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Asymmetric distribution of biotin labeling on the purple membrane

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ABSTRACT

This work examined the biotin modification of bacteriorhodopsin (BR) in the purple membrane (PM). The results of flash kinetic absorption measurements showed that photocycle was maintained in biotinylated BR. Biotinylated BR also maintained its photoelectric activity, as indicated by the photoelectric response of the bilayer lipid membrane (BLM). Atomic force microscopy (AFM) of stretavidiin-bound biotin revealed that biotin molecules covered both surfaces of the, but the amount of biotinylated BR on the extracellular (EC) surface was markedly higher than on the cytoplasmic (CP) surface. Further studies showed that, after reaction with fluorescamine (FL), biotin labeling occurred only on the CP surface. These results are informative for future work on bioconjugation of BR as well as work on oriented assembly and the design of BR-based photoelectric devices.

1. Introduction

Bacteriorhodopsin (BR) is the only protein that exists in the purple membrane (PM) isolated from Halobacterium salinarium. BR consists of a single seven alpha helix polypeptide chain and a retinal chromophore that is covalently bound to Lys-216 via a protonated Schiff base [1]. BR self-assembles into trimers and associates with lipids to form naturally two-dimensional crystalline lattices of PM [2].

Henderson et al. [3] proved that BR could be labeled with biotin *N*-hydroxy succinimide ester and concluded that, in alkaline conditions (pH 8.5–9.0), the biotin labeling occurred only on the EC surface. In general, the succinimidyl ester conjugates various molecules onto BR, such as biotin, PEG [4,5], or pH indicators [6]. Although there are seven lysine residues in the BR molecule, only K129 and K159, located on the EC surface and on the CP surface, respectively, were presumed accessible to the hydrophilic bulk phase. In addition, K159 is considered to be less exposed to the aqueous environment than K129. Using site-specific enzymatic digestion and SDS-PAGE electrophoresis, Sirokman et al. [4] demonstrated that only K129 or K159 was available for reaction with m-PEG_{5000} succinimidyl carbonate, and Heberle et al. [6] attained a similar result. Sharma et al. [5] performed a trypsin digestion of the BR-PEG_{3400}-biotin conjugate followed by MALDI-TOF-MS characterization of the resulting fragments and inferred that K129 was the most likely labeling site. The ambiguous question remained, however, of whether or not the lysine residues (K159) on the CP surface of PM could react with the succinimidyl ester and, if so, to what degree.

In this work, we directly observed the remarkable surface topography difference in biotinylation on the surfaces of both normal and FL-modified PM via atomic force microscopy (AFM). We also studied the change of function associated with biotinylated PM versus normal PM.

2. Experimental methods

2.1. Materials and reagents

PM was isolated from the R1M1 strain of *H. salinarium* according to standard procedures [7,8] and kept in double-distilled water at 4 °C. Streptavidin and other reagents were purchased from Sigma. 6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid sulfosuccinimidyl ester, sodium salt (biotin-XX, SSE) was purchased from Molecular Probes. The buffer used in the experiments was neutral Tris-(hydroxymethyl)-aminomethane buffer (250 mM KCl, 10 mM Tris, pH 7.60). A solution of 50 μg/mL streptavidin in 10 mM PBS buffer (pH 7.60) was used in the experiments.

2.2. Reaction of normal PM with FL

A solution of FL in acetone (12 mg/mL) was added to a normal PM suspension (4 mg/mL) in borate buffer (0.1 M, pH 9.0). After stirring for about 1 min at 4 °C, the mixture was subjected immediately to dialysis against distilled water.

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2.3. Biotin labeling of normal PM and FL-modified PM

The biotin labeling procedure was followed as described previously [3,9]. Briefly, 1 mL of a 4 mg/mL suspension of normal PM was added to a 2 mL reaction tube containing a stir bar and was mixed with 100 μL of a 1 M sodium bicarbonate solution (pH 8.5). Subsequently, 100 μL of 10 mg/mL freshly prepared solution of biotin-XX, SSE was added to the reaction tube and stirred for 1 h. The sample was then washed three times by centrifugation, resuspended in a 0.1 M sodium bicarbonate solution (pH 8.5), and left in the same alkaline buffer for 24 h to remove unwanted biotin. Finally, the sample was dialyzed against distilled water for 48 h at 4 °C. The biotin labeling procedure of FL-modified PM was the same as that of normal PM.

2.4. M_{412}

The flash kinetic absorption changes that reveal the transient accumulation of the intermediate M_{412} were measured using homemade instruments [10] and analyzed by the method of Govindjee et al. [11]. All measurements were carried out at room temperature (25 ± 1 °C).

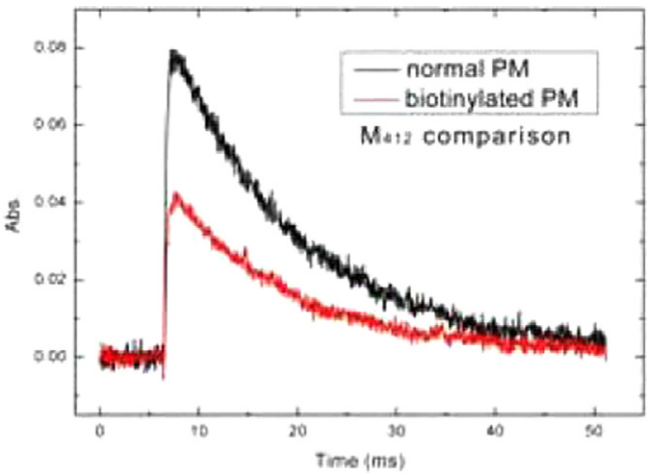

Fig. 1. The M_{412} yield of biotinylated PM was about 50% that of normal PM at the same BR concentration.

2.5. Photoelectric response

The apparatus for measuring the photovoltage signals of PM patches assembled into bilayer lipid membrane (BLM) was homemade, as described by Tien [12]. A 100 W tungsten lamp with heat protection filters was used as a light source. The calomel electrodes were protected from illumination to avoid photo effects at the electrode interfaces. The electrolytes were 100 mM KCl and 5 mM $CaCl_2$ (pH 6.4, unbuffered). The membrane-forming solution was a mixture of PM suspension in water and 50 mg/ml Lecithin in decane. The planar BLM with PM or biotinylated PM was formed over an aperture (150–200 μm in diameter) in a thin Teflon foil that separated the cell into compartments A and B [13]. After the biotinylated PM was adsorbed to the bilayer and approached equilibrium, the photovoltage signals were recorded in real time. In the control experiment, unbiotinylated PM (0.02 mg/mL) was adsorbed to the bilayer and the photovoltage was recorded.

2.6. Atomic force microscopy

A commercial AFM (NanoScope IIIa, Multi-Mode AFM, Digital Instruments) was used. The concentration of biotinylated PM stock suspension was 0.4 mg/mL in double-distilled water. For imaging, the suspension was diluted 10-fold with a buffer of pH value 7.6 to a concentration of 40 μg/mL. Afterwards, 10 μL of diluted suspension was deposited on freshly cleaved mica, which was glued onto a steel disk and magnetically mounted onto the piezoelectric scanner. The liquid cell was then set above the steel disk and 30 μL of the same buffer was injected before imaging. AFM was operated in tapping mode in room temperature buffer solution. Oxide-sharpened Si3N4 tips (NP-S) were used for all of the experiments; these tips have a spring constant of about 0.06 N/m and a resonance frequency of about 8.5 KHz. During scanning, the setpoint value was adjusted as high as possible to minimize the force and to prevent pollution of the tips. Typical scan rate was about 1 Hz. Images were processed using the software provided with the NanoScope instrument. First-order flattening and the erase scan line command were performed as needed.

3. Results

3.1. M_{412}

Under the same experimental conditions, M_{412} yield of biotinylated PM was about 50% that of PM (Fig. 1). The M_{412} lifetimes of PM

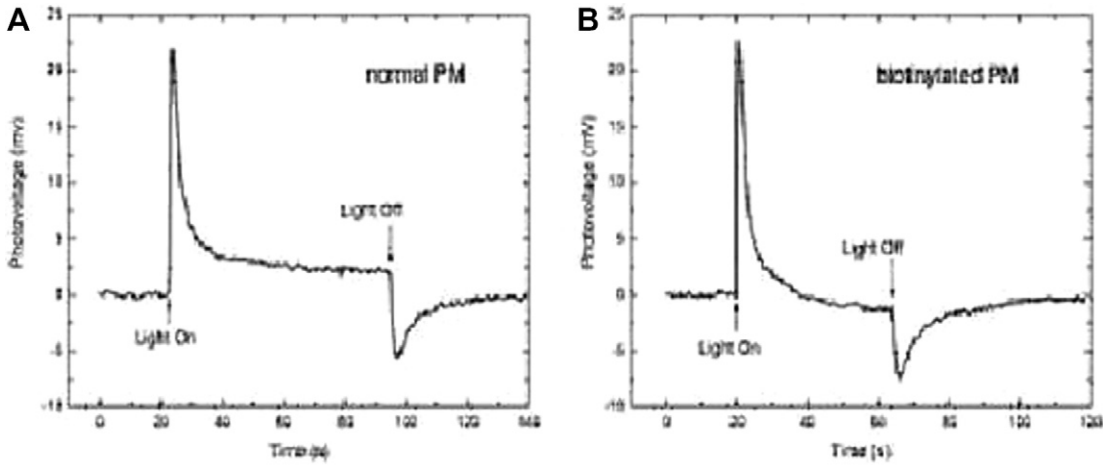

Fig. 2. (A) The photoelectric response signal of normal PM in BLM; (B) biotinylated PM. The photovoltages induced by either normal PM or biotinylated PM were quite similar.

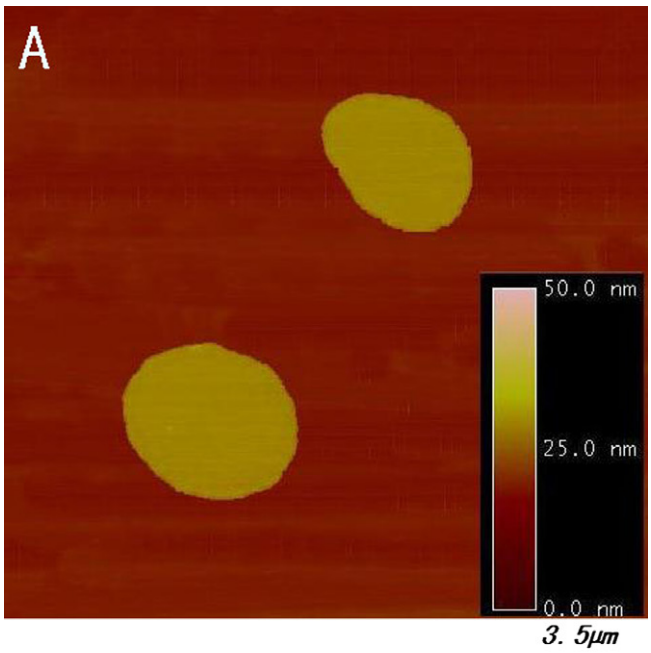

Fig. 3. Streptavidin bound to biotin, thereby labeling both the EC and CP surfaces of normal PM via K129 and K159, respectively. Since K129 is more exposed to a hydrophilic bulk phase, the EC surface of PM contained many more streptavidin molecules than the CP surface. (A) Flat surface of biotinylated PM patches before streptavidin incubation. (B) 30 min after streptavidin injection into the liquid cell.

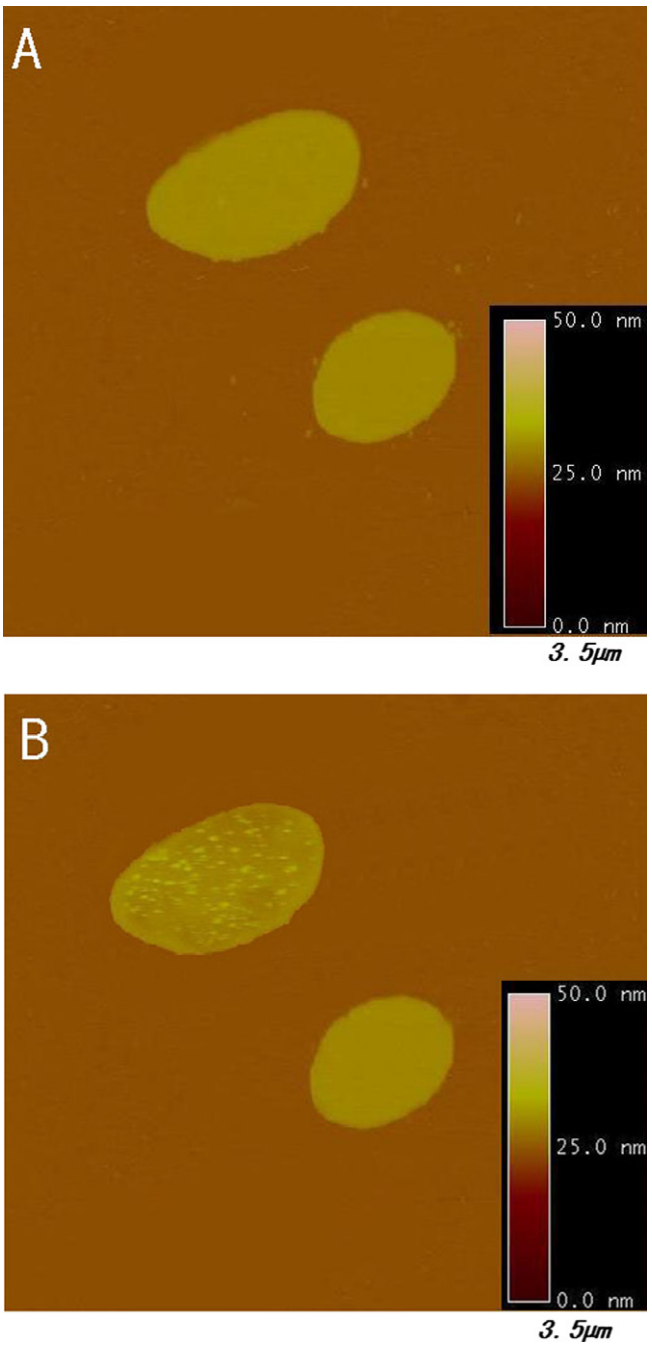

Fig. 4. Streptavidin bound to biotin, which labeled the CP surface of FL-modified PM (A) Flat surface of biotinylated and FL-modified PM patches before streptavidin incubation. (B) 30 min after streptavidin injection into the liquid cell.

and biotinylated PM were fit by exponential decay functions of time constants 13.15 ms and 12.09 ms, respectively, suggesting that the M412 and related intermediates were not disturbed by biotinylation.

3.2. Photoelectric response

The photovoltage signals of the planar bilayer formed over an aperture were recorded in real time (Fig. 2). With random orientations, the photovoltage induced by illuminating biotinylated PM was very close to that of normal PM. The similarity of the two photoelectric signals showed that biotinylated PM can also maintain its photoelectric response capacity [13–15]. Our previous work showed that oriented assembly can improve the photoelectric signal by about 20-fold [9] despite the proton pump activity of an individual BR molecule being reduced.

Circuit analysis of photocurrents induced by the light-activated proton pump of bacteriorhodopsin demonstrated that currents were dominated by oriented PM fragments on the EC surface of the bilayer, as shown in previous works [16]. It was conceivable, therefore, that there was preferential adsorption on the EC surface facing the BLM bilayer, where K129 was located. The precise reason for this preferential adsorption has not been definitively determined,

though it may be attributable to a difference in surface charge density. In any case, biotinylation of PM could preferentially enhance the orientation where the EC surface faced the BLM bilayer.

3.3. Asymmetric distribution of biotin labeling on normal PM

Using AFM, we monitored in situ the process of streptavidin binding to the biotinylated PM surface. After adsorption of biotinylated PM onto mica but prior to streptavidin incubation, topographical imaging was performed and revealed smooth, flat membrane patches with a diameter of nearly 0.5 μm (Fig. 3A). Subsequently, a 10 μl drop of 50 μg/mL streptavidin, used as an antibody for biotin, was injected into the liquid drop located between the mica and liquid cell of the AFM, and images were taken in real time during the incubation. The streptavidin binding was completed within 30 min on both membrane patch surfaces. We detected streptavidin attachment on both membrane patch surfaces, with coverage on one surface much less prominent than on the other (Fig. 3B). Streptavidin molecules appeared as bright dots roughly 3 nm in height. There was no obvious surface topography change when incubation time was increased from 30 to 60 min or by doubling the concentration of streptavidin (data not shown). To further prove that streptavidin binding was a result of biotinylation on PM as opposed to nonspecific adsorption, control experiments with normal PM and streptavidin were undertaken in the same conditions as above. No streptavidin could be detected on the surfaces of normal PM patches, even after prolonged time of antibody incubation or after the concentration of streptavidin was doubled (data not shown). Therefore, there was no nonspecific adsorption of streptavidin to PM under our experimental conditions.

This result did not coincide with Henderson et al. [3], who inferred that biotin labeling in alkaline conditions occurred only on the EC surface of the PM via K129. We inferred that one membrane patch surface in the scan area to which streptavidin was densely attached was the EC surface of the PM and the other was the CP surface.

3.4. Biotin labeling occurred only on the CP surface of FL-modified PM

Fluorescamine (FL) is a surface-labeling and amino-group selective reagent. Modification of PM with FL has been shown to modify only K129 on the EC surface [17]. After chemical modification of PM by FL and subsequent biotinylation, we monitored streptavidin binding to the sample *in situ* using AFM. The procedure was the same as described in Section 3.3 and showed similar flat, smooth patches (Fig. 4A). Subsequently, a 10 μL drop of 50 μg/mL streptavidin was injected and images were taken in real time during incubation. We detected no attachment on one membrane patch surface, but some attachment on the other (Fig. 4B). We concluded that the membrane patch with no streptavidin attachment was PM with its EC surface facing upward, while the other with some streptavidin attachment was PM with its CP surface facing upward. On the CP surface, we detected streptavidin molecules as bright dots roughly 3 nm in height. The same control experiments with FL-modified PM and streptavidin were undertaken as in Section 3.3. We also found that there was no nonspecific adsorption of streptavidin to FL-modified PM.

In Fig. 4B, the membrane patch attached by streptavidin, with its CP surface facing upward, had a similar coverage to the one in Fig. 3B, which was less densely attached by streptavidin. Because the experimental conditions in Fig. 3 were the same as those in Fig. 2, we could conclude that, in Fig. 3B, the membrane patch surface in the scan area to which streptavidin was less densely attached was the CP surface of the PM and that the other membrane patch surface was the EC surface of the PM. This supported our former conclusion.

4. Discussion

Previous research has seldom addressed whether or not biotinylation of BR could affect the protein's structure and function. After biotinylation, the UV-VIS absorption spectrum of PM suspensions hardly shifted (data not shown), suggesting that the tertiary structure of BR and the microenvironment of the retinal had not been disturbed. The fitting results of the flash kinetic spectrum of M_{412} suggest that the essential photocycle properties of BR had only slightly been affected by biotinylation [11,18], but the decrease in M412 by about 50% after biotinylation was probably due to modification at the biotinylated sites.

The photoelectric response of oriented patches of biotinylated PM mediated by streptavidin was 20 times that of the control patches [9]. Because there was no nonspecific adsorption of streptavidin onto PM, the imperfect degree of orientation of about 80% caused by molecular recognition was mainly due to the biotinylation of K159 [9,19]. Based on our experimental results, it was possible to achieve a higher degree of orientation for a biotinylated PM assembly by modifying or mutating K159. Moreover, a K129 mutant could also provide specific modification to the CP surface of PM due to the limited labeling accessibility of K159 so that different surfaces of oriented PM patches could be assembled. Combining the oriented assemblies of biotin-labeled PM with other technologies may enable the use of such applications as photoelectronics devices with long-lifetimes, mechanical stability and high efficiencies.

In contrast to Henderson's results, we detected biotin molecules covering both surfaces of PM using streptavidin binding, but the amount of biotinylated BR on the EC surface was remarkably higher than on the CP surface. Further studies showed that after reaction of BR with FL, biotin labeling occurred only on the CP surface supporting our conclusion that biotinylation could occur on both surfaces of normal PM. Although there were seven lysine residues in the BR molecule, only K129 and K159, located in the DE loop on the EC surface and in the EF loop on the CP surface, respectively, were presumably exposed to hydrophilic bulk phase. In addition, K159 was considered to have been less exposed to the aqueous environment than K129. Therefore, in normal PM, both K129 and K159 were available for reaction with biotin.

In summary, three conclusions can be made from this study: (1) biotinylation of BR in PM reduces the yield of M_{412} by about 50%; (2) biotinylation of BR in alkaline conditions occurs not only on the EC surface of PM via K129 but also on the CP surface via K159, with the amount of biotin labeling on the former surface remarkably higher than on the latter; and (3) after reaction of BR with FL, biotinylation of BR occurs only on the CP surface via K159.

5. Abbreviations

PM purple membrane from *Halobacterium salinarium*
BR bacteriorhodopsin
AFM atomic force microscopy
Biotin-XX, SSE 6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid sulfosuccinimidyl ester sodium salt
EC extracellular
CP cytoplasmic
FL fluorescamine
BLM bilayer lipid membrane

Acknowledgments

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