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Isolation of an H_2-dependent electron-bifurcating CO_2-reducing megacomplex with MvhB polyferredoxin from *Methanothermobacter marburgensis*

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Keywords
CO_2 reduction; flavin-based electron bifurcation; formylmethanofuran dehydrogenase; heterodisulfide reductase; methanogenesis

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In the hydrogenotrophic methanogenic pathway, formylmethanofuran dehydrogenase (Fmd) catalyzes the formation of formylmethanofuran through reducing CO_2. Heterodisulfide reductase (Hdr) provides two low potential electrons for the Fmd reaction using a flavin-based electron-bifurcating mechanism. [NiFe]-hydrogenase (Mvh) or formate dehydrogenase (Fdh) complexes with Hdr and provides electrons to Hdr from H_2 and formate, or the reduced form of F_{420}, respectively. Recently, an Fdh-Hdr complex was purified as a 3-MDa megacomplex that contained Fmd, and its three-dimensional structure was elucidated by cryo-electron microscopy. In contrast, the Mvh-Hdr complex has been characterized only as a complex without Fmd. Here, we report the isolation and characterization of a 1-MDa Mvh-Hdr-Fmd megacomplex from *Methanothermobacter marburgensis*. After anion-exchange and hydrophobic chromatography was performed, the proteins with Hdr activity eluted in the 1- and 0.5-MDa fractions during size exclusion chromatography. Considering the apparent molecular mass and the protein profile in the fractions, the 1-MDa megacomplex was determined to be a dimeric Mvh-Hdr-Fmd complex. The megacomplex fraction contained a polyferredoxin subunit MvhB, which contains 12 [4Fe-4S]-clusters. MvhB polyferredoxin has never been identified in the previously purified Mvh-Hdr and Fmd preparations, suggesting that MvhB polyferredoxin is stabilized by the binding between Mvh-Hdr and Fmd in the Mvh-Hdr-Fmd complex. The purified Mvh-Hdr-Fmd megacomplex catalyzed electron-bifurcating reduction of [^{13}C]-CO_2 to form [^{13}C]-formylmethanofuran in the absence of extrinsic ferredoxin. These results demonstrated that the subunits in the Mvh-Hdr-Fmd megacomplex are electronically connected for the reduction of CO_2, which likely involves MvhB polyferredoxin as an electron relay.

Abbreviations
BV, benzyl viologen; CoB-SH, coenzyme B; CoM-SH, coenzyme M; CoM-S-S-CoB, heterodisulfide of CoM-SH and CoB-SH $F_{420}H_2$, the reduced form of F_{420}; DOC, deoxycholate; Fdh, formate dehydrogenase; Fmd, formyl-MFR dehydrogenase or its molybdopterin isoenzymes; FprA, A-type flavoprotein; Fwd, tungstopterin isoenzymes of Fmd; H_4MPT, tetrahydromethanopterin; Hdr, heterodisulfide reductase; Hmd or [Fe]-hydrogenase, H_2-forming methylene-H_4MPT dehydrogenase; IAA, iodacetamide; iBAQ, intensity-based absolute quantification; Mcr, methyl-coenzyme M reductase; MFR, methanofuran; Mtd, F_{420}-dependent methylene-H_4MPT dehydrogenase; Mvh, Hdr-associating [NiFe]-hydrogenase; PSM, peptide spectra matched for the protein; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl)phosphine; Tris, tris(hydroxymethyl)aminomethane.

Introduction

In the hydrogenotrophic methanogenic pathway of *Methanothermobacter* species, two isoenzymes of formylmethanofuran (formyl-MFR) dehydrogenase (Fmd or Fwd) have been characterized [1–6]. Both isoenzymes catalyze the reduction of CO_2 to formyl-MFR (Fig. 1). Fmd contains molybdopterin as the prosthetic group at the active site for CO_2 reduction, and Fwd contains tungstopterin at the active site. The *fwdABCDFG* gene cluster encodes the full set of subunits of Fwd, and both isoenzymes commonly use the FwdAFG subunits [7,8]. The *fmdBC* gene cluster encodes the Fmd-specific subunits, in which the *fmdC* gene fuses with a part of the *fwdD* gene homologue [7,9]. Thus, Fmd consists of the FwdAFG and FmdBC proteins. Fwd is constitutively produced in culture media containing molybdate and/or tungstate; however, the production of Fmd is upregulated by the presence of molybdate in the medium [9]. Crystal structures of the tetrameric and dimeric forms of FwdABCDFG have been reported [10]. The crystal structures showed that FwdBD forms the tungstopterin active site, at which CO_2 is reduced to formate and then transferred to the second active site in FwdA via a channel. In the second dinuclear zinc active site in FwdA, formate is conjugated to the amino group of MFR to form formyl-MFR [10]. Electrons for CO_2 reduction may be provided by cytosolic reduced ferredoxin [1], although the CO_2-reducing activity of Fmd was experimentally determined using titanium (III) citrate as the electron donor rather than reduced ferredoxin [11].

In the hydrogenotrophic methanogenic pathway in methanogens without cytochromes, such as *Methanothermobacter*, heterodisulfide reductase (Hdr) is the main enzyme that provides the low-potential electrons needed for the CO_2 reduction catalyzed by Fmd or Fwd [12–15]. Hdr forms a complex with [NiFe]-hydrogenase (Mvh) [16] or F_{420}-dependent--formate dehydrogenase (Fdh) [17–19], which oxidize H_2 and formate or the reduced form of F_{420} ($F_{420}H_2$), respectively. The Mvh and Fdh modules provide electrons to the Hdr module in the complexes. The Hdr module catalyzes the reduction of ferredoxin and heterodisulfide in coenzyme M and coenzyme B (CoM-S-S-CoB) using the flavin-based electron-bifurcation mechanism [14]. The reduced ferredoxin diffuses through the cytosol and functions as the electron donor for the reaction catalyzed by Fmd and Fwd. Hdr was first purified as a tetramer of heterotrimeric HdrABC from *Methanothermobacter marburgensis* [20] and was later purified as a dimer of a complex with MvhAGD from several methanogenic archaea, including *Methanothermobacter marburgensis* [15,16].

The crystal structures of the Mvh-Hdr complexes from *Methanothermococcus thermolithotrophicus* and *Methanothermobacter wolfeii* were solved [15]. Based on the high-resolution crystal structure of the Mvh-Hdr complex from *Methanothermococcus thermolithotrophicus*, the possible mechanisms of flavin-based electron bifurcation were discussed. The presence of megacomplexes of Mvh-Hdr-Fwd and Fdh-Hdr-Fmd was first demonstrated by a pull-down assay using His-tagged proteins [17]. Due to the instability of the His-tag-purified megacomplexes, the molecular size was not determined by size-exclusion chromatography, and the enzymatic activity was not reported [17,21]. Recently, an Fdh-Hdr-Fmd megacomplex was purified from *Methanospirillum hungatei* [19]. This methanogen is phylogenetically distantly related to *Methanothermobacter* and *Methanococcus* species. The Hdr activity of the Fdh-Hdr-Fmd megacomplex in the presence of CO_2 and formate was stimulated by MFR; therefore, this megacomplex catalyzes the conversion of CO_2 to formyl-MFR using low potential electrons generated by the electron-bifurcation reaction of the Hdr module [19]. The cryo-electron microscopic structure of the megacomplex indicated that the low potential electrons are directly transferred from Hdr to the CO_2-reduction active site of Fmd through the polyferredoxin subunits FmdF [19].

Here, we report for the first time the purification and characterization of the 1-MDa Mvh-Hdr-Fmd complex, which contains the polyferredoxin MvhB. MvhB polyferredoxin was not observed in the previously purified Mvh-Hdr complexes or Fmd/Fwd [2–6,15,16]. We detected the enzyme activity of the 1-MDa enzyme complex that catalyzes CO_2 conversion to formyl-methanofuran using [^{13}C]-CO_2, which supports the functional complexation of the three enzyme modules in the megacomplex.

Results and Discussion

Purification of the Hdr complexes

Three chromatographic steps were used to purify the proteins with Hdr activity from the cell extract of *Methanothermobacter marburgensis* cultivated in standard culture medium with molybdate (Fig. 2). The proteins with Hdr activity eluted mainly in the 0.48-M NaCl step gradient from the anion-exchange (Q-Sepharose) column, while in some experiments, Hdr

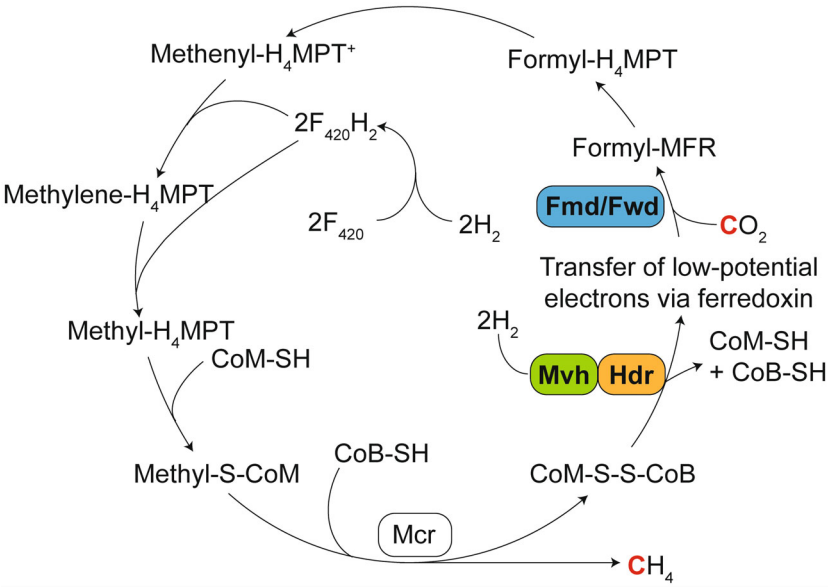

Fig. 1. The hydrogenotrophic methanogenic pathway in *Methanothermobacter*. Two low potential electrons generated by the enzyme complex of [NiFe]-hydrogenase (Mvh) and heterodisulfide reductase (Hdr) using the flavin-based electron bifurcation mechanism were used for the CO_2 reduction catalysed by formyl-MFR dehydrogenase (Fmd or Fwd). Heterodisulfide (CoM-S-S-CoB) of coenzyme M (CoM-SH) and coenzyme B (CoB-SH) is formed by the reaction catalysed by methyl-coenzyme M reductase (Mcr). H_4MPT, tetrahydromethanopterin; MFR, methanofuran.

activity eluted mainly in the 0.52-M NaCl step. The major Hdr fraction was further fractionated with a hydrophobic (phenyl-Sepharose) column, in which the Hdr activity was broadly distributed in step gradients containing 0.5-, 0.4- and 0.2-M ammonium sulfate. The total yield of Hdr activities in the three phenyl-Sepharose fractions was 66%. We used the Hdr fraction in 0.4 M ammonium sulfate for further fractionation with size-exclusion chromatography (Superose 6 Increase), from which the Hdr activity eluted as two peaks at 1 and 0.5 MDa (Fig. 3A,B). Setzke *et al.* [16] previously reported the detection of 0.5 and 0.25 MDa Hdr complexes from size-exclusion chromatography, which was the dimeric and monomeric forms of Mvh-Hdr based on SDS/PAGE protein profiles.

Proteome analysis of the fractions

SDS/PAGE in the fractions at 1.0 and 0.5 MDa of the size-exclusion chromatography revealed a similar but not identical protein profile (Fig. 4). For example, MvhB is more prominent in the 1 MDa fraction, whereas HdrA has a relative higher abundance in the 0.5 MDa fraction. Thus, the MvhB : HdrA ratio shifts in the 1 and 0.5 MDa fractions. To identify the proteins in the fractions, we performed mass spectrometric proteome analysis (Fig. 3C–F and Table 1). The proteome analysis revealed that the 1.0- and 0.5-MDa fractions consist of the subunits of Mvh (MvhAGDB), subunits of Hdr (HdrABC) and subunits of Fmd (FmdBC) and Fwd (FwdABCDFG). The sample was obtained from the cells of *Methanothermobacter marburgensis* cultivated in the presence of molybdate; therefore, Fmd and Fwd coexist. The intensity of the proteome data indicates that the Fmd-specific subunits (FmdBC) are more abundant than the respective Fwd-specific subunits (FwdBC) in this enzyme complex (Table 1). The 1-MDa fraction was reloaded on the same size-exclusion chromatography column, which reproduced the 1-MDa peak and confirmed the presence of the stable 1-MDa complex (Fig. 5). However, the reloaded chromatography also yielded small peaks at 0.5 and 0.2 MDa, which indicated that the 1-MDa megacomplex can be slightly dissociated into small subcomplexes by the reloaded chromatography step. From the apparent molecular mass of the fractions, we predicted that the 1.0-MDa megacomplex consists of the MvhAGD-MvhB-HdrABC-FwdABCDFG (or FmdBC) dimer. Hereafter, we refer to this complex as the Mvh-Hdr-Fmd megacomplex for simplicity.

The proteome data indicated that the subunits of Mvh, Hdr and Fmd broadly eluted between the size-exclusion chromatography fractions. The size of the 0.5-MDa peak corresponds with those of the

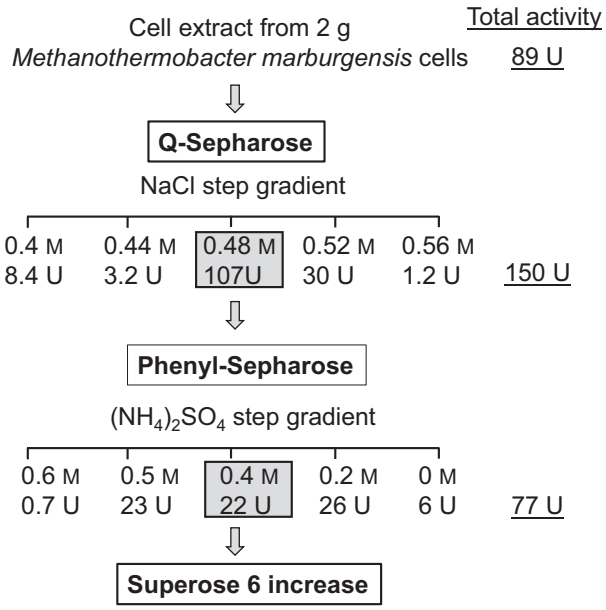

Fig. 2. Distribution of Hdr activity in the chromatography steps. The cell extract from 2 g of *Methanothermobacter marburgensis* cells was fractionated on Q-Sepharose with a NaCl step gradient. The 0.48-M NaCl fraction from Q-Sepharose was applied to phenyl-Sepharose, proteins were eluted with a $(NH_4)_2SO_4$ gradient. The activity of heterodisulfide reductase was measured. The activity in each fraction and the total activities were presented. In some experiments, the Hdr activity was mainly eluted at 0.52 M NaCl. The 0.4 M $(NH_4)_2SO_4$ fraction was applied to Superose 6 Increase size-exclusion chromatography.

MvhAGD-HdrABC dimer (0.5 MDa) and the FmdABCDFG dimer (0.4 MDa). The subunits of Mvh were found not only in the 1.0- and 0.5-MDa fractions but also in the fractions smaller than 0.2 MDa. Previously, the MvhAGD part was purified as a form without the Hdr complex [16,20,22]. The deduced molecular mass of the MvhAGD monomer is 103 kDa. Accordingly, the smaller protein fractions exhibited Mvh activity and contained the MvhAGD subunits (Fig. 3D). The proteome analysis indicated that the 0.2-MDa fraction contains a high concentration of A-type flavoprotein (FprA) as the major protein in this fraction (Table 1), which is the dominant protein in SDS/PAGE at the 0.2-MDa region (Fig. 4). The deduced size of the FprA monomer is 45 kDa, and it has a tetrameric structure of ~ 0.2 MDa [23].

MvhB is a polyferredoxin encoded in the *mvh* operon [24], which contains 12 [4Fe-4S]-clusters and may function as a diffusible electron carrier [14,25,26] or as an electron relay in an enzyme complex [27]. The association of MvhB with the Mvh-Hdr complex or Fmd/Fwd has not been reported, although Mvh (MvhAGD) was purified as a complex with MvhB [28]. MvhB is broadly distributed in size-exclusion chromatography in fractions containing 1-MDa Mvh-Hdr-Fmd, 0.5-MDa Mvh-Hdr or 0.4-MDa Fmd and 0.2-MDa Mvh (Fig. 3D), which suggests that MvhB can bind the subunits of Mvh, Hdr and Fmd.

Correlation analysis of elution profiles of the proteins

We observed a broad elution profile of Hdr activity from the phenyl-Sepharose column (Fig. 2). In a previous report, similar broad elution behaviour of Hdr activity in several chromatographic steps was observed [16]. Based on these chromatographic properties, several different types of Hdr complexes may occur. To determine the protein components in the Q- and phenyl-Sepharose fractions, we performed proteome analysis of the fractions (Fig. 6). From Q-Sepharose, the FwdA subunit eluted as more than one peak, while MvhA and HdrA were detected only in a peak. From phenyl-Sepharose, the MvhA, HdrA and FwdA subunits coeluted (Fig. 6). These data indicate that Mvh, Hdr and Fmd (or Fwd) form a stable complex and that there is no Mvh-Hdr complex lacking Fmd (or Fwd). In contrast, FwdA presents alone in the lower NaCl concentration fraction, apart from the Mvh-Hdr complex. Interestingly, FprA coeluted with the Mvh, Hdr and Fmd subunits in the Q- and phenyl-Sepharose columns (data not shown). FprA has F_{420} oxidase activity and catalyzes the reduction of molecular oxygen to water using $F_{420}H_2$ as a hydride donor [23,29,30]. Notably, FprA was copurified with Mvh and Mvh-Hdr in some experiments [28,31], and FprA was proposed as an electron carrier flavoprotein [31–33].

H_2-dependent [^{13}C]-CO_2 reduction to form [^{13}C]-formyl-MFR

Based on the composition of the Mvh-Hdr-Fmd megacomplex, the low-potential electrons provided in Hdr may be directly supplied to Fmd for the reduction of CO_2 and production of formyl-MFR. To test the possible activity, an enzyme assay was performed to detect the formation of formyl-MFR in the presence of MFR and CoM-S-S-CoB under CO_2 and H_2 in the absence of free ferredoxin. In the assay, the concentration of formyl-MFR was determined by mass spectrometry. MFR was isolated by the standard method from *Methanothermobacter marburgensis* cells [34], which yields MFR with ~ 10% contaminated formyl-MFR.

Unexpectedly, the control experiment using the 1-MDa megacomplex fraction without CoM-S-S-CoB showed a formyl-MFR degrading activity, which

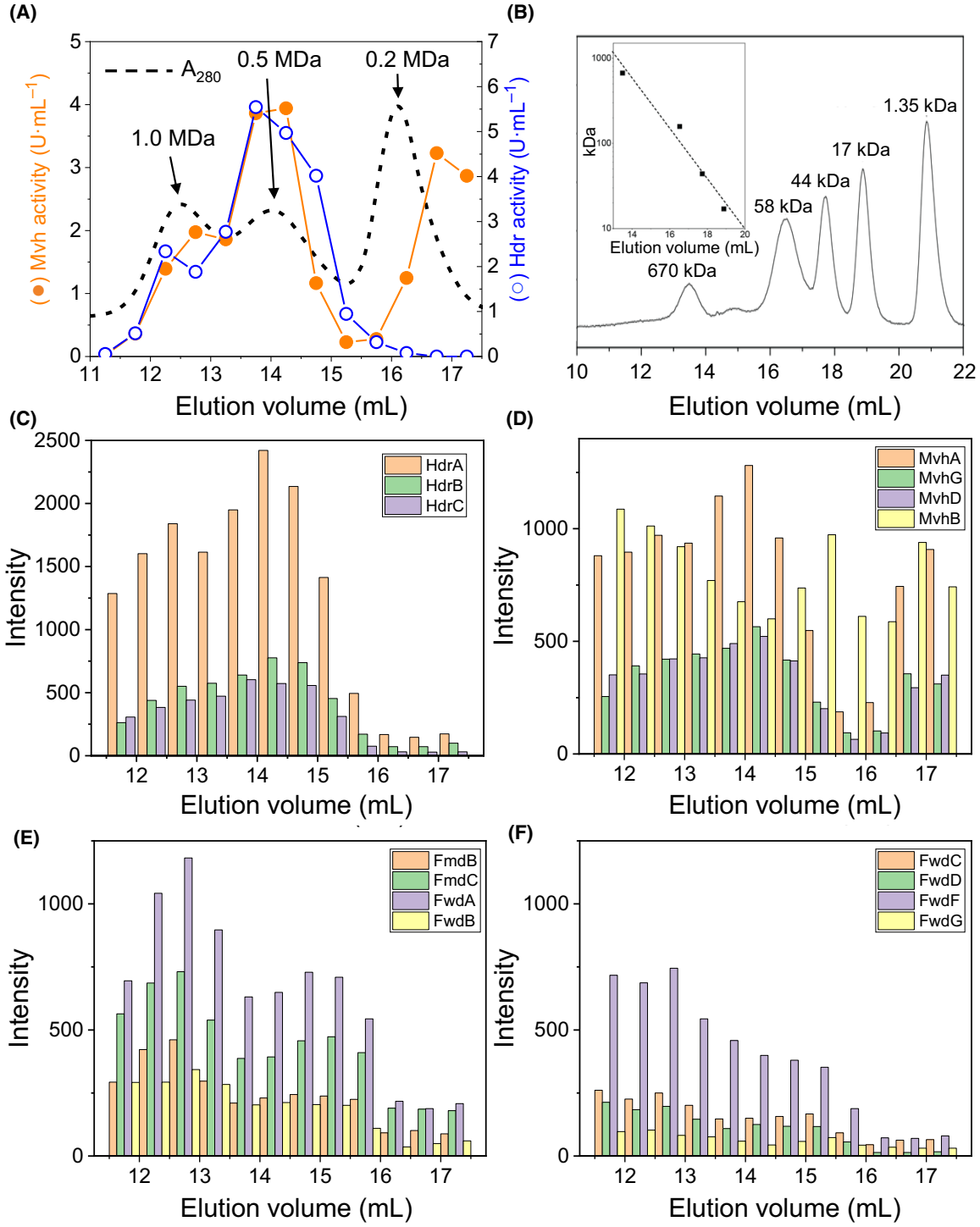

Fig. 3. Elution profile from size exclusion chromatography and proteome analysis. (A) UV–Vis absorbance (dashed line) and heterodisulfide reductase (Hdr) (open blue circle) and Hdr-associating [NiFe]-hydrogenase (Mvh) (closed orange circle) activities of the fractions (n = 3). (B) Calibration of elution profiles using protein standards (n = 2). (C–F) Proteome intensity of the protein subunits of Hdr (C), Mvh (D), and formylmethanofuran dehydrogenase isoenzymes (Fmd and Fwd) (E, F) in the fractions (n = 1). Intensity is expressed by the total number of identified peptide spectra matched for the protein (PSM).

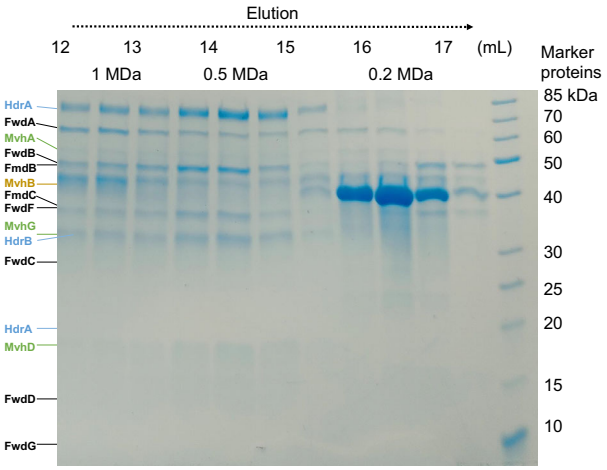

Fig. 4. SDS/PAGE of the size-exclusion chromatography (Superose 6 Increase) fractions. The proteins involved in 10 μL of the fraction at the elution volume were analysed (n = 1). The elution volumes of the fractions are indicated above the gel picture. The estimated sizes of the proteins (1, 0.5 and 0.2 MDa) in the fractions were indicated. The deduced positions of the subunits of heterodisulfide reductase (Hdr), Hdr-associating [NiFe]-hydrogenase (Mvh) and formylmethanofuran dehydrogenase isoenzymes (Fmd and Fwd) are indicated.

Table 1. Proteome data of 1-, 0.5- and 0.2-MDa fractions from size-exclusion chromatography with intensity indicated by iBAQ.

		Intensity ($\times 10^9$)		
Protein	kDa	1 MDa	0.5 MDa	0.2 MDa
HdrA	72	5.3	6.7	0.29
FwdA	63	4.0	1.3	0.57
MvhA	53	3.4	4.7	0.12
FwdB	49	0.80	0.31	0.08
FmdB	48	1.8	0.58	0.61
MvhB	44	3.4	1.2	0.79
FmdC	43	3.6	1.1	0.84
FwdF	39	8.7	4.4	1.0
MvhG	34	3.6	5.5	0.11
HdrB	33	2.8	5.0	0.08
FwdC	29	0.89	0.41	0.16
HdrC	21	1.8	4.2	0.08
MvhD	16	1.9	3.0	0.16
FwdD	14	1.2	0.50	0.10
FwdG	9	2.8	1.0	0.79

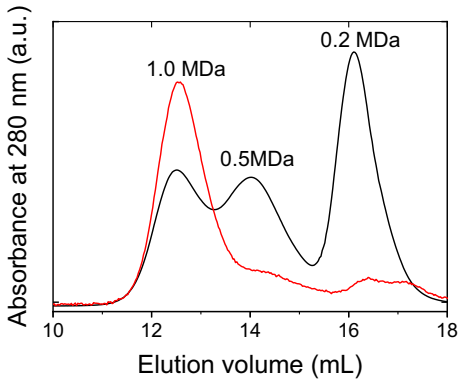

Fig. 5. Reloaded chromatography of the 1-MDa fraction from the size-exclusion column. The 100 μL of the ~ 1.0 MDa peak fraction in the first size-exclusion chromatography shown in Fig. 3A was reloaded onto the same size-exclusion column (n = 2). The absorbance at 280 nm is an arbitrary unit (a.u.) that does not represent the absolute concentration of the proteins in the two elutions. The initial elution profile (black line) and the reloaded 1-MDa fraction from the initial chromatography (red line).

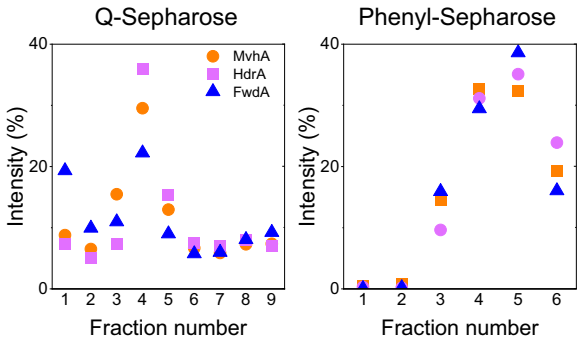

Fig. 6. Distribution of the representative subunits of the Mvh-Hdr-Fmd complex; MvhA (orange circle), HdrA (purple square) and FwdA (blue triangle) in the eluted fractions from Q-Sepharose and phenyl-Sepharose. Hdr, heterodisulfide reductase; Fwd, tungstopterin-containing formylmethanofuran dehydrogenase; Mvh, Hdr-associating [NiFe]-hydrogenase. The step-gradient fractions 1–9 in Q-Sepharose were eluted at 0–1.0 M NaCl concentrations. The step-gradient fractions 1–6 in phenyl-Sepharose eluted at 0.6–0 M ammonium sulfate concentrations. The proteome intensity of the subunits HdrA, MvhA, and FwdA is expressed as a percentage of the total number of identified peptide spectra matched for the protein (PSM) of each subunit (n = 1).

decreased the formyl-MFR concentration in the assay (Fig. 7A). Fmd/Fwd catalyzes oxidation of formyl-MFR, which is the standard assay method of this enzyme [6], in which methylviologen is added as an electron acceptor. The Mvh-Hdr-Fwd complex and/or dissociated Fmd/Fwd might catalyze oxidation of formyl-MFR using an unknown electron acceptor in the assay or the hydrolysis of formyl-MFR to formate and MFR. The formyl-MFR degrading rate decreased in the test assay containing the complete set of materials for the enzyme reaction, indicating that the formyl-MFR degrading activity also occurs in the test assay; in addition, this formyl-MFR degrading activity is higher than the formyl-MFR forming activity. To

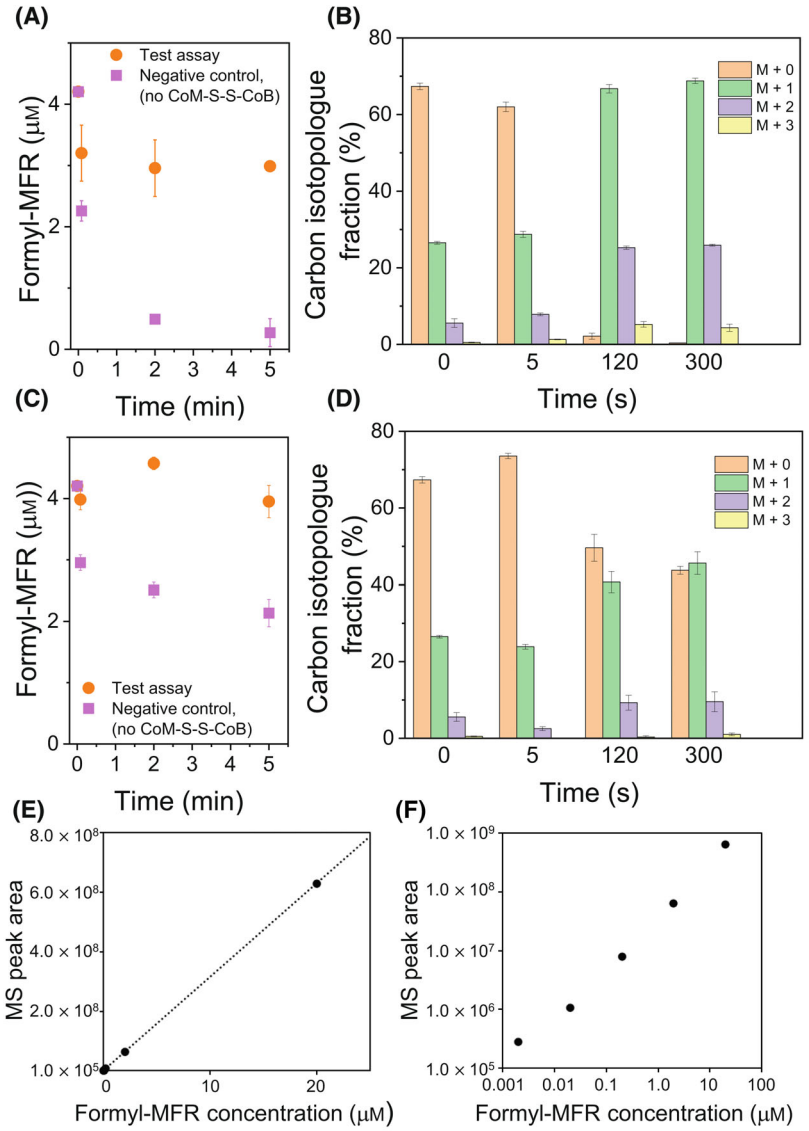

Fig. 7. H_2-dependent [^{13}C]-CO_2 conversion reaction to form [^{13}C]-formylmethanofuran (formyl-MFR). (A, C) Concentration of formyl-MFR in the enzyme assay for the 1 MDa (A) and 0.5 MDa (C) fractions. (B, D) Isotope distribution of formyl-MFR in the assay of the 1 MDa (B) and 0.5 MDa (D) fractions. The carbon isotopologue fraction (%) of formyl-MFR (771 *m/z*), including [$^{13}C_0$]-formyl-MFR (M + 0), [$^{13}C_1$]-formyl-MFR (M + 1), [$^{13}C_2$]-fromyl-MFR (M + 2), and [$^{13}C_3$]-formyl-MFR (M + 3) is shown. The carbon isotopologue fraction of the 0-min samples is the same as the natural abundance. Error bars correspond to the standard error of three independent measurements. (E, F) Standard curve of the determination of formyl-MFR using mass spectrometric (MS) peak area. The concentration of formyl-MFR was determined using the extinction coefficient of formyl-MFR ($\varepsilon 273 = 1100\ M^{-1}\cdot cm^{-1}$) [42]. The calibration line using the formyl-MFR standard with the absolute number (E) and with logarithmic scales (F) are shown.

measure the formyl-MFR-forming activity of the Mvh-Hdr-Fmd complex, we determined the incorporation of ^{13}C into formyl-MFR from [^{13}C]-CO_2. The mass spectrometric data indicated that the formyl-MFR molecule was fully labelled with ^{13}C within 120 s (Fig. 7B). In the case of the 0.5-MDa fraction, which contains a lower amount of the 1-MDa Mvh-Hdr-Fmd complex, the formyl-MFR-degrading activity was lower than that of the 1.0-MDa fraction (Fig. 7C), and the rate of ^{13}C incorporation into formyl-MFR was slower than that of the 1-MDa fraction (Fig. 7D). This result indicated that the 1-MDa Mvh-Hdr-Fmd complex catalyzes the H_2-dependent electron-bifurcating CO_2 reduction to form formyl-MFR.

Conclusion

In biological enzyme systems, reactions often occur in a large enzyme complex to increase the efficiency of sequential reactions [19,35]. In this work, we purified and characterized the Mvh-Hdr-Fmd complex from *Methanothermobacter marburgensis*. The proteome elution patterns of the Mvh, Hdr and Fmd (and Fwd) subunits in chromatography indicated that the Mvh-Hdr complex is complexed with Fmd and Fwd in *Methanothermobacter marburgensis*. The molecular mass of the 1-MDa Mvh-Hdr-Fmd complex in this study is similar to the dimeric Fdh-Hdr-Fmd from *Methanospirillum hungatei* [19]. However, the two Hdr megacomplexes exhibit differences in the phylogenetically distantly

related methanogenic archaea, that is, MvhB polyferredoxin is present in the Mvh-Hdr-Fmd complex from *Methanothermobacter marburgensis*. MvhB is not present in the *Methanospirillum hungatei* genome and is therefore not found in the purified Fdh-Hdr-Fmd complex of this methanogenic archaeon [27,36]. In size-exclusion chromatography, MvhB was broadly distributed in the fractions containing the megacomplex, subcomplexes and individual modules, which suggests that MvhB exhibits binding affinity to all components of the Mvh-Hdr-Fmd complex, forming an electron relay. The 1-MDa Mvh-Hdr-Fmd megacomplex catalyzed the reduction of CO_2 to form formyl-MFR under H_2 in the absence of external free-ferredoxin. Therefore, in the Mvh-Hdr-Fmd complex, low-potential electrons (which are generated by flavin-based electron bifurcation in Hdr) transfer to the CO_2 reduction active site in Fmd through an electron-transfer chain composed of iron–sulfur clusters, which probably includes MvhB polyferredoxin.

Materials and methods

Materials

Methanothermobacter marburgensis DSM2133 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany). Most chemicals were obtained from Sigma–Aldrich (Taufkirchen, Germany). Heterodisulfide (CoM-S-S-CoB) was synthesized as described previously with some modifications [14,37].

Cultivation methods

The medium contained 6.8 $g{\cdot}L^{-1}$ (50 mM) KH_2PO_4, 2.544 $g{\cdot}L^{-1}$ (24 mM) Na_2CO_3, 2.12 $g{\cdot}L^{-1}$ (40 mM) NH_4Cl, 0.2 mM $MgCl_2{\cdot}6H_2O$, 50 μM $FeCl_2{\cdot}4H_2O$, 5 μM $NiCl_2{\cdot}6H_2O$, 1 μM $CoCl_2{\cdot}6H_2O$, 1 μM $NaMoO_4{\cdot}2H_2O$, and 0.09 $g{\cdot}L^{-1}$ Titriplex. The concentrated trace element solution containing 0.2 M $MgCl_2{\cdot}6H_2O$, 50 mM $FeCl_2{\cdot}4H_2O$, 5 mM $NiCl_2{\cdot}6H_2O$, 1 mM $CoCl_2{\cdot}6H_2O$, 1 mM $NaMoO_4{\cdot}2H_2O$, and 90 $g{\cdot}L^{-1}$ Titriplex I was prepared separately and adjusted to pH 6.7 by the addition of NaOH. The trace element solution (0.1% v/v) was added to the medium. A 0.2% water solution of resazurin sodium salt was finally added to the medium (final concentration 0.6 $mg{\cdot}L^{-1}$). We used a 360-mL glass fermenter for the continuous cultivation of *Methanothermobacter marburgensis*. Anoxic medium was fed by a peristaltic pump with a controlled flow rate. The feeding medium was pressurized by N_2 ($\leq \sim +0.1$ bar). An 80% H_2/20% CO_2/0.2% H_2S mix gas was supplied through a glass sparger. The temperature of the glass vessel was controlled at 65 °C by circulating water from a water bath. The medium was stirred with a plastic stirrer bar at ~ 300 r.p.m. The cells were harvested by anaerobic centrifugation using Beckman JA-25.50 at 13 000 ***g*** for 15 min at 4 °C.

Preparation of cell extract for the enzyme assay

All steps were performed anaerobically in an anaerobic chamber (Coy Laboratories, Grass Lake, MI, USA). Frozen *Methanothermobacter marburgensis* cells (2 g) were suspended in 5 mL of 50 mM Tris/HCl pH 7.6 containing 2 mM dithiothreitol. The cell suspension was subjected to ultrasonication on ice/water for 2 min using a SONOPULS GM200 (Bandelin, Berlin, Germany) with a 72D tip with 30% cycles 12 times, with 2 min of braking between sonication cycles. After transferring the sample solution to centrifuge tubes, the sonicated vessel was rinsed with buffer solution, and the rinsed buffer solution was combined with the sonicated sample to make a total volume of 11 mL. The undisrupted cells were removed by centrifugation in an Avanti JXN-26 with a JA-25.50 at 13 000 r.p.m. (20 000 ***g***) for 30 min at 4 °C.

Protein purification

The supernatant containing ~ 100 mg of protein was loaded on a Q-Sepharose (HiTrap Q-HP) from Merck (Darmstadt, Germany) (5 mL) column, which was equilibrated with 50 mM Tris/HCl pH 7.6 containing 2 mM dithiothreitol (buffer A). The proteins bound on the column were eluted with a step gradient using 50 mM Tris/HCl pH 7.6 containing 2 mM dithiothreitol and 1 M NaCl (buffer B). The step gradient was 30%, 40%, 44%, 48%, 52%, 56%, 60% and 100% buffer B with a 2 $mL{\cdot}mL^{-1}$ flow rate. The proteins with Hdr activity were mainly eluted in the 48% or 52% buffer B fraction of the Q-Sepharose chromatography, depending on the experiments. The fraction that contained the greatest Hdr activity was collected and diluted with the same volume of 50 mM Tris/HCl pH 7.6, containing 2 mM dithiothreitol and 1.2 M ammonium sulfate (buffer C). The diluted sample was loaded on a phenyl-Sepharose (HiTrap Phe-HP) (5 mL) column from Merck equilibrated with buffer C. The proteins bound on the column were eluted with a step gradient using 50%, 58%, 67%, 83% and 100% buffer A with a 2 $mL{\cdot}min^{-1}$ flow rate. The elution conditions of Q-Sepharose and phenyl-Sepharose columns are according to a previous method used to purify the Mvh-Hdr complex from *Methanothermobacter marburgensis* [22]. The buffer solution of the 33% buffer C fraction of the Phenyl Sepharose column containing 0.4 M ammonium sulfate was exchanged with 50 mM Tris/HCl pH 7.6 containing 2 mM dithiothreitol and 150 mM NaCl by an Amicon Ultra 0.5 (3 kDa cut-off) filter. The sample was finally concentrated to ~ 0.5 mL and applied to a size-exclusion Superose 6 Increase column (10/300 GL) and eluted with a 0.5 $mL{\cdot}min^{-1}$ flow rate. The size-exclusion column was calibrated with thyroglobulin (bovine) at 670 kDa, γ-globulin (bovine) at

158 kDa, ovalbumin (chicken) at 44 kDa, myoglobin (horse) at 17 kDa and vitamin at B12 1.35 kDa (Fig. 3B). The standard materials were from Bio-Rad (Feldkirchen, Germany).

Enzyme assays

The activity of Mvh in the purification of the complexes was measured using 2 mM benzyl viologen (BV), and the activity was calculated using the extinction coefficient of BV at 578 nm (8.6 $mM^{-1}\cdot cm^{-1}$) [38]. For the assay, 0.7 mL of 50 mM Tris/HCl pH 7.6 containing 10 mM DTT was preincubated at 65 °C for 5 min. Typically, 7 µL of 200 mM BV were added as the substrate to a 1-mL quartz cuvette (1 cm light path) to form a 2 mM final concentration, and then 10 µL of 20 mM sodium dithionite were added to form a 290 µM final concentration. The enzyme reaction was started by adding 10 µL of diluted cell extract at 65 °C. One unit of enzyme activity is defined as the oxidation of 2 µmol of BV per minute.

Hdr activity was determined by recording the oxidation of reduced BV by CoM-S-S-CoB [16]. For the assay, 0.7 mL of 800 mM potassium phosphate buffer pH 7.0 was preincubated at 65 °C for 5 min. Seven microlitres of 200 mM BV were added to a 1 mL quartz cuvette (1 cm light path) to form a 2 mM final concentration, and then 10 µL of 20 mM sodium dithionite were added. The enzyme reaction was started by the addition of 7 µL of 100 mM CoM-S-S-CoB to form a 1 mM final concentration at 65 °C. The activity was calculated using the extinction coefficient of BV [38]. One unit of enzyme activity is defined as the oxidation of 2 µmol of BV per minute.

The activity of Mvh-Hdr-Fmd was determined by the formation of formyl-MFR from MFR and [^{13}C]-CO_2 using H_2 as an electron donor. The formation of formyl-MFR was determined by mass spectrometric analysis. As the standard, formyl-MFR was synthesized from MFR, which was isolated from *Methanothermobacter marburgensis* cells [34]. In the anaerobic chamber, a 0.11-mL reaction mixture containing 20 µM MFR and 1 mM heterodisulfide in 800 mM phosphate buffer pH 7.0 was prepared in 1.5-mL amber vials and closed with a rubber stopper. After evacuation and refilling with 100% H_2 (without overpressure), 0.3 mL [^{13}C]-CO_2 (99% from Sigma-Aldrich) was injected. The reaction was started by adding the 0.05 $mg\cdot mL^{-1}$ 1-MDa and 0.07 $mg\cdot mL^{-1}$ 0.5-MDa fractions (final concentration). In the negative control, heterodisulfide was omitted from the reaction mixture. After incubation at 65 °C, the sample vial was cooled in ice water and then exposed to air to inactivate the enzymes.

Mass spectrometric protein profile analysis

The mass spectrometric protein profile was analysed as described previously [39]. Cell pellets were lysed and reduced by 5 mM tris(2-carboxyethyl)phosphine (TCEP) in the presence of 2% deoxycholate (DOC) at 90 °C for 10 min. Based on the protein concentration measured by the bicinchoninic acid method, the amount of protein in the samples was unified for comparison. After that, the samples were incubated at 25 °C for 30 min in 100 mM ammonium bicarbonate pH 8.2 and 10 mM iodacetamide (IAA) and then digested overnight at 30 °C with trypsin, MS approved (Serva, Heidelberg, Germany). Before LC-MS analysis, the samples were desalted using Chromabond Spin C18 WP (Macherey-Nagel, Heppenheim, Germany) according to the manufacturer's instructions. Peptides were analysed using liquid chromatography-mass spectrometry using an Orbitrap Exploris 480 equipped with an Ultimate 3000 RSLC nano and a nanospray ion source (Thermo Scientific, Dreieich, Germany). A reversed-phase HPLC column (75 µm × 42 cm) packed with C18 resin (2.4 µm; Dr. Maisch, Ammerbuch, Germany) was used for peptide separation with formic acid/acetonitrile as solvents in a 45 -min gradient. MS data were searched against an in-house *Methanothermobacter marburgensis* protein database using SEQUEST embedded into PROTEOME DISCOVERER 1.4 software (Thermo Scientific). In the case of the analysis of the protein fraction by size-exclusion chromatography, the purified fraction was directly used for mass spectrometric analysis. The total number of identified peptide spectra matched for the protein (PSM) value was used to evaluate the number of peptides identified for high-scoring proteins. The PSM value could be higher than the number of peptides identified for high-scoring proteins because peptides are identified repeatedly. To compare the intensity between different proteins, we calculated intensity-based absolute quantification (iBAQ) [40].

Determination of formyl-MFR

Quantitative and qualitative determination of the mass of formyl-MFR was performed using HRES LC-MS as described in the previous publication [41]. The chromatographic separation was performed on a Thermo Scientific Vanquish HPLC System using a Kinetex Evo C18 column (150 × 0.12 mm, 100 Å, 1.7 µm; Phenomenex, Aschaffenburg, Germany) equipped with a 20 × 2.1 mm guard column of similar specificity at a constant eluent flow rate of 0.2 $mL\cdot min^{-1}$ and a column temperature of 30 °C, with eluent A being 0.1% formic acid in water and eluent B being 0.1% formic acid in MeOH (Honeywell, Morristown, NJ, USA). The injection volume was 5 µL. The mobile phase profile consisted of the following steps and linear gradients: 0–2 min constant at 0% B; 2–6 min from 0% to 90% B; 6–8 min constant at 90% B; 8–8.1 min from 90% to 0% B; 8.1–10 min constant at 0% B. A Thermo Scientific ID-X Orbitrap mass spectrometer was used in positive mode with a high-temperature electrospray ionization source and the following conditions: H-ESI spray voltage at 2500 V, sheath gas at 35 arbitrary units, auxiliary gas at 7 arbitrary units, sweep gas at 1 arbitrary unit, ion transfer tube temperature at 300 °C, vaporizer temperature at

275 °C. Detection was performed in full scan mode using the orbitrap mass analyser at a mass resolution of 240 000 in the mass range 700–850 (m/z). Extracted ion chromatograms of the $[M + H]^+$ forms were integrated using TRACEFINDER software (Thermo Scientific), applying a mass tolerance of 5 p.p.m. Detection was performed in a combined full scan/targeted MS2 mode using the orbitrap mass analyser. Full scan measurements were performed at a mass resolution of 240 000 in the mass range 700–850 (m/z). The $[M + H]^+$ mass-to-charge ratio of formyl-methanofuran was targeted for fragmentation with a mass tolerance of 25 p.p.m., fragmented using a stepped HCD collision at energies of 15%, 30% and 45%. Fragments were analysed at a mass resolution of 30 000 using the orbitrap analyser. Extracted ion chromatograms of the $[M + H]^+$ forms were integrated using TRACEFINDER software (Thermo Scientific). Absolute concentrations were calculated based on an external calibration curve (Fig. 7E,F).

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

SS contributed to study conceptualisation. SN contributed to microbiological and biochemical investigations. NP contributed to mass spectrometric analysis for the enzyme assays. JK contributed to the proteome analysis. SS contributed to funding acquisition. SN and SS contributed to analysing and interpreting the data and writing original draft. NP and JK contributed to writing the methods, reviewing and editing. SS and SN contributed to visualization.

Data availability statement

Any additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

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