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# Trimeric mutant bacteriorhodopsin, D85N, shows a monophasic CD spectrum

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The structure of mutant bacteriorhodopsin (bR), D85N, was examined by CD and X-ray diffraction at pH 7. The absorption maximum of D85N at pH 7 is located at 605 nm, which is similar to the acid-blue form of wild-type bR. D85N shows a monophasic CD band, the maximum of which is at 575 nm, although the crystalline arrangement and the trimeric structure is maintained. The acid-blue form of wild-type bR shows a biphasic CD despite the similarity in absorption spectra.

Bacteriorhodopsin; Circular dichroism; Exciton coupling; Site-specific mutant; X-ray diffraction

## 1. INTRODUCTION

Bacteriorhodopsin (bR), the sole protein of purple membrane of *Halobacterium halobium*, is a light-driven proton pump which generates a trans-membrane electrochemical gradient [1]. bR molecules are arranged in trimers which form a two-dimensional hexagonal lattice [2,3]. The three-dimensional structure of bR at moderate resolution has been solved to clarify the locations and orientations of some amino acid residues [3]. The structural changes that occur upon formation of the M intermediate have been determined [4–6]. The role of each amino acid residue in the proton pumping activity has been revealed by the site-directed mutagenesis (for review, see [7]). Studies using mutagenic techniques have revealed the importance of aspartates in the proton pumping activity and spectroscopic properties of bR [7–11].

Replacement of D85 by N shows dramatic effects on the visible spectrum and proton-pumping activity [8,9,12]. Recently, it is shown that D85N exists as three distinct spectroscopic species in equilibrium [13]. Turner et al. argued that these three species are closely related to M, N and O intermediates [13]. D85N is dominated by a blue species at pH 7, which would be related to O, and a yellowish pigment at pH 12, which would be related to M [13]. Structural studies of D85N is of great interest in the context of the structural analyses of the photo-intermediates.

We have started intensive investigation of the structure of D85N using CD and X-ray diffraction. CD has been widely used to help understand the chromophoric properties of bR [14–17]. The CD spectrum in the visible region of native bR is composed of a pair of negative and positive bands [14,15]. The origin of this biphasic shape is understood to be an exciton coupling between the chromophores in the trimeric structure of bR [16,17], although some arguments against the exciton model have been raised [18,19]. During the structural studies of D85N, we found that D85N shows a monophasic CD spectrum despite the fact that the crystalline structure is maintained. Here, we report the CD spectrum of D85N as compared with that of native bR.

## 2. MATERIALS AND METHODS

### 2.1. *Sample preparation*

Purple membrane containing wild-type bR was isolated from *H. halobium* strain R1M1 by a standard procedure [20]. *H. halobium* containing the recombinant D85N bR gene was constructed using a shuttle vector, as described [11,21]. D85N bR was prepared as purple membrane sheets, by the same method as for wild-type preparation.

### 2.2. *Measurement of optical properties*

Absorption spectra were measured with a Shimadzu UV160 spectrophotometer at room temperature. CD spectra were measured with a JASCO spectropolarimeter, model J-500A, using a quartz cell of 5 mm optical path. Each CD spectrum was collected 4 times. The purple membrane concentration was determined spectrophotometrically based on the absorbance at 280 nm of the wild-type. The temperature

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was maintained at 20°C by circulating thermostated water through the cuvette holder. Both spectrometers were individually controlled by a personal computer, NEC PC9801.

2.3. *X-ray diffraction experiment*

X-ray diffraction measurements were carried out with the MUSCLE Diffractometer at BL-15A in the Photon Factory according to [5].

## 3. RESULTS AND DISCUSSION

The absorption maximum of D85N at pH 7 is located at 605 nm, so the color is blue. Although three spectroscopically distinct species are in equilibrium in D85N, the blue pigment ($\lambda_{max}$ = 605 nm) is dominant at pH 7. According to Turner et al., at pH 7, the amount of yellow pigment is less than 5% and the amount of purple pigment is almost zero [13], although their $\lambda_{max}$ of blue pigment was reported to be 615 nm.

Fig. 1 shows CD spectra of D85N and wild-type bR at pH 7. The characteristic biphasic band is clearly seen in the wild-type spectrum. On the other hand, D85N shows a monophasic CD spectrum, which is completely different from that of the wild-type. The CD peak of D85N is located at about 575 nm, while the absorption maximum is located at 605 nm. According to the exciton coupling model, such a monophasic CD spectrum is considered to be an indication of monomeric bR. In fact, bR solubilized in Triton X-100 gave a monophasic CD and was therefore interpreted as a monomer [15].

The X-ray diffraction profile from the purple membrane composed of D85N is shown in Fig. 2. The profile is essentially identical to the X-ray diffraction pattern of the wild-type. The diffraction lines appear at the same positions, and the relative intensity of each reflection is similar to that of the wild-type. Moreover, neither broadenings of reflection lines nor an increase of diffuse background were observed. These facts indicate

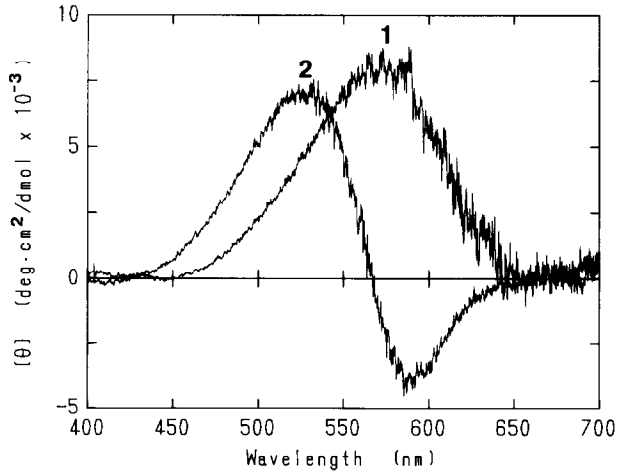


Fig. 1. Visible CD spectra of the D85N bR mutant (curve 1) and wild-type bR (curve 2) at pH 7. The purple membrane concentrations were 70.1 $\mu$M for D85N and 58.7 $\mu$M for the wild-type, respectively.

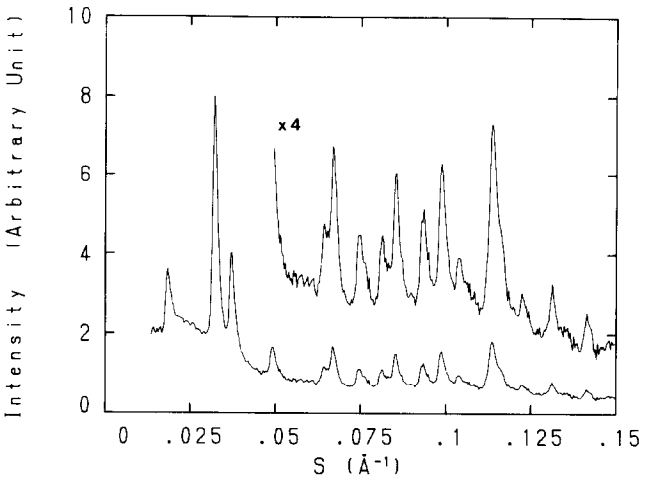


Fig. 2. X-ray diffraction profile of the purple membrane composed of D85N at pH 7. Bragg reflections up to (7,1) are clearly observed. S is defined as $2\sin\theta/\lambda$, where $2\theta$ and $\lambda$ are the scattering angle and the X-ray wavelength, respectively.

that neither the crystalline arrangement of bR nor the trimeric structure are significantly affected by the mutation. Therefore, the monophasic CD of D85N should be attributable an origin other than a monomer. However, the result does not necessarily argue against the exciton coupling model. Exciton coupling should be extremely sensitive to the arrangement of chromophore, retinal [16]. It is possible that the replacement, D-to-N affects an orientation of retinal. Another possible, but unlikely, explanation for the monophasic CD of D85N based on the exciton coupling model is that lattice vibrations are of a sufficient degree to interact with exciton. The interaction would decouple excitons to give a monophasic CD [17]. The latter explanation is given for the monophasic CD of the M intermediate and bR treated with dimethyl sulfoxide or sodium borohydride [17]. Such global structure changes occur in the M intermediate [3–5]. Chemical treatment seems to increase membrane fluidity. These changes would bring about a change in lattice vibrations [17].

Another important feature of the CD of D85N is that the maximum of the CD band is 30 nm shorter than the absorption maximum. The difference between CD maximum and absorption maximum is also seen in solubilized bR: the CD maximum is 545 nm while the absorption maximum is 558 nm [15]. Similar differences are also seen in the case of the visual pigment, rhodopsin [22]. Kakitani and Kakitani [23] demonstrated that the difference can be explained by an electron–vibration interaction. Assuming that the CD spectrum of D85N is its intrinsic CD, we consider that the difference is attributable to an electron–vibration interaction.

Wild-type bR shows an acid-induced purple-to-blue transition. The acid-induced blue form has its absorption maximum at 605 nm [24,25], which is similar to D85N at pH 7. Fig. 3 shows the comparison of CD

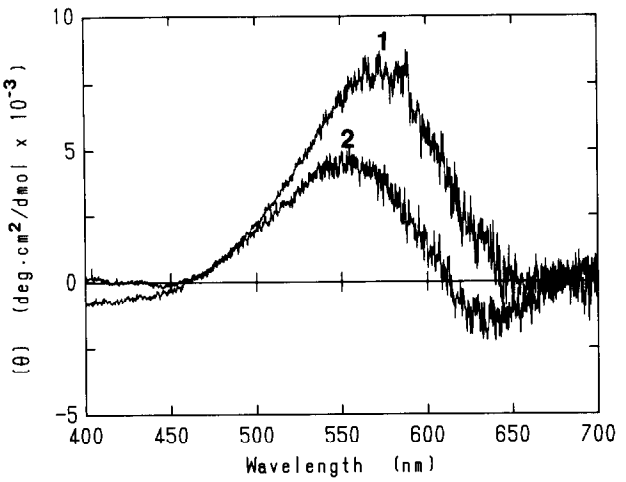


Fig. 3. Visible CD spectra of D85N bR mutant at pH 7 (curve 1) and wild-type bR at pH 3 (curve 2). The purple membrane concentrations were 70.1 $\mu$M for D85N and 48.9 $\mu$M for the wild-type.

spectra between D85N at pH 7 and the wild-type acid-blue state (pH 3). Apparently, the acid-blue form shows a biphasic CD band characteristic of wild-type bR, although the baseline around 400 nm is slightly affected by aggregation. The cross-over point (605 nm) coincides with the absorption maximum, a feature that is the same as the CD of wild-type bR at neutral pH. Thus, we can say that the wild-type acid-blue form shows an exciton coupling. However, the X-ray diffraction pattern from the acid-blue form indicates that the crystalline structure has almost disappeared (unpublished result and [26]). We have to assume that the trimeric structure of bR would be stable and independent of crystalline structure if the biphasic CD comes from exciton coupling.

The difference in CD indicates that the chromophoric orientation in D85N is quite different from the wild-type acid-blue form, despite the similarity in absorption spectra. Acid-mediated protonation of D85 would be involved in the purple-to-blue transition of the wild-type bR [13]. The small difference between protonated aspartic acid and the uncharged aspartic acid analog, aspargine, would cause the delicate orientational difference of chromophore retinal.

An argument against the exciton coupling model has been raised in [18,19]. The following feature of the present results would be appropriate evidence against the exciton coupling model: the trimeric mutant bR shows a monophasic CD, while a biphasic CD is observed for acid-blue wild-type bR despite of the loss of crystallinity. However, as mentioned above, these results may be interpretable in terms of the exciton coupling model under some assumptions for the bR structure. The X-ray diffraction shown in Fig. 2 is not sufficient to see the chromophoric orientation. Until the detailed structure of D85N becomes available, we will not be able to support or discount the exciton coupling model. Apparently, the D85N mutant bR is a good model to consider the origin of CD of bR. In order to reveal the origin of CD, further structural studies, as well as the pH dependence of CD of D85N, are now under way by our group.

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