ウェブサイト公開用

（様式 10）
（海外特別研究員事業）
令和　2年　3月31日

海外特別研究員最終報告書

独立行政法人日本学術振興会 理事長　殿

採用年度　平成30年度
受付番号　201860579
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海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：　マールブルグ　（国名：　ドイツ　）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
比較ゲノミクスから予想されたバクテリアの新規ヒドロゲナーゼの探索と機能解明

3. 派遣期間：平成30年　8月　1日～令和　2年　3月31日(609日間)

4. 受入機関名及び部局名
Max-Planck-Institute for Terrestrial Microbiology, Microbial Protein Structure

5. 所期の目的の遂行状況及び成果…書式任意　書式任意（A4 判相当 3 ページ以上、英語で記入も可）
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6.研究発表」以降については様式10－別紙1～4に記入の上、併せて提出すること。

Summary of the part 1:
Hydrogen gas is a green energy carrier. Microorganisms use dedicated enzymes, called hydrogenase to convert this gas into energy. One of the model enzyme is [Fe]-hydrogenase that catalyzes the reversible hydride transfer between hydrogen gas and the methanogenic C1-carrier tetramethanohydropterin using the unique prosthetic group, the FeGP cofactor. Up to now, [Fe]-hydrogenase, its paralogue (HmdII) and the FeGP cofactor were identified only in the domain Archaea. In this part, I report the discovery that bacteria are also able to produce HmdII and the FeGP cofactor. Notably the bacterial HmdII utilized the bacterial C1-carrier tetrahydrofolate for the hydride transfer. This finding has a great potential for biotechnological development of the [Fe]-hydrogenase variants that function in bacteria.

Aim of the part 1:
The project aimed to discover a novel hydrogenase from a bacterium in the genus *Desulfurobacterium*. All genomes from this genus contain putative genes for the paralogous protein of [Fe]-hydrogenase (HmdII) and for HcgA-G proteins involved in the biosynthesis of iron-guanylylpyridinol (FeGP) cofactor, which is the prosthetic group of [Fe]-hydrogenase. Phylogenomic analysis revealed that the emergence of Hcg proteins in the genus *Desulfurobacterium* was much earlier than that of HmdII. Therefore I hypothesized that *Desulfuronbacterium* has an unknown enzyme, which hosts the FeGP cofactor for the activation of hydrogen gas.

Result of the part 1:
First of all, I investigated the production of the FeGP cofactor in *Desulfurobacterium thermolithotrophum*, as the FeGP cofactor has only been found in methanogenic archaea. To detect the protein-bound bacterial FeGP cofactor and estimate its amount, the cell extract

of *D. thermolithotrophum* was subjected to the standard procedure for cofactor extraction with methanol and 2-mercaptoethanol combined with a subsequent [Fe]-hydrogenase-reconstitution assay. Approximately 14 mU/mg Hmd activity was reconstituted from cell extract of *D. thermolithotrophum* indicating that the amount of the FeGP cofactor in the bacterium was approximately as high as in mesophilic methanogenic archaea. The cell-extract analyses clearly demonstrated the presence of the bacterial FeGP cofactor in a protein-bound fashion. To obtain the chemical composition of the bacterial FeGP cofactor, mass spectrometric analysis was performed. Despite of repeated trials, the mass peaks originated from the FeGP cofactor were not detected in the cofactor extract, probably due to the presence of unknown compounds, which interfere with the mass spectrometric analysis. Therefore I developed the new method for the purification of the FeGP cofactor. Using the sample purified by this method, the mass originated from the FeGP cofactor was obtained. The exact mass originated from the bacterial cofactor was identical to that from archaeal cofactor. The result suggested that *D. thermolithotrophum* produces the FeGP cofactor, whose structure is same as the FeGP cofactor generated by methanogenic archaea. This fact motivated us to discover an unknown enzyme hosting the cofactor in the native cells. The most possible candidate is HmdII. His_6-tagged-HmdII from *D. thermolithotrophum* was overproduced in *Escherichia coli* and its ability to bind the FeGP cofactor was assessed by the ultrafiltration experiment and infrared spectroscopy. Both results indicated that the bacterial HmdII binds the FeGP cofactor. Heterologously produced bacterial HmdII reconstituted with the archaeal FeGP cofactor catalyzed the hydride transfer reactions using the tetrahydromethanopterin (C_1-carrier of methanogens) and and tetrahydrofolate (C_1-carrier of methanogens) derivatives, albeit with low enzymatic activities. Crystal structure revealed the same binding mode of the FeGP cofactor in the bacterial and archaeal HmdII, and provided the molecular basis for the dual substrate specificity of the bacterial HmdII. These discoveries open new possibilities for conversion of hydrogen, because it was the first demonstration of hydride transfer from hydrogen gas to tetrahydrofolate. These data were published as the original paper in the Angewandte Chemie International Edition.

On the other hand, I also tried to discover the second enzyme hosting the cofactor, as planed in my proposal (cell extract-fractionation experiments). But this was hampered by the difficulty of the cultivation of the genus *Desulfurobacterium*. I decided to use other organisms to look for new hydrogenase. The targeted organism was *Methanospirillum hungatei*. Genome analysis of this organism revealed the absence of essential [NiFe]-hydrogenase (MvhAGD) in its genome. MvhAGD forms an enzyme complex together with heterodisulfide reductase (Hdr) in the cytochrome-less methanogenic archaea. In their methanogenic pathway, heterodisulfide reductase HdrABC catalyzes reduction of ferredoxin and heterodisulfide of coenzyme M and coenzyme B (CoM-S-S-CoB) using the flavin-based electron bifurcation mechanism. Thus, *Methanospirillum* could have considerably different Hdr system involving the new type of hydrogenase, but it was not true. I could not find the new hydrogenase, although obtained highly interesting discovery unexpectedly (continued to part 2).

Summary of the part 2:
Efficient utilization of energy is highly important issue for future biotechnology. When you have a look at environmental microbes, there might be highly sophisticated enzyme systems to better extract energy and utilize energy with high efficiency. In the part 2, I report the discovery of the new electron bifurcation system highly optimized in energy-poor environments. Heterodisulfide reductase (Hdr) in methanogenic archaea is essentially important for the energy conservation of methanogens. The Hdr reaction serves as the final step of methanogenesis by coupling the exergonic reduction of heterodisulfide of coenzyme M and coenzyme B (CoM-S-S-CoB) and the endergonic reduction of ferredoxin via flavin-based electron-bifurcation. Bifurcation takes two intermediate electrons and creates one high- and one low-energy electron. High-energy electrons are needed to perform difficult chemical reactions, such as the reduction of ferredoxin. In methanogenic archaea without cytochrome, Hdr forms a complex with either [NiFe]-hydrogenase (Mvh) or formate dehydrogenase (Fdh). Each redox enzyme extracts electrons from hydrogen gas or formate, respectively, and donates electrons to the Hdr module, which bifurcates electrons to CoM-S-S-CoB and ferredoxin. The

reduced ferredoxin is the reducing power for the initial reaction of methanogenesis: reduction of CO2 to form formyl-methanofuran, which is catalyzed by formyl-methanofuran dehydrogenase (Fmd). Here, I report purification and characterization of the stable super-complex of Fdh-Hdr-Fmd from *Methanospirillum hungatei*, which lacks Mvh-encoding gene but grows on H2 and CO2. The super-complex catalyzed the individual enzyme reactions and the concerted electron-bifurcation reactions from formate to reduce CoM-S-S-CoB and CO2 to form formyl-MFR in the absence of free ferredoxin. In addition, this super-complex catalyzed the electron-bifurcating reductions using the reduced form of F_{420} ($F_{420}H_2$) as an electron donor. The structure of the super-complex revealed by the cryogenic electron microscopy discovered that the polyferredoxin serves as a nanowire between the Hdr and Fmd modules. Direct transfer of electrons via the polyferredoxin appears to be the key for the efficient electron transfer contributing on the growth of *M. hungateii* under low H_2 pressure, as this organism habitats in the hydrogen-poor environments such as syntrophic association.

Aim of the part 2:
The aim of this part is the characterization of Hdr from *M. hungateiI,* whose genome lacks the essential [NiFe]-hydrogenase in hydrogenotrophic methanoges (Mvh).

Results of the part 2:
I established the system for the large-scale cultivation of *M. hungatei*. Enzymes in the cytosolic fraction were fractionated by anion-exchange chromatography, hydrophobic-interaction chromatography and size-exclusion chromatography. During the fractionation, the enzymatic activities were always determined. The chromatography experiments indicated that Hdr forms a complex with formate dehydrogenase (Fdh) and folmylmethanofuran dehydrogenase (Fmd) rather than hydrogenase. Fmd catalyzes the first reaction of methanogenesis: reduction of carbon dioxide to formyl group with methanofuran and reduced ferredoxin, which is mainly generated by the Hdr reaction. The individual enzyme reaction of the Fdh-Hdr-Fmd complex was demonstrated. It was also found that the Fmd activity was detectable only in the presence of very high concentration of salt (i.e., 1.6 M potassium phosphate). Electron bifurcation reaction was demonstrated by using either methanofurane or ferredoxin as the terminal electron acceptor. For both reactions, electron donor was formate. Interestingly, formate could be replaced with $F_{420}H_2$ for electron bifurcation. These results indicated that Fdh is the electron-providing module; Hdr is the electron-bifurcating module and also electron-output module; Fmd is the electron-output module. The structure of the Fdh-Hdr-Fmd complex was determined with the cryogenic electron microscopy by collaborating with the Max-Planck-Institute for Biophysics in Frankfurt. Cryo-EM revealed that the Fdh-Hdr-Fmd complex forms hexamer resulting in approximately 3 mega Da in size. Based on the arrangement of the FeS clusters and active sites, the electron transfer pathway was modeled. Together with the biochemistry data, the catalytic mechanism of the Fdh-Hdr-Fmd complex is proposed as follows. In the super-complex, formate is oxidized at the molybdopterin active site of FdhA to CO_2 and a proton, and two electrons are transferred through the two [4Fe-4S]-clusters to FdhB. In the FdhB subunit, the electron transfer pathway from FdhA is split into two branches. One branch connects to FAD in FdhB for reduction of F_{420}. The presence of this electron transfer branch was confirmed by F_{420}-reducing Fdh activity using formate as the electron donor. Another electron-transfer branch in FdhB connects to a [4Fe-4S]-cluster in the HdrA subunit, from which the electrons are transferred to electron-bifurcating FAD in HdrA, where electrons are bifurcated to the [4Fe-4S]-clusters in HdrC and FmdF. The electrons from HdrC finally reach to two noncubane [4Fe-4S]-clusters on HdrB, where CoM-S-S-CoB is reduced one-by-one with two high redox potential electrons to form CoM-SH and CoB-SH. The low redox-potential electrons are transferred to FmdBD, which contains molybdopterin active-site, via [4Fe-4S]-clusters of polyferredoxin FmdF and FmdG. At the molybdopterin active-site, CO_2 is reduced to formate, which is transferred through the protein channel to react with methanofran to form formyl-methanofuran. Notably, $F_{420}H_2$ is also used as an electron donor for the electron bifurcating CO_2 reduction and formation of formyl-methanofuran. The electrons from $F_{420}H_2$ are transferred to FAD in FdhB and the electrons are transferred through the two [4Fe-4S]-active site in FdhB to HdrA and then the elections are used for electron bifurcation reaction as described above. $F_{420}H_2$ is not used

as the electron donor of formate formation at the Fdh catalytic site because the redox potential of CO_2/formate ($E^{\circ\prime}$ = -420 mV) is much lower than that of $F_{420}/F_{420}H_2$ ($E^{\circ\prime}$ = -360 mV). In *M. hungatei*, Hdr always made a complex with Fdh. Hdr was never complexed with with any hydrogenase modules. However there is still a big question: why Hdr forms complex with Fdh under hydrogenotrophic conditions (electron donor is always hydrogen gas)? I guess $F_{420}H_2$ is the phisiological electron donor of the Fdh-Hdr-Fmd complex, because it is constantly provided from the F_{420}-dependent [NiFe]-hydrogenase reaction. If formate is present in the environments, the Fdh-Hdr-Fmd super-complex can use formate as the electron donor for the electron-bifurcation reaction. However the formate concentration in environments should be kept at very low level because formate is highly attractive electron donor for many microbes. The ability of the Fdh-Hdr-Fmd super-complex to utilize the both formate and $F_{420}H_2$ provides the great physiological advantage under fluctuating environments. A part of electrons from formate might be used for reduction of F_{420} when the cellular $F_{420}/F_{420}H_2$ ratio is high. In the Fdh-Hdr-Fmd super-complex, electrons provided from protonated FAD semiquinon are directly transferred to FmdABCD without free ferredoxin. Cryo-EM revealed that the polyferredoxin FmdF is the electron connector between Hdr and FmdABCD. To be honest this is the first evidence of the function of polyferredoxin as the electron nanowire. Compared with the electron transfer mediated by the free ferredoxin, direct transfer of electrons via polyferredoxin enables to prevent the leakage of electrons. Costa et al. reported weak association of Fwd (tungsten-containing isoform of Fmd) with the Fdh-Hdr and Mvh-Hdr complexes in *Methanococcus maripaludis* by pulldown assay using the strains containing HdrB or FdhA, each has His-tag (2010, PNAS). Due to the weak association, the Fwd part was dissociated from the Fdh-Hdr-Fwd and Mvh-Hdr-Fwd complexes by gel-permeation chromatography. This previous observation and the novel data here lead us to propose that polyferredoxin-linked electron transfer between Hdr and Fmd is commonly performed in hydrotrophic methanogens without cytochrome. There is a notable difference between the Hdr-containing enzyme complexes from *M. maripaludis* and *M. hungatei*. In *M. hungatei*, the genes encoding ferredoxin species FmdF and FmdG locate within the gene cluster including HdrABC despite the FmdABCD-encoding genes are located at a different region of the genome. Cryo-EM structure indicated that the polyferredoxin FwdF subunit associated with the ferredoxin-like FwdG subunit and these iron-sulfur cluster-containing subunits transfer electrons to the tungstopterin cofactor in the FwdB subunit. The presence of the FmdF and FmdG-encoding genes in the Hdr gene cluster is in accordance with the finding that FmdF tightly associates with the Fdh-Hdr sub-complex in both Fdh-Hdr-Fmd super complex and Fdh-Hdr sub-complex from *M. hungatei*. Interestingly, the genome of other methanogenic archaea in the order Methanomicrobiales, HdrABC are encoded in a gene cluster containing the all genes encoding formylmethanofuran dehydrogenases. This system might be strengthened along with the evolution of methanogenic archaea. In the hydrogenotrophic methanogenesis, a part of the metabolic C1-intermediate (methyl-tetrahydromethanopterin) is consumed for the biosynthesis of acetyl-coenzyme A; therefore, the amount of CoM-S-S-CoB produced in the methanogenic pathway is less than the amount required for electron bifurcation reaction to produce low-redox electrons for the first CO_2 reduction reaction. To drive continuously the metabolism, external reduced ferredoxin is necessary to be produced. For this purpose, membrane bound Ech type hydrogenase will reduce ferredoxin and then the reduced ferredoxin will be used for the Fmd reaction to balance the low-redox potential electrons. The experiments indicated that the Fdh-Hdr-Fmd super-complex catalyzes the electron bifurcating reduction of the external ferredoxin. This finding indicated that electron transfer between the Fdh-Hdr-Fmd super-complex and external ferredoxin is possible. Based on the cryo-EM structure, the possible reaction site of the external ferredoxin could be proposed. In the physiological condition, the free reduced ferredoxin can be used for reduction of CO_2 to supplement electrons for the methanogenic pathway.

These data are under preparation for the publication (confidential).