which is 100-times smaller than our observed phenomena, simply calculated from the comparison of the concentrations of the reactants, BR and TNM.

Sherrer and Stoeckenius reported that the blue shift of TNM-treated BR remained in the reconstituted BR, and they also noticed that the reconstituted BR did not show a blue shift compared to TNM-treated BR [3]. Since they did not use completely bleached BR for the reconstitution to avoid reduction of nitrated tyrosine residues, they may have observed a blue shift contributed by contaminated unbleached TNM-BR. In addition, spontaneous regeneration of bleached TNM-treated BR with the modified chromophore hydrolyzed from its oxime may have occurred. Though they stated that the absorption spectrum of the chromophore extracted from TNM-treated BR was similar to native retinal, we consider that their extract consisted of the unmodified retinal and that the modified chromophore may not have been extracted by their extraction method. This inference was supported by our observation of the absorption spectrum of the extract from TNM-BR (fig.2). This gradual blue shift of the extracts was explained by the assumption that remaining native retinal was extracted at the beginning and modified chromophore later from TNM-BR. The difficulty of chromophore extraction from TNM-BR may be due to retinal's modification, but its product has not been determined yet. The reverse-phase HPLC pattern shows that it is much more hydrophilic compared to native retinal (fig.4).

As reported before, the reduction of the nitrated tyrosine residues did not return the blue shift of the absorption spectrum [1,3], this experimental result is reasonable assuming that retinal has been modified.

According to our estimate, the quantity of nitrated tyrosine residues in the longer chymotryptic fragment (Gly-72–Ser-248) prepared from the TNM-BR in the second stage was much more than that of the shorter one (Glu-1–Phe-71). This observation suggests that tyrosine residues which dissociate in the photocycle were also present in the fragment (Gly-72–Ser-248).

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