Biochemical Characterization of a Group-5 Soluble Diiron Monooxygenase Hydroxylase and Related Chaperonin-like Component

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ABSTRACT: Soluble diiron monooxygenases are observed in a wide range of microorganisms and have garnered significant interest as oxidation biocatalysts owing to their ability to catalyze the oxygenation of a variety of aliphatic/aromatic hydrocarbons. They can be categorized into six groups based on the types of substrate, sequences, and component arrangement on the gene cluster. Earlier, the expression of several groups of such soluble diiron monooxygenases in representative heterologous hosts, such as *Escherichia coli*, was considered to be difficult. However, the functional expression of group-5 hydroxylase component (MimA and MimC) in *Escherichia coli* along with its related chaperonin-like component (MimG) was reported recently. Here, we describe their purification via a heterologous expression system and the biochemical characterization of MimAC, the complex of MimA and MimC and MimG to obtain insights into their structure and exact roles. MimAC and MimG were fused with His-tags and purified using affinity chromatography in a homogenous state on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Blue native polyacrylamide gel electrophoresis demonstrated that the quaternary structure of MimG was almost identical to that of GroEL expressed in *E. coli*, indicating that its function was also similar to GroEL. Size-exclusion chromatography and inductively coupled plasma analysis demonstrated that MimAC was assembled in $(\alpha\beta)_2$ configuration and exhibited two iron atoms and at least one zinc atom per $\alpha\beta$ complex. This result indicated that MimAC possessed a dinuclear iron center, similar to other soluble diiron monooxygenase hydroxylases.

INTRODUCTION

Gaseous alkanes are abundantly found in natural gas, which is an ideal and low-cost alternative carbon source, making it an appealing feedstock for value-added chemicals and fuels.^{1,2} Due to the stability of the C-H bonds in short-chain alkanes such as methane, ethane, and propane, upgrading these compounds is one of the most significant challenges in catalysis.^{3–5} In nature, some bacteria can convert such alkanes to alcohols under ambient temperature using hydrocarbon monooxygenases. This monooxygenase family includes a variety of enzymes such as cytochrome P450, flavin-dependent monooxygenase, AlkBrelated alkane hydroxylases, soluble diiron monooxygenases (SDIMO), and particulate methane monooxygenases (pMMO) observed in organisms ranging from microorganisms to plants and animals.5-7 Among these, SDIMOs are a diverse group of non-heme diiron enzymes that activate dioxygen and have garnered significant interest owing to high sequence diversity, broad substrate range, and various applications.⁵ Notably, soluble methane monooxygenases (sMMO) and butane monooxygenases (BMO) are of primary interest owing to their ability of oxidizing short-chain alkanes.8 For this reason, sMMOs and BMOs are the most well-characterized members of this monooxygenase family with respect to their biochemistry and structure.^{9–12} On the other hands, the biochemical properties of propane monooxygenases (PMO) are not less well understood, although they can also catalyze the oxidation of light alkanes.13,14

Recently, SDIMOs were categorized into six groups of enzymes based on their structure and substrate specificity rooted in phylogenetic relationships.^{15–18} sMMOs and BMOs is belonging to group-3 of SDIMO family and contain a dimeric hydroxylase protein with three subunits in $(\alpha\beta\gamma)_2$ stoichiometry along with the reductase and regulatory proteins.¹⁹ The hydroxylase component exhibits a $(\alpha\beta\gamma)_2$ heterohexameric structure in phenol hydroxylate (group-1)²⁰ and toluene 4-monooxygenase (group-2).²¹ The alkene monooxygenase

(group-4) hydroxylase differs in owing to the presence of only the α - and β -subunits, which are arranged as $\alpha\beta$ monomers.²² There is no experimental evidence demonstrating the structures of the hydroxylase component of PMOs (group-5), although they are presumed to possess ($\alpha\beta$)₂ dimeric structure based on the analogy with the hydroxylase component of groups 1-3.¹⁶

To facilitate the understanding of SDIMO enzymology and improve their efficacy in various applications, heterologous expression systems of enzymes belonging to group-1 and -2 in hosts such as *E. coli* have been constructed.²³ It is worth noting that the coexpression of the GroEL-like protein, namely, MimG from Mycolicibacterium smegmatis, enabled the functional expression of a group-5 SDIMOs (MimABCD) from *Mycolicibacterium goodii* in *E. coli*,²⁴ despite the resistance of group-3 and -6 SDIMOs to this approach and production of insoluble and/or inactive proteins in E. coli.25,26 In the mimABCD gene cluster, mimA and mimC encode the hydroxylase component, and mimB and mimD encode the reductase and the regulatory proteins, respectively.²⁷ In addition, the gene encoding the chaperonin-like protein, mimG is present on the gene cluster.²⁸ MimG is homologous to GroEL that assists newly synthesized polypeptides is folded correctly and prevents aggregation and precipitation of the folded proteins. E. coli transformed with the plasmid containing mimA and mimC does not demonstrate any catalytic activity without the co-expression of the mimG gene.²⁴ Such a GroEL-like protein-encoding genes, mmoG and bmoG were also observed in the gene cluster involving genes encoding MMOs and BMOs (group-3), respectivly and are involved in the transcriptional activation of SDIMO genes in the native host.²⁹⁻³¹ Accordingly, MimG and MmoG have been postulated to function as molecular chaperones. Attempts to purify the GroEL-like protein have not been successful³⁰; however, purification of these GroEL-like proteins is essential for the determination of their exact role. Thus, this study aimed to isolate and biochemically characterize the GroEL-like protein and the hydroxylase component of group5 SDIMO in order to obtain significant insight into the function of the SDIMO-associated GroEL-like protein.

MATERIALS AND METHODS

Reagents

The plasmids pCDFDuet1, pRSFDuet1 were obtained from Novagen Inc. (Madison, WI, USA). The plasmid pGro7 was obtained from Takara Bio Inc. (Shiga, Japan). Iron(III) citrate and COSMOGEL His-Accept was obtained from Nacalai Tesque. (Kyoto, Japan). Iron and zinc standard solutions were obtained from Fujifilm Wako Chemicals Co. (Osaka, Japan). Ammonium iron(III) citrate was obtained from Kanto Chemicals Co. (Tokyo, Japan). All other chemicals used were of reagent grade.

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>pCDFI	mimGop																	
GCCAT	ACCGC	GAAAG	GTTTT	GCGCC	ATTCG	ATGGT	GTCCG	GGATC	TCGAC	GCTCT	CCCTT	ATGCG	ACTCC	TGCAT	TAGGA	AATTA	ATACG	ACTCA
CTATA	GGGGA	ATTGT	GAGCG	GATAA	CAATT	CCCCT	GTAGA	AATAA	TTTTG	TTTAA	CTTTA	ATAAG	GAGAT	ATACC	ATGGC	GAAGG	AACTG	CGTTT
CAACA	GCGAT	GCGCG	TGCGC	GTCTG	GAGCA	GGGTG	TGAAC	GCGCT	GGCGG	ATGCG	GTGAA	GGTTA	CCCTG	GGTCC	GAAAG	GTCGT	AACGC	GATCC
TGGAA	AAACT	GACCG	GTCCG	CCGAC	CATTA	CCAAC	GATGG	TGTTA	CCATC	GCGCG	TGAAA	TTCAG	CTGCG	TGACC	CGTTT	GCGAA	CATGG	GCGCG
CAACT	GGTGA	ANGAG	GTGGC	GATGA	AAACC	AACGG	TGTGG	TIGGT	GATGG	TACCA	COMPC	GCGAC	CGTGC	TGGCG	CAAGC	GATGG	TIUGT	GAAGG
CCTGG	CACCT	CCCTA	CCACC	ACCCA	TCTCC	CTCCT	ammoo	COTCO	TOOCA	CCCAC	COMPO	amcaa	CCCAT	GIIGI	arcro	ammer	COTAG	CCACA
GAGCA	TGTTG	GCAAG	ACCGG	TGTTG	TGACC	ACCGA	GGAAA	GCGAT	ACCCT	GGGCA	TGGCG	GTTGA	TGTTG	TGGAC	GGTAT	CGAAT	TCGAC	CACGG
CTACA	CCAGC	GGTTA	TATGG	TGACC	GATCC	GGAGC	GTATG	GAAGC	GGTTC	TGGAC	AACCC	GCTGA	TTCTG	CTGAC	CAACA	AGAAA	ATCAG	CCAGG
TGCAA	GAGAT	TATGC	CGACC	CTGGA	AGTTG	CGAAG	CGTGC	GGATC	GTCCG	CTGGT	TGTGA	TTGCG	GAGGA	TGTGG	ATGGT	CCGGC	GCTGC	AGCTG
CTGGT	TGGTG	GCAAC	ATGCA	CAAAA	CCATG	CAAAG	CGTTG	TGGTT	CGTGC	GCCGG	GTTTT	GGTCA	CCGTC	GTGTT	GCGGA	ACTGG	AAGAC	CTGGC
GGTGG	CGCTG	GGTGG	CCATG	TTATC	GCGAA	GGATA	CCGGC	ATTGA	CCTGG	GTGAG	GTTGC	GCGTG	AACAC	CTGGG	TAGCT	GCGAT	CGTCT	GACCG
CGACC	GAAAG	CGACA	CCACC	ATTGT	GGGTC	CGCGT	GGTCA	CCAGA	ACCTG	GTGGA	TGCGC	GTGTT	GCGCA	GCTGG	AAGTG	CAACG	TGAAC	GTGCG
CGTAT	DACAD	CGGAT	CGTGA	CATCC	CARCA	TRCCC	TATTG	CCCGT	COGTO	CCCCCC	CTGTG	GCGGT	CTATTC CTATT	GTGTG	CCCTC	CGCGA	ACCOC	GTTGA
CGCAA	GCGCA	CCGTG	TTCTC	GACGC	GGTGG	ACCTC	GTTGG	CGATC	AACCG	ATCGG	TCGTG	ACCTG	GTTCG	TCGTG	CGCTG	accca	ACCGC	TGCGT
TGGAT	TGCGT	TCAAC	GCGGG	TTTTG	AGGGC	GGTGA	TGTGG	TTGAT	GTGGT	TGCGG	ATCTG	CCGCT	GGGTC	ACGGT	TTCAA	CGCGC	TGACC	GGCGA
ATACG	GTGAT	ATGTT	CGAGG	AAGGC	ATCAT	TGACC	CGTTT	AAAGT	TACCC	GTGCG	GCGCT	GGAAA	GCGCG	GCGAG	CATCG	CGGCG	CTGCT	GATTA
CCACC	GAAAC	CGCGG	TGGTT	GAGGA	AATCC	TGGGT	CAGCC	GGGTG	CGATT	ATGGC	GCCGG	GTTTT	GGTGA	CCTGG	CGGAG	GGTAT	GGTTC	GTCCG
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TCACT	ATAGG	GGAAT	TGTGA	GCGGA	TAACA	ATTCC	CCATC	TTAGT	ATATT	AGTTA	AGTAT	AAGAA	GGAGA	TATA				
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>pCDFI	DmimG-1	His																
GCCAT	ACCGC	GAAAG	GTTTT	GCGCC	ATTCG	ATGGT	GTCCG	GGATC	TCGAC	GCTCT	CCCTT	ATGCG	ACTCC	TGCAT	TAGGA	AATTA	ATACG	ACTCA
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CAGTT	GGTCA	AGGAG	GTGGC	GATGA	AGACC	AACGG	CGTGG	TCGGT	GACGG	CACCA	CCACC	GCCAC	CGTGT	TGGCG	CAGGC	GATGG	TTCGC	GAGGG
CTTGG	CGGCC	GTCGA	GGCCG	GCGCC	AACCC	GATGC	GCGTG	CGTCG	CGGCA	TCGAA	CGCAC	CGTGC	CGGTG	GTCGT	CGAAT	CGCTG	CGCAG	CCACA
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GAGCA	CGTCG	GCAAG	ACCGG	CGTGG	TCACG	ACCGA	GGAGA	GTGAC	ACCCT	GGGCA	TGGCG	GTCGA	CGTCG	TCGAC	GGCAT	CGAGT	TCGAC	CACGG
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CIECCIE	CCCCCC	CANGO	amoga	CAACA	CCARC	CRAME	CORCO	mmcmc	CCDCC	CICGI	COMMO	CCACA	CCCCC	COCERC	ACGGG	acmoo	ACCAC	cmcccc
CGTCG	CGCTC	GGCGG	GCACG	TCATC	GCGAA	GGACA	CCGGC	ATCGA	CCTGG	GCGAG	GTGGC	CCGCG	AGCAT	CTGGG	ATCGT	GCGAC	CGTCT	CACCG
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TGGAT	CGCCT	TCAAC	GCCGG	CTTCG	AAGGA	GGCGA	TGTCG	TCGAT	GTTGT	CGCCG	ATCTA	CCGCT	CGGAC	ATGGC	TTCAA	TGCAC	TCACC	GGCGA
CCACC	GEGAC	ATGTT	TCGAGG	GAGGA	GATCAT	TCGACC	CARCO	AAGGT	GACCC	BUGGCC BUGGCC	GCGCT	GGAGA	GECCA	GCCTC TCTCG	CCCAN	CCGCG	CIGCT	GATCA
TCCAA	CATCT	ACGGT	AGTCA	TCATC	ATCAT	CATCA	TTGAG	AATTC	GAGCT	CGGCG	CGCCT	GCAGG	TCGAC	AAGCT	TGCGG	CCGCA	TAATG	CTTAA
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TTAGT	ATATT	AGTTA	AGTAT	AAGAA	GGAGA	TAT												
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C																		
>nRSFI	mimac	His																
GCCAT	ACCGC	GAAAG	GTTTT	GCGCC	ATTCG	ATGGT	GTCCG	GGATC	TCGAC	GCTCT	CCCTT	ATGCG	ACTCC	TGCAT	TAGGA	AATTA	ATACG	АСТСА
CTATA	GGGGA	ATTGT	GAGCG	GATAA	CAATT	CCCCT	GTAGA	AATAA	TTTTG	TTTAA	CTTTA	ATAAG	GAGAT	ATACC	ATGCA	TCATC	ATCAT	CATCA
TAGCA	GCGGT	CTGGA	AGTTC	TGTTT	CAGGG	CCCGA	GTGGT	ATGAG	CAGAC	AAAGC	CTGAC	CAAGG	CGCAC	GCCAA	GATAA	GCGAA	CTCAC	CTGGG
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GCTGG	AATGG	CAGAA	GCTCT	TCCTG	TCGAT	CATTC	CGTTC	CCCGA	GATCT	CGGCG	GCCCG	CGCCA	TGCCG	ATGGC	CATCG	ACGCC	GTGCC	GAACC
CGGAG	ATCCA	CAACG	GCCTG	GCGGT	CCAGA	TGATT	GATGA	GGTTC	GGCAC	TCGAC	GATCC	AGATG	AACCT	CAAGA	AGCTC	TACAT	GAACA	ACTAC
CGACG	CCCGG	ACCGC	CCCCA	ACATGA	TACCT	CACGO	GTTCG	CGAAC	GACCO	CGCGG	GCACC A	CACTO	GCGCC	AGTIC	GATGA	CCGAT	CACCC	ACCGG
CGAAC	GGCGA	CTACC	TGCTG	CCGAC	GGTGT	TCCAC	TCGGT	GCAGT	CCGAC	GAGTC	GCGAC	ACATC	TCGAA	CGGCT	ACTCG	ATCTT	GCTGA	TGGCG
CTCGC	CGACG	AACGC	AACCG	ACCGC	TGCTC	GAACG	CGACC	TGCGC	TACGC	CTGGT	GGAAC	AACCA	CTGCG	TGGTC	GACGC	CGCGA	TCGGC	ACCTT
CATCG	AGTAC	GGCAC	CAAGG	ACCGC	CGCAA	GGACC	GGGAG	AGCTA	CGCCG	AGATG	TGGCG	TCGCT	GGATC	TACGA	CGACT	ACTAC	CGCAG	TTACC
TTCTG	CCACT	GGAGA	AGTAC	GGACT	GACGA	TTCCG	CACGA	CCTGG	TCGAG	GAGGC	GTGGA	AGCGC	ATCGT	CGAGA	AGGGC	TACGT	GCATG	AGGTG
GCCCG	ATTCT	TCGCC	ACCGG	TIGGC	CGGTG	AACTA	CTGGC	GCATC	GACAC	CATGA	CCGAC	ACCGA	CTTCG	AGTGG	TTCGA	GCACA	AGTAT	CCGGG
CTGGT	ACAAC	AAGTT	CGGCA	AGTGG	TGGGA	GAACT	ACAAC	CGGCT	GGCCT	ACCCC	GGGCG	CAACA	AGCCG	ATCGC	GTTCG	AAGAG	GTCGG	GTACC
AGTAC	CCGCA	CCGGT	GCTGG	ACCTG	CATGG	TGCCC	GCGTT	GATCC	GCGAG	GACAT	GATCG	TCGAG	AAGGT	CGACG	GCCAG	TGGAG	GACGT	ACTGC
TUCGA GTGGC	GACCT	CTGCA	TGGAC	CGACG	GACCT	CGCCCT	ACATC	GTGAA	CGATC	TCGCC	GAGCA	CCGCC	GAACA	GGGBA	GACTO	TCGTC	GGGCA	ACCAC
ACCTG	GATCT	GGATC	CGCAG	AAGAT	GTGGA	CCCTC	GACGA	CGTGC	GCGGC	AACAC	GTTCA	ACAGC	CCCAA	CGTGC	TGCTG	AACCA	GATGA	CCAAC
GACGA	GCGGG	ACGCG	CATGT	CGCGG	CGTAC	CGCGC	CGGCG	GGGTC	CCGGC	CTGAG	AATTC	GAGCT	CGGCG	CGCCT	GCAGG	TCGAC	AAGCT	TGCGG
CCGCA	TAATG	CTTAA	GTCGA	ACAGA	AAGTA	ATCGT	ATTGT	ACACG	GCCGC	ATAAT	CGAAA	TTAAT	ACGAC	TCACT	ATAGG	GGAAT	TGTGA	GCGGA
TAACA	ammee	CCATC	TTACT	ATATT	ACTTA	ACTAT	aacaa	CCACA	TAT									

Figure 1. The DNA sequences spanning the multi-cloning site in expression plasmids. (A) pCDFDmimG_{op}, (B) pCDFDmimG-His, and (C) pRSFDmimAC-His. The *mimG* and *mimA* sequences are highlighted in pink; the His-tag gene, the recognition sequence of HRV-3C, and linker regions are highlighted in blue, green, and grey, respectively. Initiation and termination codons are indicated in bold.

Plasmids and Site-directed Mutagenesis

For the expression of *Mycolicibacterium* MimG protein, pCDFDuet1-based expression constructs containing the native DNA sequence and codon-optimized sequence were used. The DNA fragments of *mimG* were purchased from GenScript Japan Inc. (Tokyo, Japan). The fragment containing the native sequence was cloned into the pCDFDuet-1 vector between the *NcoI* and *EcoRI* sites resulting in pCDFDmimG vector, in accordance with a previously published protocol.²⁴ The fragment

containing the codon-optimized sequence was cloned into the same vector between *NcoI* and *Hind*III sites to construct the pCDFDmimGop vector (Fig. 1A).

For the expression of *Mycolicibacterium* MimAC, the previously reported plasmid based expression construct was used .²⁴ The DNA fragments of *mimA* and *mimC* containing native sequences were purchased from GenScript Japan Inc. (Tokyo, Japan). The pRSFDuet1-based expression constructs were used for the expression of native DNA sequences of *mimA* and *mimC*. The fragments containing native sequences of *mimA* and *mimC* were cloned into pRSFDuet1 vector between *NcoI* and *EcoRI* and *NdeI* and *Xho I* sites, respectively, resulting in the pRSFDmimAC vector in accordance with a previously published report.²⁴

His-tag coding sequences were introduced into the sequences of *mimG* in pCDFDmimG, *mimA* in pRSFDmimAC, and *gro*EL in pGro7 using oligonucleotide-directed mutagenesis. The pair of mutagenic oligonucleotides was purchased from Macrogen Japan (Tokyo, Japan). (5'-GAA TTC GAG CTC GGC GCG CCT GCA GGT CGA-3' and 5'-TCA ATG ATG ATG ATG ATG ATG ACT ACC GTA GAT GTT GGA CGG GCG GAC CAT GC-3' for pCDFDmimG, 5'-CT GGA AGT TCT GTT TCA GGG CCC GAG TGG TAT GAG CAG ACA AAG CCT GAC CAA GGC GCA C-3' and 5'-ACC GCT GCT ATG ATG ATGAT GAT GAT GCA TGG TAT ATC TCC TTA TTA AAG TTA AAC AAA ATT ATT TCT AC-3' for pRSFDmimAC, 5'-TAG CAG CCA TCA TCA TCA TCA TCA TTA ATT GCC CTG CAC CTC GCA GAA ATA AAC AAA CC -3' and 5'-CCC GGG CCC TGA AAC AGA ACT TCC AGA CTA CCC ATC ATG CCG CCC ATG CCA CCC ATG CC-3' for pGro7). Mutagenesis was performed with inverse PCR. This was followed by DpnI digestion and self-ligation to synthesize pCDFDmimG-His from pCDFDmimG, pRSFDmimAC-His from pRSFDmimAC (Fig. 1B, C, and Fig. 2), and pGro7-His from pGro7. The resulting gene sequences were validated by DNA sequencing using a 3730xl DNA Analyzer (Life Technologies) after the construction of the vector.

A >His-tag fused MimG

MAREL RFNSD ARARL EQGVN ALADA VKVTL GPKGR NAILE KLTGP PTITN DGVTI AREIQ LRDPF ANMGA QLVKE VANKT NGVVG DGTTT ATVLA QAMVE BGLAA VEAGA NPMRV RRGIE RTVPV VVEEL RSHSV EVGSS SULRI KALA ASDDE ALGDV IAAX ENGKG TGVVT TEESD TLGMA VDVVD GIEFD HGYTS GYMVT DPERM EAVLD NPLIL LTNKK ISQVQ EINDT LEVAK KADRP LVVIA EDVDG PALQL LVGGN HHKTM QSVVV RAPGF GHRV AELED LAVAL GGVI AKDG IDLGE VAREH LGSCD RLTAT ESDTI LVGFR GHQNL VDARV AQLEV QRERA RIDAD RDILD LRIAR LTGRV AVIRV GGATS VELKE RMLRV EDALA ATRAA LEAGI VSGGG TALAQ AHRVL AALESA ASIDA LITT ETAVV EELIG QPGAI MAPGF GDLE GMVRP SNIG SHHHH HH

>His-tag fused MimA

В

MHHH HHSG LEVLF QGPSG MSRQS LTKAH AKISE LTWEP TFATP ATRFG TDYTF EKAPK KDPLK QIMRS YFSWE EEKDN RVYGA MDGAI RGNNF RQVQQ RWLEW QKLFL SITPF PEISA ARAMP MAIDA VNNF IHNGL AVQMI DEVHK STIQM NLKL YWNNY IDPAG F9MTE KAPAN NYAGTI GROF GEGFI TGDAI TAANI YLTVV AETAF TNTLF VAMPD EAAAN GDYLL PTVFH SVQSD ESRHI SNGYS ILLMA LADER NRPLL ERDLR YAWWN NHCVV DAAIG TFIEY GTKDR KKDRE SYAEW WRKWI YDDYY RSYLL PLEKY GLTIP HDLVE EAWKH IVÆKG VYHEV ARFFA TGWPU NYKWI DTWTD DFEW FEHKY PGWYN KFGKW WENNY RLALP GRNKP IAFEE VGYQY PHRCK TCMVP ALIRE DMIVE KVDGQ WRTYC SETCY WTDAV AFRGE YEGRA TENNG RLTGF REWET LHHGK DLADI VTDLG YVRDD GKTLV GQPHL DLDPQ KMWTL DDVRG NTFNS PNVLL NQMTN DERDA HVAAY RAGGV PA

Figure 2. Amino acid sequence of the His-tag fused proteins. (A) Histag fused MimG and (B) His-tag fused MimA. The *mimG* and *mimA* sequences are indicated in black; His-tag gene, the recognition sequence of HRV-3C, and linker regions are highlighted in red, blue, and green, respectively.

Expression and purification of proteins

The chaperonin, namely, GroEL, was induced with the addition of _L-arabinose and purified from *E. coli* BL21(DE3) containing pGro7-His according to a previously published protocol.³² For the isolation of MimG, *E. coli* cells (Rosetta 2(DE3)) containing pCDFmimG-His were cultured in an autoinduction medium

(ZY-5052)³³ containing 50 µg/mL streptomycin and 20 µg/mL chloramphenicol at 37 °C and 135 rpm for 5 h, followed by further incubation at 25 °C and 135 rpm for 16 h. Overnight cultures were harvested by the centrifugation at 5000 $\times g$ for 30 min at 4 °C. Cells were disrupted in 10 mM bis-Tris (pH 7.2) and 0.5 M NaCl (buffer A) with sonication. Subsequent centrifugation for 1 h at 4°C with 30,000 $\times g$ yielded cell-free lysates containing the MimG protein. Purification was performed by affinity chromatography using a Ni-Sepharose (COSMOGEL His-Accept) 10 mL column with buffer А as equilibration/washing buffer and buffer A + 0.5 M imidazole as elution buffer.

For the isolation of the MimAC complex, E. coli cells (Rosetta 2(DE3)) were co-transformed with two plasmids, namely, pCDFmimG and pRSFmimAC-His, and cultured in lysogeny broth with 50 µg/mL streptomycin and 50 µg/mL kanamycin at 37 °C and 135 rpm until the optical density (600 nm) reached 0.5. The protein expression was induced with 0.1 mM isopropyl- β -D-thiogalactopyranoside, and *E. coli* cells were subsequently cultured at 25 °C and 135 rpm in the presence of 250 µM ammonium iron(III) citrate and 250 µM iron(III) citrate. Overnight cultures were harvested by centrifugation at 5000 $\times g$ for 30 min at 4 °C. Cells were disrupted in10 mM bis-Tris (pH 7.2) and 0.5 M NaCl (buffer A) by sonication. Subsequent centrifugation for 1h at 4°C with 30,000 $\times g$ yielded cell-free lysates containing the MimG protein. Purification was performed using affinity chromatography with a Ni-Sepharose 10 mL column (COSMOGEL His-Accept) and buffer A as the equilibration/washing buffer and buffer A + 0.5 M imidazole as elution buffer. To excise the His-tag region from the protein, aliquots of HRV3C protease were added to the collected fraction, followed by incubation in the buffer at 4 °C overnight. The protein solution was dialyzed against 10 mM Tris-HCl buffer (pH 7.2) containing 10 mM NaCl (buffer B) at 4 °C to remove excess imidazole and NaCl. Subsequently, anion exchange chromatography was performed using a Hitrap Q 5 mL column (Cytiva) with buffer B as equilibration/washing buffer. The protein was eluted with a 20-column volume linear gradient of NaCl (10-500 mM). The fractions containing the purified proteins were collected and concentrated by ultrafiltration using Vivaspin turbo 15 (Sartorius AG, Göttingen, Germany). The protein samples were stored at -80 °C until further use. Purity was determined using SDS-PAGE. Protein concentration was determined by measuring the intensity of absorbance at 280 nm.³⁴ Densitometry analysis of the gel bands was performed using the ImageJ software. Electrospray ionization-mass spectra were obtained using the Agilent LC/MSD (G6125B) system.

Analysis of subunit structures

Blue native gel electrophoresis was performed using NativePAGE 4-16 % Bis-Tris Gels according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Gel filtration was performed using a Superdex 200 10/300 GL column (Cytiva) and a neutral buffer (50 mM phosphate containing 150 mM KCl at pH 7.0) was used as an eluent. Aldorase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (14 kDa) were used as marker proteins for the determination of molecular mass.

Inductively coupled plasma atomic emission spectroscopy (ICP-AES)

The presence of metallic components was determined using ICP-AES with the ICPS-8100 emission spectrometer (Shimadzu). The calibration curves of the metal ions were constructed using the metal ion standard solution (10.0 ppm). The purified protein was dialyzed against 5 mM sodium phosphate buffer at pH 7.0 and the resulting samples was subjected to ICP analysis.

RESULTS AND DISCUSSION

Optimization of expression construct of MimG

A GroEL-like protein, namely, MimG derived from *M.* smegmatis was expressed in the soluble fraction obtained from *E. coli* in accordance with previous reports.²⁴ The protein was produced even with the use of an autoinduction medium (ZY-5052)³³ instead of the commonly used IPTG induction method (Fig. 3A). Cell lysates were subjected to SDS-PAGE. The bands corresponding to MimG whose molecular mass was calculated to be 57915 kDa based on the amino acid sequences were identified in the soluble fraction. Furthermore, we attempted to improve the expression of MimG protein by optimizing codon usage. However, *E. coli* cells containing the expression plasmid, namely, pCDFDmimG_{op}, exhibiting the rare codon-modified *mimG*, demonstrated the expression of MimG protein almost completely in the insoluble fraction (Fig. 3B). Hence, we employed the pCDFDmimG plasmid for subsequent experiments.



Figure 3. SDS-PAGE analysis of *E. coli* lysates expressing MimG with autoinduction medium (A) pCDFDmimG and (B) pCDDFmimG_{op}. M, Marker; S, Soluble fraction; P, Precipitation fraction. The arrows indicate the expressed proteins.

Characterization of MimG

The GroEL and MimG were purified by affinity chromatography. A single band corresponding to each, GroEL and MimG, was observed in SDS-PAGE results, suggesting that the proteins could be isolated in a homogenous state (Fig. 4A, B).

To confirm the subunit structure of the isolated GroEL-like protein, namely, MimG, the samples were subjected to blue native PAGE, which involves native PAGE analysis in the presence of the coomassie blue dye performed under nondenaturing conditions. A single band was observed corresponding to GroEL from *E. coli* in accordance with previous reports (Fig. 4C).³⁵ This was consistent with the report stating that the crystal structure of GroEL shows a porous cylinder of 14 subunits in the dimer of heptamers.³⁶ In contrast, two bands corresponding to MimG were observed, one of which demonstrated almost the same mobility as that by GroEL. The molecular mass of MimG was determined to be 8.5×10^5 Da (Fig. 4D). The molecular mass of one of the bands was calculated to be 4.2×10^5 Da. Hence, the band denoted its heptamer region. Thus, the observation of the additional band of MimG suggests that the binding affinity between two MimG heptamers might be weaker than that of the GroEL. MimG is responsible for the

functional expression of MimA and MimC hydroxylase components since the co-expression of MimG results in the solubility of MimA and MimC in *E. coli* cells.²⁴ Additionally, we observed that the recombinant MimG protein could be assembled in a similar structure (the dimer of heptamers) to that of the GroEL from *E. coli*, indicating that MimG serves as a molecular chaperon similar to GroEL.



Figure 4. SDS-PAGE and blue native PAGE analysis of purified proteins. SDS-PAGE analysis of (A) GroEL and His-tag fused MimG (B); Blue native PAGE analysis of (C) GroEL and (D) His-tag fused MimG. M, Marker; S, Sample; The arrows indicate expressed proteins.

Characterization of MimA and MimC

As reported previously, E. coli cells expressing MimA, MimC, and MimG are capable of the oxidation of aromatic compounds. In support of this assertion, we were able to purify the His-tag fused recombinant hydroxylase component from E. coli cells upon simultaneous expression of MimA, MimC, and MimG. For affinity chromatography with Ni-Sepharose resin, we introduced the His-tag sequence into the N-terminal site of the mimA gene in the pRSFDmimAC vector. The resulting sample of the affinity subjected chromatography was to anion-exchange chromatography for further purification. The purity was verified by SDS-PAGE, where two bands corresponding to the isolated MimA and Mim C were observed with molecular masses of 64 kDa and 41 kDa, respectively. These values are almost identical to the calculated values of 63293 kDa for MimA and 41510 kDa for Mim C, respectively. This indicated that MimA was successfully isolated, and the hydroxylase component consisted of another subunit, possibly MimC protein, resulting in the formation of MimAC complex (Fig. 5A). For further clarifications of this additional band, we conducted an LC/MS analysis of the hydroxylase component. The molecular masses corresponding to this band was determined to be 41542 kDa, which is almost identical to the calculated values of 41510 kDa. The densitometric analysis of two bands on SDS-PAGE gel showed a 1.0:1.2 ratio, demonstrating that the hydroxylase component is composed of Mim A and Mim C with an approximate ratio of 1:1.

To elucidate the subunit structure of the hydroxylase component, namely, the MimAC complex, we performed size-exclusion chromatography. The MimAC complex protein showed a single peak in the chromatogram, suggesting that the MimAC complex was isolated as a single molecule in a homogenous state (Fig. 5B). The molecular mass was determined to be 1.9×10^5 Da based on the elution volume. This suggested that the MimAC complex was a dimeric hydroxylase protein with two subunits in the $(\alpha\beta)_2$ stoichiometry, in accordance with previous predictions based on the arrangement of gene clusters.¹⁶ Based on the available structural information, the SDIMOs contain hydroxylase proteins composed of two or three subunits in a $(\alpha\beta\gamma)_2$ or $\alpha\beta$ quaternary structure.

Furthermore, no homologs to the γ subunit in the gene cluster of group-5 SDIMOs, including the operons of *M. smegmatis* and *M. godii*, have been observed.²⁷ Accordingly, it is likely that the hydroxylase component of group-5 SDIMOs differs in the quaternary structure from other SDIMOs reported to date.



Figure 5. Characterization of purified MimAC complex. (A) Sizeexclusion chromatography, (B) Chromatogram of MimAC complex (C) Sequence alignment of the zinc-binding motif of SDIMO hydroxylase α subunits (The sequences of MimA from *Mycolicibacterium goodii*, ThmA from *Pseudonocardia* sp. K1, AmoC from *Gordonia rubripertincta* B-276, PrmA from *Mycobacterium* sp. TY-6, PhN from *Pseudomonas stutzeri* OX1, TmoA from *Pseudomonas mendocina* KR1, and MmoX from *Methlococcus capsulatus* Bath were aligned using ClustalW.)

To investigate whether the dinuclear metal center was formed, ICP-AES analysis was performed to quantify the amount of transition metals binding to the purified hydroxylase component. The iron content was determined to be 1.8 atoms per $\alpha\beta$ complex, suggesting that the dinuclear iron center was embedded in this enzyme. Other metals including Co, Ni, and Cu were not detected in the analysis. However, 1.4 Zn atoms per $\alpha\beta$ complex was detected, suggesting that MimA contained at least one zinc-binding site. Among the SDIMOs, only group-1 SDIMO hydroxylase, for example, phenol hydroxylase from Pseudomonas stutzeri OX1, accommodates the zinc ion supported by four cysteine residues.²⁰ These conserved cysteine residues, namely, Cys404, Cys407, Cys430, and Cys434 were observed in the MimA sequence, (Fig. 5C), demonstrating that they coordinate to the Zn ion in MimA protein similar to phenol hydroxylase of group-1 SDIMO, possibly stabilizing the scaffold.

CONCLUSIONS

We purified MimG, which is a group-5 SDIMO-associated GroEL-like protein via heterologous expression of group-5 SDIMOs and demonstrated its quaternary structure and assembly, which is almost identical to that of GroEL protein expressed in *E. coli*, which is one of the best characterized protein among the chaperonin protein family. This indicates that MimG plays a significant role in aiding the proper folding of the hydroxylase component. This study also demonstrated that the recombinant MimAC complex, namely the hydroxylase component that is co-expressed with MimG, exhibited a distinguished feature of $(\alpha\beta)_2$ quaternary structure and the zinc-

binding site, indicating that the molecular properties of SDIMO are very diverse along with the variety of their sequences. These findings would extend our understanding of the biochemistry and enzymology of SDIMOs, which enhances the potential of the application to developing molecular tools for biocatalysis for the upgrading of light hydrocarbons as well as efficient bioremediation.

ACKNOWLEDGEMENTS

We appreciate the support from JST PRESTO (JPMJPR16S9). This work was funded by the Tonen General Sekiyu Research/Development Encouragement & Scholarship Foundation.

Author Contributions

NF conceived and designed the experiments. CI YA performed the experiments. CI YA analyzed the data. NF wrote the paper.

Competing interests

The authors have declared that no competing interests exist.

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