Volume 292, number 1,2, 73-75 FEBS 10319 November 1991

ADONIS 0014579391010294

Crystallographic characterization by X-ray diffraction of the M-intermediate from the photo-cycle of bacteriorhodopsin at room temperature

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Received 2 September 1991

The structure of the M-intermediate appearing in the photo-cycle of bacteriorhodopsin was studied with X-ray diffraction techniques at room temperature. The lifetime of the M-intermediate was prolonged by treatment with an arginine solution at alkaline pH (Nakasako et al., FEBS Lett. 254, 211-214). The diffraction profile of membranes which had accumulated the M-intermediate had small but significant differences in the intensities of Bragg reflections and the lattice constant in comparison with that of membranes having *trans*-bacteriorhodopsin. Diffraction intensities were carefully evaluated and the structural changes during the formation of the intermediate were evaluated with difference Fourier analysis. We could find structural changes around helices G and B.

X-ray diffraction; Purple membrane; Bacteriorhodopsin; M-intermediate

1. INTRODUCTION

Bacteriorhodopsin (bR) is the sole protein found in the purple membrane of *Halobacterium halobium*. The bR is composed of 248 amino acid residues and the chromophore retinal [1]. As revealed by electron microscopy [2], bR is folded into seven α-helices spanning the lipid bilayer and an additional long segment in the aqueous region. The bR molecules form trimers and the trimers are organized as a two-dimensional crystal in the purple membrane.

In its light-adapted state, the chromophore of bR is all-*trans* retinal. This form of bR, called *trans*-bR, absorbs light and undergoes a unique photoreaction cycle [3]. During the reaction cycle, bR actively transports a proton from the inside of the bacterial cell to the outside.

The mechanism of the proton transport is still obscure. The most direct approach to the transport mechanism is to study the structure of the M-intermediate, which is the key species for proton transport. X-ray diffraction techniques are suitable for the characterization of the intermediate. We began to search for suitable conditions to accumulate the intermediate for the X-ray diffraction experiments and found a arginine treatment [4], where the intermediate could be stabilized without alternation of the structure of bR and the membrane or the sequence of the reaction cycle of bR.

In this paper, we report the results of static and time-resolved X-ray diffraction experiments for the arginine-treated purple membrane and present a crystallographic characterization for membranes with the M-intermediate. The possible structural changes during the formation of the intermediate are also discussed.

Abbreviations: bR, bacteriorhodopsin; *Trans*-bR, bR having all-*trans* retinal as the chromophore.

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2. MATERIALS AND METHODS

2.1. *Sample preparation*

Purple membranes were isolated from *Halobacterium halobium* R1M1 using standard procedures [5]. The details of the arginine treatment and its effect on prolonging the life-time of the M-intermediate were previously reported [4]. Samples for X-ray diffraction experiments were prepared as follows. A droplet of 30 μl of the purple membranes suspension (300 μM bR) in an arginine solution (20 mM arginine hydrochloride (Wako Pure Chemicals, Osaka), 25 mM CAPS (3-Cyclohexylaminopropanesulfonic acid; Dojin Chemicals, Tokyo), pH 10.0) was dried on a sheet of myler (thickness 10 μm) for 1 day and the same procedure was repeated 7-10 times to obtain data with high statistic accuracy for the diffraction experiment.

2.2. *X-ray diffraction experiments*

X-ray diffraction experiments were performed with the MUSCLE Diffractometer installed at the focusing optics of BL-15A [6] in the Photon Factory of the National Laboratory for High Energy Physics, Tsukuba, Japan.

The X-ray wavelength was tuned to 1.5 Å and the sample-to-detector distance was 637 mm. Diffraction profiles were recorded with

a position-sensitive detector of a delay line type (Rigaku, Tokyo) controlled with a minicomputer (Micro-11/23, Automation System Research, Tokyo) via a CAMAC data acquisition system. The variation of the intensity of incident X-ray beam was monitored with an ionization chamber placed in front of the sample chamber.

The sample was illuminated by intense light during recording of the diffraction profile. The wavelength of the illumination light was selected with a cut-off filter 0-53 (>510 nm, Toshiba, Tokyo) to accumulate the M-intermediate or with an interference filter KL-41 (410 nm, Toshiba, Tokyo) for *trans*-bR. A 300-s exposure time was used for each recording of the diffraction profile.

The X-ray diffraction profiles were corrected with the current of the ionization chamber. After subtraction of the background scattering, Bragg peaks were corrected with respect to the geometrical factors in the experiment. The integrated intensities were calculated by a profile fitting using an non-linear asymmetric function (M. Nakasako, unpublished work) and corrected with a Lorentz factor.

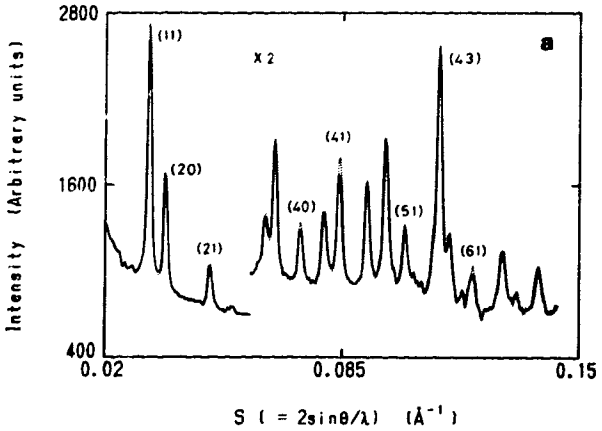
3. RESULTS AND DISCUSSION

The X-ray diffraction profiles of the membrane in both M-intermediate and *trans*-bR states are shown in Fig. 1. Although some workers had reported that the crystalline arrangement of the bR trimer in the membrane would be distorted during the formation of the M-intermediate [7,8], we had never observed any symptom of distortion or disorder, judging from the intensity and width of Bragg peaks.

There were small but significant differences in the intensities of some Bragg peaks between the M-intermediate and *trans*-bR states. For instance, the intensities of (2 0), (2 1), (3 2), (4 2) and (5 1) reflections increased and those of (1 1), (4 0), (4 1) and (6 1) reflections decreased in the M-state (Fig. 1 and Table I). The opposite change was observed after recovery of the *trans*-bR state produced by illuminating the membrane in the M-state, in the same sample. These results obviously indicate that some structural change occurred during the formation of the M-intermediate and are consistent with the results obtained with neutron diffraction experiments on the M- intermediate at low temperature [9] except for the intensity change in (2 2) reflection. The changes in intensities evaluated by the procedures described above are listed in Table I. These data can be used for the crystallographic confirmation of the accumulation of M-intermediate in the high resolution structural study using electron microscopy.

The lattice constant of the membrane was increased by 0.1 Å in the M-intermediate (Fig. 1(b) and Table I). The lattice constant change was considered to reflect that interactions between the trimers and/or between the trimer and the phospholipid were slightly weakened during the formation of the M-intermediate. As well as the intensity, the lattice constant was recovered in the *trans*-bR state.

These changes were observed with good reproducibility, as shown by a correlation coefficient value of 0.99 among various data sets obtained from 5 independent samples.



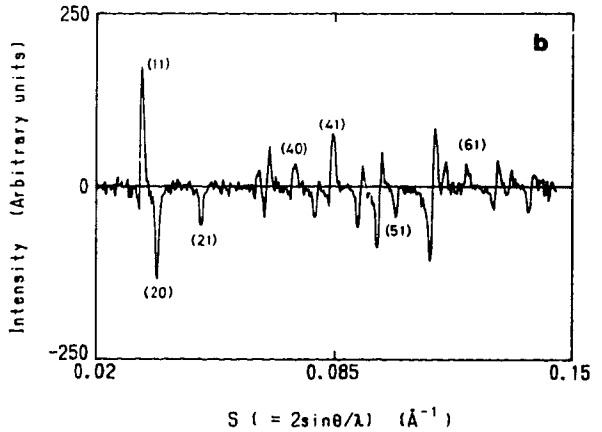


Fig. 1. (a) X-ray diffraction profiles of the membrane accumulating the *trans*-bR (·····) and M-intermediate (————) recorded at room temperature. S is the reciprocal space distance. 2θ and λ designate the diffraction angle and the wavelength of X-ray, respectively. Some indices for the two-dimensional hexagonal powder diffraction pattern are given as (h k). (b) Difference X-ray diffraction profile obtained by subtracting the profile of the M-state from that of *trans*-bR state in (a). Intensity changes are clearly seen. The biphasic profile in the region of $S > 0.1$ Å^{-1} is mainly due to the lattice constant change in the M-state.

We found that the conversion from *trans*-bR to the M-intermediate induced small changes in both intensity and lattice constant. Therefore, a difference electron density map projected to the membrane plane was used to characterize the structural change. In the calculation of the difference map, we utilized the separation ratio of intensities for the multiple reflections and the phases obtained by electron diffraction and microscopy [10], because of the small differences in the intensity and the lattice constant between both states.

Although there were many peaks in the difference map (Fig. 2), we considered pairs of most prominent positive and negative peaks around helix G and helix B, reflecting structural changes. In a model simulation, the changes of electron densities at these sites in the differ-

Table I

Integrated intensities of Bragg peaks

(h k)	I_{trans}	I_M	I_M/I_{trans}
(1 1)	6750	6400	0.95
(2 0)	3125	3681	1.18
(2 1)	1690	1964	1.16
(3 0)	343	384	1.12
(2 2)	2507	2431	0.97
(3 1)	4850	4745	0.98
(4 0)	2553	2334	0.91
(3 2)	3145	3582	1.14
(4 1)	6305	5657	0.90
(5 0)	5451	5976	1.10
(4 2)	8593	9384	1.09
(5 1)	4268	4998	1.17
(6 0)	2956	3803	1.29
(4 3)	17920	17780	0.99
(5 2)	5319	5187	0.98
(6 1)	3196	2729	0.85
(5 3)	6939	7027	1.01
(6 2)	1210	970	0.80
(7 1)	4077	4446	1.09
a (Å)	62.81 ± 0.04	62.95 ± 0.03	

The intensities were obtained from the X-ray diffraction profiles shown in Fig. 1. I_M and I_{trans} are integrated intensities for the M-intermediate and *trans*-bR state, respectively. The values for (3 0) and (6 0) reflections are less reliable. The R factor defined by the following equation gives a value of 6.9%.

$$R = \frac{\Sigma |(I_M - I_{trans})|}{\Sigma I_{trans}} \times 100$$

The a is the lattice constant of purple membrane in each state.

ence map were also required to explain the intensity change in the M-intermediate. There are some possibilities to explain these most prominent positive peaks in the difference map; (1) shift of the side chains of a few amino acid residues, (2) tilt of the α-helix by a few degrees and (3) shift of α-helix by about 1 Å. Among these possibilities, the latter two cases are favorable because of the existence of a negative peak at the opposite side of the positive one in helices B and G. A small rearrangement of the helices B and G would change the interactions of amino acid residues involved in proton translocation, such as Asp^{85}, Asp^{96} and Asp^{212} [11], located in the region.

Owing to the intense synchrotron radiation and the arginine treatment, we could record the decay kinetics of the M-intermediate with time-resolved measurement (data not shown). The results showed that the hexagonal arrangement of the bR trimer in the membrane was not distorted during the decay of the M-intermediate. Hence, we conclude that no global structural changes

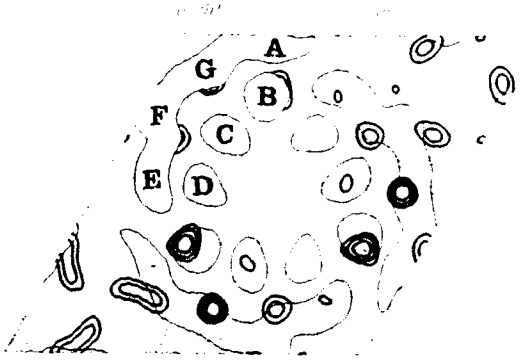


Fig. 2. Difference electron density map projected membrane normal between trans-bR and M-intermediate calculated with the modulus of (F_M-F_{trans}). Thick contour lines indicate the positive density region where the electron density is increased at the M-intermediate and thin contours indicate the negative density region. The scattering length of the most prominent positive peak corresponds to about one amino acid. The seven helices are labeled by A–G according to Engelman et al. [12].

in the membrane occurred during the photo-cycle of bR and that the proton translocation could be achieved by local small structural changes as described above.

Acknowledgments: This work was partly supported by grants from the Ministry of Education, Science and Culture of Japan to F.T. and M.K. X-ray diffraction experiments were performed by the approval of Photon Factory Advisory Committee (Proposal 87-036). The authors would like to thank Prof. T. Ebrey for his careful correction of the manuscript.

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