## Supplementary Materials: Crystal Structures of [Fe]-Hydrogenase from *Methanolacinia paynteri* Suggest a Path of the FeGP-Cofactor Incorporation Process

Gangfeng Huang, Francisco Javier Arriaza-Gallardo, Tristan Wagner and Seigo Shima



**Figure S1.** Observation of subtle rearrangements of the asymmetric pHmd when the C-terminal domain of holo-jHmd and apo-jHmd are superposed. (**a**, **b**) C-terminal domain superposition between the apo-form of the asymmetric pHmd homodimer (orange) and apo-form of jHmd (light pink, PDB: 2B0J). The chain from the next monomer of pHmd in the central domain is distinguished by green color. In panel **a**, the superposition was done on the C-terminal domain of the next monomer (orange) and in panel **b**, the superposition between the holo-form of the asymmetric pHmd homodimer (green). (**c**, **d**) C-terminal domain superposition between the holo-form of the asymmetric pHmd homodimer (green) and holo-form of jHmd (cyan, PDB: 3F47). The chain from the next monomer of pHmd in the central domain is distinguished by orange color. In panel **c**, the superposition was done on the C-terminal domain from the next monomer of pHmd in the central domain is distinguished by orange color. In panel **c**, the superposition was done on the C-terminal domain from the second monomer (orange) and in panel **d**, the superposition form the second monomer (orange) and in panel **d**, the superposition was done on the C-terminal domain from the second monomer (orange) and in panel **d**, the superposition was done on the C-terminal domain of the same monomer (in green).



**Figure S2.** Superposition of the FeGP cofactor binding sites in pHmd (PDB: 6YKA), Hmd from *Methanococcus aeolicus* (aHmd) (PDB: 6HAC) and jHmd (PDB: 3F47). The protein parts are shown as cartoon model, the FeGP cofactor and the side chain of residues related to its binding are shown in ball and stick model.



**Figure S3.** Comparison of the loop involved in the FeGP cofactor coordination in the jHmd and pHmd apo-forms. Apo-jHmd was colored in black. Apo-pHmd of the pHmd asymmetric homodimer was indicated by colors (dark blue with low B-factor to green with higher B-factor). The FeGP cofactor from the holo structure was superposed to indicate FeGP position (a transparent ball and stick model).

## Methanolacinia\_paynteri

Methanolacinia\_paynteri

Methanolacinia\_paynteri

Methanolacinia\_paynteri Methanococcus\_aeolicus

Methanolacinia\_paynteri

Methanolacinia\_payneri Methanolacinia\_payneri Methanothermococcus\_thermolithotrophicus Methanotris\_igneus Methanothermis\_fervidus Methanothermis\_fervidus Methanothermis\_fervidus Methanothermobacter\_marburgensis Methanoregula\_formicicu Methanoregula\_formicicu



α2 ηι <u>000000000</u> <u>000</u> **3**0 **4**0

α3 0000000000 50

β1

α1 200000 20





		β10	α10		α11		
Methanolacinia_paynteri	0000000	<u> </u>	<u>0000000</u>	2 2222	00000000	000000000	000000000
	230	240	250	2	260	270	280
Methanolacinia paynteri	ELGKKARG	HAFKL	PAELIGPV	DMCAALT	AITYAGL	VYRDAVMNI	LGAPAGESOMMAT
Methanococcus aeolicus	KIAKISRC	TAFKM	PANLISPVO	DMGSAVT	APVYAAII	SYRDAVINI	LGAPADEAOMMADE
Methanothermococcus thermolithotrophicus	CIAKEARG	ТАҮКМ	PANLISPVO	DMGSAVT	APVYAAVI	AYRDAVIKI	LGAPADEAOMMAD
Methanocaldococcus jannaschii	EIGKIARG	KAFKM	PANLIGPVO	DMCSAVT	ATVYAGLI	AYRDAVTKI	LGAPADEAOMMADE
Methanotorris igneus	ALAOKARG	TAYKL	PANLISPVO	DMGSAVT	ATVYAGLI	AYRDAVTKI	LGAPADEAOMMAE
Methanobrevibacter ollevae	ELGLKARG	SAFTL	PANMVGPVO	DMCSAVT	AITYAGII	SYRDIVTOI	LGAPAGEAOSMAN
Methanothermus fervidus	DIAKKARC	NAFKL	PAELLGPVC	DMCSALT	AITYAGII	SYRNSVMNI	LCAPADEAOMMAK
Methanobacterium formicicum	DWGVAARG	DAFKL	PAELLGPVC	DMCSALT	AITYAGII	SYRDSVMNI	LGAPAGEAOMMAK
Methanothermobacter marburgensis	ELGOKARG	NAYRL	PAELLGPVC	DMCSALT	AITYAGII	SYRDSVTON	LGAPASFAOMMAK
Methanoregula formicica	TIGKTARG	EAFTL	PADMLGPVO	DMCAALT	AVTYAGII	TYRESVMNV	LGAPAGE TOMMAKE
Methanocorpusculum labreanum	DLGHKARG	TAYKL	PAELLGPVC	DMCAALT	AVTYAGLI	TYRESVMNV	LGAPAGEAOMMAA
-							
	~12		m7 cr12			~14	
M-11	0.000000		17 0.15	10	- up	0.00000000	
Methanolacinia_paynteri	200	2000		222222	200	220	200000
	290	300	211			2.211	341
Methanolacinia navnteri		_ `_		_			
hechanoracrinia_payheerr	SLEQITAY	MKKV <mark>G</mark>	IKNLEENLI	PGVFLGT	ADSMNFG	IABILPTVI	KSLEKRAK
Methanococcus_aeolicus	SLEQITAY AITQMLEL	MKKV <mark>G</mark> MRNE <mark>G</mark>	IKNLEENLI IQNMENKLI	PGVFLGT	AD SMNFGE AD SMCFGE	ABILPTVI LSELLPASI	KSLEKRAK
Methanococcus_aeolicus Methanothermococcus_thermolithotrophicus	SLEQITAY AITQMLEL AITQILEL	MKKVG MRNEG MRKEG	IKNLEENLI IQNMENKL INNMEKTLI	PGVFLGT IPGALTGT PKALT <mark>GT</mark>	ADSMNFGE ADSMCFGE ADSM <mark>CFG</mark> E	IAEILPTVI LSELLPASI LSDIL <mark>P</mark> PAI	KSLEKRAK. KVLEEHKK KVLEKHAAEEES.
Methanococcus_aeolicus Methanothermococcus_thermolithotrophicus Methanocaldococcus_jannaschii	SLEQITAY AITQMLEL AITQILEL ALTQIHNL	MKKVG MRNEG MRKEG MKEKG	IKNLEENLI IQNMENKLI INNMEKTLI IANMEEALI	PGVFLGT IPGALTGT PKALTGT PAALLGT	ADSMNFGE ADSMCFGE ADSMCFGE ADSMCFGE	IAEILPTVI LSELLPASI LSDILPPAI LAEILPTAI	KSLEKRAK. KVLEEHKK. KVLEKHAAEEES. KVLEKHKVVEEGI
Methanococcus_aeolicus Methanochermococcus_thermolithotrophicus Methanocaldococcus_jannaschii Methanocorris_igneus	SLEQITAY AITQMLEL AITQILEL ALTQIHNL AITQLLEL	MKKVG MRNEG MRKEG MKEKG MKEKG	I KN LEEN LI I QNMENKL I NNMEKTLI I ANMEEALI I ANMEKVL	PGVFLGT NPGALTGT PKALTGT PAALLGT NPKALTGT	ADSMNFGE ADSMCFGE ADSMCFGE ADSMCFGE ADSMCFGE	IAEILPTVI LSELLPASI LSDILPPAI LAEILPTAI LAEILPTAI	KVEEKHAAEEES KVEEKHAAEEES KVEEKHAAEEES KVEEKHKVVEEEGI
Methanococcus_aeolicus Methanothermococcus_thermolithotrophicus Methanocaldococcus_jannaschii Methanotorris_igneus Methanotorris_cigneus	SLEQITAY AITQMLEL AITQILEL ALTQIHNL AITQLLEL ALTQLLEL ALTNVVAL	MKKVG MRNEG MRKEG MKEKG MKEEG MKKEG MQNEG	I KN LEENLI I QNMENKLI I NNMEKTLI I ANMEEALI I ANMEKVLI I ANMEKVLI I DKMDDALI	PGVFLGT NPGALTGT PKALTGT PAALLGT NPKALTGT NPGALLGT	ADSMNFGE ADSMCFGE ADSMCFGE ADSMCFGE ADSMCFGE ADSMCFGE	IAEILPTVI LSELLPASI LSDILPPAI LAEILPTAI LADILPQAI	LKSLEKRAK. KVLEEHKK KVLEKHAAEEES. KVLEKHKVVEEGI EVLKKHGVEDEGI ESLEKRSK
Methanococcus_policus Methanothermococcus_thermolithotrophicus Methanocaldococcus_jannaschii Methanotorris_igneus Methanobrevibacter_olleyae Methanothermus_fervidus	SLEQITAY AITQMLEL AITQILEL ALTQIHNL AITQLLEL ALTNVVAL SLEQLTNL	MKKVG MRNEG MRKEG MKEKG MKKEG MCNEG MEKVG	IKNLEENLI QNMENKLI INNMEKTLI IANMEEALI IANMEKVLI IDKMDDALI IDKMEEKLI	PGVFLGT NPGALTGT PKALTGT PAALLGT NPKALTGT NPGALLGT PKALLGT	ADSMNFG ADSMCFG ADSMCFG ADSMCFG ADSMCFG ADSMCFG ADSMNFG	IAEILPTVI LSELLPASI LSDILPPAI LAEILPTAI LADILPQAI LSEIVPTVI	KSTEKRAK. KVEEKRAK. KVEEKHAAEEES. KVEKHKVVEEGJ EVEKKHGVEDEGEI ESLEKRSK. KY
Methanococcus_aolicus Methanochermoccus_thermolithotrophicus Methanocaldococcus_jannaschii Methanotorris_igneus Methanotorvibacter_olleyae Methanothermus_fervidus Methanotherterium_formicium	SLEQITAY AITQMLEL AITQILEL ALTQIHNL AITQLEL ALTQUHNL SLEQLINL SLEQLINL SLTQVTDL	MKKVG MRNEG MRKEG MKEKG MKKEG MEKVG MEKVG MNSVG	IKNLEENLI QNMENKLY INNMEKTLI IANMEEALI IANMEKVLY IDKMDDALY IDKMEEKLI IDHMEEKLI	PGVFLGT PGALTGT PKALTGT PAALLGT NPKALTGT NPGALLGT PKALLGT PGALLGT	ADSMNFG ADSMCFG ADSMCFG ADSMCFG ADSMCFG ADSMNFG ADSMNFG ADSMNFG	IASILPTVI LSELLPASI LSDILPPAI LAEILPTAI LADILPQAI LSEIVPTVI LSEILPTVI AADVLPSVI	KYLEKRAK. KYLEKRAK. KYLEKHAAEES. KYLEKHKVEEGG EVKKHGVEDEGE ESEKRSK. KYLEKEKK. EVENRKGKGP.
Methanococcus_aeolicus Methanothermococcus_thermolithotrophicus Methanocaldococcus_jannaschii Methanotorris_igneus Methanothermus_fervidus Methanothermus_fervidus Methanothermobacter_marburgensis	SLEQITAY AITQMLEL AITQILEL ALTQILEL ALTQILEL ALTNVVAL SLEQLTNL SLEQITAL SLEQITAL	MKKVG MRNEG MRKEG MKEKG MKEG MEKVG MEKVG MNSVG MEKVG	IKNLEENI IQNMENKI INNMEKTI IANMEEAI IANMEKVI IDKMEEKI IDHMEEKI IDKMEENI	PGVFLGT PGALTGT PKALTGT PAALLGT PKALTGT PKALLGT PKALLGT PGALLGT	ADSMNFG ADSMCFG ADSMCFG ADSMCFG ADSMCFG ADSMNFG ADSMNFG ADSMNFG ADSMNFG	TAELPTVI LSELLPASI LSDILPPASI LAEILPTAI LAEILPTAI LSEIVPTVI LSEILPTVI AADVLPSVI	KUEEKRAK. KVIEEHKK. KVIEEHKK. KVIEKHKVVEEG EVKKHGVEDEGEI ESIEKRSK. KVIEKRSK. EVIENRKGKGP.
Methanococcus_seolicus Methanothermococcus_thermolithotrophicus Methanotaldococcus_jannschii Methanotroris_igneus Methanothermos_fervidusuus Methanothermos_fervidusuus Methanothermohacter_marburgensis Methanoregula_formicia	SLEQITAY AITQMLEL AITQILEL ALTQIHNI AITQLLEL ALTNVVAL SLEQITAL SLEQITAL	M K V G G M K N E G G M R K E K E G M K E K E G G M K V G M N S V G M N S V G M N S V G M N R V G M N R V G	I KNLEENI I QNMENKI I NNMEKTI I ANMEEAI I ANMEKVI I DKMEEKI I DKMEEKI I DKMEENI I DKMEENI	PGVFLGT PGALLGT PKALLGT PAALLGT PAALLGT PGALLGT PGALLGT PGALLGT PGALLGT	ADSMNFG ADSMCFG ADSMCFG ADSMCFG ADSMCFG ADSMNFG ADSMNFG ADSMNFG ADSMNFG ADSMNFG	TABLETVI LSELLPASI LSDILPPAI LADILPQAI LSEIVPTVI LSEIVPTVI SABILPTVI VGEILPPVI	KUEKRAK KUEEHKK. KUEEHKAEEES. KUEKHAEEES. EVEKKUVEEGEI ESEEKROK KUEEKK EVERKGKGP. EILEKRKK KAEEKKK

## Methanolacinia\_paynteri

nothermobacter\_marburgensis noregula\_formicica nocorpusculum\_labreanum

Methanolacinia_paynteri										
Methanococcus_aeolicus										,
Methanothermococcus_thermolithotrophicus	C	С	С	Ε	L	Κ	Κ			
Methanocaldococcus jannaschii	Τ	Κ	С	Е	I	М	S	Q	K	E
Methanotorris_igneus	Т	Κ	С	Е	Ι	М	s	õ	А	ľ
Methanobrevibacter olleyae										
Methanothermus fervidus										
Methanobacterium formicicum		Т	С	N	Ι					
Methanothermobacter marburgensis										
Methanoregula formicica										1
Methanocorpusculum labreanum										
	•	1	1	1	1		-	-	1	ľ

Figure S4. Alignments of Hmd amino acid sequences from different organisms. All sequences are obtained from NCBI database. Alignment was performed by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The figure was made by ESPript 3.0 [1]. Lys150 was highly conserved in [Fe]-hydrogenase except Methanobrevibacter species (e.g. M. smithii) and marked with red closed circle in the figure.



S4/S8



**Figure S5.** Comparison of the GP-binding states in the structure of Hmd from *M. marburgensis* obtained in an oxidized broken state with that in the structure of the GP bound form of pHmd. (a) Hmd from *M. marburgensis* in an oxidized broken state. (b) The structure of the GP bound form pHmd. Hmd from *M. marburgensis* was in hexamer composed of the three open-form dimers, in which a loop containing Asp189 (colored in grey) from the nearby dimer is inserted into the active-site cleft. The pHmd structure was in dimer and does not harbor such an extra loop observed in Hmd from *M. marburgensis*.



**Figure S6.** Superposition of the active sites of the apo- and holo-forms of Hmd. Lys150 was shown in ball and stick model. The holoenzyme of Hmd from the *Methanococcus aeolicus* in complex with methenyl-H<sub>4</sub>MPT<sup>+</sup> (aHmd-FeGP/methenyl-H<sub>4</sub>MPT<sup>+</sup>), the apo-form of the asymmetric homodimer of pHmd (pHmd-apo), the GMP-bound form of pHmd (pHmd-GMP) and the GP-bound form of pHmd (pHmd-GP) are shown using the color indicated in the figure. The clash point between Lys150 side chain and the phenyl ring part of methenyl-H<sub>4</sub>MPT<sup>+</sup> was highlighted by red dash open circle.

**Enzymatic Reaction:** 

$$E + S_1 + S_2 \xrightarrow{k_1} [E-S_1-S_2] \xrightarrow{k_3} E + P_1 + P_2$$

Full set of ODEs:

$$\begin{aligned} \frac{dE}{dt} &= -k_1 \cdot S_1(t) \cdot E(t) + k_{-1} \cdot [E \cdot S_1 \cdot S_2](t) \\ \frac{dS_1}{dt} &= -k_1 \cdot S_1(t) \cdot E(t) + k_{-1} \cdot [E \cdot S_1 \cdot S_2](t) \\ \frac{d[S_2]}{dt} &= 0 \\ \frac{d[E \cdot S_1 \cdot S_2]}{dt} &= k_1 \cdot S_1(t) \cdot E(t) - k_{-1} \cdot [E \cdot S_1 \cdot S_2](t) - k_3 \cdot [E \cdot S_1 \cdot S_2](t) \\ \frac{d[P_1]}{dt} &= k_3 \cdot [E \cdot S_1 \cdot S_2](t) \\ \end{aligned}$$

Michaelis-Menten approximation:

$$K_{M} \cong \frac{k_{3} + k_{-1}}{k_{1}}$$
$$V(t) = -\frac{dS_{1}(t)}{dt} = \frac{k_{3}E_{0}S_{1}(t)}{S_{1}(t) + K_{M}}$$

**Figure S7.** Equations used for the simulation of the modelled reaction and calculation of the kinetic parameters. The results are shown in Figure S8. To calculate the apparent  $V_{\text{max}}$  and apparent  $K_m$ , we fixed the concentration of H<sub>2</sub> and H<sup>+</sup>. Therefore, the rate of the changes of H<sub>2</sub> and H<sup>+</sup> is zero and the model was approximated to the Michaelis-Menten (single substrate/product) equation.



Reduction of methenyl-H₄MPT<sup>+</sup> at pH 7.5

**Figure S8.** Simulation of the progressive curves of the reactions. The substrate consumption in the reduction of methenyl-H<sub>4</sub>MPT<sup>+</sup> with H<sub>2</sub> at pH 7.5 and the product formation in the oxidation of methylene-H<sub>4</sub>MPT at pH 6.0 in the enzymatic reaction mixture was recorded and simulated using the pHmd holo-forms (the wild-type and Lys150Ala variant) reconstituted under standard conditions. The kinetic parameters (apparent  $V_{max}$  and apparent  $K_m$ ) were obtained by simulation of the curves fitting using Michaelis-Menten equation as shown in Figure S7. Continuous line represents simulation of the modelled reaction, (+) shows experimental data. Simulations were performed in Python 3.7 using Spyder 4.1 and the SciPy library.

Enzymatic Reaction:

$$\begin{array}{c} \mathsf{FeGP} + \mathsf{apo-Hmd} \xleftarrow{k_2} \mathsf{holo-Hmd} + \mathsf{S}_1 + \mathsf{S}_2 & \xleftarrow{k_1} [\mathsf{Hmd-S}_1 - \mathsf{S}_2] \xrightarrow{k_3} \mathsf{holo-Hmd} + \mathsf{P}_1 + \mathsf{P}_2 \\ & \swarrow \\ \mathsf{FeGP} \end{array}$$

Full set of ODEs:

$$\begin{aligned} \frac{d[FeGP]}{dt} &= -k_2 \cdot [FeGP](t) \cdot [apo-Hmd](t) + k_{-2} \cdot [holo-Hmd](t) - k_4 \cdot [FeGP] \\ \frac{d[apo-Hmd]}{dt} &= -k_2 \cdot [FeGP](t) \cdot [apo-Hmd](t) + k_{-2} \cdot [holo-Hmd](t) \\ \frac{d[holo-Hmd]}{dt} &= k_2 \cdot [FeGP](t) \cdot [apo-Hmd](t) - k_{-2} \cdot [holo-Hmd](t) - k_1 \cdot [S_1](t) \cdot [holo-Hmd](t) + k_{-1} \cdot [Hmd-S_1 \cdot S_2] \\ \frac{d[S_1]}{dt} &= -k_1 \cdot [S_1](t) \cdot [holo-Hmd](t) + k_{-1} \cdot [Hmd-S_1 \cdot S_2] \\ \frac{d[S_2]}{dt} &= 0 \\ \frac{d[Hmd-S_1 \cdot S_2]}{dt} &= k_1 \cdot [S_1](t) \cdot [holo-Hmd](t) - k_{-1} \cdot [Hmd-S_1 \cdot S_2] - k_3 \cdot [Hmd-S_1 \cdot S_2](t) \\ \frac{d[P_1]}{dt} &= k_3 \cdot [Hmd-S_1 \cdot S_2] \\ \frac{d[P_2]}{dt} &= 0 \end{aligned}$$

**Figure S9.** Equations used for the simulation of the binding constant of the FeGP cofactor to the pHmd apoenzyme. The results are shown in Figure S10. For the calculation, the fixed concentrations of H<sub>2</sub> and H<sup>+</sup> were used. Therefore, the rate of the changes of H<sub>2</sub> and H<sup>+</sup> is zero and the model was approximated to the Michaelis-Menten equation. The decomposition rate (*k*<sub>4</sub>) of the FeGP cofactor to guanylylpyridinol (GP) in the reconstitution assay was experimentally determined (0.01  $\mu$ M·s<sup>-1</sup> at pH 7.5 and 0.007  $\mu$ M·s<sup>-1</sup> at pH 6.0). We assumed *k*-2 is zero and defined *k*<sub>2</sub> as reconstitution rate in the main text.



Reduction of methenyl-H₄MPT<sup>+</sup> at pH 7.5

**Figure S10.** Simulation of the change of the activity in the reconstitution assay of pHmd. Experimental data (+) is the same shown in Figure7c, d, g, h. The apparent  $V_{max}$  value was adjusted by the ratio between the specific activity of the reconstituted enzyme at 18 µM substrate concentrations and that of the maximum activity obtained in the reconstitution kinetic assays. Simulations were done using formulas described in Figure S9 and coded in Python 3.7 using Spyder 4.1 and the SciPy library. The simulated curves fit to the experimental data without considering cooperativity of the two binding sites of the apoenzyme. The simulation curves fit to the experimental data other than the reduction of methenyl-H4MPT<sup>+</sup> of the wild-type enzyme at pH 7.5 with higher FeGP cofactor concentrations (700 nM and 300 nM).

## Reference

1. Robert, X.; Gouet, P., Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res.* **2014**, *42*, W320-W324.