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

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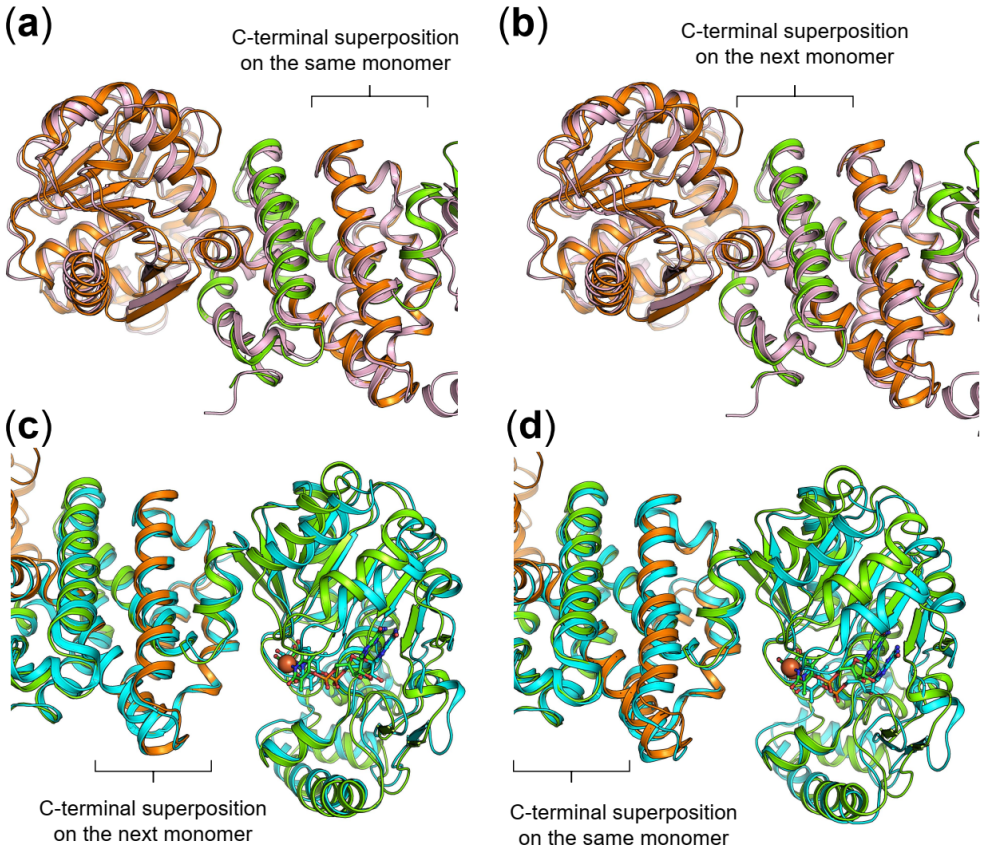


Figure S1. Observation of subtle rearrangements of the asymmetric pHmd when the C-terminal domain of holo-jHmd and apo-jHmd are superposed. (**a**, **b**) C-terminal domain superposition between the apo-form of the asymmetric pHmd homodimer (orange) and apo-form of jHmd (light pink, PDB: 2B0J). The chain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

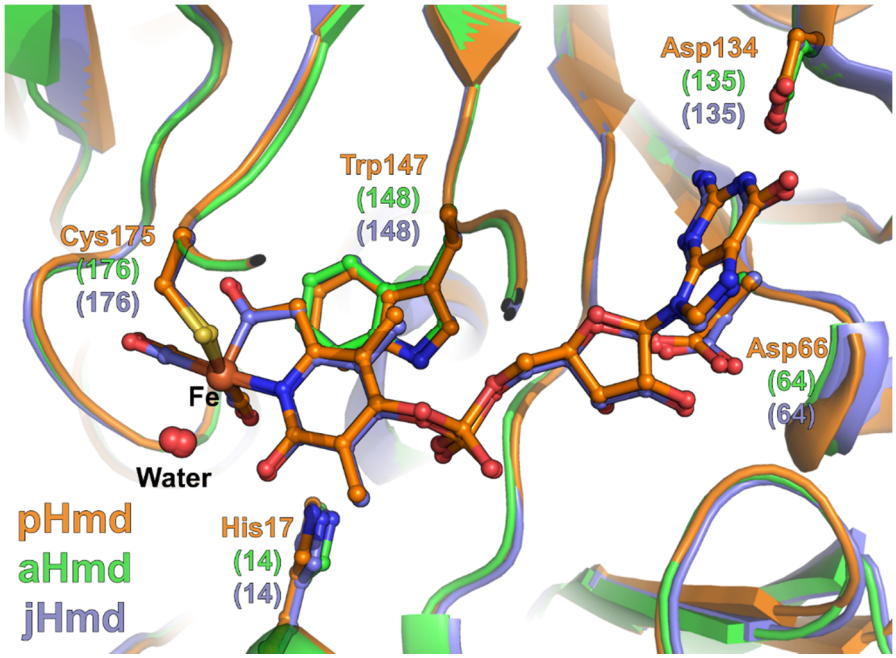


Figure S2. Superposition of the FeGP cofactor binding sites in pHmd (PDB: 6YKA), Hmd from *Methanococcus aeolicus* (aHmd) (PDB: 6HAC) and jHmd (PDB: 3F47). The protein parts are shown as cartoon model, the FeGP cofactor and the side chain of residues related to its binding are shown in ball and stick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

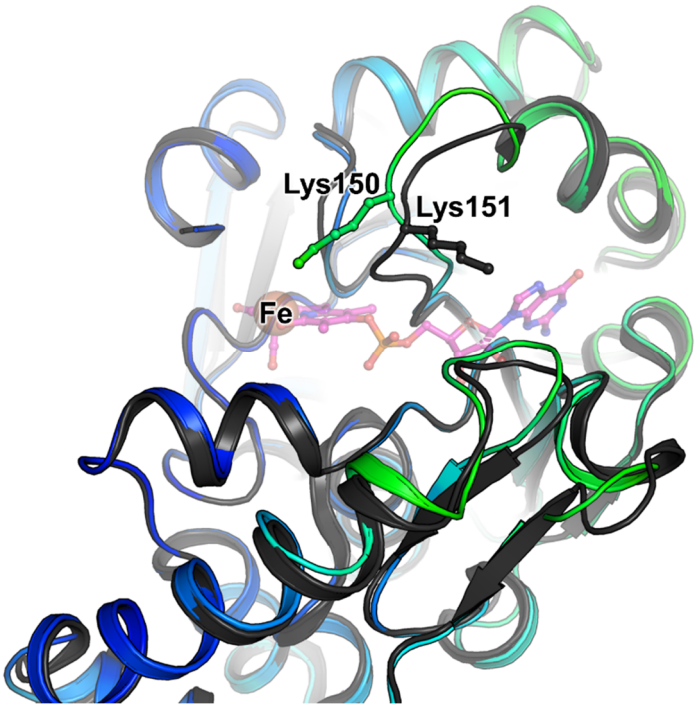


Figure S3. Comparison of the loop involved in the FeGP cofactor coordination in the jHmd and pHmd apo-forms. Apo-jHmd was colored in black. Apo-pHmd of the pHmd asymmetric homodimer was indicated by colors (dark blue with low B-factor to green with higher B-factor). The FeGP cofactor from the holo structure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

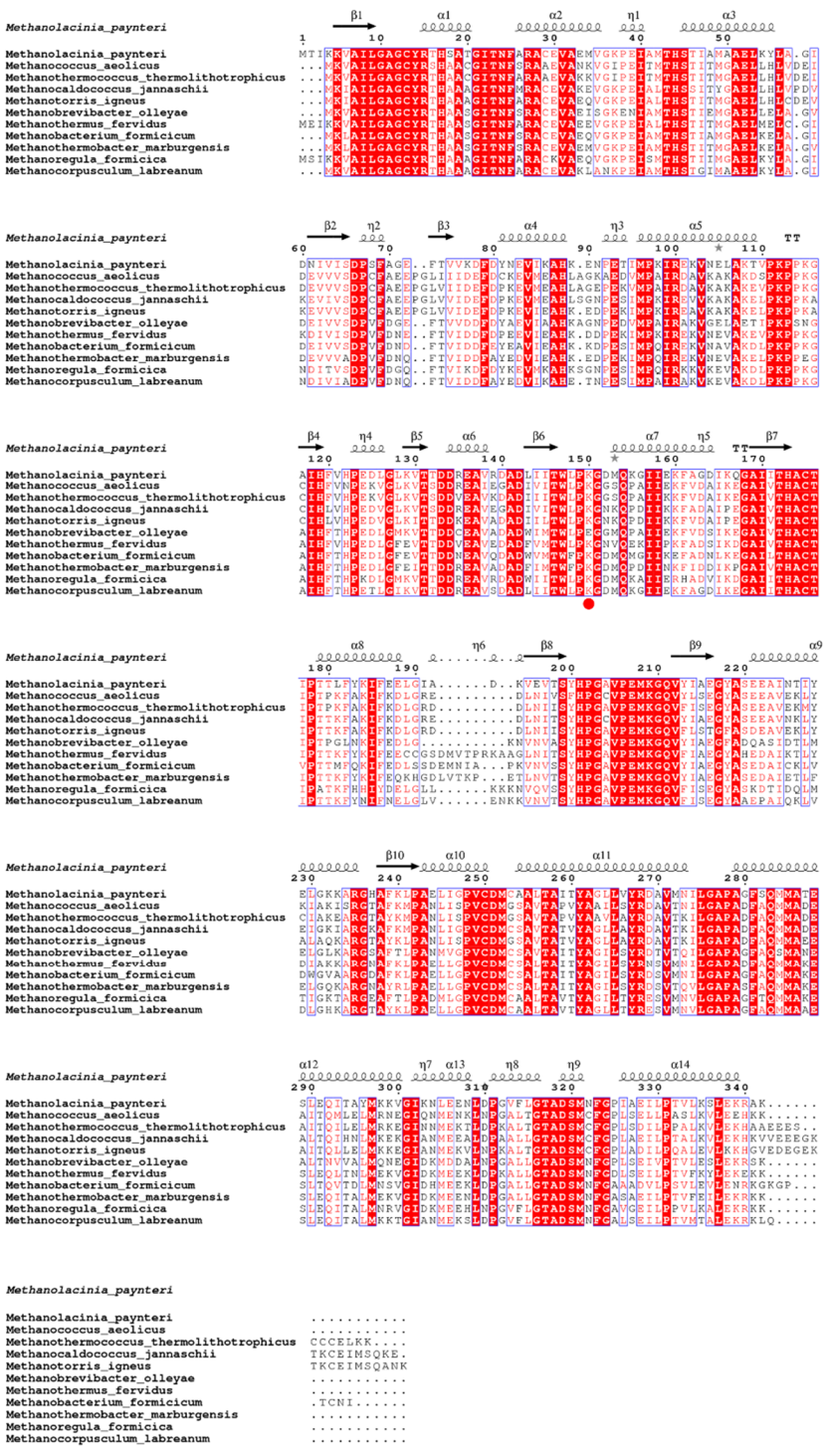


Figure S4. Alignments of Hmd amino acid sequences from different organisms. All sequences are obtained from NCBI database. Alignment was performed by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The figure was made by ESPript 3.0 [1]. Lys150 was highly conserved in [Fe]-hydrogenase except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

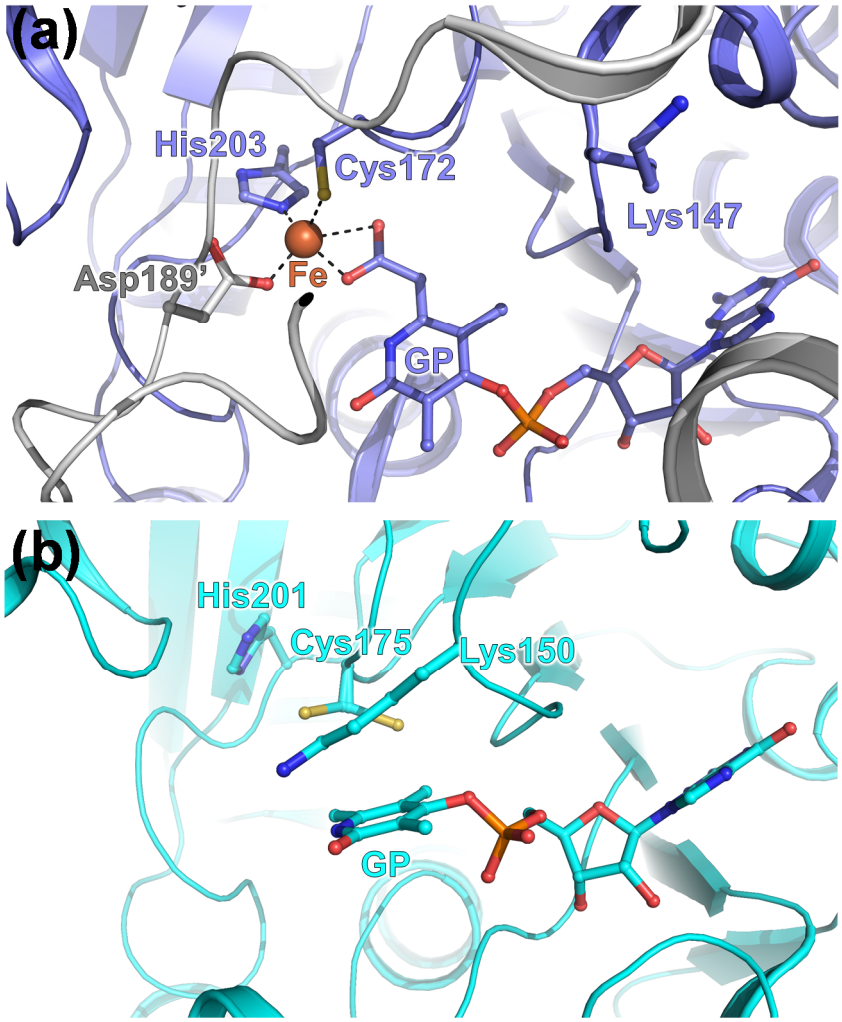


Figure S5. Comparison of the GP-binding states in the structure of Hmd from *M. marburgensis* obtained in an oxidized broken state with that in the structure of the GP bound form of pHmd. (**a**) Hmd from *M. marburgensis* in an oxidized broken state. (**b**) The structure of the GP bound form pHmd. 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*.

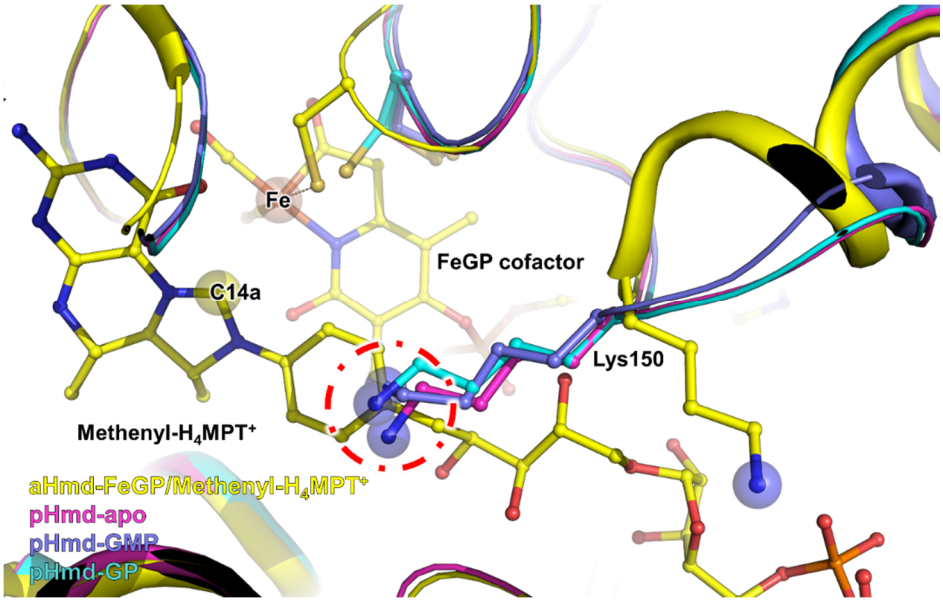


Figure S6. Superposition of the active sites of the apo- and holo-forms of Hmd. Lys150 was shown in ball and stick model. The holoenzyme of Hmd from the *Methanococcus aeolicus* in complex with methenyl-H4MPT+ (aHmd-FeGP/methenyl-H4MPT+), the apo-form of the asymmetric homodimer of pHmd (pHmd-apo), the 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-H4MPT+ was highlighted by red dash open circle.

Enzymatic Reaction:

$$E + S_1 + S_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [E\text{-}S_1\text{-}S_2] \xrightarrow{k_3} E + P_1 + P_2$$

Full set of ODEs:

$$\frac{dE}{dt} = -k_1 \cdot S_1(t) \cdot E(t) + k_{-1} \cdot [E\text{-}S_1\text{-}S_2](t)$$

$$\frac{dS_1}{dt} = -k_1 \cdot S_1(t) \cdot E(t) + k_{-1} \cdot [E\text{-}S_1\text{-}S_2](t)$$

$$\frac{d[S_2]}{dt} = 0$$

$$\frac{d[E\text{-}S_1\text{-}S_2]}{dt} = k_1 \cdot S_1(t) \cdot E(t) - k_{-1} \cdot [E\text{-}S_1\text{-}S_2](t) - k_3 \cdot [E\text{-}S_1\text{-}S_2](t)$$

$$\frac{d[P_1]}{dt} = k_3 \cdot [E\text{-}S_1\text{-}S_2](t)$$

$$\frac{d[P_2]}{dt} = 0$$

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_{max} and apparent K_m, we fixed the concentration of H_2 and H^+. Therefore, the rate of the changes of H_2 and H^+ is zero and the model was approximated to the Michaelis-Menten (single substrate/product) equation.

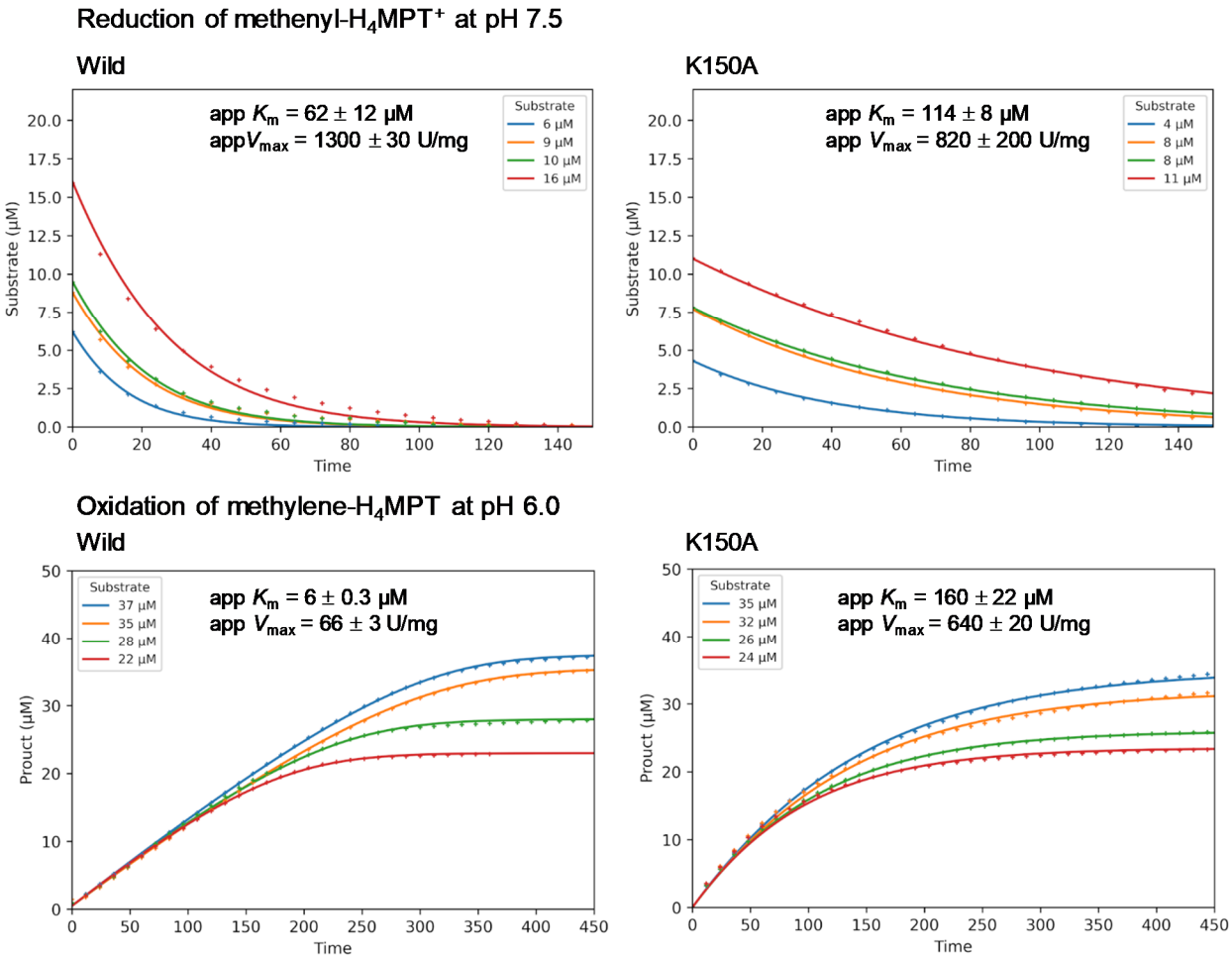
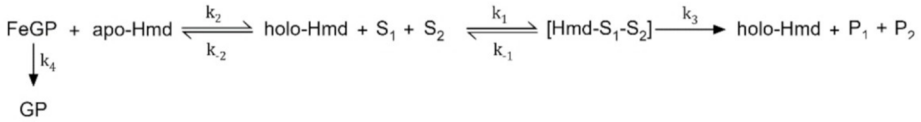


Figure S8. Simulation of the progressive curves of the reactions. The substrate consumption in the reduction of methenyl-H_4MPT$^+$ with H_2 at pH 7.5 and the product formation in the oxidation of methylene-H_4MPT at pH 6.0 in the enzymatic reaction mixture was recorded and simulated using the pHmd holo-forms (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:



Full set of ODEs:

$$\frac{d[FeGP]}{dt} = -k_2 \cdot [FeGP](t) \cdot [apo\text{-}Hmd](t) + k_{-2} \cdot [holo\text{-}Hmd](t) - k_4 \cdot [FeGP]$$

$$\frac{d[apo\text{-}Hmd]}{dt} = -k_2 \cdot [FeGP](t) \cdot [apo\text{-}Hmd](t) + k_{-2} \cdot [holo\text{-}Hmd](t)$$

$$\frac{d[holo\text{-}Hmd]}{dt} = k_2 \cdot [FeGP](t) \cdot [apo\text{-}Hmd](t) - k_{-2} \cdot [holo\text{-}Hmd](t) - k_1 \cdot [S_1](t) \cdot [holo\text{-}Hmd](t) + k_{-1} \cdot [Hmd\text{-}S_1\text{-}S_2]$$

$$\frac{d[S_1]}{dt} = -k_1 \cdot [S_1](t) \cdot [holo\text{-}Hmd](t) + k_{-1} \cdot [Hmd\text{-}S_1\text{-}S_2]$$

$$\frac{d[S_2]}{dt} = 0$$

$$\frac{d[Hmd\text{-}S_1\text{-}S_2]}{dt} = k_1 \cdot [S_1](t) \cdot [holo\text{-}Hmd](t) - k_{-1} \cdot [Hmd\text{-}S_1\text{-}S_2] - k_3 \cdot [Hmd\text{-}S_1\text{-}S_2](t)$$

$$\frac{d[P_1]}{dt} = k_3 \cdot [Hmd\text{-}S_1\text{-}S_2]$$

$$\frac{d[P_2]}{dt} = 0$$

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_2 and H^+ were used. Therefore, the rate of the changes of H_2 and H^+ is zero and the model was approximated to the Michaelis-Menten equation. The decomposition rate (k_4) of the FeGP cofactor to guanylylpyridinol (GP) in the reconstitution assay was experimentally determined (0.01 μM·s^{-1} at pH 7.5 and 0.007 μM·s^{-1} at pH 6.0). We assumed k_{-2} is zero and defined k_2 as reconstitution rate in the main text.

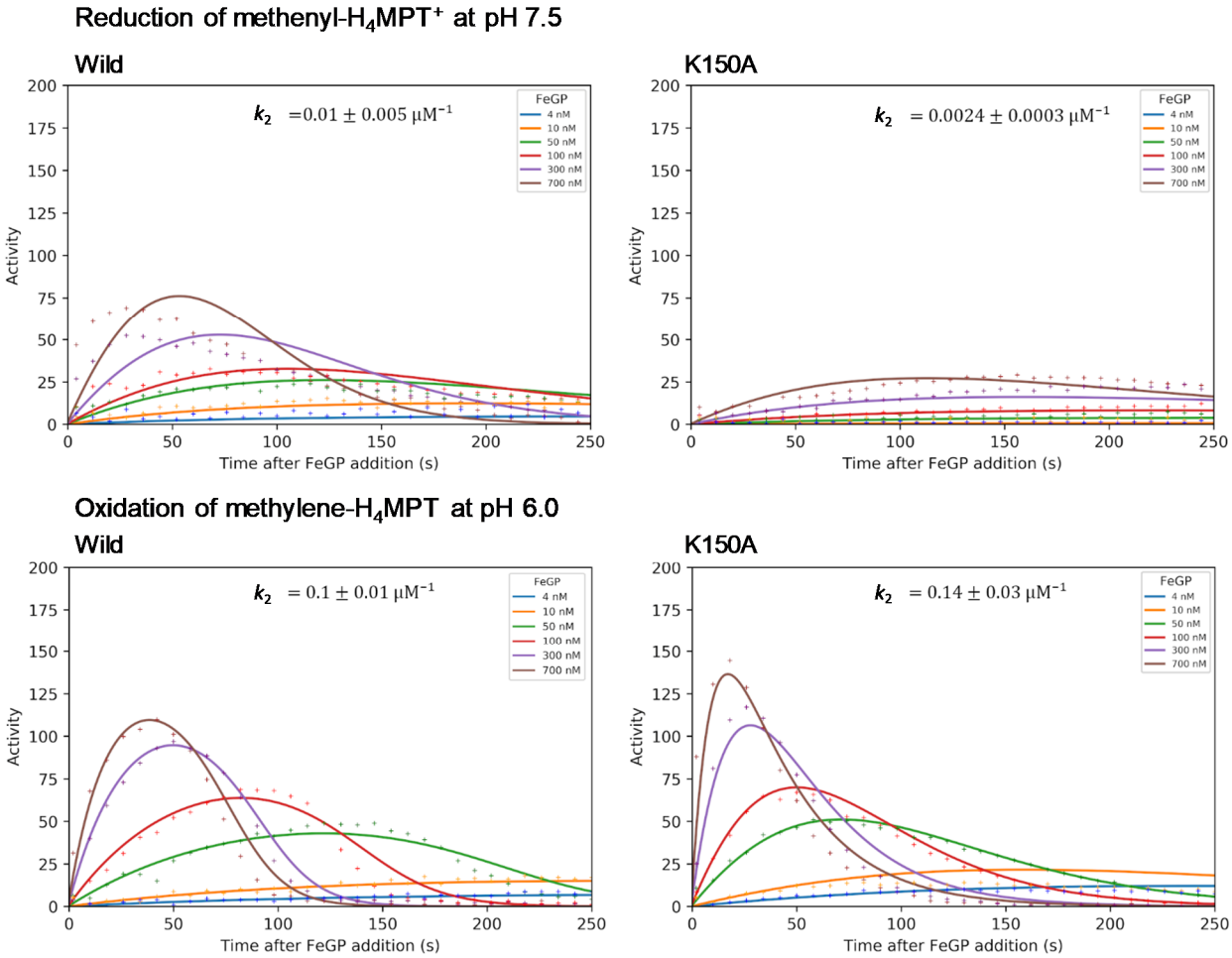


Figure S10. Simulation of the change of the activity in the reconstitution assay of pHmd. Experimental data (+) is the same shown in Figure7c, d, g, h. The apparent V_{max} value was adjusted by the ratio between the specific activity of the reconstituted enzyme at 18 μM substrate concentrations and 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+ 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.