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研究課題名（和文）Structural Dynamics of ABC Transporter MsbA during Functional Activity

研究課題名（英文）Structural Dynamics of ABC Transporter MsbA during Functional Activity

研究代表者
NGO　XUANKIEN（NGO, XUANKIEN）

金沢大学・ナノ生命科学研究所・特任助教

研究者番号：60778190

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研究成果の概要（和文）：高速原子力顕微鏡を使用した細菌ABCトランスポーターMsbAの構造ダイナミクスに関する最近の正確な分析は、内膜を反転した後のリピドA/リポ多糖が多くのATPaseサイクルの間でもMsbAの膜貫通ドメインコアに残ることを示しています。 結合ATPは、2つのNBDを閉じたコンフォメーションにロックして、IM全体でのリピドA/LPSの反転を容易にする外向きのコンフォメーションを維持します。 リピドA/LPSがMsbA結合ATPのTMDコア内にトラップされた場合、前の仮説で報告されたように、内向きと外向きの間のコンフォメーション変化は観察されません。

研究成果の学術的意義や社会的意義
Our study is important to precisely understand the working mechanism of lipid A transport from the IM to OM by MsbA in bacteria.

研究成果の概要（英文）：Our recent precise analyses on structural dynamics of bacterial ABC transporter MsbA using High-speed Atomic Force Microscopy (HS-AFM) show that lipid A/lipopolysaccharide (LPS) after flipping across inner membrane (IM) remains in the transmembrane domains (TMDs) core of MsbA even during many ATPase cycles. Bound ATP locks two NBDs in closed conformation for maintaining an outward-facing conformation that facilitates the flipping of lipid A/LPS across IM. ATPase activity of MsbA (ATP→ADP.Pi) may always go on during and after flipping of lipid A/LPS across membrane. When lipid A/LPS is trapped inside TMDs core of MsbA bound ATP, we do not observe the conformational transitions between inward-facing and outward-facing as reported in the previous hypotheses. Currently, we continue this study to unravel the correlation between the structural dynamics and transport activity of MsbA.

研究分野：Biophysics

キーワード：ABC transporter　Lipid A　High-speed AFM

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１．研究開始当初の背景(Background at the beginning of the Research)

MsbA, an ATP-binding cassette (ABC) transporter, transports and flips lipid A and lipopolysaccharides (LPS) from the inner leaflet to the outer leaflet of the cytoplasmic (inner) membrane of Gram-negative bacteria. MsbA also transports multiple drugs, conferring the drug resistance. The cryo-EM structures of MsbA obtained under different conditions have suggested that large conformational changes possibly take place in the transmembrane domains (TMDs) coupled to the ATPase reaction in the ATPase sites formed by two nucleotide binding domains (NBDs). However, as in the case of other ABC transporters, dynamic processes during the functional activity have never been elucidated. In this project, the applicant will visualize and analyse function-linked dynamic motion of MsbA molecules reconstituted in lipid membranes containing lipid A or LPS using high speed atomic force microscopy (HS-AFM). Thereby, this study will provide deeper mechanistic insights into the molecular processes in substrate transport by ABC transporters.

[MAIN TEXT]

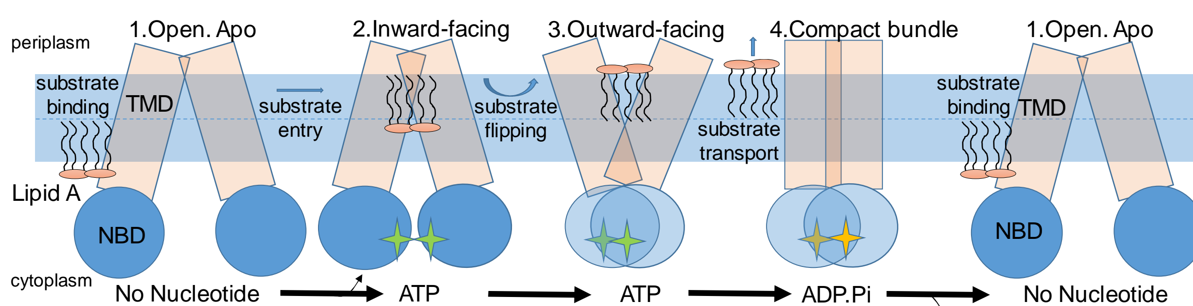

Figure 1: Trap-and-flip model for lipid A and/or LPS transport by ABC transporter MsbA during its ATPase cycle (adapted from Mi et al., Nature 2017).

All ABC transporters (importers/exporters) that operate in all species from bacteria to human share a common structural organization, consisting of two distinct domains (i.e., TMDs and cytosolic NBDs). This structural arrangement appears to be well-suited for their function of substrate transport across the cell membrane. The two ATP-binding cassettes come together to form two nucleotide-binding sites with two nucleotides sandwiched at the interface of the two NBDs. Therefore, the two NBDs must repeat association and dissociation during multiple ATPase cycles. These dynamic events are very likely to be transmitted to the two α-helical bundles connected to the NBDs, so that the TMDs (major parts of the α-helical bundles) undergo different conformations including inward-facing and outward-facing conformations that facilitate substrate entry, migration and release (see **Fig. 1**). Nevertheless, this plausible (hypothetical), general view has never been verified experimentally, despite several structural studies of ABC transporters that suggest this scenario. This situation of slow progress of understanding the molecular process also remains for other proteins. And yet, the use of HS-AFM is gradually changing this situation. This microscopy enables real time, high-resolution imaging of individual biological nano-machines during their functional activity, as demonstrated by recent studies on walking myosin V (Kodera et al., Nature 2010), rotor-less F1-ATPase (Uchihashi et al., Science 2011), actin filaments interacting with cofilin (Ngo et al., e-Life 2015), and a few others.

In this project, the applicant will study MsbA, a bacterial membrane protein belonging to the large family of ABC transporters, using HS-AFM and other methods. MsbA is supplied energy by ATP hydrolysis for transporting lipid A and lipopolysaccharides (LPS). LPS, also known as endotoxins found in Gram-negative bacteria, are the major component of the outer membrane protecting bacteria against harsh environments. LPS are large molecules consisting of lipid A (an actual virulence factor) and a long polysaccharide. LPS synthesized in the cytoplasm constitute the inner membrane and are further transported to the outer membrane via transporters (not well identified). In the inner membrane, LPS in the inner leaflet (cytoplasmic side) are transported to the outer leaflet (periplasmic side) and flipped by MsbA (see **Fig. 1**). The crystal structure of MsbA has been resolved by X-ray crystallography (Chang et al., Science 2001, Ward et al., PNAS 2007). MsbA is a homodimer and its each long arm linked to the NBD is composed of six α-helices. The cryo-EM structure of MsbA bound to LPS (Mi et al., Nature 2017) revealed that the LPS binding pocket is surrounded by the transmembrane helices. It also suggested that the pocket is deformed during the ATPase cycle. However, how the chemical and structural states of the NBDs dynamically affect the TMDs is not straightforwardly revealed in this study. That is, it is not clear whether the substrate binding pocket assumes the inward-facing and outward-facing conformations depending on the different nucleotide states in the NBDs. In addition, although MsbA can transport both lipid A, LPS and multiple drugs, it is currently elusive whether or not a common mechanism is employed for different substrates (Siarheyeva et al., Biochem. J. 2009). Moreover, a recent study reported that an electrochemical gradient across the membrane stimulates the ATPase activity of MsbA and both forms of energy are possibly used for substrate transport (Singh et al., Nat. Commun. 2016). **The key scientific question of this study** is how ATP binding and hydrolysis in NBDs allosterically regulate the conformational transitions in TMDs to drive the substrate transport across the lipid membrane.

２．研究の目的(Purpose of the Research)

1. To clarify how the ATPase activity of MsbA is affected by different substrates and a proton gradient.
2. To reveal the dynamic conformational changes of MsbA during functional activity and to determine its rate as a function of [ATP] and [substrates].
3. To clarify whether or not conformational changes of MsbA can also be induced by an electrochemical proton gradient established between both sides of the lipid membrane, in a way similar to the case of ATPase cycle.
4. To clarify how ATP and a proton gradient show a synergistic effect on the size and rate of conformational changes of MsbA.

３．研究の方法(Research Methods)

The first year: The applicant will focus on the optimization of experiments for the incorporation of MsbA into lipid membranes in the forms of MsbA proteoliposomes (MsbA-PL) and MsbA proteonanodiscs (MsbA-ND). Then, the ATPase activity of MsbA will be measured for MsbA-PL and MsbA-ND in the presence or absence of lipid A, LPS or drugs to clarify how different substrates affect the ATPase activity of MsbA (***Purpose 1***). Using MsbA-PL, the ATPase activity will be measured under different proton gradients and substrates (***Purpose 1***). Dynamic HS-AFM images of MsbA-ND on mica

in the side-on orientation and MsbA embedded in planar lipid bilayers supported by streptavidin (or tamavidin) 2D crystals will be obtained under different nucleotides (e.g. ATP, ATPγS, AMPPNP, ADP, ADP vanadate and nucleotide free) and substrate conditions. The method to prepare the supported lipid bilayers is well established in our lab. The method to put proteonanodiscs on mica in the side-on orientation is also established (Haruyama et al., Structure 2018).

The second year: The *purpose 2* will be focused. HS-AFM imaging of dynamic structures of MsbA embedded in lipid membranes will be continued under different nucleotide and substrate conditions. From the crystal structure of MsbA, the periplasmic side surface next to the TMDs including loops connecting transmembrane helices are protruded only slightly above the membrane surface (1 nm) and the height of NBDs protruded from the membrane is 6 nm. The association and dissociation of the NBDs during ATP hydrolysis are expected to result in a several-nanometer change in their separation, which should be detected precisely in AFM images. We expect that HS-AFM imaging of MsbA at the periplasmic side surface will be able to resolve individual α-helices and/or loops because of its low height from the membrane, as demonstrated in bacteriorhodopsin imaging (Shibata et al., Nat. Nanotechnol. 2010). However, we will take precautions against sample-substrate surface interactions. When the NBDs make contact with mica or 2D crystals of streptavidin (or tamavidin), the dynamic motion of the NBDs (and hence the motion of the TMDs) may be hampered. In this case, we will use membranes suspended above nano-chambers created in the tamavidin 2D crystals directly formed on mica (Noshiro and Ando, manuscript in preparation; see Fig. 2a,b). Note that transported lipid A or LPS may be able to be detected by HS-AFM imaging if the transport and flip of lipid A or LPS occur as a group of several molecules.

The third year: The *Purpose 3 and 4* will be focused. To form electrochemical proton gradients across planar lipid bilayers in which MsbA proteins are embedded, we will use nano-chambers mentioned above. The partial removal of tamavidin molecules from the 2D crystals of tamavidin by its ionic treatment will form numerous nano-chambers (Noshiro and Ando, manuscript in preparation). Lipid bilayers can be formed on this crystal surface, producing small suspended membrane areas that are only slightly indented (0.1−0.4 nm) by the contact with the AFM tip (central darker area, Fig. 2b). A proton gradient is formed between the nano-chambers and the bulk solution across the membrane by changing pH. MsbA-PL containing a biotin lipid will be ruptured on the surface of tamavidin 2D crystals, resulting in MsbA embedded in planar lipid bilayers. Either cytoplasmic or periplasmic side of the embedded MsbA will be accommodated by chance in the nano-chambers. Note that HS-AFM can search the target MsbA proteins embedded in the suspended membrane by measuring their tip load-dependent height. After establishing this method, HS-AFM imaging will be performed to observe dynamic structural changes of MsbA during substrate transport powered by a proton gradient alone or both forms of energy.

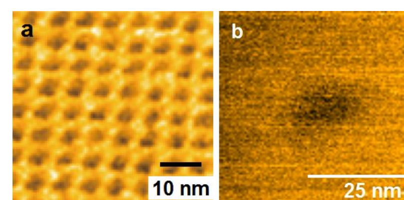

Figure 2: AFM images of tamavidin 2D crystal (a) and lipid bilayer suspended above a nano-chamber created in the 2D crystal (b).

４．研究成果(Research results)

Our recent precise analyses on structural dynamics of bacterial ABC transporter MsbA using high-speed atomic force microscopy (HS-AFM) show that lipid A/LPS after flipping across inner membrane (IM) remains in the transmembrane domains (TMDs) core of MsbA even in the presence of many ATPase cycles. Bound ATP locks two NBDs in closed conformation for maintaining an outward-facing conformation that facilitates the flipping of lipid A/LPS across IM. ATPase activity of MsbA (ATP→ADP.Pi) may always go on during and after flipping of lipid A/LPS across membrane. When lipid

A/LPS is trapped inside TMDs core of MsbA bound ATP, we do not observe the conformational transitions between inward-facing and outward-facing as reported in the previous hypotheses. Currently, we continue this study to unravel the correlation between the structural dynamics and transport activity of MsbA.

5．主な発表論文等

〔雑誌論文〕　計7件（うち査読付論文　7件／うち国際共著　7件／うちオープンアクセス　7件）

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3．雑誌名	**6．最初と最後の頁**
BiorXiv	0
掲載論文のDOI（デジタルオブジェクト識別子）	**査読の有無**
10.1101/2020.04.14.041046	有
オープンアクセス	**国際共著**
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Journal of Cell Biology	1, 19
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10.1083/jcb.202007033	有
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オープンアクセスとしている（また、その予定である）	該当する

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3．雑誌名	**6．最初と最後の頁**
Phytopathology	0
掲載論文のDOI（デジタルオブジェクト識別子）	**査読の有無**
10.1094/phyto-09-21-0397-r	有
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3．雑誌名 Proceedings of the National Academy of Sciences	6．最初と最後の頁 0
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3．雑誌名 Bioprotocol	6．最初と最後の頁 0
掲載論文のDOI（デジタルオブジェクト識別子） なし	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計4件（うち招待講演　2件／うち国際学会　1件）

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2．発表標題 Structural dynamics of bacterial ABC transporter MsbA during functional activity.
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The 57th Annual Meeting of the Biophysical Society of Japan, Miyazaki, Japan
４．発表年
2019年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

－

６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計1件

国際研究集会	開催年
Biophysical Society 64th Annual Meeting	2020年～2020年

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関