# Site-Specific Incorporation of a Cu<sup>2+</sup> Spin-Label into Proteins for Measuring Distances by Pulsed ESR Spectroscopy

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## Content

## A. Supplemental Figures

- **S1.** *DEER spectroscopy pulse and detection frequencies*
- **S2.** Cu-NO time domain data and processing for CFP15PyTyr in  $D_2O$ .
- **S3.** Factors affecting Cu-NO dipolar interactions CFP
- **S4.** *Mass Spectrometry of CheY41PyTyr*
- **S5.** SEC-MALS traces for CheY-41PyTyr-D54K
- **S6.** *Cu-NO primary time domain data and processing for CheY41PyTyr:CheA-P2-NO*
- **S7.** *Cu-Cu primary time domain data and processing for CheY41PyTyr:CheA-P2-NO*
- **S8.** *Cu-NO primary time domain data and processing for CheY41PyTyr:CheA-P1-NO*

## **B.** Supplemental Tables

**S1**. Diffraction Data Collection and Refinement Statistics for CheY-PyTyr



**Figure S1**. DEER spectroscopy pulse and detection frequencies (**Left**) The 4-pulse DEER PDS pulse sequence was used in this work to measure distances between unpaired electron spins of  $Cu^{2+}$  ions bound to the unnatural amino acid PyTyr and MTSSL spin labels (R1). (**Right**) The field-swept primary echo recorded at 60 K on CFP151PyTyr orthogonally-labeled with  $Cu^{2+}$  and R1. The insert is the horizontal (×2) spectrum expansion. The filled circle is the pumped position on R1 and the empty circle is at the point of the  $Cu^{2+}$  spectrm corresponding to the detection frequency. The asterisk indicates a  $Cr^{3+}$  line from the external reference.



**Figure S2**. PDS data analysis for CFP151PyTyr in D<sub>2</sub>O. Background corrected Cu-NO time domain signals (upper left) treated by the Tikhonov Regularization-Maximum Entropy (TIKR-MEM) method (middle) or SVD (right), or denoised by wavelet transform (lower left) followed by P(r) reconstruction by TIKR+MEM (middle) or the SVD method (right), with the red areas showing the uncertainty.



**Figure S3**. Factors affecting the Cu<sup>2+</sup>-NO dipolar signal in CFP. (A) Denoised background corrected time domain signals (left) and resulting P(r) calculated by SVD (right) for (i) Cu<sup>2+</sup>-NO DEER of CFP labeled with PyTyr(151) and R1(208) in D<sub>2</sub>O and MES buffer, (ii) Cu<sup>2+</sup>-NO DEER of CFP labeled with PyTyr and R1 in H<sub>2</sub>O and MES buffer, (iii) NO-NO DEER of CFP labeled at residue Cys151 and Cys208 both by R1 in H<sub>2</sub>O and MES buffer. (iv) Cu<sup>2+</sup>-NO DEER of CFP labeled with PyTyr and R1 in H<sub>2</sub>O and Tris buffer, (v) NO-NO DEER of CFP labeled with PyTyr and R1 in H<sub>2</sub>O and MES. Red areas show the uncertainty in SVD.



**Figure S4.** Deconvoluted mass-spectrum for CheY41PyTyr, showing incorporation. Expected MW: 13,598 Da. Found MW: 13,598 Da.



**Figure S5.** SEC-MALS traces for CheY-41PyTyr-D54K. The protein is a monomer in solution with and without 1 equivalent of  $Cu^{2+}$ .



**Figure S6**. PDS data analysis for CheA (P1-P2, P2 label) + CheY-41PyTyr-D54K. Background corrected Cu-NO time domain signals (upper left) treated by the Tikhonov Regularization-Maximum Entropy method (upper right), or denoised by wavelet transform (lower left) followed by P(r) reconstruction by the SVD method (lower right), with the red areas showing the uncertainty.



**Figure S7**. PDS data analysis for CheA (P2 label) + CheY-41PyTyr-D54K. Background corrected Cu-Cu time domain signals (upper left) treated by the Tikhonov Regularization-Maximum Entropy method (upper right), or denoised by wavelet transform (lower left) followed by P(r) reconstruction by the SVD method (lower right), with the red areas showing the uncertainty.



**Figure S8**. PDS data analysis for CheA (P1-P2, P1 label) + CheY-41PyTyr-D54K. Background corrected Cu-NO time domain signals (upper left) treated by the Tikhonov Regularization-Maximum Entropy method (upper right), or denoised by wavelet transform (lower left) followed by P(r) reconstruction by the SVD method (lower right), with the red areas showing the uncertainty.

Data Collection Metrics	
Space Group	P1
Unit Cell Dimensions, (Å)	a = 33.63
	b = 34.81
	c = 58.54
Unique Reflections	6,240
Resolution, (Å)	33.50-2.70
Last Shell, (Å)	2.79-2.70
Redundancy, (%/last shell)	2.0/1.7
Completeness, (%/last shell)	91.7/79.5
I/σ, (overall/last shell)	9.9/1.2
Linear R-fac (R <sub>Merge</sub> ), (overall/last shell)	.189/.639
CC <sup>1</sup> / <sub>2</sub> , (overall/last shell)	.807/.693
Refinement Metrics	
R-Work, (%/last shell)	.219/.289
R-Free, (%/last shell)	.316/.425
No. atoms	1,794
No. water molecules	6
Mean B value	69.8
RMSD from ideal bond lengths, (Å)	.015
RMSD from ideal bond angles, (Å)	1.24

 Table S1. Crystallographic data collection and refinement statistics.