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Arginine remarkably prolongs the lifetime of the M-intermediate in the bacteriorhodopsin photocycle at room temperature

Masayoshi Nakasako, Mikio Kataoka and Fumio Tokunaga

Department of Physics, Faculty of Science, Tohoku University, Sendai, Miyagi 980, Japan

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The lifetime of the M-intermediate in the bacteriorhodopsin photocycle was remarkably prolonged on drying a suspension of purple membrane, in arginine solution at alkaline pH. The M-intermediate could be completely accumulated under illumination (> 530 nm) at room temperature. The crystalline structure of purple membranes was retained after this treatment. The lifetime of the M-intermediate was found to be longer than 100 s and depended on the pH of the purple membrane suspension before drying. It was suggested that an interaction between the guanidium group of arginine and an amino acid residue played an important role in the prolongation.

Bacteriorhodopsin; Purple membrane; M-intermediate; Arginine

1. INTRODUCTION

BR is the sole protein in PM of *Halobacterium halobium* and functions as a light-driven proton pump [1]. The photoreaction cycle of BR is characterized by several reaction intermediates [2]. Of these, the most important for function is considered to be the M-intermediate [3].

Although the structure of the M-intermediate has been studied using various techniques, the results are inconsistent with each other [4–6]. We have begun a structural study on the M-intermediate employing the technique of X-ray diffraction under conditions where accumulation of the M-intermediate can be confirmed visually. However, two disadvantages are encountered under the reported conditions for the M-intermediate being stabilized and accumulated [2,7,8]. One is that Cl^- is present at a high concentration under such conditions; this is unsuitable for an X-ray diffraction investigation, since Cl^- strongly absorbs hard X-rays. The other is that the sample should be cooled to low temperature for the complete accumulation of intermediate. Below 0°C, the lattice constant of PM is decreased by 1 Å [9]. Hence, this is unfavorable for structural analysis on the basis of X-ray or neutron diffraction, since we cannot use the phases determined by electron microscopy for the sample which has a lattice constant of 62.7 Å [10].

We therefore examined several sets of conditions that met the following criteria: (i) the duration of the lifetime can be prolonged to more than 10 s at room temperature; (ii) the crystalline structure remains and (iii) large quantities of reagents that are strong absorbers of hard X-rays are not required. We found that treatment with arginine satisfied these criteria and markedly prolonged the lifetime of the M-intermediate. Here, we describe the effects of this treatment and discuss the possible mechanism underlying the prolongation.

Correspondence address: F. Tokunaga, Department of Physics, Faculty of Science, Tohoku University, Aobayama, Sendai, Miyagi 980, Japan

Abbreviations: Arg-PM, arginine-treated purple membranes; BR, bacteriorhodopsin; PM, purple membranes

2. MATERIALS AND METHODS

PM were isolated from *H. halobium* strain R1M1 according to [11]. The PM were washed with Arg-HCl solution

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(75–250 mM). The pH of Arg-HCl solution was adjusted with Mes, Hepes, Taps and Caps (Dojin Chemical) and NaOH solution. The concentration of PM in each experiment is given in the appropriate figure legend. After centrifugation, the pellet was resuspended and dialyzed vs the same solution for 1 or several days. A droplet of the PM suspension was dried on a sheet of mylar or quartz glass under room humidity for 1 day. The sample obtained was wet and viscous.

Spectroscopic measurement was carried out on a Shimadzu UV-160 spectrophotometer at room temperature. The sample was irradiated with yellow light from a 1 kW slide projector (Master) for accumulation of the M-intermediate. The wavelength of the light (>530 nm) was selected using a cut-off filter (O-53, Toshiba).

The X-ray diffraction experiment was performed on the MUSCLE diffractometer [12] at BL-15A in the Photon Factory of the National Laboratory for High Energy Physics, Tsukuba. The wavelength of the X-rays was tuned in 1.5 Å, the sample-to-detector distance being 80 cm. A 4 mm receiving slit was used in front of the position-sensitive detector. The exposure time employed was 300 s. No radiation damage to PM was found to be caused by the incident X-ray beam.

3. RESULTS AND DISCUSSION

Drying of the PM suspension was required for sufficient prolongation of the lifetime and complete accumulation of the M-intermediate (table 1). Denaturation was not observed in the absorption spectra of samples obtained by drying PM suspensions of pH within the range pH 5–11. Even in the case of a sample prepared from a PM suspension of pH 11, the absorption spectrum was the same as that of the native form, indicating that the solvent environment was unaltered by drying. Complete accumulation of the M-intermediate occurred with suspensions of which the pH was above 8 before drying.

Table 1

Prolongation of lifetime of the M-intermediate

Reagent	Half lifetime (s)
Arginine-HCl[d]	40
Arginine-HCl[s]	<1
Guanidine-HCl[s]	5
Methylguanidine-HCl[s]	2
Dimethylguanidine-H_2SO_4[s]	8
Tetramethylguanidine-HCl[s]	ND

[d] Dried sample; [s] solution sample. These samples were denatured by drying except for arginine. ND: accumulation of M-intermediate not observed in the absorption spectrum. The concentrations of each reagent and BR in the sample were adjusted to 0.75 M and 8 μM, respectively. pH values of all PM suspensions before drying were adjusted to 10.5 with 100 mM Caps and NaOH solution

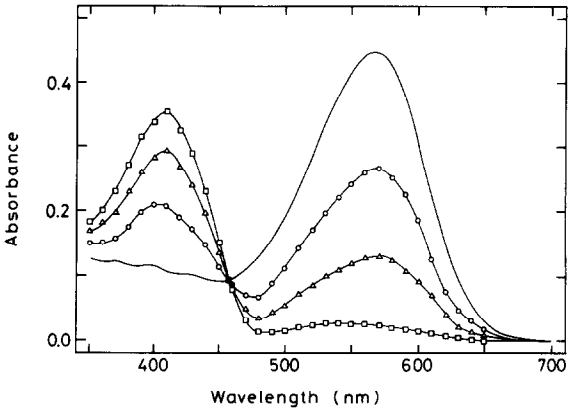

Fig.1. Absorption spectra of Arg-PM at various times during conversion of the M-intermediate to the original pigment (*trans*-BR) at room temperature. The sample was prepared by drying a PM suspension (16 μM BR in 100 mM Arg-HCl and 25 mM Caps, pH 9.4). (——) Spectrum of light-adapted BR; (○—○, △—△, □—□) spectra for the conversion reaction. These spectra were obtained by time-scanning at each wavelength after turning off the irradiation light; (△—△, ○—○) spectra 5 and 20 s later, respectively; (□—□) spectrum of the M-intermediate obtained by extrapolation of each scan to 0 s.

Fig.1 shows the absorption spectra of Arg-PM at various times during conversion of the M-intermediate to *trans*-BR. The absorption maximum of the light-adapted form was located at 568 nm and the profile of the spectrum was the same as that of the native PM, indicating that this treatment did not change the local structure around the chromophoric retinal. *Trans*-BR underwent complete and reversible conversion to the M-intermediate at room temperature on irradiation with yellow light. An isosbestic point was found at about 460 nm in the process of conversion of the intermediate to the *trans* state. These aspects are in complete agreement with observations at low temperature [2]. It has been reported that conversion of the M-intermediate to *trans*-BR involves three different reaction components under alkaline conditions [13]. In the case of Arg-PM, it was suggested that all of three components became slower. We therefore conclude that treatment with arginine retarded the rate of conversion reaction but had no effect on the pathway of the photoreaction cycle.

The structure of Arg-PM in the light-adapted form was examined in X-ray diffraction investigations. The diffraction pattern was observed to be composed of sharp Bragg peaks as shown in fig.2.

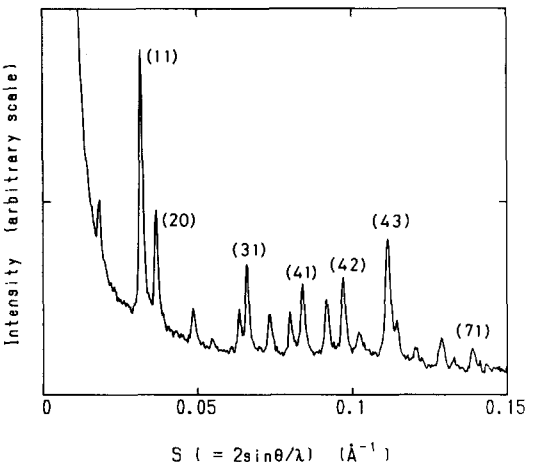

Fig.2. X-ray diffraction pattern from Arg-PM. A PM suspension of 210 μl (256 μM BR in 125 mM Arg-HCl and 25 mM Caps, pH 10.5) was dried on a mylar sheet. Sample diameter was about 5 mm. The incident X-ray beam was normal to the sample plane. *S*, reciprocal space distance. 2θ and λ designate the diffraction angle and X-ray wavelength, respectively. Some indices for a two-dimensional hexagonal powder pattern are given as (*hk*).

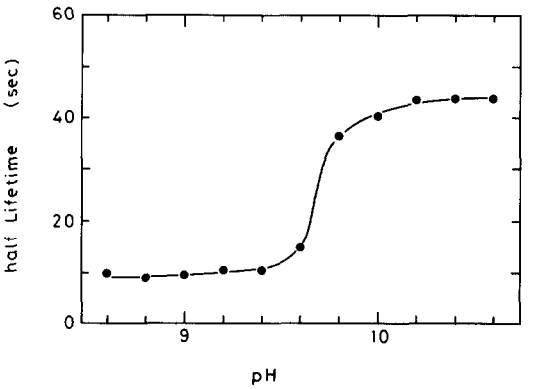

Fig.3. Dependence of half lifetime on pH of the suspension before drying. The half lifetime is defined as the time point at which the absorbance at 412 nm attained the value corresponding to half of the difference absorbance between the M-intermediate and *trans* state. The Arg-PM concentration was adjusted to about 8 μM for all samples.

The integrated intensities and integral widths of the Bragg peaks were found to be consistent with those of native PM in the region observed, taking into account the Lorentzian factor of the sample. The lattice constant was determined to be 62.7 Å, being referred to the diffraction pattern of a wet native PM. This also indicated that the solvent environment around the PM in the wet, viscous sample was identical to that in the suspension. We concluded that the treatment did not affect the structure of PM, although the crystalline structure of PM has been observed to show distortion in some cases under the conditions reported thus far [7,8]. This treatment therefore enables one to study the structure of the M-intermediate in the native configuration at room temperature by means of various techniques. We have obtained a diffraction pattern for the M-intermediate which exhibited a number of differences compared to the *trans*-BR state.

The lifetime of the M-intermediate produced in Arg-PM is dependent on the pH value. The duration of the half lifetime was maintained at about 10 s over the range pH 8–9.6 and became 4-fold greater beyond pH 9.8 as shown in fig.3. It was suggested that the critical point around pH 9.7 was the result of an amino acid residue having a pK_a value around 10, similar to tyrosine.

Since guanidine can also increase the lifetime, as reported by Yoshida et al. [8], this pointed to the guanidium group of arginine mainly affecting the prolongation. Hence, we examined the prolongation effect arising from a number of reagents with structures similar to that of arginine. The results are summarized in table 1. In the case of 1-methylguanidine and 1,1-dimethylguanidine, the M-intermediate was accumulated on irradiation and its lifetime was lengthened. However, we were unable to detect accumulation of the M-intermediate in a preparation with 1,1,4,4-tetramethylguanidine which corresponds to the four hydrogen atoms in the guanidium group being replaced by methyl groups. It is clearly seen that the guanidium moiety interacts with BR and retards the rate of conversion of the M-intermediate to *trans*-BR. Since the guanidium group is positively charged under our experimental conditions and is able to undergo interaction with a negative charge, the present data provide strong support for the prolongation effect originating from an interaction between the guanidium group and an amino acid residue, such as aspartic acid, glutamic acid or tyrosine, whose side chain can be negatively charged.

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