Pulse Dipolar EPR Reveals Double-Histidine Motif Spin-labelling is Robust Against Competitor Ions

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Pulse-dipolar EPR is an appealing strategy for structural characterization of complex systems in solution that complements other biophysical techniques. Significantly, the emergence of genetically encoded self-assembling spin labels exploiting exogenously introduced double-histidine motifs in conjunction with Cu\textsuperscript{II}-chelates offers high precision distance determination in systems non-permissive to thiol-directed spin labelling. However, the non-covalency of this interaction exposes potential vulnerabilities to competition from adventitious divalent metal ions, and pH sensitivity. Herein, a combination of room-temperature isothermal titration calorimetry (ITC) and cryogenic relaxation-induced dipolar modulation enhancement (RIDME) measurements are applied to the model protein Streptococcus sp. group G protein G, B1 domain (GB1). Results demonstrate double-histidine motif spin labelling using Cu\textsuperscript{II}-nitrilotriacetic acid (Cu\textsuperscript{II}-NTA) is robust against the competitor ligand Zn\textsuperscript{II}-NTA at >1000-fold molar excess, and high nM binding affinity is surprisingly retained under acidic and basic conditions even though room temperature affinity shows a stronger pH dependence. This indicates the strategy is well-suited for diverse biological applications, particularly metalloproteins with divalent metal ion cofactors.

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Pulse Dipolar EPR Reveals Double-Histidine Motif
Spin-labelling is Robust Against Competitor Ions

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ABSTRACT. Pulse-dipolar EPR is an appealing strategy for structural characterization of complex systems in solution that complements other biophysical techniques. Significantly, the emergence of genetically encoded self-assembling spin labels exploiting exogenously introduced double-histidine motifs in conjunction with Cu\textsuperscript{II}-chelates offers high precision distance determination in systems non-permissive to thiol-directed spin labelling. However, the non-covalency of this interaction exposes potential vulnerabilities to competition from adventitious divalent metal ions, and pH sensitivity. Herein, a combination of room-temperature isothermal titration calorimetry (ITC) and cryogenic relaxation-induced dipolar modulation enhancement (RIDME) measurements are applied to the model protein *Streptococcus sp.* group G. protein G, B1 domain (GB1). Results demonstrate double-histidine motif spin labelling using Cu\textsuperscript{II}-nitrilotriacetic acid (Cu\textsuperscript{II}-NTA) is robust against the competitor ligand Zn\textsuperscript{II}-NTA at >1000-fold molar excess, and high nM binding affinity is surprisingly retained under acidic and basic conditions even though room temperature affinity shows a stronger pH dependence. This indicates the strategy is well-suited for diverse biological applications, particularly metalloproteins with divalent metal ion cofactors.

**KEYWORDS** RIDME; Competitive Binding; GB1; EPR; Dissociation Constant; ITC
As the complexity of biomolecular assemblies implicated in health and disease has increased, so too has interest in pulse-dipolar EPR (PD-EPR) as a robust strategy for solution-state structural characterization of proteins\textsuperscript{1,2} and nucleic acids\textsuperscript{3,4} in the nanometer distance regime.\textsuperscript{5,6} PD-EPR is a powerful tool that complements X-ray crystallography, NMR, cryo-EM and Förster Resonance Energy Transfer (FRET) data by providing structural insight outwith crystallization, size-limitation, or structurally perturbative labels. Hence, PD-EPR has been applied to study conformational equilibria,\textsuperscript{7} oligomerization degree,\textsuperscript{8,9} complexation events,\textsuperscript{10–12} and competing structural models.\textsuperscript{13}

Pairs of paramagnetic moieties are commonly introduced into diamagnetic systems of interest using thiol-based site-directed spin labelling.\textsuperscript{14} Cysteine residues are typically covalently modified, as for the nitroxide R1 sidechain (figure 1a top). This strategy is suboptimal in systems containing essential cysteine residues, non-permissive to post-translational reduction. However, Cu\textsuperscript{II}-based genetically-encodable self-assembling spin labels using double-histidine motifs have emerged as an alternative labelling strategy.\textsuperscript{15,16} Additionally, the bipedal mode of Cu\textsuperscript{II}-chelate attachment at the double-histidine motif (figure 1a bottom) results in significantly improved precision and accuracy in the distance domain. Cu\textsuperscript{II}-nitrilotriacetic acid (Cu\textsuperscript{II}-NTA) spin labelling of double histidine motifs for PD-EPR has been applied successfully to enzymes,\textsuperscript{17} metalloproteins,\textsuperscript{18} and nucleoprotein complexes.\textsuperscript{19}
Figure 1. Spin label structures and the GB1 constructs used in this work. a) MTSL nitroxide conjugated to a cysteine residue, resulting in the R1 sidechain (top) and Cu$^{II}$-NTA coordinated to a double-histidine motif (bottom). b) Cartoon representation of the I6R1/K28H/Q32H GB1 construct, with the R1 nitroxide and Cu$^{II}$-NTA spin labels shown in stick representation. c) Cartoon representation of the K28H/Q32H GB1 construct, with the Cu$^{II}$-NTA shown in stick representation.

Despite this success, optimization of the spin labelling approach is non-trivial, because the non-covalency of the interaction predisposes sensitivity to variations in binding affinity. For instance, different buffer conditions influence the double-histidine motif labelling efficiency with Cu$^{II}$-NTA. Furthermore, while the influence of pH upon formation of Cu$^{II}$-chelates has been characterized by CW-EPR previously, current literature has not shown how pH variations influence binding at the double-histidine motif, particularly under cryogenic temperatures. Similarly, current literature has not addressed competition for double-histidine motif sites by adventitious divalent metal ions, and so warrants investigation. In the current study, *Streptococcus sp.* Group G. protein G, B1 domain (GB1) constructs (I6R1/K28H/Q32H (figure 1b) and K28H/Q32H (figure 1c) were used as biological model systems, in Cu$^{II}$-nitroxide relaxation-induced dipolar modulation enhancement (RIDME) pseudo-titrations,$^{10,23}$ and isothermal titration calorimetry (ITC) measurements, respectively.
Measurements were first performed in presence of the model competitor ligand, Zn\textsuperscript{II}-NTA, which was chosen because i) it is a weak ligand for double-histidine motifs compared to Cu\textsuperscript{II}-NTA, and ii) it is diamagnetic, so does not contribute to the detected EPR signal. An EPR silent competitor ligand is desirable because analysis of pseudo-titration data is simplified (see SI section 1.6). Room temperature ITC data (figure 2a) fitted to a one-site model where binding stoichiometry could vary, indicated a binding affinity of 513 μM. Using the determined enthalpy change (ΔH), the binding affinity was extrapolated to 235 K (i.e., the temperature at which the binding equilibrium is found to freeze out in our samples, such that diffusional processes cease, meaning our EPR data reflects equilibria at 235 K),\textsuperscript{22} to determine the influence of the competitor ligand upon double-histidine loading efficiency with Cu\textsuperscript{II}-NTA under PD-EPR conditions.
Figure 2. Zn$^{II}$-NTA competitor RIDME pseudo-titration. a) ITC data performed at 298 K, 800 μM 28H/32H GB1 titrated against 12 mM Zn$^{II}$-NTA. b) RIDME dipolar evolution functions, with the corresponding fits shown in dotted black. Modulation depths (Δ) are indicated. c) Validated RIDME distance distributions, corresponding to the dipolar evolution functions shown in b). The colour scheme is the same in b) and c). The concentrations of Zn$^{II}$-NTA are indicated. Colour bars represent reliability ranges (green: shape reliable; yellow: mean and width reliable; orange: mean reliable; red: no quantification possible). d) A univariate fit of the competitor dissociation constant (32 μM) is shown in solid black. Experimental points are shown as the blue scatter, and 95% confidence intervals are shown as the red error bars.

The corresponding RIDME pseudo-titration was performed at 1 μM protein concentration in presence of 10 μM Cu$^{II}$-NTA (to ensure quantitative loading and negligible ligand depletion\textsuperscript{24} (see SI section 1.6)) and varying Zn$^{II}$-NTA concentrations. Importantly, the dipolar evolution functions (figure 2b) and distance distributions (figure 2c) show that in all cases, the expected
peak at ~2.5 nm is retrieved as the only significant feature following data validation. The fitted competitor \(K_D\) value (32 \(\mu\)M) is within 2-fold of that determined from ITC when extrapolated to 235 K (48 \(\mu\)M) (figure 2d). This suggests that Cu\(^{II}\)-NTA is robust against adventitious divalent metals in vast excesses, >1000-fold, even at low \(\mu\)M protein concentrations. Additionally, this benchmarks quantitation of Cu\(^{II}\)-nitroxide RIDME modulation depths for remotely determining binding affinities of EPR silent ligands, in a competition assay format.

Next, the influence of pH upon double-histidine motif loading efficiency with Cu\(^{II}\)-NTA was investigated by measuring ITC and RIDME at pH 6.4. Since only deprotonated histidine residues can coordinate Cu\(^{II}\)-NTA, it follows that binding affinity should decrease under acidic conditions. Indeed, room-temperature ITC performed at pH 5, below the approximate pK\(_A\) of solvent-exposed histidine,\(^{25}\) shows negligible binding (see SI section 2.3), and measurements at pH 6.4, fitted to a one-site model, indicated a 20-fold reduction in affinity compared to previous work\(^{23}\) (figure 3a). Extrapolating \(\Delta H\) to 235 K suggested a binding affinity of ~4 \(\mu\)M.
Figure 3. pH 6.4 RIDME pseudo-titration. a) ITC data performed at 298 K, 75 μM K28H/Q32H GB1 titrated against 2 mM Cu$^{II}$-NTA. b) RIDME dipolar evolution functions, with the corresponding fits shown in dotted black. Modulation depths (Δ) are indicated. c) Validated RIDME distance distributions, corresponding to the dipolar evolution functions shown in b). The colour scheme is the same in b) and c). The concentrations of Cu$^{II}$-NTA are indicated. d) A bivariate fit of the dissociation constant (0.31 μM) is shown in solid black. Experimental points are shown as the blue scatter, and 95% confidence intervals are shown as the red error bars.

A RIDME pseudo-titration was performed at 5 μM protein concentration to validate the room-temperature ITC prediction of reduced affinity under PD-EPR conditions. Significantly, the dipolar evolution functions (figure 3b) show Cu$^{II}$-NTA binding is only marginally reduced at lower pH, with one equivalent Cu$^{II}$-NTA saturating ~70% of available double-histidine motifs. This is further borne out by the fitted dissociation constant (figure 3d), 0.31 μM compared to 0.14 μM in previous work at pH 7.4.$^{23}$ The affinity reduced by only 2-fold, indicating that the
influence of pH upon double-histidine motif loading may be attenuated at lower temperatures. A possible explanation is that histidine protonation is endothermic,\textsuperscript{26} driving the equilibrium towards the deprotonated state at lower temperatures, compensating for reduced pH and facilitating double-histidine loading. Importantly, this would also imply significantly tighter binding at higher pH, where histidine deprotonation is already favored.

**Figure 4.** pH 8.4 RIDME pseudo-titration. a) ITC data performed at 298 K, 75 μM K28H/Q32H GB1 titrated against 2.5 mM Cu\textsuperscript{II}-NTA. b) RIDME dipolar evolution functions, with the corresponding fits shown in dotted black. Modulation depths (Δ) are indicated. c) Validated RIDME distance distributions, corresponding to the dipolar evolution functions shown in b). The colour scheme is the same in b) and c). The concentrations of Cu\textsuperscript{II}-NTA are indicated. d) A bivariate fit of the dissociation constant (0.091 μM) shown in solid black. Experimental points are shown as the blue scatter, and 95% confidence intervals are shown as the red error bars.
To clarify the disparity between ITC and PD-EPR data at pH 6.4, room-temperature ITC was also performed at pH 8.4 (figure 4a), fitted to a one-site model, where a 20-fold increase in affinity was predicted (via improved thermodynamic favorability of binding) compared to previous work. Another RIDME pseudo-titration was performed at 2 μM protein concentration, with dipolar evolution functions (figure 4b) suggesting modest improvement in binding affinity. The fitted dissociation constant (figure 4d) of 0.091 μM indicates binding affinity is approximately 2-fold higher than at pH 7.4, consistent with observation at pH 6.4 that the influence of pH upon binding affinity is attenuated with decreasing temperature. While an endothermic protonation process would suggest much tighter binding is to be anticipated at pH 8.4, consider that at this pH <1% of histidine δ-nitrogen atoms should remain protonated. This may explain why the relative increase in binding affinity is smaller than expected, since the deprotonation is already driven toward completion by the high pH.

While the data suggests that spin-labelling and measurement at pH 8.4 will afford enhanced loading and sensitivity, it should be noted that the stoichiometry of binding is ~2, compared to ~1 at pH 6.4. This may arise from deprotonation of the protein surface that promotes non-specific binding. This would explain the increased exothermic nature of the binding, if non-specific or additional binding events contributed to the isotherm and would further inflate the binding affinity when extrapolated to cryogenic temperatures. However, the corresponding distance distributions (figure 4c) do not contain additional peaks to support this hypothesis.

Perhaps most significantly, these results clearly show that Cu^{II}-NTA binding affinity for double-histidine motifs is not strongly perturbed from the high nM concentration regime by fluctuations of pH between 6.4-8.4. Coupled with measurements in presence of competitor ligand Zn^{II}-NTA, findings support that Cu^{II}-NTA is a highly robust spin label when combined with α-helical
double-histidine motifs. This is encouraging for the widespread application of double-histidine motifs in metalloproteins, or in systems where divalent metal cofactors are necessary. Additionally, the benchmarking of a competition assay using PD-EPR is particularly exciting because it allows remote detection of binding interactions with diamagnetic ligands. This will be promising in cases where paramagnetic ligand analogues are not available or cause structural perturbation. PD-EPR also has greater sensitivity than ITC and the coupling of thermodynamic and structural information allows for the facile monitoring of non-specific and competitor ligand interactions.27 Traditionally, monitoring competitive ligand binding has required expensive radio-labelling and judicious selection of appropriate isotopes.24,28 PD-EPR may complement these strategies, while obviating potential cost and safety considerations.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

I Experimental Procedures: Construct Design, Expression and Purification; Pulse EPR Sample Preparation; Metal Chelate Spin Label Preparation; Mass Spectrometry; Pulse EPR Measurements; Competitive Binding Model; Isothermal Titration Calorimetry; UV-visible Spectroscopy. II Results and Discussion: Inversion Recovery Measurements; 5-pulse RIDME Measurements; Influence of Differential pH upon Double-Histidine Motif Affinity; Influence of Differential pH upon CuII-NTA Complex Formation; Optimization of CuII-IDA Complex Formation. III References (PDF)

Notes

The authors declare no competing financial interests.
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Supplementary Material

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## III References
Experimental Procedures:

1.1 Construct Design, Expression and Purification:

Constructs of *Streptococcus sp.* group G, protein G B1 domain (GB1) were designed, expressed and purified as previously described. For completeness the protein sequences for the K28H/Q32H and I6C/K28H/Q32H GB1 constructs are given below in figures S1 and S2, respectively. The positions of the cysteine and double-histidine motif are indicated in cyan and green, respectively.

K28H/Q32H GB1 Protein Sequence:

\[
MQYKLNLGKTLKGETTEAVDAATAEVFKHYANDNGVDGKWDDATKFTVTE
\]

Figure S1. Full amino-acid sequence for the K28H/Q32H GB1 construct used in this work, with each histidine residue of the double-histidine motif shown in green; and residue number indicated above the sequence.

I6C/K28H/Q32H GB1 Protein Sequence:

\[
MQYKCLNLGKTLKGETTEAVDAATAEVFKHYANDNGVDGKWDDATKFTVTE
\]

Figure S2. Full amino-acid sequence for the I6C/K28H/Q32H GB1 construct used in this work, with the cysteine residue shown in cyan and each histidine residue of the double-histidine motif shown in green; and residue numbers indicated above the sequence.

1.2 Pulse EPR Sample Preparation:

All material was exchanged into pH-adjusted deuterated buffer A (42.4 mM Na$_2$HPO$_4$, 7.6 mM KH$_2$PO$_4$, 150 mM NaCl) by first freeze-drying and then re-dissolving in D$_2$O. For Q-band RIDME samples of 6R1/28H/32H GB1, a total volume of 70 μL was used, with protein concentrations of 1, 2, or 5 μM as stated. All EPR samples were frozen by direct immersion into liquid nitrogen. Zn$^{II}$-NTA and Cu$^{II}$-NTA stock solutions were prepared as previously described; and for labelling, Zn$^{II}$-NTA and Cu$^{II}$-NTA stock solutions with nominal concentrations of 100 and 10 mM were used, respectively.
1.3 **Metal Chelate Spin Label Preparation:**

For preparation of all metal-NTA labels: Cu$^{II}$-NTA, and Zn$^{II}$-NTA, ZnCl$_2$, CuCl$_2$ and NTA were weighed in a glove-box and aliquoted into 1.5 mL reaction tubes. Stock solutions of 100 mM were prepared as previously described. Complete dissolution was ensured by vortexing until solutions were clear of precipitate. For the optimization of Cu$^{II}$-IDA preparation, solutions of Cu$^{II}$-IDA spin labels were prepared via three distinct methods, i) mixed in a 1:1 equivalence, producing a solution of Cu$^{II}$-IDA with nominal concentration of 50 mM, before being diluted in milliQ H$_2$O, ii) mixed in a 1:1 equivalence before dilution with buffer A (150 mM NaCl, 42.4 mM Na$_2$HPO$_4$, 7.6 mM KH$_2$PO$_4$, pH 7.4), and iii) IDA stock solution was added in 1:8 equivalence with buffer, before addition of 1 equivalent of CuCl$_2$. A dilution series of 20, 10, 5, 2.5 and 1 mM Cu$^{II}$-IDA was prepared. To simulate spin label loading of protein double-histidine motifs, absorbance spectra were also recorded for the Cu$^{II}$-IDA dilution series in presence of two equivalents of imidazole. A stock solution of 200 mM imidazole was prepared in buffer A and subsequently diluted upon addition to pre-neutralized 50 mM Cu$^{II}$-IDA stock solution to 100 mM. Further dilution with buffer yielded the nominal concentrations given above.

1.4 **Mass Spectrometry:**

Mass spectrometry data was collected in-house using a Sciex Matrix Assisted Laser Desorption/Ionization (MALDI) TOF/TOF 4800 mass-spectrometer, with samples crystallized using a matrix of α-cyano-4-hydroxycinnamic acid. I6R1/K28H/Q32H and K28H/Q32H GB1 samples were both prepared at 20 μM concentration in buffer A (42.4 mM Na$_2$HPO$_4$, 7.6 mM KH$_2$PO$_4$, 150 mM NaCl, pH 7.4), and mass spectra were recorded in the absence of Cu$^{II}$-chelate.

1.5 **Pulse EPR Measurements:**

All pulse EPR experiments were performed using a Bruker ELEXSYS 580 pulse EPR spectrometer. Temperatures were maintained using a cryogen-free variable temperature cryostat (Cryogenic Ltd) operating in the 1.8-300 K temperature range. All measurements of the electron spin longitudinal relaxation times ($T_1$) of Cu$^{II}$-NTA, and all 5-pulse dead-time free RIDME measurements were performed at 30 K, using a high-power 150 W travelling-wave tube (TWT; Applied Systems
Engineering) at Q-band (34 GHz) in a critically coupled 3 mm cylindrical resonator (Bruker ER 5106QT-2w in TE012 mode).

All RIDME measurements were performed as 6-point pseudo-titration series and used the pulse sequence \((\pi/2 - \tau_1 - \pi - (\tau_2 + t) - \pi/2 - T_{mix} - \pi/2 - (\tau_2 - t) - \pi - \tau_2 - \text{echo})\). Detection pulse lengths of 12 and 24 ns (\(\pi/2\) and \(\pi\)) and a detection position at the maximum of the nitroxide spectrum. Unless otherwise stated, each trace was acquired using an SRT of 30 ms, a \(\tau_1\) of 400 ns, a \(\tau_2\) of 1500 ns, with 122 points, 2 shots-per-point, and varying number of scans, as stated. Deuterium ESEEM was suppressed using a 16-step tau-averaging cycle, and unwanted echoes were eliminated using an 8-step phase cycle, for a total of 128 steps per scan. Each measurement was acquired with a short \((T_{ref})\) and long \((T_{mix})\) mixing time, of 5 and 200 \(\mu\)s to allow suppression and observation of the dipolar coupling, respectively. Deconvoluted RIDME data was background corrected assuming a stretched exponential background function, with dimension 3-6. Data was processed and validations were performed using DeerAnalysis2018. Background dimension and start-time parameters for data processing were determined by an initial validation, consisting of 56 trials; 8 iterations of background start position (between 5-30% of the total RIDME trace length), and 7 iterations of background dimension (between 3-6 in increments of 0.5). Subsequently, a second round of validations was performed. A total of 896 trials were performed for the second validation round, consisting of 16 white noise iterations (noise level of 1.5), 8 iterations of background start position (between 5-30% of the total RIDME trace length), and 7 iterations of background dimension (between 3-6 in increments of 0.5). These validation trials were also pruned, where trials exceeding the RMSD of the global minimum by \(\geq 15\%\) were discarded. Distance distributions are based on the dipolar coupling calculated for the free electron \(g\)-value and this has not been corrected throughout the manuscript. If the distributions were analyzed and interpreted in detail the x-axes would need to be scaled by a factor 0.938. Furthermore, background imperfections from using a short \(\tau_1\) were compensated by dividing by reference traces and did not hamper modulation depth analysis.

All inversion recovery measurements were performed using detection pulse lengths of 16 and 32 ns (\(\pi/2\) and \(\pi\)), and an ELDOR \(\pi\)-pulse length of 32 ns. The detection position was consistently placed at the maximum of the Cu\(^{II}\)-NTA spectrum. Unless otherwise stated, each trace was acquired using
an SRT of 2 ms, with 2560 points, 25 shots-per-point, and 1-5 scans, as stated. A τ of 800 ns was used, with an approximate time-window length of 500 µs, incremented in steps of 200 ns.

1.6 Competitive Binding Model:

Let us begin by considering a binding equilibrium in presence of a competitor ligand (A), with the following two approximations: i) the binding of the ligand (L) and (A) to a protein (P) is mutually exclusive, and ii) the competitor (A) binds to (P) weakly with respect to (L). Here we can define a dissociation constant for both the ligand ($K_{DL}$) and for the competitor ($K_{DA}$), given in (1) and (2) respectively:

$$K_{DL} = \frac{[P][L]}{[PL]}$$

(1)

$$K_{DA} = \frac{[P][A]}{[PA]}$$

(2)

Rearranging (1) and (2) for equilibrium concentrations of protein and protein-competitor complex, given as $[P]$ and $[PA]$ respectively, yields (3) and (4):

$$[P] = \frac{K_{DL}[PL]}{[L]}$$

(3)

$$[PA] = \frac{[P][A]}{K_{DA}}$$

(4)

Here, an expression for total protein, $[P]_0$, can be defined:

$$[P]_0 = [P] + [PL] + [PA]$$

(5)

Substitution of (3) and (4) into (5) yields (6):

$$[P]_0 = \frac{K_{DL}[PL]}{[L]} + [PL] + \frac{K_{DL}[PL][A]}{[L]K_{DA}}$$

(6)

Symbolic substitution and rearrangement of (6) to solve for $[PL]$ yields (7):

$$[PL] = \frac{[P]_0[L]}{[L] + K_{DL} \left(1 + \frac{[A]}{K_{DA}}\right)}$$

(7)

Hence the effect of the competitor ligand can be subsumed into an apparent dissociation constant defined as:

$$K_{DLAPP} = K_{DL} \left(1 + \frac{[A]}{K_{DA}}\right)$$

(8)
Owing to assumption ii) (that the competitor ligand A binds to protein P weakly with respect to ligand L), in (8) we can assume that (9) is also well met:

\[ [A] = [A]_0 \]  \\

\[ (9) \]

In the simple case of a single ligand-binding site, Cu
\( ^{1} \)-nitroxide RIDME modulation depths (\( \Delta \)) can be related to the fractional saturation of the protein in terms of total protein and ligand concentrations and ligand dissociation constant \( K_{DL} \), given in (10):

\[ \Delta \times \Delta_{T_{mix}}^{-1} = \frac{[PL]}{[P]_0} = \frac{(K_{DL} + [P]_0 + [L]_0) - \sqrt{(K_{DL} + [P]_0 + [L]_0)^2 - 4[P]_0[L]_0}}{2[P]_0} \]  \\

\[ (10) \]

where \( \Delta \) is the observed modulation depth, and \( \Delta_{T_{mix}} \) is the asymptotic limit of modulation depth for a given ratio of the mixing time interval \( T_{mix} \), and the longitudinal relaxation time constant \( T_1 \) under the mono-exponential approximation, given by (11):

\[ \Delta_{T_{mix}} = \left(1 - \exp \left(\frac{-T_{mix}}{T_1}\right)\right) \]  \\

\[ (11) \]

Substitution of (8) and (9) into (10) yields (12):

\[ \Delta \times \Delta_{T_{mix}}^{-1} = \frac{[PL]}{[P]_0} = \frac{(K_{DL} \left(1 + \frac{[A]_0}{K_{DA}}\right) + [P]_0 + [L]_0) - \sqrt{(K_{DL} \left(1 + \frac{[A]_0}{K_{DA}}\right) + [P]_0 + [L]_0)^2 - 4[P]_0[L]_0}}{2[P]_0} \]  \\

\[ (12) \]

This gives an expression for modelling modulation depths as a function of total competitor concentration, in presence of fixed concentrations of protein and non-competitor ligand. For a diamagnetic competitor, \( \Delta \times \Delta_{T_{mix}}^{-1} \) is a continuously decreasing function, though this condition is not always well met for a paramagnetic competitor. Therefore, the analysis of the pseudo-titration data is simplified for diamagnetic competitor ligands. Importantly, (12) is not corrected for ligand depletion effects of the non-competitor ligand.\(^{10}\) This ligand depletion phenomenon can be defined as:

\[ \partial = \frac{[PL]}{[L]_0} \]  \\

\[ (13) \]
Particularly under conditions of low concentrations of ligand, ligand-depletion can be approximated as:

\[ \vartheta = \frac{[P]_0}{(K_{DL} + [P]_0)} \quad (14) \]

This implies that to keep ligand depletion below 10%, the concentration of \([P]_0\) should not exceed \(K_{DL} \times 0.1\), however under the conditions of our system (given the high affinity of ligand-binding) this is impractical. Instead, all our measurements and simulations were performed assuming a 10-fold excess of ligand with respect to protein so that in the limiting case of complete saturation:

\[ \vartheta = \frac{[PL]}{[L]_0} = \frac{[PL]}{10 \times [PL]} \leq 0.1 \quad (15) \]

This is also part of the rationale for measuring and simulating RIDME pseudo-titrations of increasing competitor concentration with a constant ligand and protein concentration. Under our measurement conditions, both ligand and competitor depletion effects are held below 10%, and therefore are precluded from further considerations.

1.7 Isothermal Titration Calorimetry:

All isothermal titration calorimetry experiments used a Malvern MicroCal ITC200 instrument, and were optimized and performed over 19 injections of 2 μL titrant, with an equilibration time of 120 seconds between injections, at 298 K. All solutions were degassed before the use and the final samples were centrifuged immediately before measurements. For the Zn\(^{II}\)-NTA measurements, K28H/Q32H GB1 concentration was 800 μM, and titrant concentration was 12 mM. For the variable pH measurements, K28H/Q32H GB1 concentration was 75 μM and titrant concentration was 2.5 mM. Blank conditions of either buffer A (42.4 mM Na\(_2\)HPO\(_4\), 7.6 mM KH\(_2\)PO\(_4\), 150 mM NaCl, pH 7.4) titrated against addition of 12 mM titrant, or pH adjusted buffer A titrated against addition of 1.0 or 2.0 mM titrant, were recorded for the competitor and variable pH ITC measurements, respectively. Subtraction of the blank measurements from the raw data mitigated the heat of dilution. All data analyses were performed in MicroCal Origin 7 (OriginLab, Northampton, MA) and thermodynamic parameters were derived using a single-site fitting model.
1.8  **UV-visible Spectroscopy:**

All UV-visible absorbance spectra were recorded using a Jenway 67 series UV-vis spectrophotometer, in plastic cuvettes with a path length of 10 mm, in single-beam mode. All spectra are recorded at ambient temperature, and with a wavelength resolution of 1 nm, in the wavelength range 320-800 nm. Each sample was blanked before measurement, and all were repeated in triplicate. Data was processed using the Jenway 67-series software suite, and were exported to ASCII format for plotting and analysis in Matlab. For quantitation of Cu$^{II}$-IDA and Cu$^{II}$-NTA concentration, molar extinction coefficients of 62 M$^{-1}$cm$^{-1}$ and 59 M$^{-1}$cm$^{-1}$ at 726 nm, and 800 nm were used, respectively, taken from previous literature.$^{1,11}$
Results and Discussion:

2.1 Inversion Recovery Measurements:

Inversion recovery measurements were performed to estimate the longitudinal relaxation time of the Cu\(^{II}\)-NTA, and the raw data is shown below in figures S3-5, for pH 6.4 and 8.4, and in presence of the competitor Zn\(^{II}\)-NTA, respectively. The corresponding mono- and bi-exponential fits are shown as red and blue traces, respectively. The estimates of \(T_1\) fitted under the mono- and bi-exponential approximations, as well as the reciprocal e-times are given in tables S1-3.

Figure S3. Inversion recovery data at pH 6.4 for 0.98, 2.0 and 4.9 \(\mu\)M Cu\(^{II}\)-NTA (top row), and 9.5, 21 and 85 \(\mu\)M Cu\(^{II}\)-NTA (bottom row) in presence of 5 \(\mu\)M 16R1/28H/32H GB1 shown left-to-right, respectively. The experimental data is shown in black, with the mono-exponential and bi-exponential fits shown as red and blue dotted lines, respectively.
Table S1. Mono- and bi-exponential $T_1$ estimates, and $1/e$ times for the inversion recovery data shown in figure S3.

| Sample                                      | Mono-exponential $T_1$ [$\mu$s] | Bi-exponential $T_1$ [$\mu$s] | $1/e$ time [$\mu$s] |
|---------------------------------------------|---------------------------------|-------------------------------|---------------------|
| 5 µM 6R1/28H/32H + 0.98 µM Cu$^{2+}$-NTA    | 37.1 ± 0.34                     | 31.6 (0.91) / 159 (0.09)      | 44.6                |
| 5 µM 6R1/28H/32H + 2.0 µM Cu$^{2+}$-NTA     | 38.7 ± 0.28                     | 22.1 (0.50) / 55.6 (0.50)     | 47.0                |
| 5 µM 6R1/28H/32H + 4.9 µM Cu$^{2+}$-NTA     | 38.6 ± 0.19                     | 24.2 (0.61) / 63.9 (0.39)     | 46.8                |
| 5 µM 6R1/28H/32H + 9.5 µM Cu$^{2+}$-NTA     | 35.0 ± 0.21                     | 21.0 (0.63) / 60.9 (0.37)     | 42.4                |
| 5 µM 6R1/28H/32H + 21.0 µM Cu$^{2+}$-NTA    | 27.5 ± 0.16                     | 16.2 (0.65) / 49.5 (0.35)     | 31.6                |
| 5 µM 6R1/28H/32H + 85.0 µM Cu$^{2+}$-NTA    | 22.6 ± 0.12                     | 14.2 (0.67) / 40.9 (0.33)     | 27.0                |

Figure S4. Inversion recovery data at pH 8.4 for 0.22, 0.45 and 0.91 µM Cu$^{2+}$-NTA (top row), and 1.4, 2.2 and 4.5 µM Cu$^{2+}$-NTA (bottom row) in presence of 2 µM 16R1/28H/32H GB1 shown left-to-right, respectively. The experimental data is shown in black, with the mono-exponential and bi-exponential fits shown as red and blue dotted lines, respectively.
Table S2. Mono- and bi-exponential $T_1$ estimates, and $1/e$ time for the inversion recovery data shown in figure S4.

| Sample | Mono-exponential $T_1$ [µs] | Bi-exponential $T_1$ [µs] | $1/e$ time [µs] |
|--------|----------------------------|---------------------------|----------------|
| $2 \mu M$ 6R1/28H/32H + 0.22 $\mu M$ Cu$^{2+}$-NTA | 46.6 ± 2.0 | 9.57 (0.30) / 56.6 (0.70) | 41.6 |
| $2 \mu M$ 6R1/28H/32H + 0.45 $\mu M$ Cu$^{2+}$-NTA | 53.6 ± 1.2 | 29.7 (0.62) / 104 (0.38) | 59.4 |
| $2 \mu M$ 6R1/28H/32H + 0.91 $\mu M$ Cu$^{2+}$-NTA | 51.9 ± 0.9 | 29.2 (0.60) / 92.5 (0.40) | 56.4 |
| $2 \mu M$ 6R1/28H/32H + 1.4 $\mu M$ Cu$^{2+}$-NTA | 50.9 ± 0.4 | 27.8 (0.56) / 82.9 (0.44) | 58.4 |
| $2 \mu M$ 6R1/28H/32H + 2.2 $\mu M$ Cu$^{2+}$-NTA | 49.1 ± 0.4 | 28.6 (0.58) / 81.0 (0.42) | 55.4 |
| $2 \mu M$ 6R1/28H/32H + 4.5 $\mu M$ Cu$^{2+}$-NTA | 43.2 ± 0.3 | 24.9 (0.63) / 79.6 (0.37) | 48.8 |

Figure S5. Inversion recovery data for 10 $\mu M$ Cu$^{2+}$-NTA in presence of 0, 0.77, 2.2 mM Zn$^{2+}$-NTA (top row), and 5.0, 15 and 30 mM Zn$^{2+}$-NTA (bottom row), in presence of 1 $\mu M$ 6R1/28H/32H GB1 shown left-to-right, respectively. The experimental data is shown in black, with the mono-exponential and bi-exponential fits shown as red and blue dotted lines, respectively.
Table S3. Mono- and bi-exponential $T_1$ estimates, and $1/e$ time for the inversion recovery data shown in figure S5.

| Sample | Mono-exponential $T_1$ [µs] | Bi-exponential $T_1$ [µs] | $1/e$ time [µs] |
|--------|----------------------------|---------------------------|-----------------|
| 1 µM 6R1/28H/32H + 0 mM Zn$^{II}$-NTA | 22.7 ± 0.14 | 13.7 (0.66) / 40.9 (0.34) | 27.0 |
| 1 µM 6R1/28H/32H + 0.77 mM Zn$^{II}$-NTA | 23.7 ± 0.15 | 14.8 (0.71) / 48.3 (0.29) | 28.0 |
| 1 µM 6R1/28H/32H + 2.2 mM Zn$^{II}$-NTA | 23.2 ± 0.14 | 14.4 (0.68) / 43.3 (0.32) | 27.8 |
| 1 µM 6R1/28H/32H + 5.0 mM Zn$^{II}$-NTA | 25.7 ± 0.17 | 15.5 (0.68) / 49.5 (0.32) | 29.4 |
| 1 µM 6R1/28H/32H + 15 mM Zn$^{II}$-NTA | 29.3 ± 0.19 | 17.5 (0.67) / 55.5 (0.33) | 33.6 |
| 1 µM 6R1/28H/32H + 30 mM Zn$^{II}$-NTA | 28.7 ± 0.17 | 17.3 (0.67) / 53.7 (0.33) | 34.2 |
2.2 5-pulse RIDME Measurements:

RIDME traces recorded with a mixing time interval of 200 μs were deconvoluted with traces recorded with a reference mixing time of 5 μs. Traces and corresponding distance distributions, with shaded regions indicating the ± 2σ confidence intervals are shown below in figures S6-S11, S12-17, and S18-23 for pH 6.4, pH 8.4 and competitor pseudo-titration series, respectively. The color bars represent the reliability ranges described in the DeerAnalysis manual; green indicates shape is reliable, yellow indicates mean and width are reliable, orange indicates mean is reliable, red indicates no quantification is possible. Parameters for the stretched exponential background correction are given in tables S4-6, respectively.

**Figure S6**: RIDME data of 5 μM 6R1/28H/32H GB1 in presence of 0.98 μM Cu²⁺-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right respectively.

**Figure S7**: RIDME data of 5 μM 6R1/28H/32H GB1 in presence of 2.0 μM Cu²⁺-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.
**Figure S8**: RIDME data of 5 µM 6R1/28H/32H GB1 in presence of 4.9 µM Cu²⁺-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.

**Figure S9**: RIDME data of 5 µM 6R1/28H/32H GB1 in presence of 9.5 µM Cu²⁺-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right respectively.
Figure S10: RIDME data of 5 µM 6R1/28H/32H GB1 in presence of 21 µM Cu²⁺-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.

Figure S11: RIDME data of 5 µM 6R1/28H/32H GB1 in presence of 85 µM Cu²⁺-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.

| Sample                             | Zero-time [ns] | Background Start [ns] | Background Cut-off [ns] | Background Dimension | Modulation depth (Δ) |
|------------------------------------|----------------|-----------------------|-------------------------|----------------------|-----------------------|
| 5 µM 6R1/28H/32H + 0.98 µM Cu²⁺-NTA| 206            | 372                   | 1236                    | 6.00                 | 0.075 ± 3.0 x 10⁻³   |
| 5 µM 6R1/28H/32H + 2.0 µM Cu²⁺-NTA | 206            | 151                   | 1236                    | 6.00                 | 0.149 ± 5.0 x 10⁻³   |
| 5 µM 6R1/28H/32H + 4.9 µM Cu²⁺-NTA | 206            | 62                    | 1236                    | 6.00                 | 0.355 ± 1.1 x 10⁻²   |
| 5 µM 6R1/28H/32H + 9.5 µM Cu²⁺-NTA | 206            | 151                   | 1236                    | 6.00                 | 0.407 ± 1.3 x 10⁻²   |
| 5 µM 6R1/28H/32H + 21 µM Cu²⁺-NTA  | 205            | 62                    | 1236                    | 6.00                 | 0.420 ± 1.1 x 10⁻²   |
| 5 µM 6R1/28H/32H + 85 µM Cu²⁺-NTA  | 205            | 151                   | 1236                    | 6.00                 | 0.436 ± 1.4 x 10⁻²   |

Table S4. Parameters for the stretched exponential background correction and associated modulation depths of the RIDME pseudo-titration shown in figures S6-11.
Figure S12: RIDME data of 2 µM 6R1/28H/32H GB1 in presence of 0.22 µM Cu²⁺-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right respectively.

Figure S13: RIDME data of 2 µM 6R1/28H/32H GB1 in presence of 0.45 µM Cu²⁺-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right respectively.
Figure S14: RIDME data of 2 µM 6R1/28H/32H GB1 in presence of 0.91 µM Cu\(^{II}\)-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.

Figure S15: RIDME data of 2 µM 6R1/28H/32H GB1 in presence of 1.4 µM Cu\(^{II}\)-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.
Figure S16: RIDME data of 2 µM 6R1/28H/32H GB1 in presence of 2.2 µM Cu\textsuperscript{II}-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.

Figure S17: RIDME data of 2 µM 6R1/28H/32H GB1 in presence of 4.5 µM Cu\textsuperscript{II}-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right respectively.

| Sample                       | Zero-time [ns] | Background Start [ns] | Background Cut-off [ns] | Background Dimension | Modulation depth (Δ)       |
|------------------------------|----------------|-----------------------|------------------------|----------------------|----------------------------|
| 2 µM 6R1/28H/32H + 0.22 µM Cu\textsuperscript{II}-NTA | 206            | 194                   | 1236                   | 6.00                 | 0.032 ± 2.8 x 10\textsuperscript{-3} |
| 2 µM 6R1/28H/32H + 0.45 µM Cu\textsuperscript{II}-NTA | 207            | 328                   | 1236                   | 6.00                 | 0.061 ± 3.4 x 10\textsuperscript{-3} |
| 2 µM 6R1/28H/32H + 0.91 µM Cu\textsuperscript{II}-NTA | 205            | 151                   | 1236                   | 6.00                 | 0.144 ± 4.6 x 10\textsuperscript{-3} |
| 2 µM 6R1/28H/32H + 1.4 µM Cu\textsuperscript{II}-NTA | 205            | 62                    | 1236                   | 6.00                 | 0.363 ± 1.1 x 10\textsuperscript{-2} |
| 2 µM 6R1/28H/32H + 2.2 µM Cu\textsuperscript{II}-NTA | 204            | 62                    | 1248                   | 6.00                 | 0.365 ± 1.3 x 10\textsuperscript{-2} |
| 2 µM 6R1/28H/32H + 4.5 µM Cu\textsuperscript{II}-NTA | 206            | 62                    | 1236                   | 6.00                 | 0.413 ± 1.4 x 10\textsuperscript{-2} |

Table S5. Parameters for the stretched exponential background correction and associated modulation depths of the RIDME pseudo-titration shown in figures S12-17.
Figure S18: RIDME data of 1 µM 6R1/28H/32H GB1 in presence of 10 µM Cu²⁺-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.

Figure S19: RIDME data of 1 µM 6R1/28H/32H GB1 in presence of 10 µM Cu²⁺-NTA and 0.77 mM Zn²⁺-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.
Figure S20: RIDME data of 1 µM 6R1/28H/32H GB1 in presence of 10 µM Cu^{II}-NTA and 2.2 mM Zn^{II}-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.

Figure S21: RIDME data of 1 µM 6R1/28H/32H GB1 in presence of 10 µM Cu^{II}-NTA and 5.0 mM Zn^{II}-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.
Figure S22: RIDME data of 1 µM 6R1/28H/32H GB1 in presence of 10 µM Cu\(^{2+}\)-NTA and 15.0 mM Zn\(^{2+}\)-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.

Figure S23: RIDME data of 1 µM 6R1/28H/32H GB1 in presence of 10 µM Cu\(^{2+}\)-NTA and 30.0 mM Zn\(^{2+}\)-NTA. The experimental trace, background corrected data, and distance distribution are shown left-to-right, respectively.

| Sample | Zero-time [ns] | Background Start [ns] | Background Cut-off [ns] | Background Dimension | Modulation depth (Δ) |
|--------|---------------|------------------------|-------------------------|----------------------|----------------------|
| 1 µM 6R1/28H/32H + 0 mM Zn\(^{2+}\)-NTA | 206 | 151 | 1236 | 6.00 | 0.419 ± 1.1 x 10\(^{-2}\) |
| 1 µM 6R1/28H/32H + 0.77 mM Zn\(^{2+}\)-NTA | 207 | 328 | 1236 | 6.00 | 0.364 ± 1.5 x 10\(^{-2}\) |
| 1 µM 6R1/28H/32H + 2.2 mM Zn\(^{2+}\)-NTA | 205 | 151 | 1236 | 6.00 | 0.214 ± 8.0 x 10\(^{-3}\) |
| 1 µM 6R1/28H/32H + 5.0 mM Zn\(^{2+}\)-NTA | 206 | 106 | 1236 | 6.00 | 0.128 ± 8.8 x 10\(^{-3}\) |
| 1 µM 6R1/28H/32H + 15 mM Zn\(^{2+}\)-NTA | 206 | 62 | 1236 | 6.00 | 0.054 ± 4.6 x 10\(^{-3}\) |
| 1 µM 6R1/28H/32H + 30 mM Zn\(^{2+}\)-NTA | 208 | 106 | 1236 | 6.00 | 0.032 ± 4.8 x 10\(^{-3}\) |

Table S6: Parameters for the stretched exponential background correction and associated modulation depths of the RIDME pseudo-titration shown in figures S18-23.
2.3 Influence of Differential pH upon Double-Histidine Motif Affinity:

Spin labelling with Cu$^{II}$-NTA and double-histidine motifs is a coordination-based approach, therefore it follows that affinity of labelling should be dependent on local pH; if the δ-nitrogen of histidine is protonated it cannot coordinate Cu$^{II}$-chelates effectively, and so affinity of binding should steeply decrease at pH values below the pK$\alpha$ of histidine. To test this hypothesis and investigate the influence of pH upon labelling efficiency at double-histidine motifs, isothermal titration calorimetry measurements were performed at pH 5.0 and 9.0, (and pH 6.4 and 8.4 in the main text) with results shown in figures S24 and S25, respectively. Thermodynamic parameters of each measurement are summarized in table S7.

Figure S24: ITC data recorded for 75 μM K28H/Q32H GB1 protein in presence of 2 mM Cu$^{II}$-NTA at pH 5.0. Repeats are shown in the left and right panels, respectively.
It is seen from figure S24 that at pH 5.0 there is negligible binding at the double histidine motif, which is consistent with the expectation that at pH < 6.0 (the approximate pKₐ of histidine), the binding affinity is reduced. This is likely the combination of two effects that preclude coordination of Cu²⁺-NTA at double histidine sites; first complexation of Cu²⁺ with the NTA chelator will reduce with lower pH, and similarly, histidine protonation will disrupt coordination by the double histidine. Less Cu²⁺-NTA complex will be available to bind, and fewer histidine residues will be deprotonated and susceptible to coordination at the imidazole δ-nitrogen. At pH 6.4 (figure 3 main text) there is a recovery of double-histidine loading, albeit with a reduced affinity, approximately an order of magnitude weaker binding than previously observed at pH 7.4. This is again in keeping with the expected trend of increasing affinity with increasing histidine deprotonation. Furthermore, it is noted that at pH 8.4 (figure 4 main text), the binding affinity is approximately the same as at pH 7.4, suggesting the histidine residues are already approaching complete deprotonation at pH 7.4. If the pKₐ of histidine is taken as 5.5-6.0, then this observation is consistent, since < 1% of histidine residues will be protonated at pH 7.4, and < 0.01% of neighboring histidine pairs will be protonated.
Table S7: Fit parameters taken from the ITC data shown in figures S24-25 above, and in the main text. (*) indicates that the pH 7.4 ITC measurement was performed using 75 μM 6R1/28H/32H in presence of 2 mM CuII-NTA ligand.

As can be seen from table S7, there is also an apparent influence of pH on the enthalpy of binding. With increasing pH binding becomes more exothermic, and subsequently affinity increases more steeply with decreasing temperature. This is particularly relevant for pulse EPR applications, since measurements are typically performed at cryogenic temperatures. There is also an increase in the stoichiometry of binding (n) with increasing pH, suggesting a possible role in determining the relative specificity of binding; it should be noted that WT GB1 has been observed to natively bind CuII via coordination to acidic residues, D40 and E56 at the C-terminal domain of the protein.12 Propensity for this native binding event may increase as acidic residues become deprotonated at higher pH.

2.4 Influence of Differential pH upon CuII-NTA Complex Formation:

Nominal stock solutions of 100 mM CuCl2 x 6 H2O and NTA were prepared from aliquots weighed in a glovebox, dissolved in 1 mL pH adjusted milliQ H2O, (to pH 2.0 or 12.0 respectively), using 2 M HCl and 5 M NaOH. Stock solutions of 10 mM CuII-NTA were prepared from these stock solutions by being mixed in a 1:1 equivalence before dilution with pH-adjusted buffer A (150 mM NaCl, 42.4 mM Na2HPO4, 7.6 mM KH2PO4). Respective dilution series were performed at pH 5.0, 6.4, 8.4 and 9.0, and were measured in triplicate, at nominal concentrations of 10, 7.5, 5.0, 2.5 and 1.0 mM CuII-NTA. CuII-NTA has an extinction coefficient of 63 M-1 cm-1 at A800nm at pH 7.4. Spectra are shown for series performed at pH 5.0, 6.4, 8.4 and 9.0 in figures S26-29, respectively. Comparison of the observed A800nm values and those predicted from theory are shown in figure S30. Observed absorbance at 800 nm, and the calculated concentrations are given in tables S8-11.
Figure S26. Absorbance spectra recorded for the pH 5.0 Cu\textsuperscript{II}-NTA dilution series, repeated in triplicate, with ± 2σ confidence intervals indicated as the shaded regions. Magenta, cyan, red, blue and black traces correspond to 1, 2.5, 5.0, 7.5, and 10.0 mM Cu\textsuperscript{II}-NTA concentration.

Figure S27. Absorbance spectra recorded for the pH 6.4 Cu\textsuperscript{II}-NTA dilution series, repeated in triplicate, with ± 2σ confidence intervals indicated as the shaded regions. Magenta, cyan, red, blue and black traces correspond to 1, 2.5, 5.0, 7.5, and 10.0 mM Cu\textsuperscript{II}-NTA concentration.
Figure S28. Absorbance spectra recorded for the pH 8.4 Cu\textsuperscript{II}-NTA dilution series, repeated in triplicate, with ± 2σ confidence intervals indicated as the shaded regions. Magenta, cyan, red, blue and black traces correspond to 1, 2.5, 5.0, 7.5, and 10.0 mM Cu\textsuperscript{II}-NTA concentration.

Figure S29. Absorbance spectra recorded for the pH 9.0 Cu\textsuperscript{II}-NTA dilution series, repeated in triplicate, with ± 2σ confidence intervals indicated as the shaded regions. Magenta, cyan, red, blue and black traces correspond to 1, 2.5, 5.0, 7.5, and 10.0 mM Cu\textsuperscript{II}-NTA concentration.
Figure S30. Concentration of Cu$^{II}$-NTA vs absorbance at 800 nm for pH 5.0, 6.4, 8.4 and pH 9.0, from left-to-right, and top-to-bottom. Experimental data for each repeat set is shown as a red, blue or cyan scatter, with the literature value for the extinction coefficient plotted in black.

Table S8. The observed absorbance at 800 nm for the pH 5.0 Cu$^{II}$-NTA series, taken from the spectra shown in figure S26.

| Sample     | Observed $A_{800\text{nm}}$ (a.u.) | Calculated Concentration (mM) |
|------------|-----------------------------------|------------------------------|
| Repeat     | 1       | 2      | 3      | 1      | 2      | 3      |
| 10 mM Cu$^{II}$-NTA | 0.621   | 0.621  | 0.621  | 9.9    | 9.9    | 9.9    |
| 7.5 mM Cu$^{II}$-NTA | 0.484   | 0.485  | 0.486  | 7.7    | 7.7    | 7.7    |
| 5.0 mM Cu$^{II}$-NTA | 0.328   | 0.328  | 0.328  | 5.2    | 5.2    | 5.2    |
| 2.5 mM Cu$^{II}$-NTA | 0.142   | 0.142  | 0.142  | 2.3    | 2.3    | 2.3    |
| 1.0 mM Cu$^{II}$-NTA | 0.065   | 0.065  | 0.065  | 1.0    | 1.0    | 1.0    |
It can be seen from figures S26-29 that the baseline in the region 400-550 nm is not entirely flat, suggesting the presence of precipitate. Qualitatively, precipitate was not observed upon dilution in pH-adjusted buffer for any series, however for the pH 5.0 series, some precipitate was observed at the 1:1 mixing of the acidified CuCl$_2$ and alkaninized NTA stock solutions, suggesting the neutralization reaction was not complete; this may have contributed to the sloping baseline in the region 400-550 nm. From figures S28 and S29 it is seen that the pH 8.4 and 9.0 absorbance series have reasonably flat baselines, and so precipitation does not appear to be problematic, even at alkaline pH. Absorbance at 800 nm seems to be consistently higher for the series recorded at pH

Table S9. The observed absorbance at 800 nm for the pH 6.4 Cu$^{II}$-NTA series, taken from the spectra shown in figure S27.

| Sample          | Observed A$_{800nm}$ (a.u.) | Calculated Concentration (mM) |
|-----------------|-----------------------------|--------------------------------|
| Repeat          | 1   | 2 | 3 | 1 | 2 | 3 |
| 10 mM Cu$^{II}$-NTA | 0.648 | 0.648 | 0.648 | 10.3 | 10.3 | 10.3 |
| 7.5 mM Cu$^{II}$-NTA | 0.463 | 0.464 | 0.464 | 7.3 | 7.4 | 7.4 |
| 5.0 mM Cu$^{II}$-NTA | 0.323 | 0.323 | 0.324 | 5.1 | 5.1 | 5.1 |
| 2.5 mM Cu$^{II}$-NTA | 0.154 | 0.154 | 0.154 | 2.4 | 2.4 | 2.4 |
| 1.0 mM Cu$^{II}$-NTA | 0.066 | 0.069 | 0.072 | 1.0 | 1.1 | 1.1 |

Table S10. The observed absorbance at 800 nm for the pH 8.4 Cu$^{II}$-NTA series, taken from the spectra shown in figure S28.

| Sample          | Observed A$_{800nm}$ (a.u.) | Calculated Concentration (mM) |
|-----------------|-----------------------------|--------------------------------|
| Repeat          | 1   | 2 | 3 | 1 | 2 | 3 |
| 10 mM Cu$^{II}$-NTA | 0.598 | 0.597 | 0.597 | 9.5 | 9.5 | 9.5 |
| 7.5 mM Cu$^{II}$-NTA | 0.445 | 0.445 | 0.446 | 7.1 | 7.1 | 7.1 |
| 5.0 mM Cu$^{II}$-NTA | 0.299 | 0.300 | 0.301 | 4.7 | 4.8 | 4.8 |
| 2.5 mM Cu$^{II}$-NTA | 0.173 | 0.173 | 0.173 | 2.7 | 2.7 | 2.7 |
| 1.0 mM Cu$^{II}$-NTA | 0.061 | 0.061 | 0.061 | 1.0 | 1.0 | 1.0 |

Table S11. The observed absorbance at 800 nm for the pH 9.0 Cu$^{II}$-NTA series, taken from the spectra shown in figure S29.

| Sample          | Observed A$_{800nm}$ (a.u.) | Calculated Concentration (mM) |
|-----------------|-----------------------------|--------------------------------|
| Repeat          | 1   | 2 | 3 | 1 | 2 | 3 |
| 10 mM Cu$^{II}$-NTA | 0.655 | 0.655 | 0.656 | 10.4 | 10.4 | 10.4 |
| 7.5 mM Cu$^{II}$-NTA | 0.497 | 0.498 | 0.498 | 7.9 | 7.9 | 7.9 |
| 5.0 mM Cu$^{II}$-NTA | 0.336 | 0.337 | 0.337 | 5.3 | 5.3 | 5.3 |
| 2.5 mM Cu$^{II}$-NTA | 0.175 | 0.175 | 0.176 | 2.8 | 2.8 | 2.8 |
| 1.0 mM Cu$^{II}$-NTA | 0.074 | 0.074 | 0.074 | 1.2 | 1.2 | 1.2 |
9.0, when compared to pH 5.0, however this does not manifest in a significant shift of the extinction coefficients, and plots in figure S30 show the theoretical absorbance values for an extinction coefficient of 63 M$^{-1}$cm$^{-1}$. When the absorbance values are linearly fitted, extinction coefficients of 63 and 64 M$^{-1}$cm$^{-1}$ are found for Cu$^{II}$-NTA at pH 5.0 and 9.0, respectively.

2.5 Optimization of Cu$^{II}$-IDA Complex Formation:

Previous literature has indicated that RIDME pseudo-titration modulation depths, particularly for the I$6$H/N$8$H/K$28$R$1$ series in presence of Cu$^{II}$-IDA are consistently lower than anticipated.\(^1\) This led to speculation that the equilibrium concentration of Cu$^{II}$-IDA was also lower than expected, leading to a reduced availability of the chelate to coordinate the double-histidine motif. Interestingly, this effect was not observed with the Cu$^{II}$-NTA chelate, suggesting the problem does not stem from the protein construct, but rather is specific to the Cu$^{II}$-IDA label. It is known from literature that the complexation constant of Cu$^{II}$-NTA is approximately three orders of magnitude greater than for Cu$^{II}$-IDA,\(^2\) and it has been shown that forming the complex in presence of a tetra-histidine protein can increase PELDOR modulation depth,\(^2\) for otherwise identical experimental conditions. Therefore, to optimize the preparation of Cu$^{II}$-IDA spin label, UV-visible spectroscopy measurements were performed using three different preparatory conditions, as described in section 1.3.

Results are shown below in figures S31-33 for preparation modes i), ii) and iii). Method i) corresponds to 1:1 mixing of CuCl$_2$ and IDA to produce 50 mM Cu$^{II}$-IDA, before diluting in milliQ H$_2$O, method ii) instead dilutes with buffer A (150 mM NaCl, 42.4 mM Na$_2$HPO$_4$, 7.6 mM KH$_2$PO$_4$, pH 7.4), and method iii) first dilutes the IDA buffer in 1:8 equivalence with buffer A, before addition of 1 equivalent of CuCl$_2$. Methods i), ii) and iii) are discussed subsequently as ‘H$_2$O series’, ‘neutralized series’ and ‘buffer series’, respectively, to distinguish them. Comparison of the observed $A_{726nm}$ values and those predicted from theory are shown in figure S34. Observed absorbance at 726 nm, and the calculated concentrations are given in tables S12-14 overleaf.
Figure S31. Absorbance spectra recorded for the ‘$\text{H}_2\text{O}$’ Cu$^{II}$-IDA dilution series (method i), repeated in triplicate, with ± 2σ confidence intervals indicated as the shaded regions. Magenta, cyan, red, black and orange traces correspond to 20, 10, 5, 2.5, and 1.0 mM Cu$^{II}$-IDA concentration.

Figure S32. Absorbance spectra recorded for the ‘neutralized’ Cu$^{II}$-IDA dilution series (method ii), repeated in triplicate, with ± 2σ confidence intervals indicated as the shaded regions. Magenta, cyan, red, black and orange traces correspond to 20, 10, 5, 2.5, and 1.0 mM Cu$^{II}$-IDA concentration.
Figure S33. Absorbance spectra recorded for the ‘buffer’ Cu\textsuperscript{II}-IDA dilution series (method iii), repeated in triplicate, with ±2σ confidence intervals indicated as the shaded regions. Magenta, cyan, red, black and orange traces correspond to 20, 10, 5, 2,5, and 1.0 mM Cu\textsuperscript{II}-IDA concentration.

Figure S34. Concentration of Cu\textsuperscript{II}-IDA vs absorbance at 726 nm for the ‘H\textsubscript{2}O’, ‘neutralized’, and ‘buffer’ series, from left-to-right. Experimental data for each repeat set is shown as a red, blue or cyan scatter, with the literature value for the extinction coefficient plotted in black. For the ‘H\textsubscript{2}O’ and ‘neutralized’ series, there is reasonable agreement (within ~20%), with the values predicted from theory.
Table S12. The observed absorbance at 726 nm for each Cu\textsuperscript{II}-IDA solution taken from the spectra shown in figure S31.

| Sample        | Observed A\textsubscript{726}\textsuperscript{(a.u.)} | Calculated Concentration (mM) |
|---------------|------------------------------------------------------|-------------------------------|
|               | Repeat 1 2 3                                       | 1 2 3                         |
| 20 mM Cu\textsuperscript{II}-IDA | 1.075 1.075 1.075                                   | 17.3 17.3 17.4               |
| 10 mM Cu\textsuperscript{II}-IDA | 0.569 0.569 0.568                                   | 9.2 9.2 9.2                  |
| 5.0 mM Cu\textsuperscript{II}-IDA | 0.287 0.287 0.287                                   | 4.6 4.6 4.6                  |
| 2.5 mM Cu\textsuperscript{II}-IDA | 0.145 0.145 0.145                                   | 2.3 2.3 2.4                  |
| 1.0 mM Cu\textsuperscript{II}-IDA | 0.052 0.052 0.052                                   | 0.8 0.8 0.8                  |

Table S13. The observed absorbance at 726 nm for each Cu\textsuperscript{II}-IDA solution taken from the spectra shown in figure S32.

| Sample        | Observed A\textsubscript{726}\textsuperscript{(a.u.)} | Calculated Concentration (mM) |
|---------------|------------------------------------------------------|-------------------------------|
|               | Repeat 1 2 3                                       | 1 2 3                         |
| 20 mM Cu\textsuperscript{II}-IDA | 1.302 1.294 1.295                                   | 21.0 20.8 20.9               |
| 10 mM Cu\textsuperscript{II}-IDA | 0.678 0.681 0.684                                   | 10.9 11.0 11.0               |
| 5.0 mM Cu\textsuperscript{II}-IDA | 0.380 0.384 0.390                                   | 6.1 6.2 6.3                  |
| 2.5 mM Cu\textsuperscript{II}-IDA | 0.155 0.157 0.160                                   | 2.5 2.5 2.6                  |
| 1.0 mM Cu\textsuperscript{II}-IDA | 0.080 0.088 0.084                                   | 1.3 1.4 1.4                  |

Table S14. The observed absorbance at 726 nm for each Cu\textsuperscript{II}-IDA solution taken from the spectra shown in figure S33.

| Sample        | Observed A\textsubscript{726}\textsuperscript{(a.u.)} | Calculated Concentration (mM) |
|---------------|------------------------------------------------------|-------------------------------|
|               | Repeat 1 2 3                                       | 1 2 3                         |
| 20 mM Cu\textsuperscript{II}-IDA | 1.532 1.570 1.580                                   | 24.7 25.3 25.5               |
| 10 mM Cu\textsuperscript{II}-IDA | 1.352 1.380 1.370                                   | 21.8 22.3 22.1               |
| 5.0 mM Cu\textsuperscript{II}-IDA | 0.780 0.780 0.790                                   | 12.6 12.6 12.7               |
| 2.5 mM Cu\textsuperscript{II}-IDA | 0.301 0.295 0.283                                   | 4.9 4.8 4.6                  |
| 1.0 mM Cu\textsuperscript{II}-IDA | 0.113 0.112 0.109                                   | 1.8 1.8 1.8                  |

Figure S31 shows that in the absence of phosphate buffer, the region of the spectrum between 320 and ~500 nm wavelength there is minimal absorbance, regardless of the measured Cu\textsuperscript{II}-IDA concentration. This implies that Cu\textsuperscript{II}-IDA does not contribute to absorbance in this region. In figure S32, the baseline is no longer flat, and shows strong absorbance across a wide range of wavelengths; since the resulting solution is not black, it is possible this is instead the result of Rayleigh scattering, due to precipitation. In figure S33, this effect seems to be further exacerbated, with spectra also lacking the pronounced local maximum at ~750 nm. Instructively, the difference between spectra
in figures S32 and S33 indicates the significance of first allowing the Cu\textsuperscript{II}-IDA complex to form before the addition of phosphate; this is particularly relevant since IDA is not a strong chelator of Cu\textsuperscript{II}, therefore the reaction equilibrium may lie to the left, and result in a population of free Cu\textsuperscript{II}. This likely explains the emergence of the sloping baseline, since a percentage of the free Cu\textsuperscript{II} will precipitate through interaction with the phosphate buffer, or due to alkaline pH. In the case of figure S33, this may be more pronounced, since the IDA chelate is first diluted in buffer, meaning the equilibrium will shift towards the protonated state. Therefore, upon addition of the CuCl\textsubscript{2} there will be less IDA available to first form the complex, leaving free Cu\textsuperscript{II}, which can form copper phosphate precipitate.

Measurements were then reproduced in the presence of a 2-fold molar excess of imidazole, to emulate conditions of forming Cu\textsuperscript{II}-IDA chelator in the presence of a double-histidine motif. As above, series are distinguished in discussion as ‘\textsubscript{H\textsubscript{2}O series}’ and ‘buffer series’ and spectra are shown respectively in figures S35 and S36. Comparison of the observed A\textsubscript{726nm} values and those predicted from theory are shown in figure S37. Observed absorbance at 726 nm, and the calculated concentrations are given in tables S15 and S16 overleaf.

**Figure S35.** Absorbance spectra recorded for the ‘\textsubscript{H\textsubscript{2}O’ Cu\textsuperscript{II}-IDA + imidazole dilution series, repeated in triplicate, with ± 2σ confidence intervals indicated as the shaded regions. Magenta, cyan, red, black and orange traces correspond to 20, 10, 5, 2.5, and 1.0 mM Cu\textsuperscript{II}-IDA concentration (each measured in presence of 2 equivalents of imidazole).
Figure S36. Absorbance spectra recorded for the ‘buffer’ Cu\(^{II}\)-IDA + imidazole dilution series, repeated in triplicate, with ± 2\(\sigma\) confidence intervals indicated as the shaded regions. Magenta, cyan, red, black and orange traces correspond to 20, 10, 5, 2.5, and 1.0 mM Cu\(^{II}\)-IDA concentration (each measured in presence of 2 equivalents of imidazole).

Figure S37. Concentration of Cu\(^{II}\)-IDA vs absorbance at 726 nm for the ‘H\(_2\)O’ and ‘buffer’ series, from left-to-right. Experimental data for each repeat set is shown as a red, blue or magenta scatter, with the literature value for the extinction coefficient plotted in black. For the ‘H\(_2\)O’ series, there is a discrepancy with the values predicted from theory, particularly at higher concentrations.
Table S15. The observed absorbance at 726 nm for each solution taken from the spectra shown in figure S35.

| Sample          | Observed A_{726nm} (a.u.) | Calculated Concentration (mM) |
|-----------------|---------------------------|-------------------------------|
| Repeat          | 1            | 2            | 3            | 1            | 2            | 3            |
| 20 mM Cu^{II}-IDA | 0.912       | 0.913       | 0.914       | 14.7         | 14.7         | 14.7         |
| 10 mM Cu^{II}-IDA | 0.488       | 0.490       | 0.489       | 7.9          | 7.9          | 7.9          |
| 5.0 mM Cu^{II}-IDA | 0.264       | 0.264       | 0.265       | 4.3          | 4.3          | 4.3          |
| 2.5 mM Cu^{II}-IDA | 0.150       | 0.149       | 0.149       | 2.4          | 2.4          | 2.4          |
| 1.0 mM Cu^{II}-IDA | 0.062       | 0.063       | 0.063       | 1.0          | 1.0          | 1.0          |

Table S16. The observed absorbance at 726 nm for each solution taken from the spectra shown in figure S36. (*) indicates that the spectra were obtained the following day, using a different blank solution, this may explain the disparity between the repeats.

| Sample          | Observed A_{726nm} (a.u.) | Calculated Concentration (mM) |
|-----------------|---------------------------|-------------------------------|
| Repeat          | 1            | 2            | 3            | 1            | 2            | 3            |
| 20 mM Cu^{II}-IDA | 1.22        | 1.22        | 1.22        | 19.7         | 19.7         | 19.7         |
| 10 mM Cu^{II}-IDA | 0.737       | 0.738       | 0.737       | 11.9         | 11.9         | 11.9         |
| 5.0 mM Cu^{II}-IDA | 0.366       | 0.366       | 0.366       | 5.9          | 5.9          | 5.9          |
| 2.5 mM Cu^{II}-IDA | 0.183       | 0.183       | 0.180*      | 3.0          | 3.0          | 2.9          |
| 1.0 mM Cu^{II}-IDA | 0.065       | 0.072*      | 0.072*      | 1.0          | 1.2          | 1.2          |

In presence of an excess of imidazole the region of the spectra between 320 and ~500 nm wavelength is similar to spectra recorded in the absence of phosphate buffer (figure S31). Furthermore, the presence of imidazole appears to completely remove precipitate, suggesting that free Cu^{II} forms adducts with the phosphate buffer. Imidazole co-ordinates both free Cu^{II} and likely stabilizes Cu^{II}-IDA such that the equilibrium lies further to the right, leading to a reduction in the population of free Cu^{II}, as well as reducing the availability of free Cu^{II} to interact with phosphate and precipitate. To ensure that the imidazole did not contribute to absorbance in the visible range, a complete imidazole dilution series was performed, and as seen from figure S38, absorbance is flat in the range 550-800 nm. The λ_{max} value of the imidazole absorbance spectrum occurs at 320 nm, and values are given below in table S17, and plotted as a function of concentration in figure S39.
Figure S38. Absorbance spectra recorded for an imidazole dilution series, repeated in triplicate, with ± 2σ confidence intervals indicated as the shaded regions. Magenta, cyan, red, blue, black, red and blue traces correspond to 200, 100, 80, 60, 40, 20, and 10 mM imidazole concentration.

| Sample          | Observed A<sub>320nm</sub> (a.u.) |
|-----------------|-----------------------------------|
|                 | Repeat 1 | 2 | 3 |
| 200 mM Imidazole| 0.399    | 0.398 | 0.397 |
| 100 mM Imidazole| 0.190    | 0.190 | 0.190 |
| 80 mM Imidazole | 0.150    | 0.150 | 0.150 |
| 60 mM Imidazole | 0.113    | 0.113 | 0.112 |
| 40 mM Imidazole | 0.078    | 0.078 | 0.077 |
| 20 mM Imidazole | 0.037    | 0.037 | 0.037 |
| 10 mM Imidazole | 0.026    | 0.024 | 0.024 |

Table S17. The observed absorbance at 320 nm for each solution taken from the spectra shown in figure S38.
Figure S39. Concentration of Imidazole vs absorbance at 320 nm, the theoretical curve is given using an extinction coefficient of 2 M$^{-1}$cm$^{-1}$. Experimental data for each repeat set is shown as a red, blue or magenta scatter.
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