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Supplemental Information

EPR Spectroscopy Targets Structural Changes in the *E. coli* Membrane Fusion CusB upon Cu(I) Binding

Aviv Meir, Ahmad Abdelhai, Yoni Moskovitz, and Sharon Ruthstein

Supporting information

EPR spectroscopy targets conformational and topological changes in the *E.coli* membrane fusion CusB dimer upon Cu(I) binding

Aviv Meir, Ahmad Abdelhai, Yoni Moskovitz, Sharon Ruthstein*

Department of Chemistry, Faculty of Exact Sciences, Bar Ilan University, Ramat-Gan, Israel 5290002

Figures S1-S10

Verifying 100% spin labeling of the protein:

Double integration of the EPR spectra is related to the spin concentration. Therefore, we first prepared a calibration curve using various concentrations of free MTSSL spin label in chitin×1 lysis buffer (1.5M NaCl, 250mM $Na_2PO_4{\cdot}2H_2O$; pH 8.85). The protein concentration was determined by Lowry assay. Figure S1 compares CusB integrated area with the free spin-labels calibration curve, indicating almost 100% spin labeling and no remaining free spin for all CusB samples.

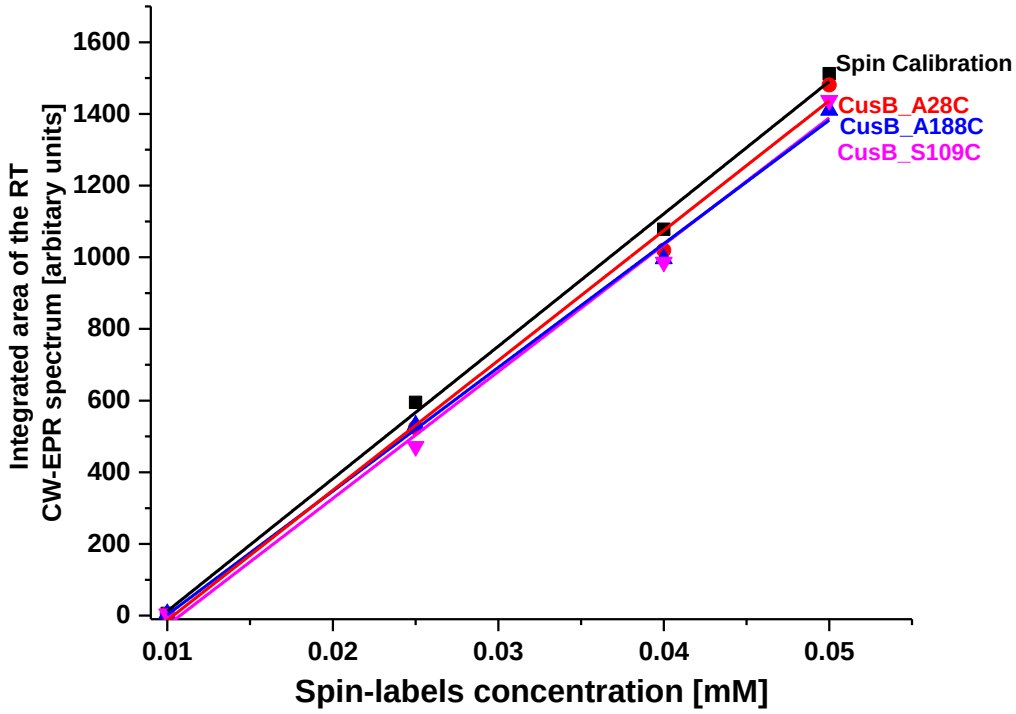

Figure S1: Integrated area of the CW-EPR spectrum corresponding to the free spin-label concentration (black line) and the CusB mutants concentration (colored lines).

RT CW-EPR spectra as a function of [Cu(I)]

RT CW-EPR spectra were acquired for the various mutants as a function of [Cu(I)]. We observed a decrease in the hyperfine coupling for all mutants upon Cu(I) coordination. The stabilization in the reduction of a_N occurred at a ratio of [Cu(I):[CusB] 3:1. Higher Cu(I) concentrations led to aggregation of the protein, which resulted in a reduction of the EPR signal (see 5:1 [Cu(I):[CusB]).

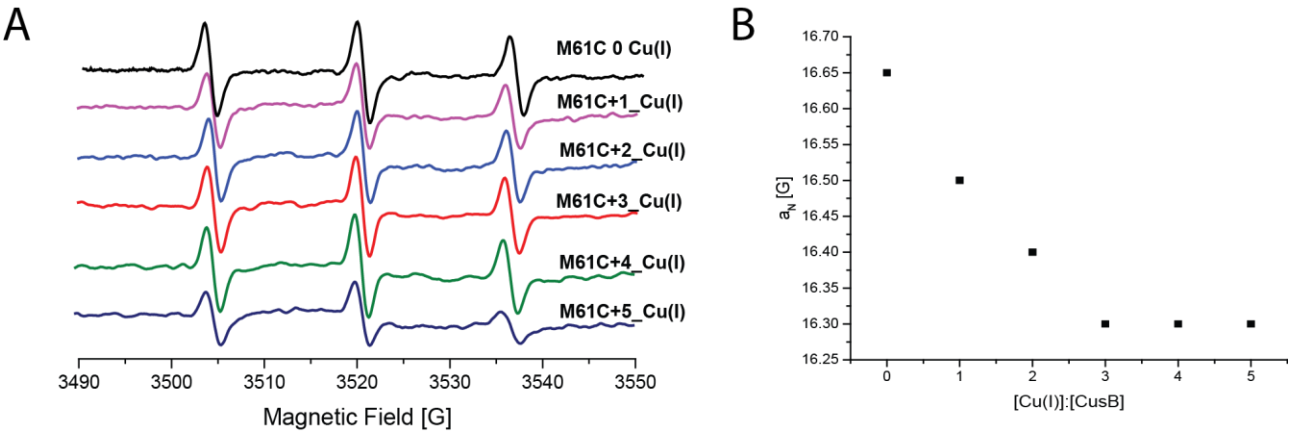

Figure S2: **A**. RT CW-EPR spectra for CusB_M61C mutant in the presence of various Cu(I) concentrations. **B**. The change in the a_N coupling. [CusB]=0.04mM.

Raw-DEER data

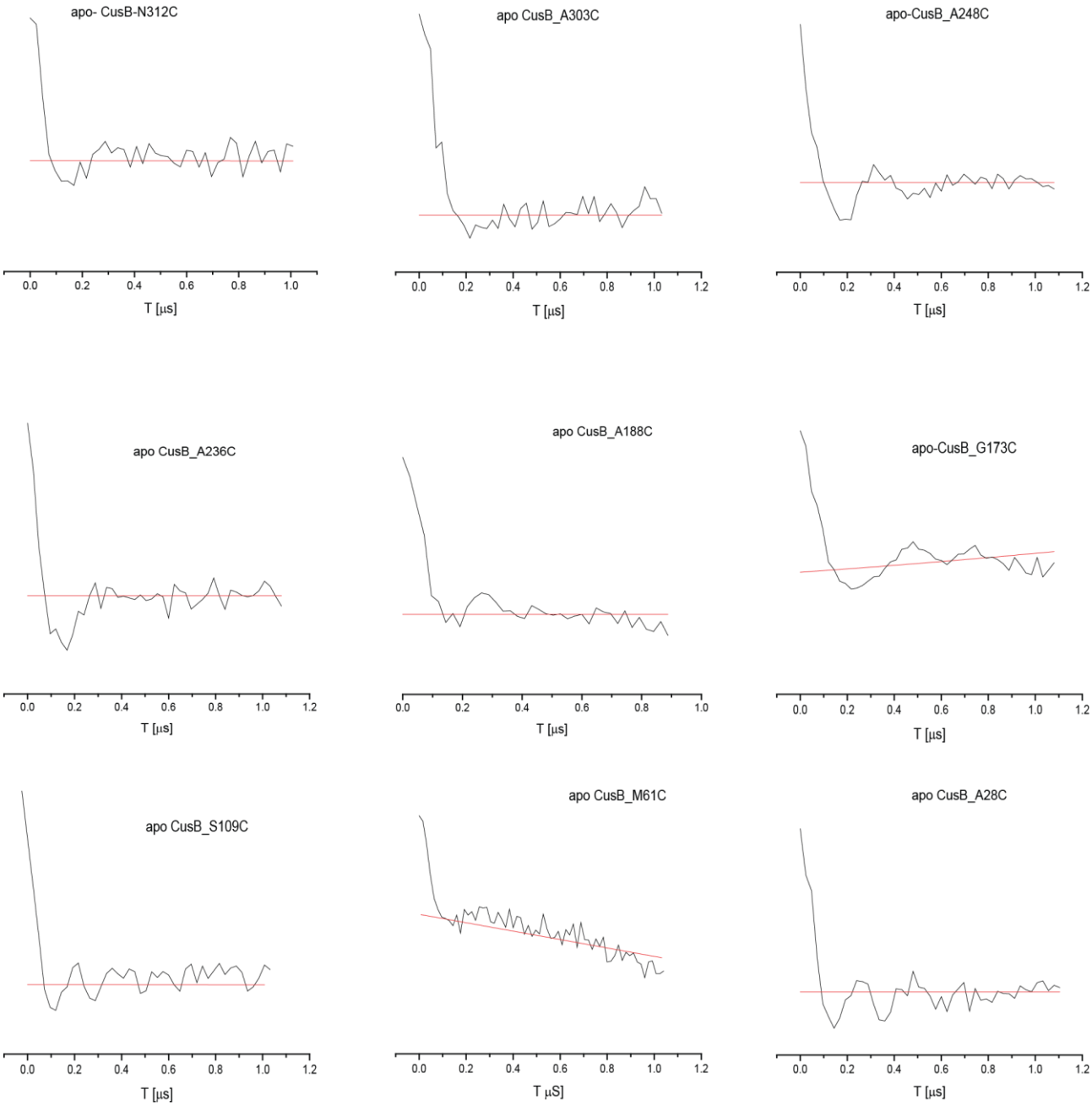

Figure S3: DEER raw data for the various CusB mutants in the apo state (black lines); the red lines denote the homogeneous background contribution.

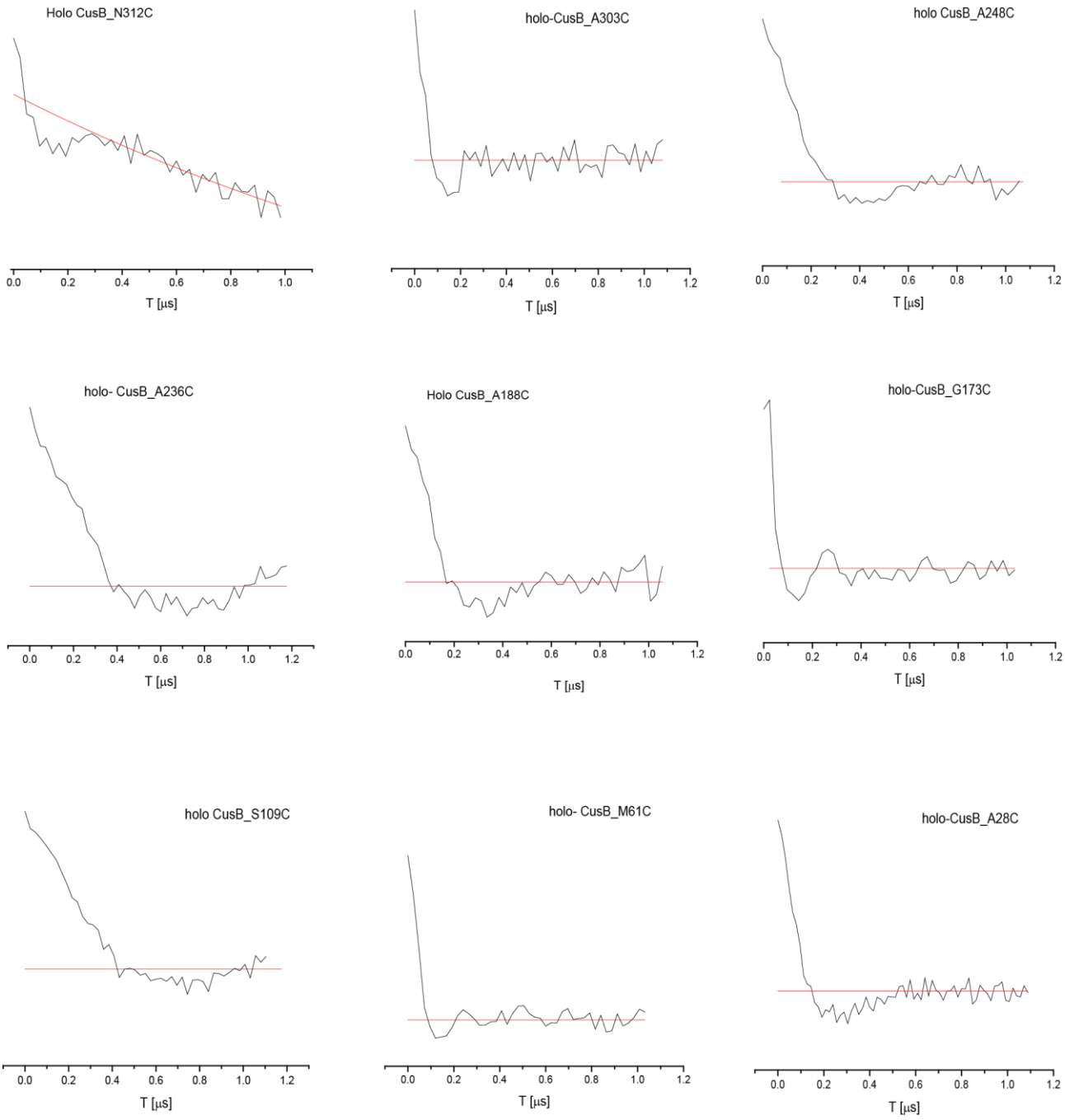

Figure S4: DEER raw data for the various CusB mutants in the holo state (black lines); the red lines denote the homogeneous background contribution.

Room temperature CW-EPR experiments

CW-EPR spectra were recorded using an E500 Elexsys Bruker spectrometer operating at 9.0–9.5 GHz. The spectra were recorded at room temperature using a microwave power of 20.0 mW, a modulation amplitude of 1.0 G, a time constant of 60 ms, and a receiver gain of 60.0 dB. The samples were measured in 0.8 mm capillary quartz tubes (VitroCom).

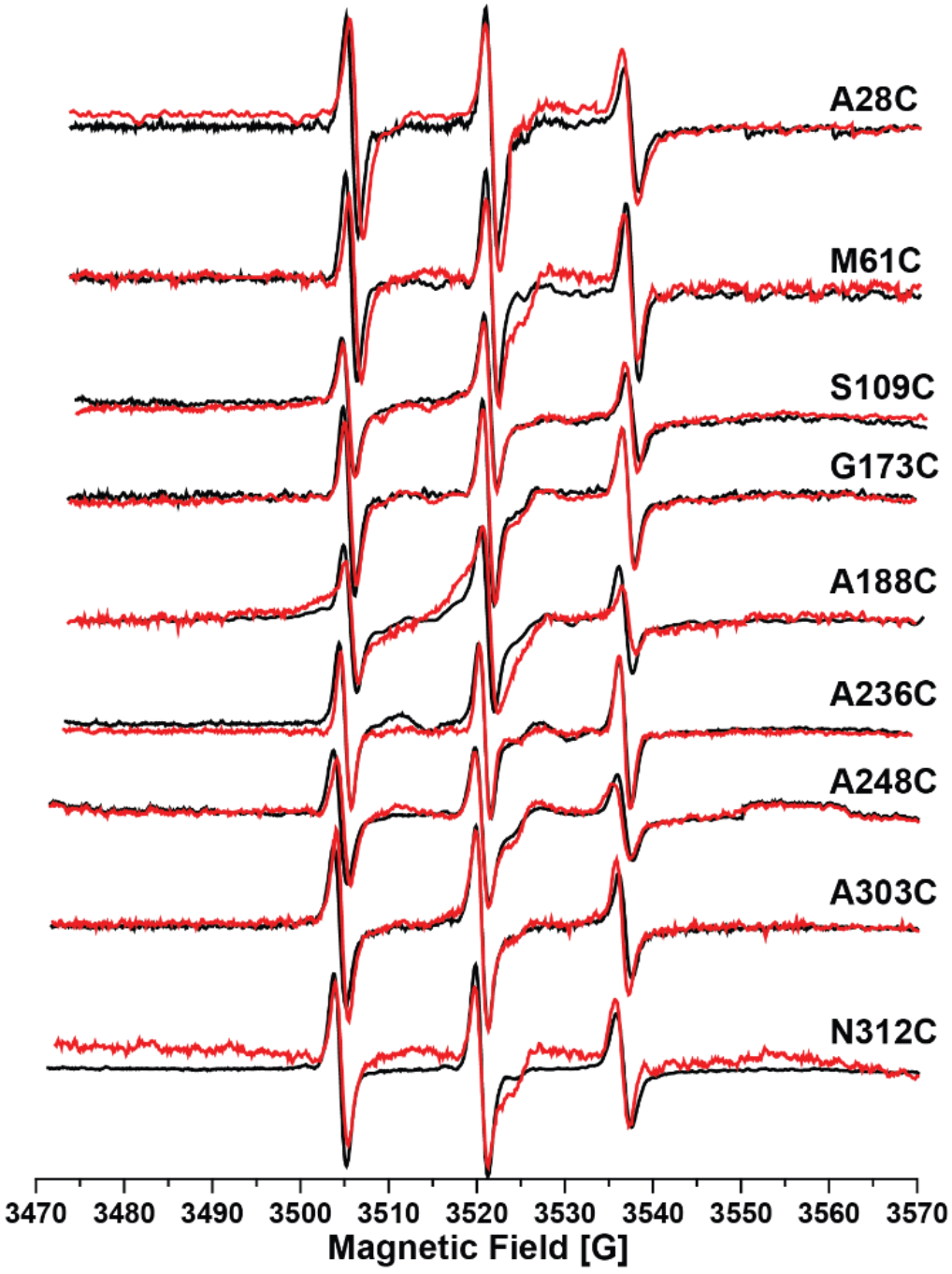

Figure S5: Room temperature X-band CW-EPR spectra of the various CusB mutants in the absence of Cu(I) (black solid line) and in the presence of Cu(I) at a ratio of 3:1 Cu(I):CusB (red). [CusB]=0.04mM.

At room temperature at higher protein concentration, after about 10-20 min, aggregation of the protein appeared, and the CW-EPR spectra become immobilized as observed in Figure S6.

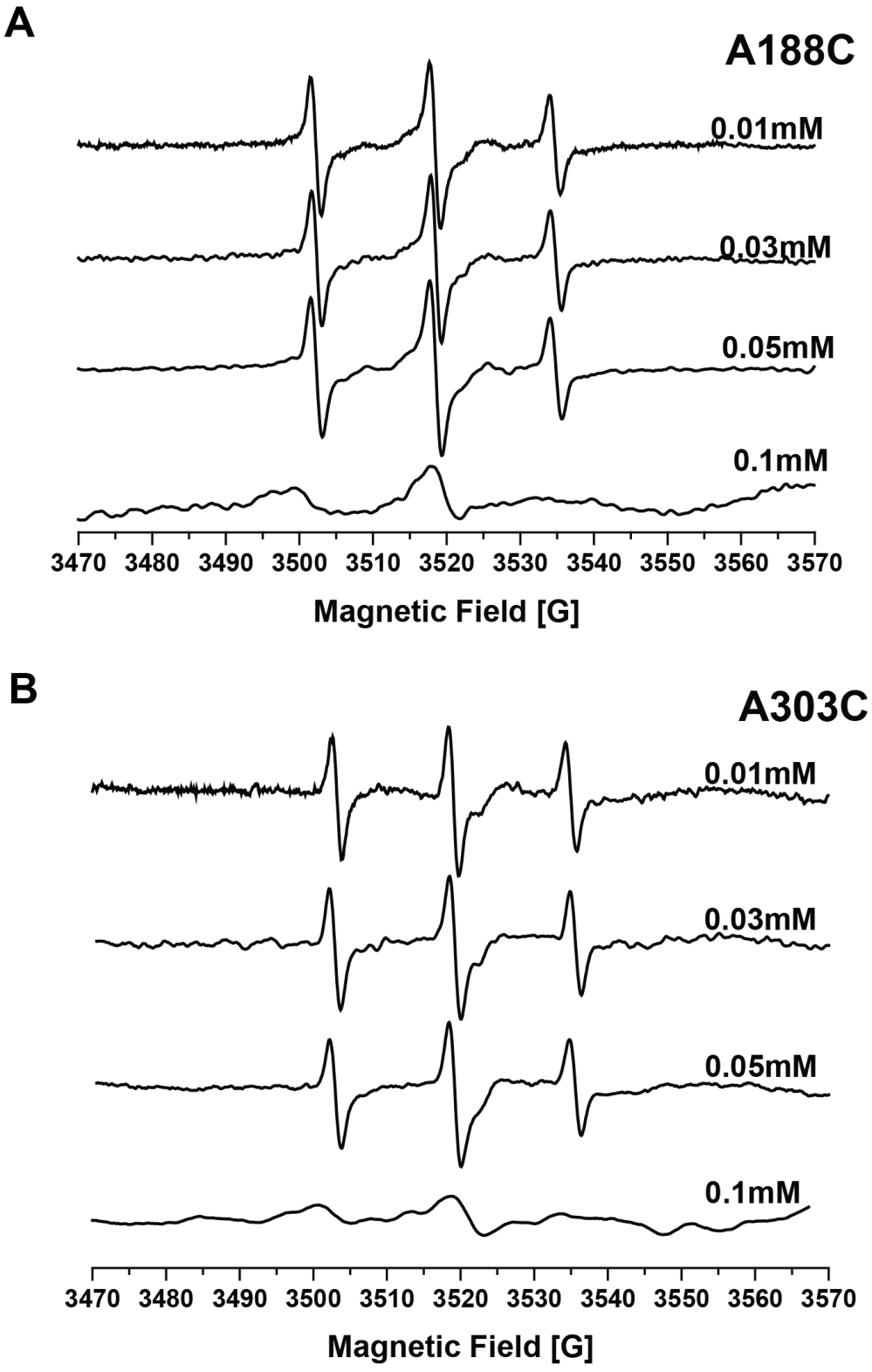

Figure S6: Room temperature X-band CW-EPR spectra of (**A**) CusB_A188C and (**B**) CusB_A303C at various protein concentrations.

Verifying dimerization using Glutaraldehyde crosslinking:

In order to ensure that the highest oligomerization form of CusB is a dimer, glutaraldehyde crosslinking was performed in the presence of high concentrations of Glutaraldehyde up to 54 times the concentration of CusB ([CusB]=0.02mM).

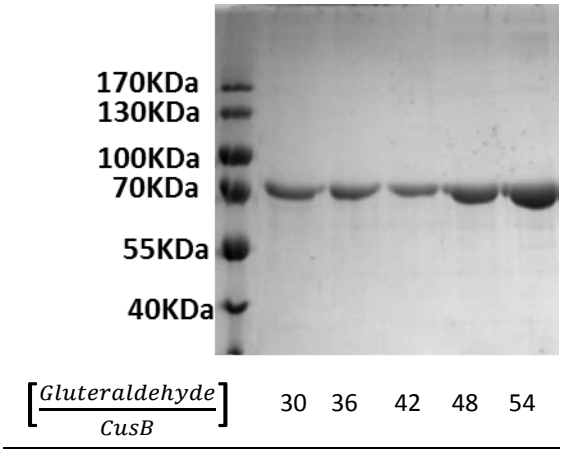

Figure S7: Glycine 10% native gel of CusB_M61C crosslinked in various ratios of Glutaraldehyde concentration, confirming a single oligomeric species. [CusB]=0.015mM.

Dimerization of CusB in solution

Native gel at various concentrations of CusB and Cu(I) was performed in the presence of two standard proteins: Staphylococcal protein A (SPA, PROSPEC) and Human serum albumin (HSA, Sigma-Aldrich). The existence of monomers and dimers in similar ratios can be observed in Figure S2. Since the gel does not include denaturation of the protein or SDS shielding, the protein's location is subjected both to the protein size as well as to its isoelectric point (pI). Having a nearly identical isoelectric point but double in size, both markers efficiently served for distinguishing between CusB's monomeric and dimeric states.

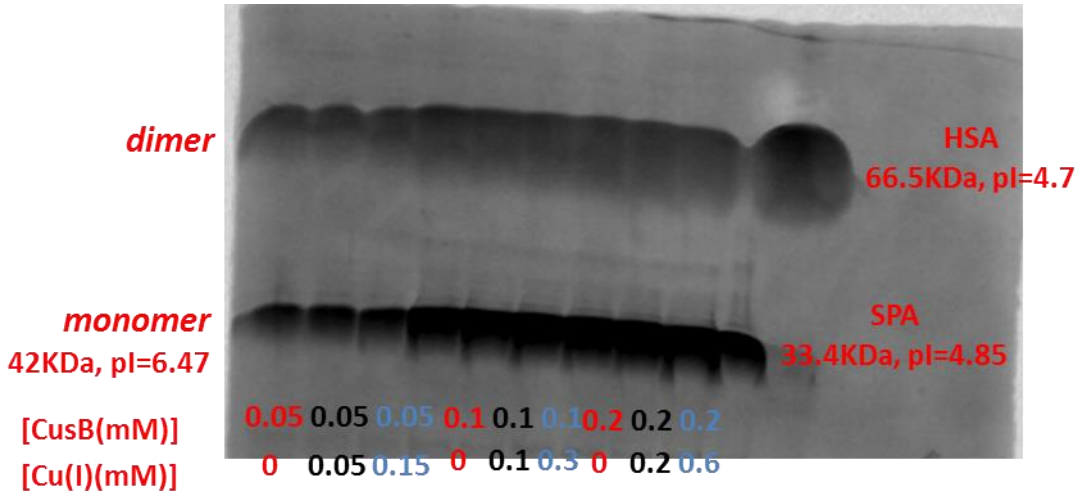

Figure S8: Glycine 10% native gel of CusB_M61C at various concentrations as a function of [Cu(I)]. In the presence of two standard proteins (HSA- 66.5KDa pI=4.7, SPA – 33.4KDa pI=4.85).

Verifying dimerization stability upon spin labeling:

In order to ensure that labeling CusB with MTSSL spin label does not disrupt CusB's dimerization, we ran different mutants on a glycine 10% native gel. Each mutant was run in its labeled and non-labeled form, in identical concentration of 0.2 mM, as determined by Lowry assay. The mutants were run along the commercially available protein human serum albumin (HSA; Sigma-Aldrich), with a known monomeric oligomerization state of monomer, 66.5 KDa in size and with an isoelectric point of 4.7.

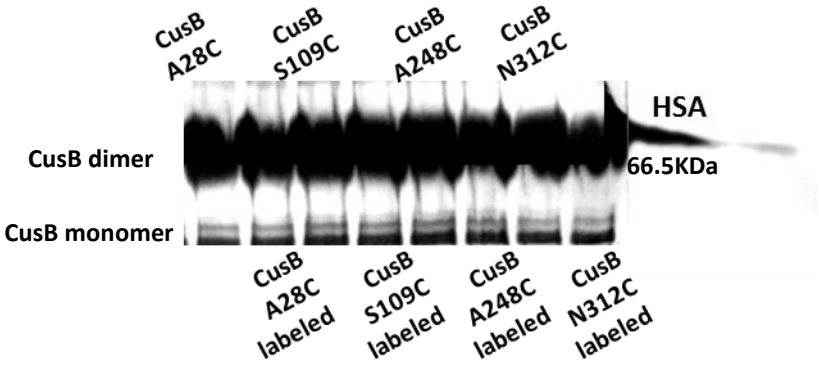

Figure S9: Glycine 10% native gel of CusB spin-labeled and non-labeled CusB mutants confirming no changes in dimerization upon spin labeling. [CusB] = 0.110 mM.

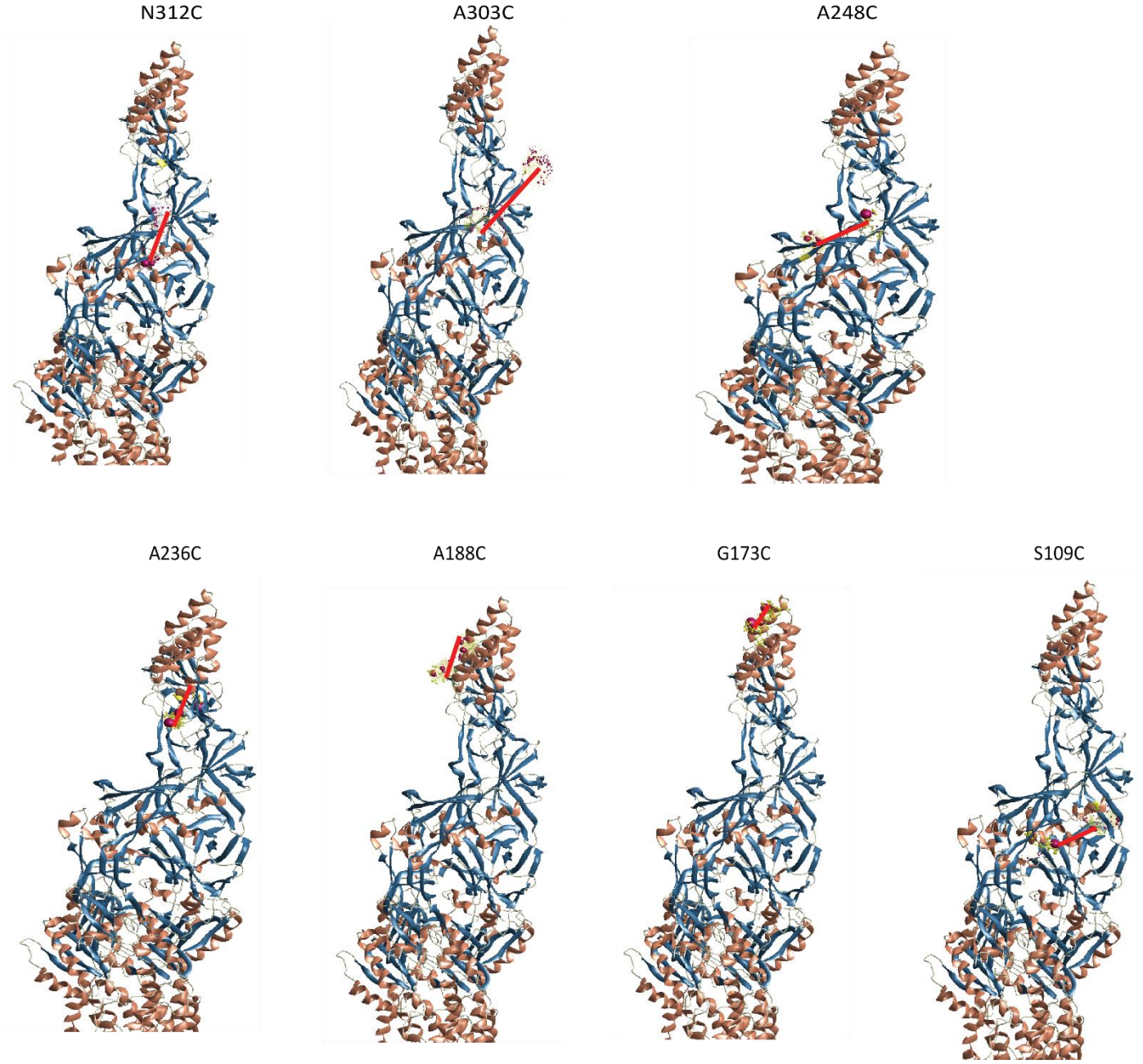

Figure S10: The orientation distribution of the various spin-labeled residues attached to CusB (PDB 3NE5) as derived from MMM simulations (magnification of Figure 4D).

Protein nucleotide sequence and amino acid translation:

CusB nucleotide sequence and amino acid translation

```
   1  ATGAAAAAAATCGCGCTTATTATCGGCAGCATGATCGCGGGCGGTATTATTTCTGCGGCA
   1   M  K  K  I  A  L  I  I  G  S  M  I  A  G  G  I  I  S  A  A

  61  GGTTTTACCTGGGTTGCAAAGGCGGAACCGCCTGCAGAAAAAACGTCGACCGCAGAACGT
  21   G  F  T  W  V  A  K  A  E  P  P  A  E  K  T  S  T  A  E  R

 121  AAAATCTTATTCTGGTACGACCCAATGTATCCCAATACGCGGTTCGATAAACCAGGTAAA
  41   K  I  L  F  W  Y  D  P  M  Y  P  N  T  R  F  D  K  P  G  K

 181  TCGCCGTTTATGGATATGGATCTGGTGCCGAAATATGCCGATGAAGAGAGTTCTGCGTCT
  61   S  P  F  M  D  M  D  L  V  P  K  Y  A  D  E  E  S  S  A  S

 241  GGTGTGCGCATTGACCCGACTCAGACGCAGAATCTGGGGGTGAAAACGGCTACCGTCACG
  81   G  V  R  I  D  P  T  Q  T  Q  N  L  G  V  K  T  A  T  V  T

 301  CGCGGACCGCTGACTTTTGCCCAGAGTTTCCCGGCGAATGTCAGTTACAACGAGTATCAG
 101   R  G  P  L  T  F  A  Q  S  F  P  A  N  V  S  Y  N  E  Y  Q

 361  TATGCCATTGTGCAGGCTCGCGCTGCCGGGTTTATCGACAAGGTGTATCCGCTTACCGTG
 121   Y  A  I  V  Q  A  R  A  A  G  F  I  D  K  V  Y  P  L  T  V

 421  GGCGATAAAGTACAAAAGGGCACACCGCTTCTCGACCTGACCATTCCTGACTGGGTGGAA
 141   G  D  K  V  Q  K  G  T  P  L  L  D  L  T  I  P  D  W  V  E

 481  GCGCAGAGTGAGTATTTACTGCTGCGCGAAACCGGCGGTACGGCGACCCAGACTGAAGGC
 161   A  Q  S  E  Y  L  L  L  R  E  T  G  G  T  A  T  Q  T  E  G

 541  ATTCTTGAGCGGCTGCGACTGGCGGGAATGCCGGAGGCGGATATTCGCCGCCTGATCGCC
 181   I  L  E  R  L  R  L  A  G  M  P  E  A  D  I  R  R  L  I  A

 601  ACGCAAAAAATCCAGACTCGCTTTACGCTCAAAGCGCCCATTGATGGCGTGATCACCGCG
 201   T  Q  K  I  Q  T  R  F  T  L  K  A  P  I  D  G  V  I  T  A

 661  TTTGATCTGCGCGCGGGAATGAATATCGCCAAAGATAACGTGGTAGCGAAAATTCAGGGT
 221   F  D  L  R  A  G  M  N  I  A  K  D  N  V  V  A  K  I  Q  G

 721  ATGGACCCGGTGTGGGTCACTGCTGCGATCCCGGAGTCTATCGCCTGGCTGGTGAAAGAT
 241   M  D  P  V  W  V  T  A  A  I  P  E  S  I  A  W  L  V  K  D

 781  GCCTCGCAGTTTACCCTCACCGTTCCGGCGCGACCGGATAAAACACTCACCATCCGCAAA
 261   A  S  Q  F  T  L  T  V  P  A  R  P  D  K  T  L  T  I  R  K

 841  TGGACGCTGCTACCTGGCGTGGATGCCGCGACCCGCACGCTGCAGCTGCGTCTGGAAGTC
 281   W  T  L  L  P  G  V  D  A  A  T  R  T  L  Q  L  R  L  E  V

 901  GACAACGCCGACGAGGCGCTAAAACCGGGCATGAACGCCTGGTTGCAACTCAACACCGCC
 301   D  N  A  D  E  A  L  K  P  G  M  N  A  W  L  Q  L  N  T  A

 961  AGCGAACCGATGCTGCTCATTCCGTCACAAGCGCTGATTGATACCGGCAGCGAACAGCGG
 321   S  E  P  M  L  L  I  P  S  Q  A  L  I  D  T  G  S  E  Q  R

1021  GTGATTACCGTTGATGCCGACGGGCGCTTTGTACCGAAACGCGTTGCTGTTTTCCAGGCA
 341   V  I  T  V  D  A  D  G  R  F  V  P  K  R  V  A  V  F  Q  A

1081  TCGCAAGGCGTCACCGCATTACGCTCTGGTCTGGCGGAAGGTGAAAAGGTGGTTTCCAGC
 361   S  Q  G  V  T  A  L  R  S  G  L  A  E  G  E  K  V  V  S  S

1141  GGCCTGTTCCTGATTGATTCCGAAGCCAATATTTCTGGCGCACTGGAGCGGATGCGCTCT
 381   G  L  F  L  I  D  S  E  A  N  I  S  G  A  L  E  R  M  R  S

1201  GAAAGTGCTACCCATGCGCATTGA
 401   E  S  A  T  H  A  H  *
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