Resource

Structure

Single-Unit Imaging of Membrane Protein-Embedded Nanodiscs from Two Oriented Sides by High-Speed Atomic Force Microscopy

Graphical Abstract

Highlights
- Membrane protein-embedded nanodiscs were used for high-speed (HS)-AFM observation
- Nanodiscs in an end-up and side-on orientation were observed by HS-AFM in real time
- HS-AFM combined with nanodiscs is a powerful tool for membrane protein dynamics

Authors
Takamitsu Haruyama, Yasunori Sugano, Noriyuki Kodera, ..., Yoshiki Tanaka, Hiroki Konno, Tomoya Tsukazaki

Correspondence
hkonno@se.kanazawa-u.ac.jp (H.K.), ttsukazaki@mac.com (T.T.)

In Brief
Haruyama et al. established a procedure for the observation of membrane proteins in nanodiscs by high-speed AFM in real time. Using the two-dimensional crystal of streptavidin as well as mica as a solid support surface, dynamic structural changes of membrane proteins in the lipid environment were visualized from two distinct angles.

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Single-Unit Imaging of Membrane Protein-Embedded Nanodiscs from Two Oriented Sides by High-Speed Atomic Force Microscopy

Takamitsu Haruyama,1,4 Yasunori Sugano,1,4 Noriyuki Kodera,2 Takayuki Uchihashi,3 Toshio Ando,2 Yoshiki Tanaka,1 Hiroki Konno,2,* and Tomoya Tsukazaki1,5,*
1Graduate School of Science and Technology, Nara Institute of Science and Technology, Nara 630-0192, Japan
2Nano Life Science Institute (WPI-NanoLSI), Kanazawa University, Kanazawa 920-1192, Japan
3Department of Physics, Nagoya University, Nagoya 464-8602, Japan
4These authors contributed equally
5Lead Contact
*Correspondence: hkonno@se.kanazawa-u.ac.jp (H.K.), ttsukazaki@mac.com (T.T.)
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SUMMARY

Membrane proteins play important roles in various cellular functions. To analyze membrane proteins, nanodisc technology using membrane scaffold proteins allows single membrane protein units to be embedded into the lipid bilayer disc without detergents. Recent advancements in high-speed atomic force microscopy (HS-AFM) have enabled us to monitor the real-time dynamics of proteins in solution at the nanometer scale. In this study, we report HS-AFM imaging of membrane proteins reconstituted into nanodiscs using two membrane protein complexes, SecYEG complex and MgtE dimer. The observed images showed single particles of membrane protein-embedded nanodiscs in an end-up orientation whereby the membrane was fixed parallel to the supporting solid surface and in a side-on orientation whereby the membrane plane was vertically fixed to the solid surface, enabling the elucidation of domain fluctuations in membrane proteins. This technique provides a basic method for the high-resolution imaging of single membrane proteins by HS-AFM.

INTRODUCTION

Many membrane proteins, including drug targets, show inherent activity when properly embedded in specific lipid bilayers. Previous atomic force microscopy (AFM) studies of membrane proteins have employed lipid bilayers spread onto the measurement surface; these lipid bilayers have been prepared from extracted cell membrane fractions or membrane protein-reconstituted liposomes (Bahatyrova et al., 2004; Fotiadis et al., 2003; Ruan et al., 2017; Seelert et al., 2000; Shibata et al., 2010). In some cases, purified membrane proteins solubilized by detergent were directly reconstituted into the supported lipid bilayer onto a supporting solid surface, such as mica and glass (Milhiet et al., 2006; Sumino et al., 2017). Therefore, the orientation of AFM observations for membrane protein dynamics is fixed to only one side (Figure S1A). To overcome this limitation, a monitoring system that can be used to visualize both sides of the membrane is needed for dynamic analyses of certain membrane proteins (Figures S1B and S1C). For example, in many membrane proteins, such as those involved in signal transduction, functional domains on both sides undergo mutual structural changes. Moreover, steric hindrance due to the large size of precursor substrate proteins may prevent protein translocation across the spread membrane. Hence, sufficient space on both sides is necessary to monitor the movement of precursor proteins and evaluate conformational changes in domains on both sides simultaneously. Nanodiscs stably hold the disc-like lipid bilayer with minimal diffusion of the lipids, and provide sufficient space on both sides of the membrane when placed in a side-on orientation (Rouck et al., 2017; Bayburt et al., 2002). Additionally, since nanodiscs can stably retain one functional unit of the membrane protein embedded in the membrane, they are suitable for single-molecule dynamic observation in real time by high-speed (HS)-AFM (Kodera et al., 2010; Miyagi et al., 2016; Shibata et al., 2017; Uchihashi et al., 2011). Single-unit imaging of membrane protein-embedded nanodiscs loaded onto solid surfaces may be achieved from various orientations using HS-AFM under appropriate conditions. Here, two membrane proteins, i.e., the protein-conducting channel SecYEG (Tanaka et al., 2015) and Mg2+ transporter MgtE (Hattori et al., 2007), were embedded into nanodiscs and observed by HS-AFM from two oriented sides.

The evolutionarily conserved SecY/SecE/SecG membrane protein complex (SecYEG) forms a complex with the cytosolic motor SecA ATPase to drive protein translocation (du Plessis et al., 2011; Rapoport et al., 2017; Tsirigotaki et al., 2017). The proton-driven motor SecDF also facilitates protein translation at the extracytoplasmic side (Tsukazaki, 2018), and the minimum necessary protein translocation activity can be achieved only by SecA and SecYEG embedded in the lipid bilayer (Akimaru et al., 1991). Therefore, we previously determined the crystal structures of SecYEG and SecA from Thermus thermophilus, a
A \quad B

C \quad D

E \quad F

G

(legend on next page)
Evidence of SecYAEG-NDs and SecYEG-NDs in the End-Up Orientation

In this study, we first used HS-AFM to observe the SecYEG-reconstituted nanodiscs (SecYEG-NDs) on mica surfaces but could not distinguish the orientation of the nanodiscs, mainly owing to the small size of the observed spherical particles, with diameters of 10 nm, as well as nonspecific binding. Hence, we prepared samples in which all spherical SecYEG-NDs were in the end-up orientation. Purified SecYEG was labeled with biotin at two positions on the periplasmic loops using a double-cysteine mutant (Figure S2), embedded in nanodiscs, and anchored onto the two-dimensional crystal of streptavidin (Yamamoto et al., 2009) (Figure 1A). The nanodiscs were presumed to be oriented as shown in Figure 1B (right). HS-AFM observations showed the presence of ~10-nm spherical objects on the two-dimensional crystal of streptavidin (Figures 1A and 1E [left]). The average height of the spherical molecule (7.7 ± 0.6 nm; n = 277, 277 molecules) was consistent with the height of SecYEG estimated from the crystal structure (Tanaka et al., 2015), suggesting that the observed particles represented SecYEG in the nanodiscs (SecYEG-NDs) (Figure 1B). Next, we observed SecYAE9-NDs and found that the average height (10.9 ± 0.8 nm; n = 305, 305 molecules) was 3.2 nm greater than that of SecYEG-NDs, possibly due to the additional SecA region. This is consistent with the expected height based on the crystal structure (Zimmer et al., 2008) (Figures 1C–1E [right]). Images of SecYAE9-NDs showed round X shapes, similar to the simulated AFM image from the crystal structure (Figure 1F), suggesting that the X-shaped particles are the domains of SecA. Close-up observations indicated that each SecA transitioned among several different conformations, as partially shown in structural analyses (Chatzi et al., 2014) (Figure 1G). Although the differences in SecA particles may reflect the functional and structural changes in solution, we could not clearly distinguish each domain of SecA. Notably, the images showed high resolution, particularly considering that SecA particles did not directly interact with the supporting surface but were positioned on the nanodisc apart from the surface. If we introduced at least three cysteine residues per unit or used shorter biotin-mal- eimide reagents for fixation to reduce the fluctuation of nanodiscs, we would obtain higher-resolution images. Thus, we established a procedure in which biotin-labeled SecYEG-NDs and SecYAE9-NDs were anchored onto the two-dimensional streptavidin surface in the end-up orientation. Moreover, after biotin labeling via introduced cysteines, we can use HS-AFM as an imaging technique to evaluate membrane-embedded proteins from arbitrary orientations.

RESULTS

SecYEG-NDs and SecYAEG-NDs in the End-Up Orientation

To explore the samples from another angle, we observed the SecYAE9-NDs adsorbed on a mica surface. Unexpectedly, we found similarly shaped particles in the same orientation. Each particle consists of one small and one large elliptically shaped object, both of which were clearly distinguishable based on cleavage (Figure 2A). The particle shapes are essentially consistent with the simulated AFM image from the crystal structure, indicating that the SecYAE9-NDs are immobilized with a side-on orientation on the mica surface (Figures 2B and 2C). Furthermore, to reliably confirm the orientation, mixed samples of SecYAE9-NDs with streptavidins were observed on the mica surface (Figure 2D). Compared with the condition without streptavidin, the HS-AFM image showed two additional smaller particles (size of ~5 nm) attached to the side of the large elliptical molecule, implying that the large and small particles correspond to the SecYEG-ND and soluble SecA regions, respectively. Previous AFM studies have shown that membrane-protein-embedded nanodiscs are immobilized with the end-up orientation on mica (Koch et al., 2018; Zocher et al., 2012). In our case, the flexibility of SecA and the biotin label may hamper the close interaction between the mica surface and lipid bilayer. Our observations demonstrate the presence of SecYAE9-NDs in the side-on orientation on the mica surface and SecYAE9-NDs in the end-up orientation on the streptavidin crystal plane using biotin-labeled samples. Although it is difficult to further elucidate the molecular mechanisms of Sec translocation machinery owing to the resolution of the HS-AFM observation system, nanodisc systems may allow the observation of protein...
transport reactions across the membrane from at least two directions.

**MgtE-NDs in the End-Up Orientation**

Next, to address whether the system using nanodiscs is applicable to other membrane proteins, we applied this approach to the Mg$^{2+}$ transporter MgtE. Dimeric MgtE regulates Mg$^{2+}$ uptake into the cells in response to Mg$^{2+}$ concentrations in the cytoplasm (Hattori et al., 2007, 2009; Ishitani et al., 2008). When the Mg$^{2+}$ concentration in the cytoplasm is low, the assembly of the cytoplasmic region, consisting of N and CBS domains, weakens, inducing structural changes in the transmembrane domain for Mg$^{2+}$ transport. In contrast, when sufficient Mg$^{2+}$ exists, the cytoplasmic region is tightly packed, blocking Mg$^{2+}$ uptake. We purified dimeric MgtE labeled with biotin on the periplasmic loop using a cysteine mutant (Figure S2) and observed MgtE-reconstituted nanodiscs (MgtE-NDs) anchored onto the streptavidin crystal in the presence of Mg$^{2+}$ (Figures 3A–3C and Video S1). The HS-AFM images showed spherical structures, consistent with the simulated AFM image based on the crystal structure (Hattori et al., 2007). The highest region (average height = 9.6 ± 1.0 nm; n = 2,576, 24 molecules) in the center of the nanodisc is considered the dimeric MgtE, consistent with the estimated height. During the observation, the height of the MgtE-NDs was nearly constant (Figures 3D and 3E), suggesting that MgtE in nanodiscs in solution in the presence of Mg$^{2+}$ was a hard, compact spherical structure, similar to the crystal structure. In contrast, the HS-AFM images of MgtE-NDs in the absence of Mg$^{2+}$ showed a jagged topography, indicating that the cytosolic domains are constantly fluctuating (Figures 3F and 3G; Video S2). In the absence of Mg$^{2+}$, the height distribution showed two peaks, which were fitted using two Gaussian curves (Figure 3H). Although the larger average height (10.4 ± 2.0 nm; n = 2,156, 21 molecules) was approximately similar to the peak observed in the presence of Mg$^{2+}$ (Figure 3E), the distribution became broader compared with that in the presence of Mg$^{2+}$ (Figures 3E and 3H). The peak at 7.8 ± 0.5 nm corresponds to the height of MgtE-ND without CBS domains (7.4 nm), presumably due to the fluctuation of the cytoplasmic domains, including CBS domains. These phenomena of the cytoplasmic region observed by HS-AFM in the absence of Mg$^{2+}$ were consistent with the flexibility of the N and CBS domains, which are distant from each other in the crystal structure and easily dissociated without Mg$^{2+}$ (Hattori et al., 2007).
Figure 3. Fluctuation of MgtE-NDs in the End-Up Orientation, Depending on Mg$^{2+}$
(A) Mg$^{2+}$-bound MgtE-ND models constructed based on the crystal structure of MgtE (PDB: 2YVX) and the nanodisc model (PDB: 2MSC). The N and CBS domains are shown in cyan and blue, respectively. Side view showing a schematic illustration of MgtE-NDs fixed on SA 2D crystals via biotin tags. The estimated height of MgtE is shown.
(B) Simulated AFM image of the Mg$^{2+}$-bound MgtE-ND model, as shown in (A) (right).
(C) Successive AFM images of Mg$^{2+}$-bound MgtE-NDs on SA 2D crystals.
(D) Time course of the height of five independent particles of Mg$^{2+}$-bound MgtE-NDs.
(E and F) Height distribution of Mg$^{2+}$-bound MgtE-NDs (E). The red line represents the single-Gaussian fitting curve (9.6 ± 1.0 nm; n = 2,576, 24 molecules). The original maximum heights of MgtE-NDs on SA 2D crystals were the highest point within the particle images of each frame. The actual heights were calculated by subtracting the background (the highest point within the SA 2D crystals images for each frame). (F) Successive AFM images of Mg$^{2+}$-unbound MgtE-NDs on SA 2D crystals.
(G) Time course of the height of five independent particles of Mg$^{2+}$-unbound MgtE-NDs.
(H) Height distribution of Mg$^{2+}$-unbound MgtE-NDs. The blue line represents the two Gaussian fitting curves (10.4 ± 2.0 nm and 7.8 ± 0.5 nm; n = 2,156, 21 molecules). The actual heights were calculated as in (E).
See also Figure S2; Videos S1 and S2.
Figure 4. MgtE-NDs Placed on the Mica Surface in the Side-On Orientation in the Presence and Absence of Mg$^{2+}$
(A) Side view of the MgtE-ND model (left). Simulated AFM image of the MgtE-ND model (right).
(B) Magnified Mg$^{2+}$-bound MgtE-ND particles on the mica surface.

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et al., 2007, 2009; Ishitani et al., 2008). Thus, we could place MgtE-reconstituted nanodiscs in the end-up orientation, similar to SecYEG-reconstituted nanodiscs, and detect real-time fluctuations in the cytoplasmic domain of MgtE in nanodiscs.

MgtE-NDs in the Side-On Orientation

We next observed images of MgtE-NDs adsorbed on mica. All particles showed the elliptical nanodisc architecture not related to the Mg\(^{2+}\) concentration, whereas the cytoplasmic domains appeared different depending on the presence of Mg\(^{2+}\) (Figures 4C and 4D; Videos S3 and S4). We recognized the tightly assembled N and CBS domains in the presence of Mg\(^{2+}\), consistent with the simulated AFM image of the crystal structure (Figures 4A–4C). In contrast, in the absence of Mg\(^{2+}\), the cytoplasmic regions were highly flexible and ambiguous (Figure 4D). These observations are also consistent with the aforementioned results using the two-dimensional crystal. Additionally, we observed MgtE-NDs on the Ni\(^{2+}\)-coated mica surface, which is positively charged, strengthening the interaction between the negatively charged cytoplasmic region and the Ni\(^{2+}\)-coated mica (Figure 4E and Video S3). The HS-AFM images showed clearer cytoplasmic regions in the absence of Mg\(^{2+}\), and successive images demonstrated the association (e.g., at 0.0 and 33.2 s) and disassembly (e.g., at 11.2 and 60.0 s) of the N and CBS domains in solution. Using HS-AFM, we observed the flexibility of the cytoplasmic domain of MgtE from two controlled directions in real time.

DISCUSSION

In this study, we employed nanodiscs containing membrane proteins that were biotin labeled via introduced cysteine residues for HS-AFM observation. By optimizing the supporting surface, membrane protein-embedded nanodiscs were immobilized to the end-up or side-on orientation in a well-controlled manner. The streptavidin crystal surface can specifically and tightly bind to biotin and prevent other nonspecific interactions, thereby allowing for the imaging of uniform particles in the end-up orientation. The mica surface enabled us to observe the membrane protein-embedded nanodiscs in the side-on orientation. The nanodisc lipid surface in the side-on orientation was vertically fixed on the mica surface, although the hydrophilic head groups of lipids are likely to favor interactions with the hydrophilic mica surface. The biotin label and large cytoplasmic region of the membrane protein protruding at both nanodisc lipid surfaces could prevent interactions between the lipid bilayer and the mica surface reported in previous studies (Koch et al., 2016; Zocher et al., 2012). This combination of HS-AFM and nanodisc technologies enabled us to observe real-time, dynamic structural changes in membrane proteins in the lipid environment from two distinct orientations. This method can be used for the dynamic imaging of other membrane proteins with a large cytoplasmic region, such as ABC transporters and receptor tyrosine kinases (Endres et al., 2014; Thomas and Tampe, 2018). Moreover, drastic conformational transitions in the soluble domains of membrane proteins could be inferred from static snapshots obtained by crystallography and electron microscopy (Gutmann et al., 2018; Ho et al., 2018; Menting et al., 2013; Mi et al., 2017), and real-time movies such as HS-AFM observation provide complementary dynamic information regarding the molecular mechanism underlying membrane protein functions. In addition, this method can be applied to elucidate the dynamics of inherently disordered soluble regions of membrane proteins. Thus, this strategy may have applications in the analysis of important biological processes involving membrane protein dynamics to help understand their molecular mechanism in detail.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and five videos and can be found with this article online at https://doi.org/10.1016/j.str.2018.09.005.

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AUTHOR CONTRIBUTIONS

DEARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES


STAR METHODS

KEY RESOURCES TABLE

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EXPERIMENTAL MODEL AND SUBJECT DETAILS

Each E. coli transformant altered from the E. coli strains BL21 (DE3) (Laboratory stock) and Rosetta 2(DE3) (Novagen, Darmstadt, Germany) was isolated and stored in LB media containing 20% glycerol at –80°C as a glycerol stock.

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to, and will be fulfilled by the Lead Author, Tomoya Tsukazaki (ttsukazaki@mac.com).
METHOD DETAILS

Preparation of SecYEG/SecYAEG-reconstituted Nanodiscs
Mutations in SecY of pAK24 and pTT610 were introduced by site-directed mutagenesis. The resulting plasmids encoded SecYL2V, S148C, R208C, R252G)-His$_6$, SecE, and SecG and SecYL2V, S148C, R208C, R252G)-(GGSG)$_2$-SecA(C648S)$_1$, His$_{10}$, SecE, and SecG, respectively. SecYEG was purified, as described previously (Tanaka et al., 2015). SecYAEG was prepared by Ni-NTA column chromatography, as described previously (Sugano et al., 2017). After decreasing the concentration of NaCl to 50 mM using an Amicon Ultra filter (Ultra-15, MWCO 100 kDa; Merck Millipore, Darmstadt, Germany), a reductant, b-mercaptoethanol (b-ME), was added to the protein solution with 15 mM. To remove the reductant, the reduced protein solution was purified using a HiTrap SP HP column (GE Healthcare, Chicago, IL, USA) (Sugano et al., 2017). Biotin-PEAC5-maleimide (Dojindo, Kumamoto, Japan) dissolved in dimethyl sulfoxide was added to the purified protein at a molar ratio of Biotin-PEAC5-maleimide:SecYEG = 20:1. The solution was incubated at room temperature for 1 h and then at 4°C for 12 h. To remove excess Biotin-PEAC5-maleimide, the SecYEG and SecYAEG solutions were loaded on a Superdex 200 Increase 5/150 GL column and a Superose 6 Increase 10/300 GL column (GE Healthcare), respectively, equilibrated with a buffer (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, 5% glycerol, and 0.1% n-dodecyl-b-D-maltoside [DDM]). The labeled Sec complex was reconstituted into nanodiscs as described previously (Sugano et al., 2017).

Preparation of MgtE-reconstituted Nanodiscs
The mgtE gene from T. thermophilus was cloned into modified pET16b (Daimon et al., 2017) and mutated by site-directed mutagenesis. The resulting plasmid, encoding MG-His$_{10}$-SSGENLYFQGSH-MgtE(A312C), was introduced into E. coli strain Rosetta 2(DE3) (Novagen, Darmstadt, Germany). The transformants were cultivated at 37°C to an A600 of approximately 0.7 in LB medium supplemented with 50 µg/mL ampicillin and 20 µg/mL chloramphenicol. The expression of MgtE was induced with 0.5 mM isopropyl-b-D-1-thiogalactopyranoside at 20°C for 16 h. The cells were harvested by centrifugation at 5000 x g for 10 min, and pellets were resuspended in a buffer (10 mM Tris-HCl [pH 8.0], 1 mM ethylenediaminetetraacetic acid and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]) and disrupted by sonication with a Q500 Sonicator (QSONICA). After centrifugation at 25,000 x g for 30 min, the supernatant was ultracentrifuged (138,000 x g for 60 min at 4°C), and the membrane fraction was collected. The membrane fraction was solubilized in a buffer (50 mM HEPES-NaOH [pH 7.0], 300 mM NaCl, 20 mM imidazole, 1.5% DDM, and 0.1 mM PMSF) after centrifugation (138,000 x g for 30 min at 4°C). The supernatant was mixed with Ni-NTA Agarose (Qiagen, Valencia, CA, USA) equilibrated with buffer A (50 mM HEPES-NaOH [pH 7.0], 300 mM NaCl, and 0.1% DDM) containing 20 mM imidazole for 30 min at 4°C. The resin was washed with buffer A containing 50 mM imidazole and then eluted with buffer A containing 300 mM imidazole. The Histag tag was cleaved by His-tagged TEV protease, and the sample was reloaded onto the Ni-NTA column with 20 mM imidazole to remove the TEV protease. The flowthrough fraction was collected and b-ME was added to a final concentration of 15 mM. The mixture was concentrated using Amicon Ultra filter (Ultra-4, MWCO 50 kDa; Merck Millipore, Darmstadt, Germany), a reductant, b-mercaptoethanol (b-ME), was added to the protein solution with 15 mM. To remove the reductant, the reduced protein solution was purified using a HiTrap SP HP column (GE Healthcare, Chicago, IL, USA) (Sugano et al., 2017). Biotin-PEAC5-maleimide (Dojindo, Kumamoto, Japan) dissolved in dimethyl sulfoxide was added to the purified protein at a molar ratio of Biotin-PEAC5-maleimide:SecYEG = 20:1. The solution was incubated at room temperature for 1 h and then at 4°C for 12 h. To remove excess Biotin-PEAC5-maleimide, the SecYEG and SecYAEG solutions were loaded on a Superdex 200 Increase 5/150 GL column and a Superose 6 Increase 10/300 GL column (GE Healthcare), respectively, equilibrated with a buffer (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, 5% glycerol, and 0.1% n-dodecyl-b-D-maltoside [DDM]). The labeled Sec complex was reconstituted into nanodiscs as described previously (Sugano et al., 2017).

HS-AFM Observation
HS-AFM imaging of SecYEG-NDs, SecYAEG-NDs, and MgtE-NDs was performed using a laboratory-built HS-AFM, as previously described (Ando et al., 2001, 2008; Uchihashi et al., 2012). The HS-AFM was equipped with small cantilevers (BL-AC10DS-A2 or BL-AC7DS-KU4; Olympus, Tokyo, Japan) and operated in the tapping mode. The free oscillation amplitude was approximately 1.5 nm, and the set-point amplitude was 80% of the free amplitude. Imaging rate, scan size, and feedback parameters were optimized during observation to enable visualization with the minimum tip force.

Prior to observations of the SecYEG-NDs, SecYAEG-NDs, and MgtE-NDs on SA 2D crystals, streptavidin molecules were twodimensionally crystallized on the mica surface as previously described (Yamamoto et al., 2009), except SA 2D crystals were formed within 15–30 min of incubation. SecYEG-NDs and SecYAEG-NDs were incubated for 5 min on SA 2D crystals after SecYEG-ND and SecYAEG-ND samples were diluted in buffer A (50 mM HEPES-NaOH [pH 7.0] and 300 mM NaCl). The surface of SA 2D crystals was then washed with buffer A (50 mM HEPES-NaOH [pH 7.0] and 150 mM NaCl, 100 mM KCl) for SecYAEG-NDs. To prepare Mg$^{2+}$-bound MgtE-NDs, purified MgtE-NDs were diluted in buffer C (50 mM HEPES-NaOH [pH 7.0], 300 mM NaCl, 100 mM KCl, and 50 mM MgCl$_2$) incubated for 5 min on SA 2D crystals, and then washed in buffer C (HS-AFM observations were carried out in buffer C). After imaging Mg$^{2+}$-bound MgtE-NDs, the surface was washed in buffer B, not including MgCl$_2$, to observe Mg$^{2+}$-unbound MgtE-NDs. HS-AFM imaging was performed in buffer B.

For observation on mica, SecYAEG-NDs were incubated for 5 min on the mica surface after SecYAEG-ND samples were diluted into 50 mM HEPES-NaOH [pH 7.0] and 100 mM NaCl. The mica surface was then washed in 50 mM HEPES-NaOH [pH 7.0]. HS-AFM
imaging was carried out in 50 mM HEPES-NaOH [pH 7.0]. To address the orientation of SecYAEG-NDs on the mica surface, 100 nM streptavidin in 50 mM HEPES-NaOH [pH 7.0] and 100 mM NaCl was loaded on the mica surface after imaging SecYAEG-NDs. After incubation for 5 min, the surface was washed in 50 mM HEPES-NaOH [pH 7.0] and observed in 50 mM HEPES-NaOH [pH 7.0] using HS-AFM. To prepare Mg\textsuperscript{2+}-bound and Mg\textsuperscript{2+}-unbound MgtE-ND samples on mica, purified MgtE-NDs were diluted in 50 mM HEPES-NaOH [pH 7.0] with or without 50 mM MgCl\textsubscript{2}. Mg\textsuperscript{2+}-bound and Mg\textsuperscript{2+}-unbound MgtE-NDs were incubated for 5 min on the mica surface and then washed in 50 mM HEPES-NaOH [pH 7.0] with or without 50 mM MgCl\textsubscript{2}. HS-AFM imaging was carried out in 50 mM HEPES-NaOH [pH 7.0] with or without 50 mM MgCl\textsubscript{2}. To observe the cytoplasmic domain of MgtE-NDs, Mg\textsuperscript{2+}-unbound MgtE-NDs were loaded on the mica surface coated with Ni\textsuperscript{2+} ion as follows. First, 100 mM NiSO\textsubscript{4} was deposited on the mica surface and incubated for 5 min. The surface was then successively washed in MilliQ water and 50 mM HEPES-NaOH [pH 7.0]. Mg\textsuperscript{2+}-unbound MgtE-NDs were incubated for 5 min on the Ni-coated mica surface and then washed in 50 mM HEPES-NaOH [pH 7.0]. HS-AFM observation was performed in 50 mM HEPES-NaOH [pH 7.0].

Simulation of AFM Images
Simulated AFM images of SecYAEG-NDs and MgtE-NDs were calculated using an AFM probe modeled by a cone shape with a radius of 0.5–1 nm and half cone of 10° as previously described (Kozai et al., 2017; Uchihashi et al., 2011). The source code for Igor Pro 6.36 (Wave Metrics Inc., Lake Oswego, OR, USA) is available on request. The structural models of SecYAEG-ND and MgtE-ND structures were manually constructed by incorporating the SecA-SecYEG crystal structure (PDB: 3DIN) and MgtE crystal structure (PDB: 2YVX) into the nanodisc model (PDB: 2MSC) (Mazhab-Jafari et al., 2015) using Pymol (Schrodinger). The calculated simulation images were smoothed by fast-Fourier transform frequency filtering (cut-off wavelength of 0.77–2.50 nm).

QUANTIFICATION AND STATISTICAL ANALYSIS
The single Gaussian curve or two Gaussian curves fitting was performed using Origin2018 (OriginLab, Northampton, MA, USA) to calculate the average height of molecules. N is the number of counted height data as described in figure legends of Figures 1 and 3.

DATA AND SOFTWARE AVAILABILITY
HS-AFM image acquisition and analysis in this paper was carried out by laboratory-developed software based on Igor Pro 6.36 (Wave Metrics Inc., Lake Oswego, OR, USA). The source codes are available upon reasonable request. Kodec 4.4.7.39 (the latest version of Kodec 4.5.7.22 for cross-sectional analysis), a HS-AFM image viewer, coded in Visual C# (Visual Studio 2010, Microsoft, USA) is available (Ngo et al., 2015).
Supplemental Information

Single-Unit Imaging of Membrane Protein-Embedded Nanodiscs from Two Oriented Sides

by High-Speed Atomic Force Microscopy

Takamitsu Haruyama, Yasunori Sugano, Noriyuki Kodera, Takayuki Uchihashi, Toshio Ando, Yoshiki Tanaka, Hiroki Konno, and Tomoya Tsukazaki
Figure S1. Schematic interpretation of typical membrane proteins embedded in the lipid bilayer, Related to Figure 1.

a. Conventional measurement of a membrane protein on the mica-supported lipid bilayer by AFM. The membrane protein directly interacts with the mica surface; observations from only one side of the membrane can be performed.

b. A model of a membrane protein possessing functional domains on both sides of the membrane, such as certain kinases. Using the method described in a, the cooperative conformational changes on both sides of the membrane could not be observed at the same time.

c. Diagram of protein translocation via a protein-conducting channel. To complete the protein translocation across the membrane, sufficient space is required on both sides of the membrane. Using the system in a, steric hindrance of the translocated protein hampers the translocation of the substrate protein.
Figure S2. Positions of Cys mutations in the SecYEG complex and MgtE, Related to Figure 1 and 3.

a, Crystal structure of *T. thermophilus* SecYEG at 2.7 Å resolution (PDB: 5AWW).
b, Crystal structure of dimeric MgtE in the presence of Mg^{2+} (PDB: 2YVX). The spheres (violet) show the positions of cysteine substitution for biotin labeling.