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Atomic force microscopy imaging of the structure of the motor protein prestin reconstituted into an artificial lipid bilayer

Shun Kumano, Michio Murakoshi, Koji Iida, Hiroshi Hamana, Hiroshi Wada *

Department of Bioengineering and Robotics, Tohoku University, 6-6-01 Aoba-yama, Sendai 980-8579, Japan

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ABSTRACT

Prestin is the motor protein of cochlear outer hair cells and is essential for mammalian hearing. The present study aimed to clarify the structure of prestin by atomic force microscopy (AFM). Prestin was purified from Chinese hamster ovary cells which had been modified to stably express prestin, and then reconstituted into an artificial lipid bilayer. Immunofluorescence staining with anti-prestin antibody showed that the cytoplasmic side of prestin was possibly face up in the reconstituted lipid bilayer. AFM observation indicated that the cytoplasmic surface of prestin was ring-like with a diameter of about 11 nm.

1. Introduction

The basis of electromotility of outer hair cells (OHCs) which realizes the high sensitivity of mammalian hearing is considered to be the motor protein prestin [1]. Several characteristics of prestin have been gradually clarified [2]. Murakoshi et al. [3] detected prestin in the plasma membrane of prestin-transfected Chinese hamster ovary (CHO) cells using Qdots as topographic markers and observed ring-like structures, possibly prestin, by atomic force microscopy (AFM). Mio et al. [4] observed prestin purified from prestin-transfected insect cells by transmission electron microscopy (TEM) and found prestin to be a bullet-shaped molecule. Although those two studies are significant, their observed images differed, indicating that the structure of prestin was unclear. Thus, the aim of the present study was to clarify such structure by reconstitution of purified prestin into an artificial lipid bilayer and observation of the prestin-reconstituted lipid bilayer by AFM.

Abbreviations: OHC, outer hair cell; CHO, Chinese hamster ovary; AFM, atomic force microscopy; TEM, transmission electron microscopy

* Corresponding author. Hiroshi Wada, Department of Bioengineering and Robotics, Tohoku University, 6-6-01 Aoba-yama, Sendai 980-8579, Japan. Fax: +81 22 795 6939.
E-mail address: wada@cc.mech.tohoku.ac.jp (H. Wada).

2. Materials and methods

2.1. Purification of prestin

The purification of prestin was performed by the method established in our previous study with some modifications [5]. CHO cells which had been modified to stably express C-terminal 3×FLAG-tagged prestin were suspended in Tris–KCl buffer (10 mM Tris, 150 mM KCl, pH 7.4) and sonicated, followed by centrifugation at 1000×*g* for 7 min at 4 °C to remove nuclei and undisrupted cells. The obtained supernatant was centrifuged at 20360×*g* at 4 °C for 2 h to collect the membrane fraction of the cells. Membrane proteins were solubilized by resuspending the obtained membrane fraction in Tris–KCl buffer containing 10 mM *n*-nonyl-β-D-thiomaltopyranoside (NTM, Dojindo, Kumamoto, Japan). After 3-h incubation on ice, samples were centrifuged at 20360×*g* at 4 °C for 3 h to remove non-solubilized proteins. The supernatant was applied to a column filled with anti-FLAG affinity gel (Sigma–Aldrich, St. Louis, MO). The column was then washed with Tris–KCl buffer containing 0.065 mM Fos-Cholin-16 (Anatrace, Maumee, OH) to replace the detergent NTM with Fos-Choline-16. Afterward, prestin was competitively eluted with 1 ml of that buffer containing 500 μg/ml of 3×FLAG peptide (Sigma–Aldrich). Whether prestin was purified or not was confirmed by SDS–PAGE, followed by Western blotting with anti-FLAG antibody and HRP-conjugated anti-mouse IgG antibody and by silver staining.

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2.2. Reconstitution of prestin into a preformed lipid bilayer

The method of direct reconstitution of membrane proteins into a preformed lipid bilayer was applied in the present study [6]. An artificial lipid bilayer was formed on mica using dioeoyl-phosphatidylcholine (DOPC) and dipalmitoyl-phosphatidylcholine (DPPC) (Avanti Polar Lipids, Alabaster, AL). The lipid bilayer was preincubated for 30 min at 4 °C with Tris–KCl buffer containing 5 mM $CaCl_2$ and 0.0065 mM Fos-cholin-16 for equilibration of the detergent within the lipid bilayer. Afterward, such bilayer was incubated with Tris–KCl buffer containing purified prestin, 5 mM $CaCl_2$ and 0.039 mM Fos-cholin-16 for 15 min at 4 °C. Excess prestin was then removed by extensive rinsing with Tris–KCl buffer. As a negative control, the lipid bilayer treated with detergent but without prestin was also prepared.

2.3. Staining of prestin in the reconstituted lipid bilayer

The existence of prestin in the lipid bilayer was confirmed by immunofluorescence staining. The prestin-reconstituted lipid bilayer was incubated with Block Ace (Dainippon Pharmaceutical Co. Osaka, Japan) for 30 min at 37 °C to avoid non-specific binding of antibodies. Afterward, that bilayer was stained with goat anti-prestin N-terminus primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100 in PBS overnight at 4 °C and with anti-goat IgG Texas Red (Santa Cruz Biotechnology) at a dilution of 1:200 in PBS at 37 °C for 60 min. The stained lipid bilayer was observed by confocal microscopy.

2.4. AFM imaging

The height images of the lipid bilayer were acquired in Tris–KCl buffer filtered with a 0.2-μm nylon filter by Multimode V AFM with a Nanoscope V controller (Veeco, Santa Barbara, CA) at 24–26 °C. V-shaped Si_3N_4 cantilevers (OMCL-TR400PSA-2, Olympus, Tokyo, Japan) with a spring constant of 0.06 N/m were used. The AFM was operated in the oscillation imaging mode (Tapping mode™, Digital Instruments) at a scan frequency of 1–0.5 kHz. In the present study, three types of images were obtained by AFM, namely, low- (5.0 × 5.0 μm), middle- (1.0 × 1.0 μm) and high-magnification images (300 × 300 nm). Each scan line has 256 and 512 points of data and an image consists of 256 and 512 scan lines for low magnification images and for middle- and high-magnification images, respectively. Obtained AFM images were flattened by use of a software program (NanoScope v7.00, Veeco) to eliminate background slopes and to correct dispersions of individual scanning lines. In addition, only high-magnification images were low-pass filtered to reduce high frequency noise. When the observed structure was ring-like, the distance between two peaks based on the cross sections was taken to be its diameter, as was done in our previous study [3].

3. Results

3.1. Purification of prestin

Whether prestin was indeed purified or not was investigated by SDS–PAGE, followed by Western blotting and silver staining. Results of Western blotting and silver staining are shown in Fig. 1A and B, respectively. In the Western blotting image, the 100 kDa band, probably showing prestin, was detected. In the results of silver staining, only one band corresponding to the band observed in Western blotting was recognized.

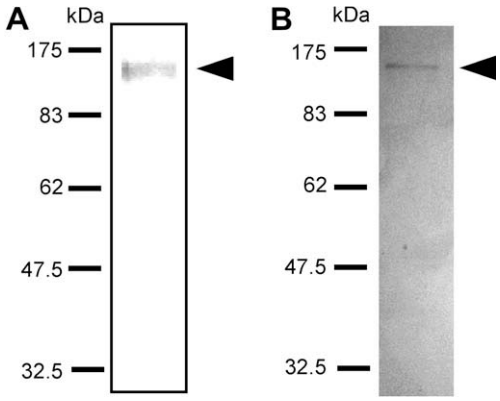

Fig. 1. Results of Western blotting and silver staining. (A) Western blotting data. A 100 kDa band probably showing prestin is seen. (B) Result of silver staining of SDS–PAGE gel. Only one band at 100 kDa, which was thought to correspond to the band detected in the result of Western blotting, is recognized.

3.2. Immunofluorescence staining of the prestin-reconstituted lipid bilayer

After the reconstitution process, immunofluorescence staining was employed to investigate whether prestin had been incorporated into the preformed lipid bilayer. Representative immunofluorescence images of the prestin-reconstituted lipid bilayer and negative control sample are shown in Fig. 2. Red fluorescence was detected in the prestin-reconstituted lipid bilayer but not in the negative control sample.

3.3. AFM imaging of the lipid bilayer

The AFM height image of the lipid bilayer without treatment showed two kinds of flat domains (Fig. 3A). A similar image was also obtained from the negative control sample (Fig. 3B). Unlike those two images, in addition to the flat domain, bumpy domains indicated by white arrows were detected in the low magnification AFM image of the prestin-reconstituted lipid bilayer (Fig. 3C). The boxed area in Fig. 3C was scanned by AFM and the obtained image is depicted in Fig. 3D. Dense small particles, some of which were recognized as ring-like structures, can be observed in that image. To clearly visualize the observed particles, the boxed area in Fig. 3D was scanned by AFM, the acquired image being shown in Fig. 3E. Moreover, three-dimensional representation of Fig. 3E is depicted in Fig. 4. Many ring-like structures were confirmed to be densely embedded in the lipid bilayer. The average diameter of such structures in Fig. 3E and other AFM images which are not shown here is 11.0 ± 1.3 nm (n = 42).

4. Discussion

4.1. Reconstitution of prestin into an artificial lipid bilayer

After the purification process, only the 100 kDa band corresponding to the band in Western blotting data was detected by silver staining of SDS–PAGE gel, indicating that prestin had been purified. The 100 kDa band probably shows the monomer of prestin. As SDS possibly affects the binding between prestin molecules, to clearly confirm the oligomerization of purified prestin, a mild detergent such as perfluoro-octanoic acid should be used as in the study by Zheng et al. [7]. The AFM height image of the lipid bilayer without treatment shows two types of flat domains (Fig. 3A), as seen in previous studies [6,8–11]. At 24–26 °C, DOPC forms fluid-phase domains, while DPPC forms gel-phase domains. The thickness of DPPC in the gel-phase is larger than that of DOPC in

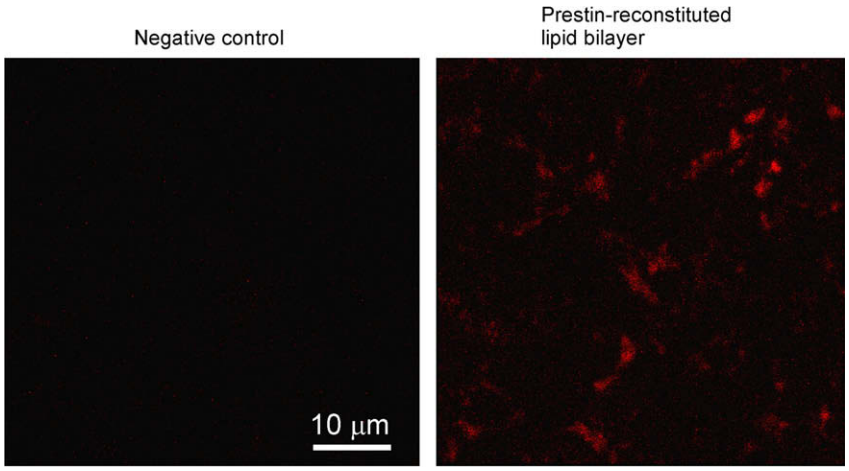

Fig. 2. Immunofluorescence staining of prestin-reconstituted lipid bilayer. Negative control sample and the prestin-reconstituted lipid bilayer were stained with anti-prestin antibody and anti-goat IgG Texas Red. Red fluorescence indicating the existence of prestin is only found in the prestin-reconstituted lipid bilayer.

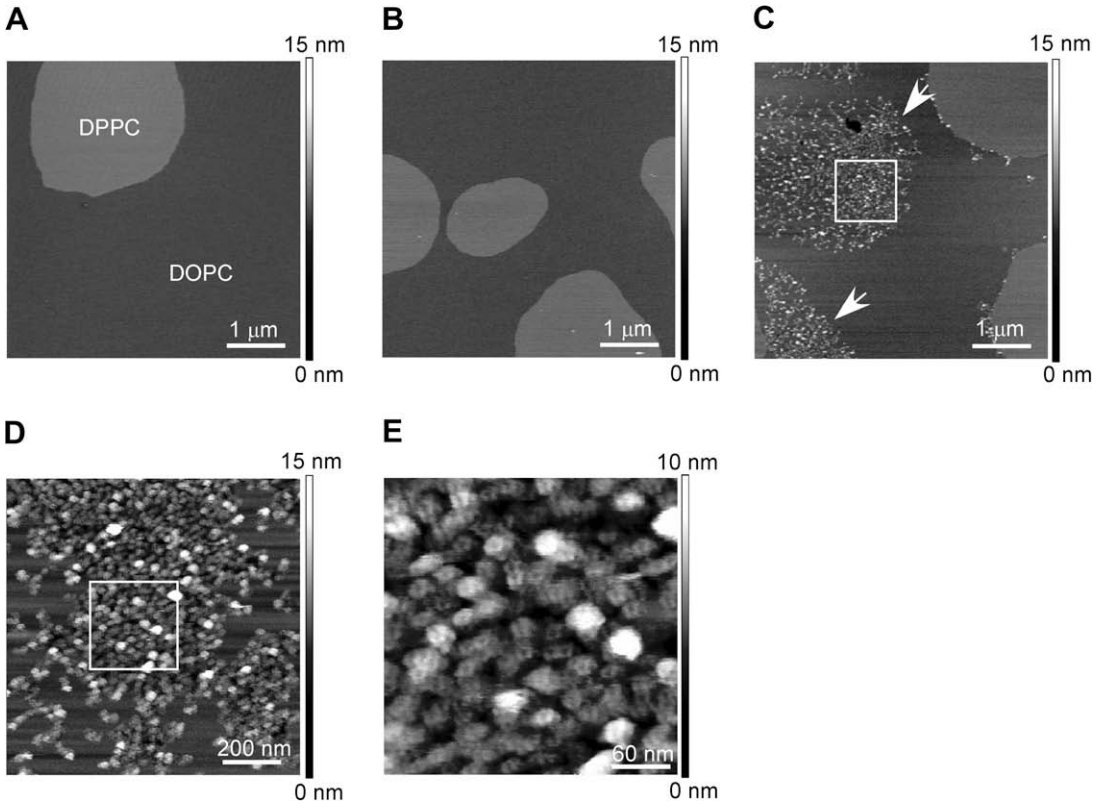

Fig. 3. AFM height images of the lipid bilayer. (A) AFM image of the lipid bilayer without treatment at low magnification. (B) AFM image of the negative control sample at low magnification. (C) AFM image of prestin-reconstituted lipid bilayer at low magnification. (D) Middle-magnification image obtained by scanning of the boxed area shown in (C). (E) High-magnification image obtained by scanning of the boxed area shown in (D). Two kinds of flat domains in (A) and (B) probably represent the domain of DPPC in the gel-phase and that of DOPC in the fluid-phase. Bumpy domains indicated by white arrows can be detected in the prestin-reconstituted lipid bilayer shown in (C). Many small particles can be found in the middle-magnification AFM images and those particles were recognized as ring-like in the high-magnification image. Such ring-like structures probably show prestin molecules.

the fluid-phase, thus indicating that the two types of observed domains were due to the difference in the thickness between the two lipids. After the reconstitution process, immunofluorescence staining using anti-prestin antibody showed that prestin existed in the reconstituted lipid bilayer (Fig. 2). In the AFM image, the bumpy domains, which probably corresponded to prestin, were recognized only in DOPC domains of the prestin-reconstituted lipid bilayer. Milhiet et al. [6] have also suggested that proteins of interest were reconstituted only into the DOPC domains in the fluid state. Thus, the present study and their study imply that proteins tend to be reconstituted into the DOPC domains in the fluid state. In the AFM image at high-magnification, ring-like structures probably showing prestin were densely reconstituted into the lipid bilayer. However, the alignment of such structures as found in the OHC plasma membrane by Sinha et al. [12] was not detected, which might have resulted from differences in the environment

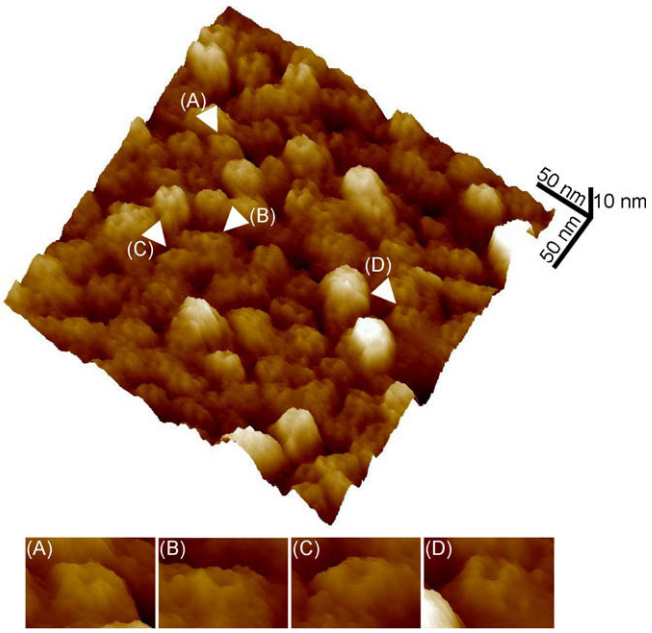

Fig. 4. Three-dimensional AFM height image of the prestin-reconstituted lipid bilayer. This figure was created from Fig. 3E. Representative examples of ring-like structures were digitally magnified and are shown (A and B). Ring-like structures, considered to be similar to those observed in the study of Murakoshi et al. [3], are found to be densely embedded in the lipid bilayer.

Table 1
Comparison of the size of prestin.

Sample	Method	Diameter (nm)	References
OHC plasma membrane	AFM	11–25	Le Grimellec et al. [15]
Purified prestin	TEM	7.7–9.6	Mio et al. [4]
Prestin-expressing CHO cell plasma membrane	AFM	9.6/13.0	Murakoshi et al. [3]
OHC plasma membrane	AFM	10	Sinha et al. [12]
Prestin-reconstituted lipid bilayer	AFM	11.0 ± 1.3	This study

between the OHC plasma membrane and the artificial lipid bilayer. The existence of actin cytoskeleton in OHCs and that of mica in the present study would affect the alignment of prestin.

4.2. Orientation of prestin

The orientation of prestin should be considered to confirm which side of prestin was observed by AFM, the extracellular side or the cytoplasmic side. Previous reports have suggested that when membrane proteins were reconstituted into a preformed lipid bilayer as done in the present study, their unidirectional orientation was obtained [6,13,14], indicating that all prestin molecules reconstituted into the lipid bilayer might be oriented in the same direction. In the present study, the standard deviation of the diameter of the observed ring-like structure, 1.3 nm, was small. Small standard deviation might increase the possibility that only either prestin molecules whose extracellular side was exposed or such molecules whose cytoplasmic side was exposed existed in the reconstituted lipid bilayer. Data showed in the previous reports, small standard deviation of the diameter and successful staining of such bilayer with anti-prestin antibody which binds to the cytoplasmic side of prestin possibly implied that the cytoplasmic side of prestin was face up. Although the possibility that the extracellular side of a few prestin molecules was exposed was not completely ruled out, it was considered that AFM possibly visualized the cytoplasmic side of prestin in the present study.

4.3. Structure and size of prestin

The AFM image of the prestin-reconstituted lipid bilayer showed dense ring-like structures, each with a diameter of 11.0 ± 1.3 nm, which were probably the surface structure of the cytoplasmic side of prestin. The previously reported sizes of prestin obtained by observation of the cytoplasmic side of prestin are listed in Table 1 [3,4,12,15]. Although it is unclear whether the particles detected in OHC plasma membranes are only comprised of prestin or not, our result is consistent with the previously reported sizes, supporting the assumption that the observed structures were prestin.

Le Grimellec et al. [15] found structures with a central depression in the cytoplasmic side of the OHC plasma membrane by AFM. Murakoshi et al. [3] showed by AFM that prestin might form a ring-like structure. On the other hand, Mio et al. [4] suggested that prestin is a bullet-shaped molecule which protrudes into the cytoplasmic side. Although Sinha et al. [12] found 10-nm particles in the cytoplasmic side of the OHC plasma membrane by AFM, whether those particles were ring-like or not was not specified. Thus, the structure of prestin has been a controversial issue. Our results demonstrate that prestin may form a ring-like structure with a diameter of about 11 nm, which agrees with results of Le Grimellec et al. [15] and Murakoshi et al. [3].

In summary, the present study attempted to visualize prestin purified and reconstituted into the artificial lipid bilayer by AFM. From the obtained AFM image, the cytoplasmic surface of prestin was indicated to be ring-like with a diameter of about 11 nm.

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