# Introducing a 2-His-1-Glu Non-heme Iron Center into Myoglobin Confers Nitric Oxide Reductase Activity

Ying-Wu Lin,[†, Δ] Natasha Yeung,[†] Yi-Gui Gao,[‡] Kyle D. Miner,[§] Lanyu Lei,[†] Howard Robinson[¶] and Yi Lu[†, §, *]

[†]*Department of Chemistry,* [‡]*George L. Clark X-Ray Facility & 3M Materials Laboratory,* [§]*Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA and* [¶]*Department of Biology, Brookhaven National Laboratory, Upton, New York 11973, USA*

[Δ]*Present address: School of Chemistry and Chemical Engineering, University of South China, Hengyang 421001, China*

## Supporting Information

### Experimental methods

**Protein Construction, Expression and Purification.** The $Fe_BMb$(-His) gene (swMb L29E/F43H) was constructed using the QuickChange Site Directed Mutagenesis Kit (Stratagene, CA). The Leu29Glu and Phe43His mutations in the swMb gene of pMbt7-7 were confirmed by DNA sequencing at the Biotechnology Center at the University of Illinois. $Fe_BMb$(-His) was expressed and purified using a protocol described previously.[1] The protein purity was assessed by SDS-PAGE and electrospray mass spectrometry (ESI/MS); the latter was performed at the Mass Spectroscopy Laboratory, University of Illinois. The observed molecular weight (17332 ± 1 Da) corresponds to the calculated molecular weight (17333 Da) of $Fe_BMb$(-His) within experimental error.

**UV-vis Spectroscopy.** UV-vis spectra were collected on a Cary 5000 (Varian) spectrometer equipped with a Quantum Northwest peltier temperature controller and stirring module at 25 °C. The reduced protein was prepared in a glovebox using dithionite as reluctant. Excess dithionite was removed using a PD-10 gel filtration column (GE healthcare). For NO studies, NO gas (Matheson Trigas, 99%) was purged of $N_2O_3$, $NO_2$, and higher $N_xO_y$ impurities by passing it through a bubbler of 1 M NaOH and a second bubbler of water before passing it into a reaction vessel filled with ultrapure water. The kinetic NO reduction by Cu(I)-$Fe_BMb$(-His), Fe(II)-$Fe_BMb$(-His) or Zn(II)-$Fe_BMb$(-His) at room temperature was monitored by a Hewlett-Packard 8453 UV-vis spectrometer,

equipped with a circulating water bath and a Polyscience digital temperature controller. Control experiments of deoxy E-$Fe_B$Mb(-His) and NO without metal were performed under the identical conditions. The molar extinction coefficient of the Soret band of ferric E-$Fe_B$Mb(-His), $\varepsilon_{415nm}$ = 135 $mM^{-1}\cdot cm^{-1}$, calculated using the standard hemochromagen method,[2] was used to determine protein concentration. The metal sources of Cu(I), Cu(II), Zn(II) and Fe(II) were $[(CH_3CN)_4Cu]PF_6$, $CuSO_4$, $ZnSO_4\cdot 7H_2O$ and $FeCl_2$, respectively.

**EPR Spectroscopy.** EPR spectra were recorded on a Bruker ESP 300 equipped with an Oxford liquid helium cryostat and an ITC4 temperature controller. The samples of ferric E-$Fe_B$Mb(-His) and Cu(II)-$Fe_B$Mb(-His) were prepared as described previously.[1] The samples of NO bound deoxy E-$Fe_B$Mb(-His), Cu(I)-$Fe_B$Mb(-His), Fe(II)-$Fe_B$Mb(-His) and Zn(II)-$Fe_B$Mb(-His) were prepared by injecting 1 mL of purified NO gas (1 atm) into the EPR tube containing 300 μL of protein solution ($Fe_B$Mb(-His), 0.5 mM, 10% glycerol, in 50 mM Bis-Tris pH 7.0), which were then flash frozen in liquid $N_2$ after incubation for 1 min, 5 min or 15 min.

**Gas Chromatography and Mass Spectroscopy (GC/MS).** Previous GC/MS NO reduction studies catalyzed by Cu-bound $Cu_B$Mb were performed under multiple turnovers conditions using ascorbate as the reductant.[3] Since Fe(II)-ascorbate can non-enzymatically reduce NO to $N_2O$,[4] giving high background NO activity, we used one turnover conditions to assay the NOR activities of metal bound $Fe_B$Mb(-His), Cu(I)-$Fe_B$Mb(-His) and Fe(II)-$Fe_B$Mb(-His). The protein was first degassed and reduced under an inert atmosphere glove box with excess dithionite. Excess reductant was removed using a PD-10 gel filtration column (GE healthcare), then 2 eq metal, Cu(I), Fe(II) or Zn(II) was added to the protein solution (0.6 mM, 3 mL in Bis-Tris buffer, pH 7.0). Control experiments were also performed for Cu(I), Fe(II) or $Fe_B$Mb(-His) only. Other procedures are the same as described previously.[3]

**X-ray Crystallographic Studies.** $Fe_B$Mb(-His) with a purity of R/Z > 4 ($A_{415nm}/A_{208nm}$) was exchanged into 20 mM Tris·$H_2SO_4$ (pH 8.0) and concentrated to ~ 1.6-1.8 mM. Then 2 eq KCN and 1.5 eq Cu(II) were added. The vapor diffusion hanging drop technique was used to crystallize the protein. The well buffer contained 0.2 M sodium acetate trihydrate, 0.1 M sodium cacodylate trihydrate pH 6.5, and 30% w/v PEG 8,000 (buffer 28#, Crystal Screen kits, Hampton Research). Crystal trays were set up by transferring 250 μL of well

buffer into each well. Then, 2 μL of well buffer and 2 μL of protein were mixed and placed on a siliconized glass slide. Crystallization can be achieved at 4 °C after ~2-3 weeks. Diffractable crystals were soaked in a cryoprotectant solution of 30% PEG 400, mounted onto cryogenic loops, and frozen quickly in liquid nitrogen. Diffraction data were collected at the Brookhaven National Lab (Upton, NY) Synchrotron Light Source X12C beamline. The crystal structure was solved by the molecular replacement method using MOLREP in the CCP4 Package[5] and then refined using X-plor[6] and SHELX'97.[7] The crystal and refinement data are summarized in Table S1. The structure has been checked by Procheck[8], with all the residues in allowed regions.

## References:


(1) (a) Yeung, N.; Lin, Y. -W.; Gao, Y. -G.; Zhao, X.; Russell, B. S.; Lei, L.; Miner, K. D.; Robinson, H.; Lu, Y. *Nature* **2009**, *462*, 1079-1082. (b) Sigman, J. A.; Kwok, B. C.; Lu, Y., *J. Am. Chem. Soc.*, **2000,** *122*, 8192-8196.

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## Complete ref.7 in the text:


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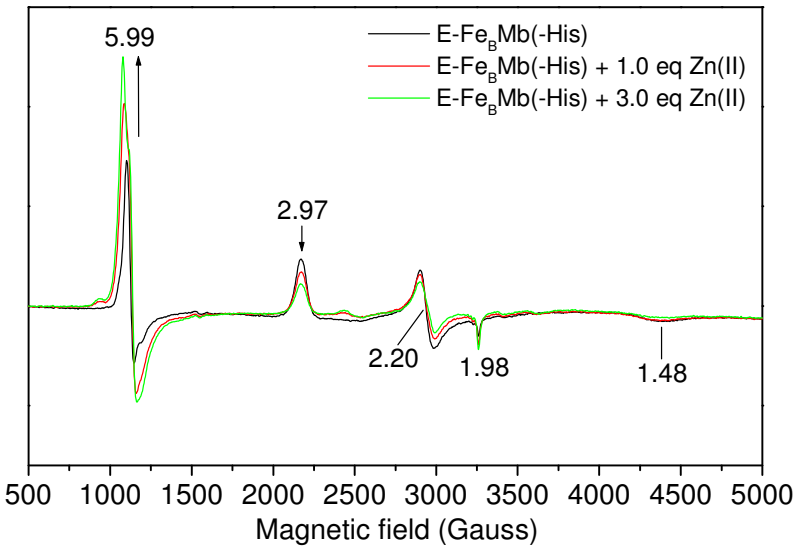



***Figure S1:*** X-band EPR spectra of ferric E-Fe$_B$Mb(-His) in 25 mM $KH_2PO_4$, 75 mM KCl, pH 7.0 and that in the presence of 1.0 or 3.0 eq Zn(II). The spectra were collected at 20 K, 5 mW power, and 9.05 GHz.

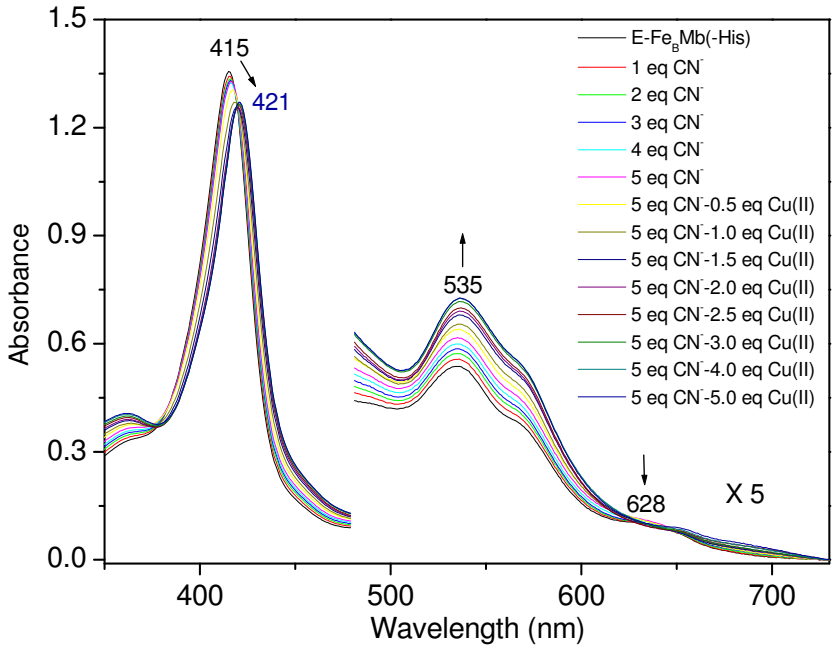



***Figure S2:*** (A) UV-vis titration of ferric E-Fe$_B$Mb(-His) with cyanide and Cu(II) in 100 mM $KH_2PO_4$, pH 7.0, 25 °C. The absorption changes of Soret band and visible bands

were indicated by arrows.

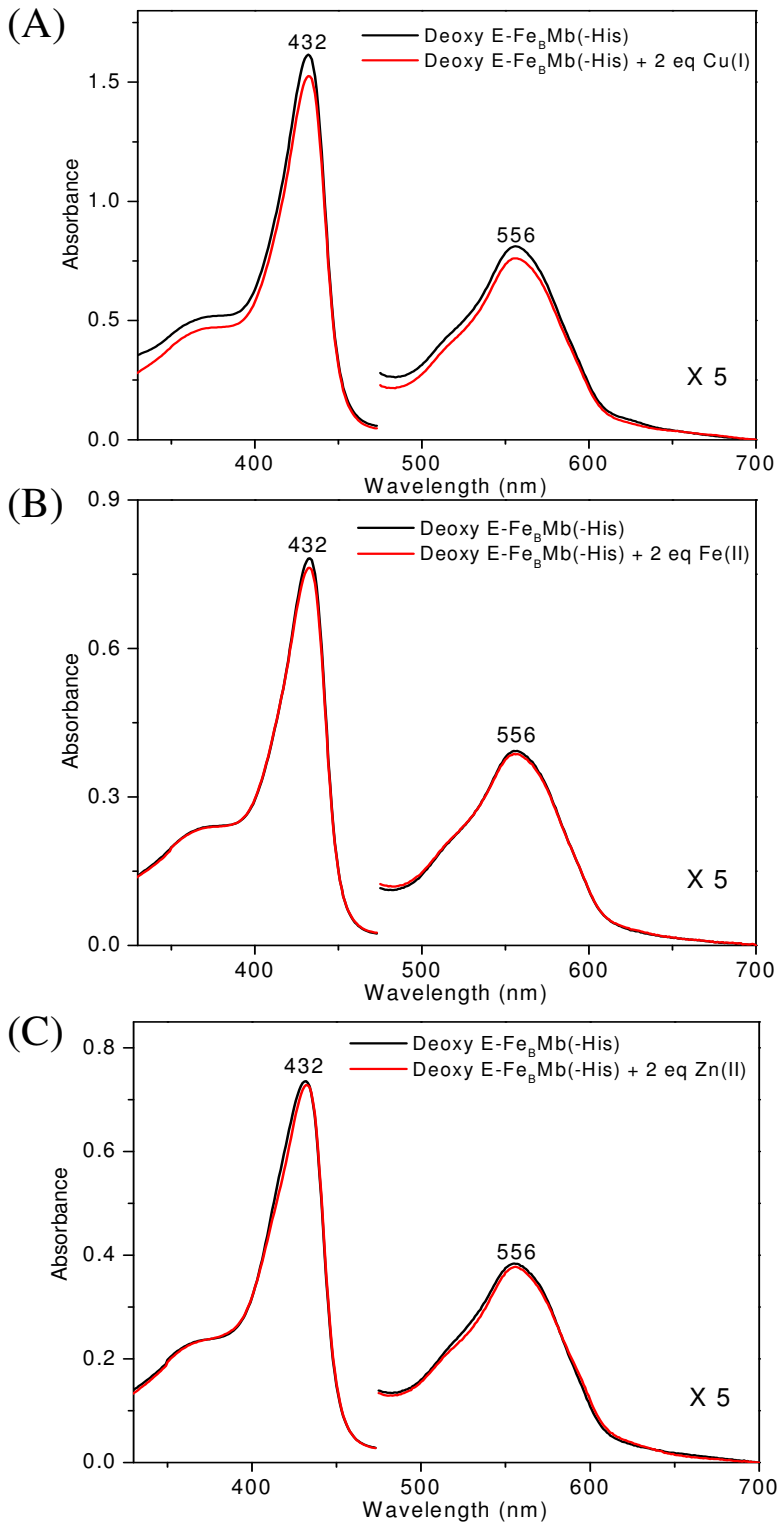



***Figure S3:*** UV-vis spectra of deoxy E-Fe$_B$Mb(-His) in absence or presence of 2 eq Cu(I) (A), Fe(II) (B) or Zn(II) (C) in 50 mM Bis-Tris pH 7.0. The dilution effects of adding

metal ions were corrected by adding the same volume of buffer containing no metal ions.

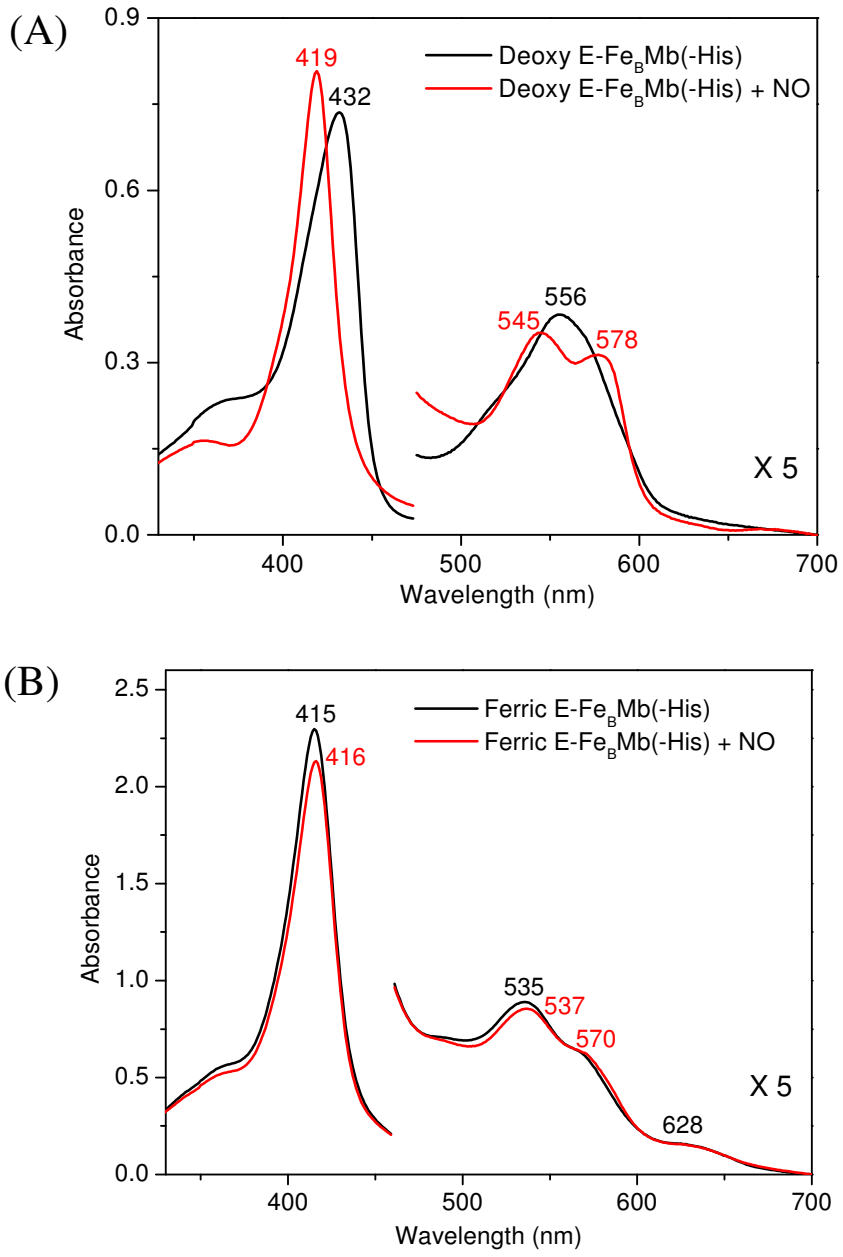
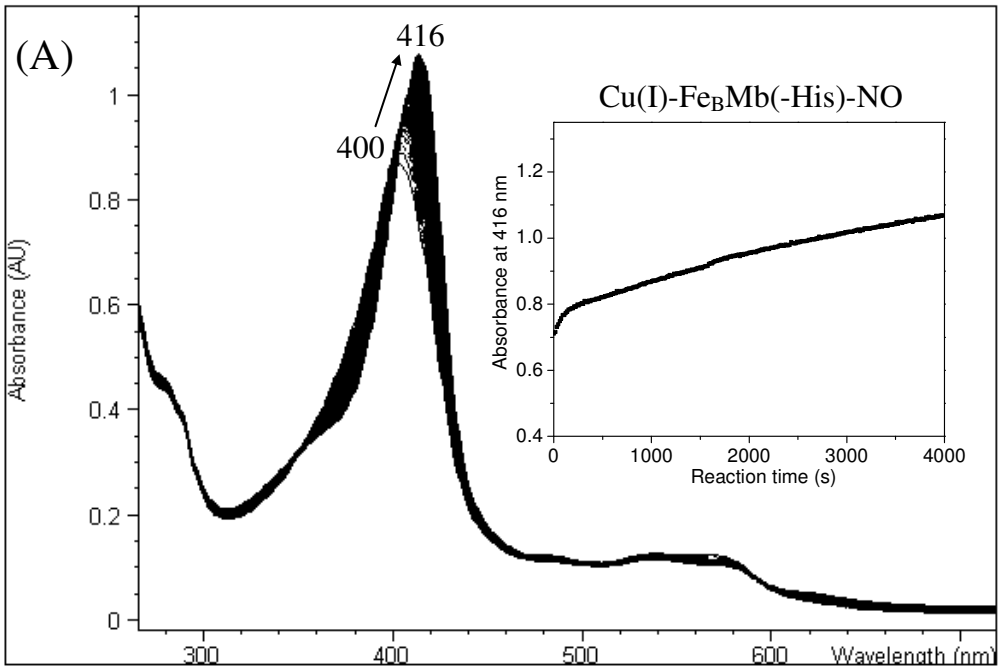
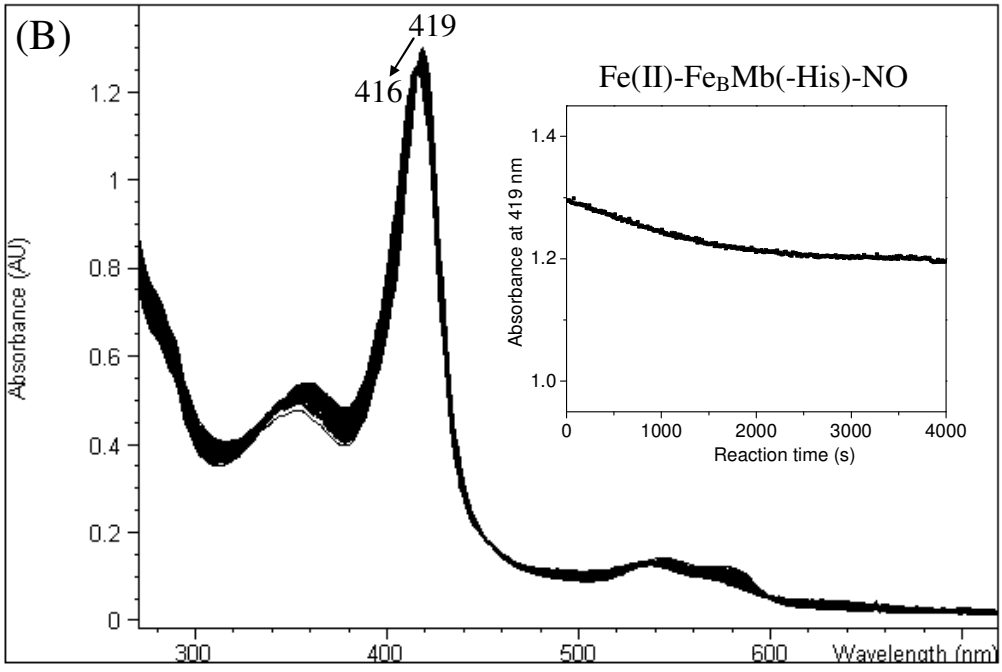



***Figure S4:*** UV-vis spectra of NO binding to deoxy (A) or ferric (B) E-Fe$_B$Mb(-His).


(A)
416
400
Cu(I)-Fe$_B$Mb(-His)-NO
Absorbance (AU)
Wavelength (nm)
Absorbance at 416 nm
Reaction time (s)


(B)
419
416
Fe(II)-Fe$_B$Mb(-His)-NO
Absorbance (AU)
Wavelength (nm)
Absorbance at 419 nm
Reaction time (s)

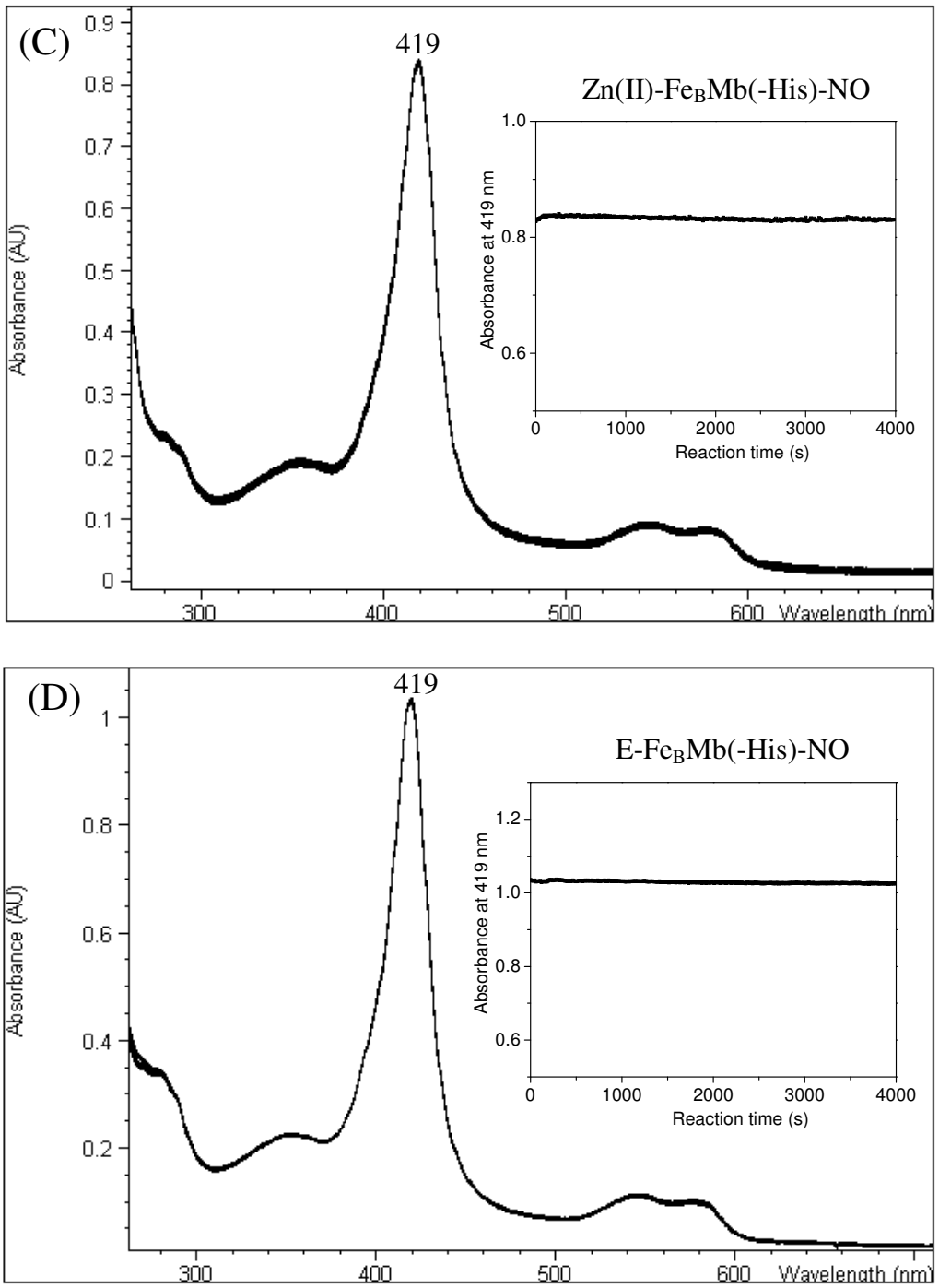



***Figure S5:*** Kinetic trace of NO reduction by Cu(I)-$Fe_B$Mb(-His) (A) or Fe(II)-$Fe_B$Mb(-His) (B) under one turnover conditions monitored for 4000 seconds with a cycle time of 15 sec at 25 °C. The control experiments of Zn(II)-$Fe_B$Mb(-His) (C) or E-$Fe_B$Mb(-His) without metal in the $Fe_B$ site (D) were performed under identical conditions. The Soret band changes are indicated by arrows.

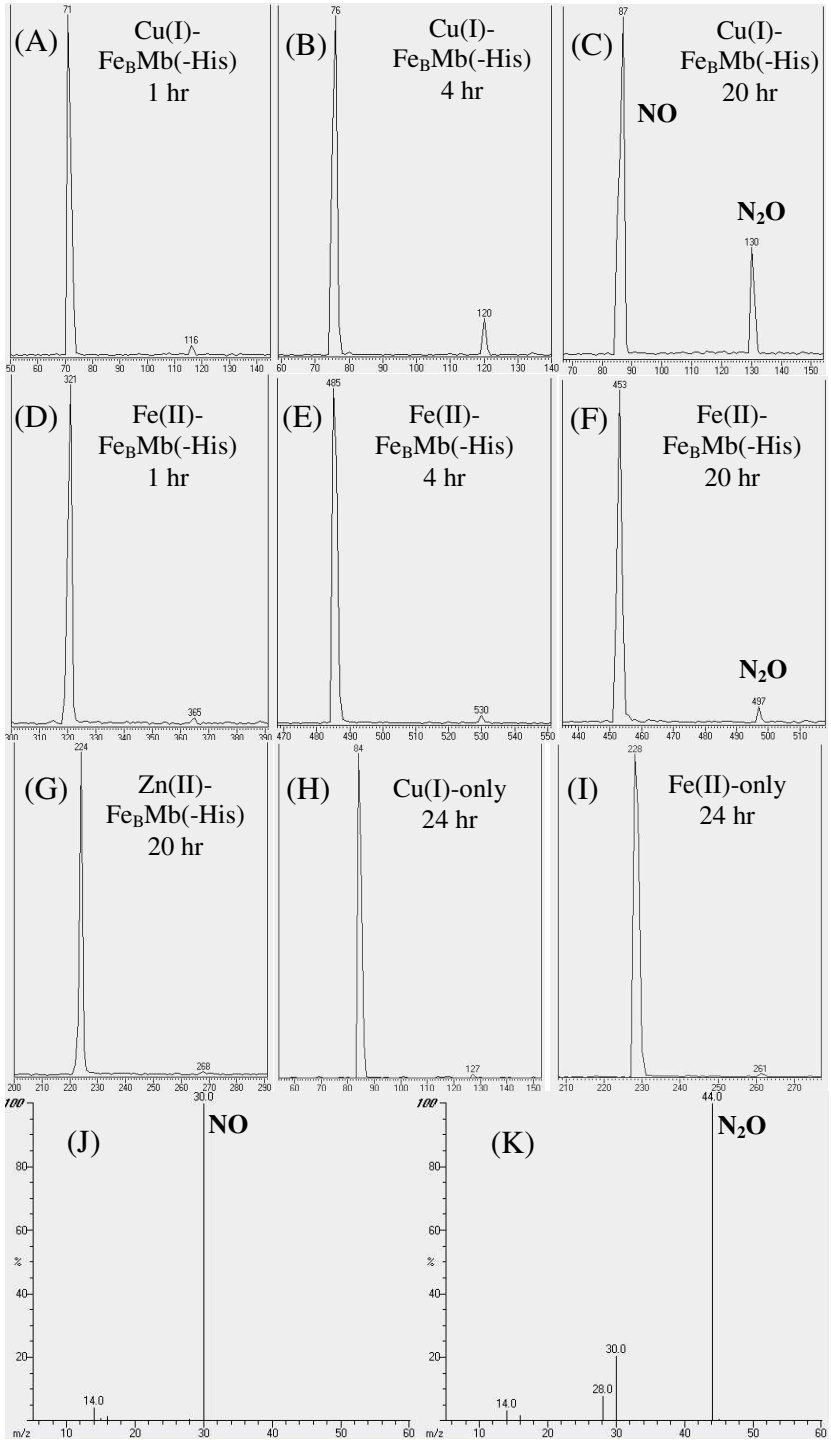



***Figure S6:*** GC/MS of NO (~100 eq) reduction at different times by Cu(I)-$Fe_B$Mb(-His) (A-C), Fe(II)-$Fe_B$Mb(-His) (D-F) ($Fe_B$Mb(-His), 0.6 mM; 2 eq Cu(I) or Fe(II)). Control experiments under identical conditions are shown for Zn(II)-$Fe_B$Mb(-His) (G), Cu(I) (H) or Fe(II) only. Typical MS results of NO (J) and $N_2O$ (K) are shown as examples.

**Table S1:** Diffraction and refinement data of the crystal structure of Cu(II)-$CN^-$-$Fe_B$Mb(-His).

| | Cu(II)-$CN^-$-$Fe_B$Mb(-His) |
|---|---|
| **PDB entry** | 3MN0 |
| **Data collection** | |
| Wavelength (Å) | 1.0988 |
| Space Group | $P2_12_12_1$ |
| Cell dimension | |
| *a, b, c* (Å) | 39.60, 48.42, 77.63 |
| $\alpha, \beta, \gamma(o)$ | 90.0, 90.0, 90.0 |
| Resolution (Å) | 1.32 (1.37 – 1.32) |
| R-merge | 0.063 (0.215) |
| I/σI | 25.0(12.5) |
| Redundancy | 14.6 (13.6) |
| Completeness (%) | 100.0(100.0) |
| **Refinement** | |
| Resolution (Å) | 8.0 – 1.65 |
| Rwork/Rfree | 0.245/0.273 |
| No. reflections | 17,420 |
| No. atoms | |
| Protein | 1,216 |
| Hem/Ion | 42/1FE,1Cu,1CN |
| Water | 186 |
| B-factor | |
| Protein | 22.0 |
| Hem/Ion | 20.8/15.2/22.9 |
| Water | 33.2 |
| Rms. Deviations | |
| Bond lengths(Å) | 0.007 |
| Bond angles (°) | 2.142 |