論 文 の 内 容 の 要 旨

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論文題目

Studies on function and structure of ferredoxin-dependent oxidoreductases from hyperthermophilic archaea

（超好熱性古細菌由来フェレドキシン依存性酸化還元酵素の機能と構造に関する研究）

Introduction

Hyperthermophilic organisms are related to primitive form of life, and their metabolism shows unique aspects such as use of ferredoxin (Fd) as an electron carrier. Their proteins are highly stable but often oxygen-sensitive when iron-sulfur cluster is included. *Sulfolobus tokodaii* is an aerobic hyperthermophile and contains a large amount of unique Zn-binding Fd. But the metabolic role of Fd is still unclear. This study deals mainly with two Fd-dependent enzymes from *S. tokodaii*, 2-oxoacid:Fd oxidoreductase (OFOR) and Fd:$NADP^+$ oxidoreductase (FNR).

OFOR catalyzes CoA and Fd dependent oxidative decarboxylation of 2-oxoacid, such as pyruvate and ketoglutarate. The thiamine pyrophosphate (TPP) and iron-sulfur cluster(s) are intrinsic cofactors. Homodimeric pyruvate:ferredoxin oxidoreductase from *Desulfovibrio africanus* (DaPFOR) is the only OFOR whose crystal structures have been determined. The 135-kDa subunit is composed of seven domains; TPP binds between domains I and VI, one [4Fe-4S] cluster (“proximal”) binds to domain VI, and two [4Fe-4S] clusters (“median” and “distal”) bind to domain V (called intramolecular Fd). Structure of other OFOR with different subunit composition has never been reported.

In addition to the classical oxidative decarboxylation reaction, OFORs can also catalyze non-oxidative decarboxylation of pyruvate to acetaldehyde, which was reported to require CoA for aldehyde production.

Reaction mechanism of OFOR1 from *Sulfolobus tokodaii* (StOFOR1)

StOFOR1 is of $(ab)_2$ type, composed of two 70 kDa a-subunits (corresponding to a fusion of domains III-I-II of DaPFOR) and two 34 kDa b-subunits (corresponding to domain VI of DaPFOR) (Fig.1). The intramolecular Fd is lacking in StOFOR1, that is, StOFOR1 contains only one [4Fe-4S] cluster/heterodimer, and is the simplest member in OFOR family. Therefore, this archaeal enzyme is a good model for studying the redox mechanism of OFOR. StOFOR1 shows broad 2-oxoacid specificity not only for pyruvate but ketoglutarate, and so on, playing important roles in the central metabolism of glycolysis (or gluconeogenesis) and the TCA cycle. The [4Fe-4S] in StOFOR1 is ligated by four Cys residues, C12, C15, C46, and C197 in b-subunit, and the enzyme has no other Cys.

To elucidate the role of these Cys residues in holding of the iron-sulfur cluster, these Cys residues were substituted with Ala to yield C12A, C15A, C46A, C197A and C12/15A mutants. All the mutants showed loss of the iron sulfur cluster, except the C197A, which retained some unidentified type of iron-sulfur cluster. On addition of pyruvate to StOFOR1, the wild type enzyme exhibited a chromophore at 320 nm and a stable large EPR signal corresponding to a hydroxyethyl-TPP radical, while the mutant enzymes did not show formation of any radical intermediate or production of acetyl-CoA, suggesting the intact [4Fe-4S] cluster is necessary for these processes. The stable radical intermediate in wild type OFOR was rapidly decomposed upon addition of CoA in the absence of an electron acceptor. Non-oxidative decarboxylation of pyruvate, yielding acetaldehyde, has been reported to require CoA for an $(abcd)_2$-type OFOR from *Pyrococcus furiosus*, but StOFOR1 catalyzed CoA-independent acetaldehyde production from pyruvate, regardless of whether the iron-sulfur cluster is intact [4Fe-4S] type or not. A comprehensive reaction scheme for StOFOR1 with a single [4Fe-4S] cluster was proposed.

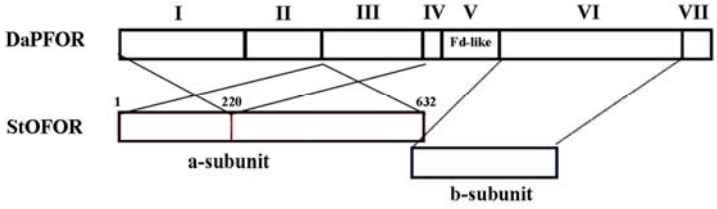

Fig.1 Corresponding domains of StOFOR and DaPFOR in primary structure.

Crystal structure of OFOR2 from *S. tokodaii* (StOFOR2)

The crystal structure of $(ab)_2$ type OFOR was determined for StOFOR2, which shows high amino acid identity with StOFOR1. The crystal structure of StOFOR2 was solved by the Se-SAD method and substrate free and pyruvate complex structure were determined at 2.1 and 2.2 Å resolution, respectively. The overall structure of StOFOR2 consists of two protomers, each of which comprises one a- and b-subunit. Residues 1-220 of both a-subunits (corresponding to domain III of DaPFOR) are stretched at both ends seemingly to keep balance

for the enzyme as two wings (wing-like domain). Somehow expectedly, TPP is located at the interface between residues 221-632 of a-subunit (corresponding to domain I-II of DaPFOR) and b-subunit (corresponding to domain VI of DaPFOR) derived from different protomers (Fig.1 and 2A). StOFOR2 exhibits a high degree of structural similarity around the TPP-binding site with other TPP-dependent enzymes. Conserved amino acids in all known crystal structures including the Gly-Asp-Gly motif and a Glu are also present in StOFOR2. The Gly-Asp-Gly motif (Residues 89-91 of b-subunit) is involved in interactions with Mg^{2+}, which are found around the pyrophosphate of TPP. The conserved Glu294 in a-subunit is hydrogen-bonded to the N1' atom of 4-aminopyrimidine group of TPP, which plays an important role in promoting C2-H ionization of thiazolum ring and activates TPP into ylide form. In contrast to the proximal [4Fe-4S] cluster of DaPFOR, the sole [4Fe-4S] cluster of StOFOR2 ligated by the four Cys residues, is located nearby the surface of b-subunit (Fig.2B). Based on the binding site geometry of TPP, the overall structure could be divided into two catalytic units, which are a_1-b_2 unit and a_2-b_1 unit. Interestingly, the catalytic unit of StOFOR2 is pretty similar with DaPFOR except for the lack of the intramolecular Fd-like domain V. Hence, the structure of StOFOR2 in complex with Fd from *S. tokodaii* (StFd) was easily modeled according to the domain V of DaPFOR (Fig.2A). Fd fitted well in a pocket of StOFOR2 to form a ternary complex of subunit-a, subunit-b and Fd just like the two molecules of StFd are protected by the wing-like domains of StOFOR2. The two [4Fe-4S] clusters of StFd, which are superimposed with median and distal clusters of DaPFOR, are geometrically adjacent to the sole [4Fe-4S] cluster of StOFOR2. The unique N-terminal domain holding a Zn^{2+} of StFd is located outside of the StOFOR2.

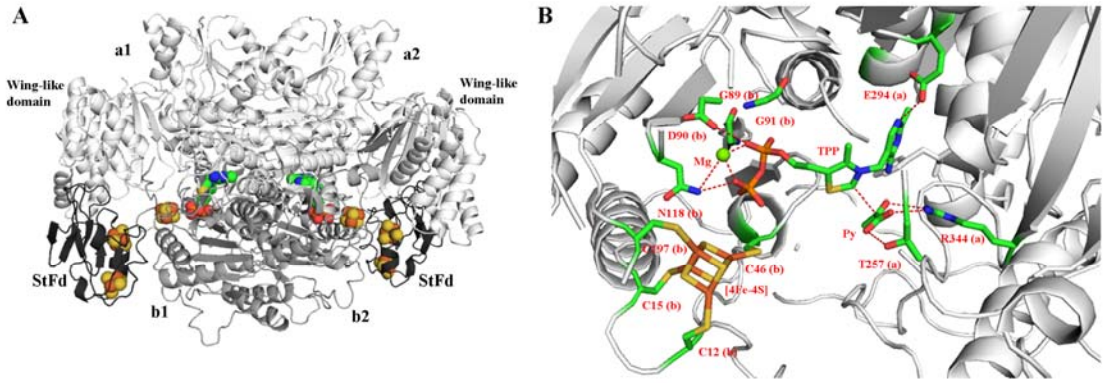

Fig.2 (A) Possible association model of StOFOR2 and StFd. The crystal structures of StOFOR2 (this study) and StFd (Fujii et al,1997) were manually docked. (B) Active site of StOFOR2

The structure of StOFOR2 in complex with pyruvate was obtained by soaking with pyruvate (Fig.2B). Similarly with DaPFOR, the substrate is bound in a buried pocket neighboring the thiazolum ring of TPP. Moreover, Arg344 and Thr257 residues in a-subunit interact with the carboxylate of pyruvate. In the direction of methyl group of pyruvate, four hydrophilic amino

acids (Thr349, Asp468, and Ser42 in a-subunit, and Lys49 in b-subunit) are present and probably recognize the C_γ-carboxylate of ketoglutarate. In the case of DaPFOR, the corresponding residues are hydrophobic amino acids (Leu121, Ala219, Met1202 and Ile843). A previous study demonstrated that replacement of Lys49 in b-subunit by Ile resulted in loss of the activity to ketoglutarate but activity to pyruvate remained.

Ferredoxin:NADP$^+$ oxidoreductase from *S. tokodaii* (StFNR)

Ferredoxin:NADP$^+$ oxidoreductases (FNRs) are ubiquitous FAD-containing enzymes that catalyze the electron transfer of reducing equivalents between Fd and NADP$^+$. FNRs are generally classified into two types: the classical plant-type family and the glutathione reductase (GR) type family. Recently, a novel type of FNR was reported, that is thioredoxin reductase like FNRs (TRLFs), which physiologically function as FNRs, but exhibit a structural homology to NADPH-dependent thioredoxin reductases. We are interested in whether any of the three types of FNRs exist in *S. tokodaii* and function in the recycling of reduced Fd produced by StOFOR. A gene *st2133* belonging to TRLF was proposed to encode FNR. Homologs of ST2133 were reported as NADH oxidase and thioredoxin reductase. A sole example of FNR from archaea is a heterodimeric enzyme from an anaerobe with distant homology to ST2133.

To elucidate the function and structure of ST2133, the gene was expressed in *Escherichia coli*, and the purified product was characterized. The oligomeric state, species and content of enzyme-bound flavin in the recombinant ST2133 were analyzed. A comprehensive biochemical analysis of the enzyme activities using the combination of NAD(P)H as an electron donor, and oxygen, hydrogen peroxide, 2,6-dichlorophenolindophenol, ferricyanide, cytochrome *c*, dithiobisnitrobenzoate, or ferredoxin as an electron acceptor, were carried out. Electron transfer from reduced StFd to NADP$^+$, and vice versa, was confirmed. The possible role of ST2133 as a member of the redox cycle for ferredoxin is discussed (Fig.3).

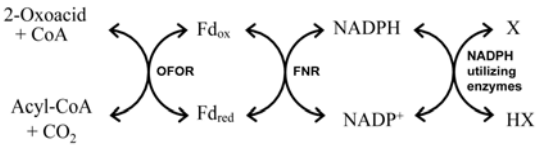

Fig.3 Putative physiological status of ST2133

Publications

1) Yan Z, Nam Y, Fushinobu S, Wakagi T (2013). *Sulfolobus tokodaii* ST2133 is characterized as a thioredoxin reductase-like ferredoxin:NADP+ oxidoreductase. *Extremophiles*. In press.

2) Yan Z, Fushinobu S, Wakagi T. Four Cys residues in heterodimeric 2-oxoacid:ferredoxin oxidoreductase are required for CoA-dependent oxidative decarboxylation but not for non-oxidative decarboxylation. Submitted.