Tuning reduction potentials of type 1 copper center in azurinby replacing a histidine ligand with its isostructural analogues

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Type 1 copper proteins have a conserved ligand set of one cysteine and two histidines, with many proteins, such as azurin, also containing an axial methionine. While the cysteine and methionine in azurin have been replaced with their respective isostructural analogues of unnatural amino acids to reveal their roles in tuning electronic structures and functional properties, such as reduction potentials ($E^{\circ'}$), the histidine ligands have not been probed in this way. We herein report the substitution of His117 in azurin with three unnatural isostructural analogues, 5-nitrohistidine(Ntr), thiazolylalanine(SHis) and 1-methylhistidine(MeH) by expressed protein ligation. While UV–vis absorption and electron paramagnetic resonance spectroscopies confirm that isostructural replacement results in minimal structural change in the Cu(II) state, the $E^{\circ'}$ of these variants increases with increasing pKa of the δ nitrogens of the imidazole. This counter-intuitive relationship between $E^{\circ'}$ of the protein and pKa of the sidechain group suggests additional factors may play a role in tuning $E^{\circ'}$.

1. Introduction

Type 1 copper (T1Cu) proteins are a family of mononuclear copper proteins with a conserved metal coordination environment and similar spectroscopic characteristics that serve electron transfer functions. A primary example is azurin from *Pseudomonas aeruginosa* (Az) that has a T1Cu coordinated by one Cys and two His residues in a trigonal plane and an axial Met above the trigonal plane (Fig. 1). This class of T1Cu proteins is interesting to study because they have been shown to display low reorganization energy, fast electron transfer (ET) rates, and a wide range of reduction potentials ($E^{\circ'}$) to meet the needs of its redox partner for different ET functions [1–7]. Understanding factors that determine and precisely tune $E^{\circ'}$ can result in deeper insight into this important class of ET proteins and the development of better redox reagents for applications such as solar energy capture and bio-fuel cells [8]. Toward this goal, we and other groups have probed the role of hydrophobicity and hydrogen bond networks in the secondary coordination sphere in tuning $E^{\circ'}$ of Az beyond the natural range of T1Cu protein, with minimal purtubation of its primary coordination sphere [9–12].

In addition to understanding the roles of the secondary coordination sphere in tuning $E^{\circ'}$ of T1Cu, it is also important to probe the roles of the primary coordination sphere ligand. However, because site-directed mutagenesis is limited to the 20 natural amino acids, it is difficult to investigate individual factors as changing a primary coordinating ligand will perturb both electronic and steric effects [6]. For example, mutating equatorial Cys or His to other natural amino acids such as glutamate or glycine will generally convert Az to a type zero or type 2 copper (T2Cu) protein [13,14]. To overcome this limitation, we and others have used expressed protein ligation (EPL) [15] to replace Cys112 and Met121 in Az with unnatural amino acids (Fig. 1) [16–18]. For example, by changing Met121 to a series of its unnatural isostructural analogues, such as oxomethionine (OxM), selenomethionine(SeM), difluoromethionine(DFM), trifluoromethionine (TFM) or norleucine (Nle), we have found a trend between $E^{\circ'}$ and the hydrophobicity of the axial ligand, confirming the role of the axial ligand in $E^{\circ'}$ tuning [19,20]. Similarly, an isostructural replacement of Cys112 with selenocysteine (SeC) results in some spectroscopic changes but has little impact on $E^{\circ'}$ [21,22].

While the roles of Cys112 and Met121 in tuning $E^{\circ'}$ and other redox properties such as ET rate have been investigated by replacement with isostructural analogues for more than a decade, replacement of the equatorial His ligands has yet to be reported. Histidine is a common

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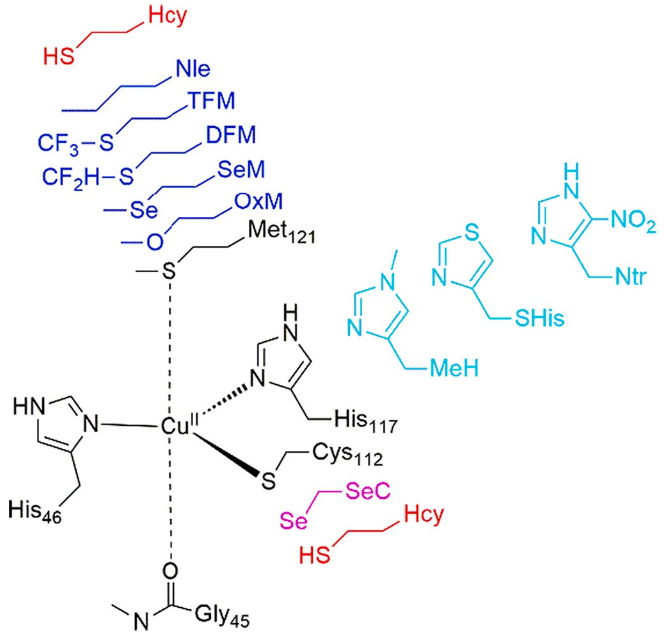

Fig. 1. Copper ligands of Az and unnatural amino acids incorporated into Az. Three histidine analogues in cyan were used in this study to replace His117, one of the equatorial histidines. Hcy: homocysteine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ligand for metalloproteins. Due to its unique sidechain structure, fine-tuning its properties through natural amino acid mutagenesis is very difficult, if not impossible to achieve because any site-directed mutagenesis with another natural amino acid will result in changes in both geometric and electronic structures. Schultz and co-workers have developed genetic codon expansion systems to incorporate four His analogues into protein [23]. Hilvert and co-workers have used N^{δ}-methylhistidine to construct an artificial metalloenzyme for carbene transfer reaction [24]. Despite this progress, these and other methods have not been applied to probe the roles of His ligand in T1Cu proteins.

As shown in Fig. 1, His117 is an equatorial T1Cu ligand in the hydrophobic patch of protein and thought to be a possible ET entry point [1,25]. Mutating His46 or His117 to Gly has creates a vacant coordination site at copper. Depending on the type of exogeneous ligand, the mutants will be either T1 or T2 copper protein [13,26,27]. As indirect perturbation of His117 by introduction of a positive charge near His117 can increase $E^{\circ\prime}$ [28], we hypothesized that directly modulating His117 by replacement with its isostructural analogues could tune the properties of the T1Cu center, including $E^{\circ\prime}$.

To evaluate this hypothesis, we chose three isostructural analogues of histidine for incorporation, including 5-nitrohistidine (Ntr), thiazolylalanine (SHis) and 1-methylhistidine (MeH) (Fig. 2) to replace His117 in azurin in other to probe the role of this histidine in tuning $E^{\circ\prime}$ more closely. The reported andpredicted pK_a values of the δ nitrogens of the imidazole, 1-methylimidazole, thiazole, 5-nitroimidazole are 7.03 [29], 7.12 [30], 2.53 [29], and 0.49 [31], respectively (Table 1). As unnatural amino acids with different pK_a values have been used in both native and artificial metalloproteins to probe the ET process [32–34], we herein report preparation by EPL, spectroscopic characterization by UV–vis and electron paramagnetic resonance (EPR) and electrochemical studies by cyclic voltammetry (CV) of Az variants containing each of the three aforementioned His analogues. We have found an interesting trend between the pK_a of the δ nitrogens and their abilities to coordinate to Cu (II) and $E^{\circ\prime}$ of the T1Cu center.

1.1. *Experimental methods*

All natural amino acids and resins were purchased from Chem-Impex Int. Co. Unnatural amino acids except 4(5)-nitrohistidine were purchased from Chem-Impex or Peptech. 4(5)-nitrohistidine was synthesized by nitration of L-Histidine by nitrate acid as reported [35]. The yield is 62%. Other chemicals were obtained from Acros or Sigma-Aldrich. Protecting groups used for fluorenylmethoxycarbonyl solid-phase peptide synthesis (Fmoc-SPPS) were Cys(Trt), Ser(OtBu), Glu (OtBu), Cys(StBu), Asn(Trt), His(Trt), Lys(Boc), Thr(OtBu) (Trt = trityl; OtBu = tert-butyl ester; StBu = S-tert-butylthio; Boc = tert-butoxycarbonyl).

Mass spectral data was collected at the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois by Electrospray Ionization (ESI) mass spectrometry using either Waters Quattro II or Waters ZMD Quadrupole Instrument.

UV–vis spectra were taken on an 8453 HP diode array spectrometer or a Cary 5000 spectrometer. X-band EPR spectra were collected on a Varian E-122 spectrometer at the Illinois EPR Research Center (IERC). The samples were run at ~30 K using liquid He and an Air Products Helitran cryostat with 20% glycerol. Magnetic fields were calibrated with a Varian NMR gaussmeter, and the frequencies were measured with an EIP frequency counter.

Peptides: The C-terminal peptides with Histidine analog (H2N-CysThrPheProGly***His(MeH, SHis or Ntr)***SerAlaLeuMetLysGlyThrLeuThrLeuLys-COOH) were synthesized on a 0.175 mmol scale with Advanced ChemTech Model 90 peptide synthesizer using standard Fmoc-based chemistry. Wang resin preloaded with Fmoc-Lys(Boc) was

Table 1
pK_a of His analogues, the absorption maximum of Az mutants and their reduction potentials.

	pK_a	λ_{max}/nm	Reduction potential /mV vs. NHE
WT-Az	7.03 [29] (7.18)[a]	627	352 ± 2
His117MeH-Az	7.12 [30] (7.01)[a]	630	380 ± 4
His117SHis-Az	2.53 [29] (2.44)[a]	633	313 ± 5
His117Ntr-Az	(0.49)[a]	625	276 ± 4

[a] . Values calculated with the use of Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (© 1994–2009 ACD/Labs) are shown in parenthesis.

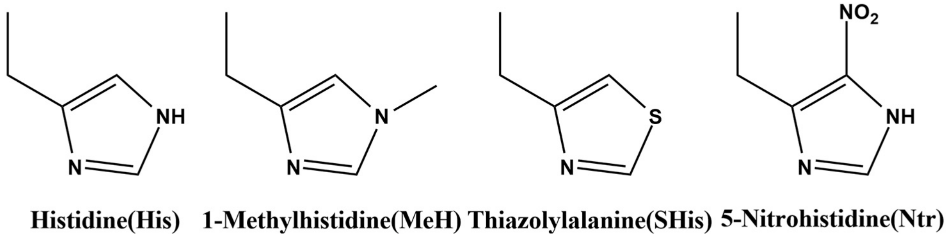

Fig. 2. Sidechains of histidine and its analogues used in this study.

pre-swelled in DMF (6×10 min, 6 mL). All Fmoc-deprotections were accomplished using 20% piperidine/DMF (*v*/*v*) (3×3 min, 6 mL). Synthesis of the peptide was completed using a five-fold excess of amino acids pre-activated in 0.4 M *N*-methylmorpholine (NMM) (6 mL) and O-(1H-6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HCTU) (4 equiv.) for 3 min and coupled to the resin for 45 min under N_2 sparging. Amino acids were double coupled when necessary and the completion of the couplings were monitored by qualitative Kaiser test [36].

Peptide cleavage from the resin is achieved by incubating the resin with a cocktail with 92.5% (*v*/*v*) trifluoroacetic acid (TFA), 2.5% triisopropylsilane, 2.5% thioanisole, 2.5% H_2O at 25 °C for 2 h. Preparative RP-HPLC was done with a Waters Delta 600 system and a DeltaPak 300 × 19 mm prep column. Solution A was 0.1% TFA in H_2O, and solution B was 80% acetonitrile/20% H_2O with 0.1% TFA. Unless otherwise stated, a linear gradient of 20% to 70% B over 23 min was used for all runs.

Expressed Protein Ligation: Expressed protein ligation is performed as described previously [17]. Cultures of *E. coli* BL21 (DE3) cells containing the NEB pTXB1 plasmid to express the azurin(L111)-Intein-cellulose-binding domain fusion protein were grown in lysogeny broth (LB) media for 8 h at 37 °C and used to inoculate eight 2 L flasks of LB media containing 100 mg/mL ampicillin. The cells were grown at 37 °C for 16 h with shaking at 210 rpm and harvested at 9000 x g.

The cell pellet was re-suspended in a lysis buffer containing 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 8.0, 250 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton-X-100 and 1 M urea (50 mL). The suspension was then lysed using sonication (Misonix Sonicator 4000, 0.5 in. diameter probe) for a work time of 6 min (6 s on, 12 s rest). Additional urea was added to bring the final concentration to 1.5 M and the lysate was subjected to a second round of sonication for 3 min work time (6 s on, 12 s off). The crude lysate was centrifuged at 20,000 x*g* for 30 min. The supernatant was carefully decanted, and the fusion protein bound by batch absorption to 70 mL of chitin resin pre-equilibrated with 20 mM HEPES, pH 7.2, 250 mM NaCl, and 1 mM EDTA (buffer 1) for 1–2 h at 4 °C. The chitin resin was then poured into a column and the column headspace was purged with Ar. The column was then washed with 5 column volumes of buffer 1 by cannulation under Ar pressure.

Ligations were initiated by the addition of the 17-mer peptide (1.02 mM, 60 mg) and 2 Eq. tris-(2-carboxyethyl)phosphine (TCEP) (3 mM, 30 mg) in 40 mL of degassed buffer 1 (35 mL) containing ~50 mM mercaptophenyl acetic acid (MPAA) (50 mM, 350 mg) to the column under Ar pressure via cannulation. The chitin resin was re-suspended in the column and the entire column was agitated gently at 4 °C for 64 h. After ligation, the column was eluted under Ar pressure and washed with 1 column volume of buffer 1. The eluent was centrifuged at 20,000 x*g* for 30 min and the supernatant was concentrated using 10,000 MWCO Millipore Centricon concentration spin tubes to a final volume of ~10 mL. The concentrated protein was then exchanged into 50 mM ammonium acetate buffer, pH 6.35 via a short desalting column with Sephadex G-25 resin.

Cu titration and anion exchange purification. The desalted sample was titrated with 0.1 equivalence of freshly made 1 mM $CuSO_4$ solution in H_2O until the ligand-to-metal charge transfer band (~625 nm) is saturated. The resulting solution was purified by anion exchange chromatography. The holo protein came with Buffer 1 (40 mM 3-(N-Morpholino)propanesulfonic acid (MOPS), pH 6.0), and apo protein came after wash with buffer 2 (1 M NaCl). The holo protein was exchanged into 50 mM ammonium acetate pH 6.35 for further study.

Electrochemical study: The reduction potential of each mutant was determined by cyclic voltammetry after verifying the WT azurin reduction potential using a CH Instruments 617A potentiostat equipped with a picoamp booster and a Faraday cage. A pyrolytic graphite edge (PGE) electrode was polished, and 2–3 μL of protein solution was applied directly to the electrode following previously described methods [20]. After a short incubation time, the electrode was immersed in either 50 mM NH_4OAc, 40 mM MOPS, 40 mM 2-(N-morpholino)ethanesulfonic acid (MES), 40 mM Tris, pH 4.0 with 100 mM NaCl, 50 mM NH_4OAc, 40 mM MOPS, 40 mM MES, 40 mM Tris, pH 7.0 with 100 mM NaCl before data collection. The reduction potentials were measured against Ag/AgCl and converted to NHE.

2. Results and discussion

The unnatural amino acids highlighted in Fig. 1 were incorporated into the 117th position of Az through expressed protein ligation, as described previously [20,21]. The identity of metal-free apo-proteins were verified by the match of the calculated and measured molecular weights (MWs) determined by electrospray ionization mass spectrometry (Fig. S1) .

To metallate the purified apo-proteins, the samples were titrated with sub-equivalents of $CuSO_4$ and monitored by UV–vis absorption spectroscopy until the spectra stopped growing. As shown in Fig. 3, the UV–Vis spectra of holo WT-Az and the three variants, all display a strong absorption band at ~625 nm which has been previously assigned to a S (*Cys*)p_π → Cu d^2_{xy} charge transfer band [5,37]. Additionally, His117Ntr Az displays an absorption centered ~350 nm, due to the presence of the nitro group [38].

To further probe the electronic structures of these Az variants, we exchanged protein into temperature-independent pH 7 (TIP7) buffer [39] (30 mM HEPES, 20 mM K_2HPO_4, pH 7) with 20% glycerol, and recorded X-band electron paramagnetic resonance (EPR) spectra on a Varian E-122 spectrometer at ~30 K (Fig. 4). The EPR spectra were simulated with SIMPOW6 to resolve different species and to give *g* tensors and hyperfine splitting values (Table S1) [40]. The EPR spectra of all variants contain an axial species similar to that of WT-Az. The axial species of His117MeH, His117SHis, His117Ntr Az display $A_{||}$ of 56, 59, 68 × 10^{-4} cm^{-1}, and $g_{||}$ of 2.260, 2.283, 2.268, respectively, comparable to those of WT-Az (60 × 10^{-4} cm^{-1} and 2.253). These results, along with the UV–vis spectroscopic data strongly suggest that replacing the His117 with its isostructural analogues has a minimal perturbation to the T1Cu characteristics. On the other hand, the EPR spectra of His117SHis-Az and His117Ntr-Az display another species with $A_{||}$ of 177 and 18× 10^{-4} cm^{-1}, respectively, which resembles the $A_{||}$ of the Type 2 copper (T2Cu) center in His117Gly Az mutant [13]. The EPR spectrum of His117MeH-Az is best simulated as T1Cu only, but we cannot rule out the existence of a small portion of T2Cu signal. Interestingly, we found that the proportion of the T2Cu species increases from ~0% to 70% and

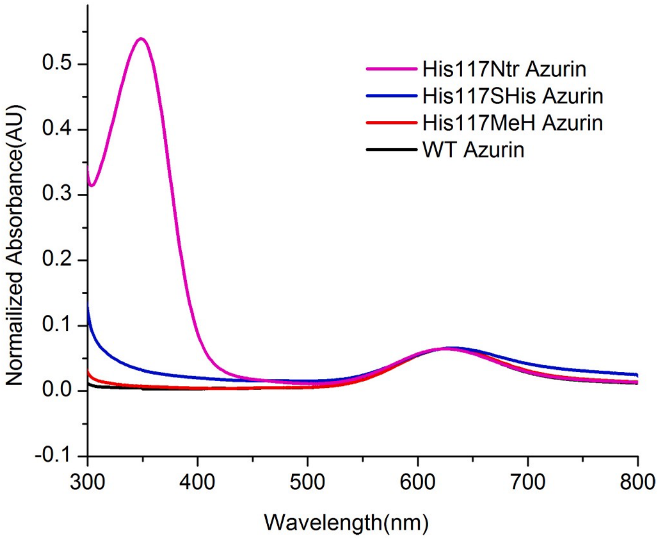

Fig. 3. UV–Vis spectra of WT-Az and the three His analogue variants. Spectra were collected at 298 K in 50 mM ammonium acetate buffer, pH 6.35.

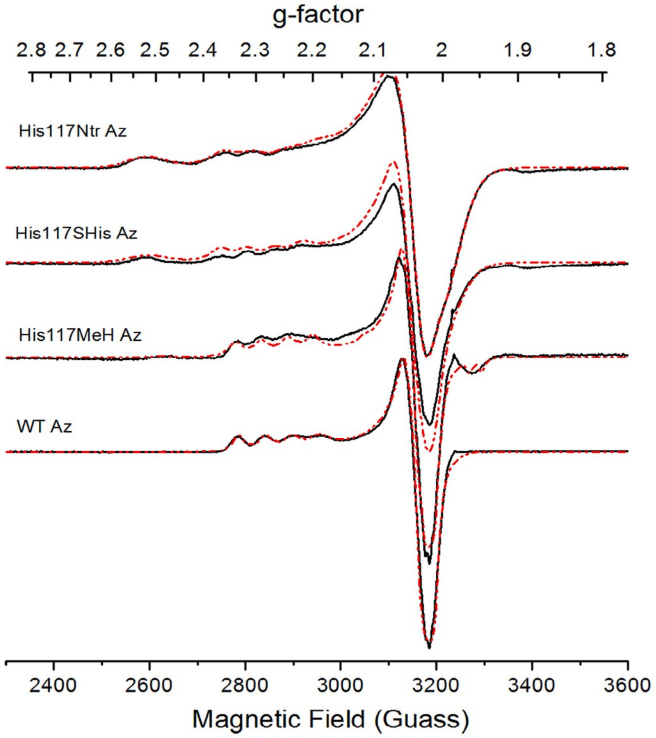

Fig. 4. X-band EPR spectra of holo Az with histidine and histidine analogues incorporated. From top to bottom: His117Ntr-Az, His117SHis-Az, His117MeH-Az, and WT-Az. Black line: experimental spectra, red dotted line: simulated spectra. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

then 82%, as pK_a values of the sidechain in His117MeH-Az, His117SHis-Az and His117Ntr-Az decrease. These results indicate that the more acidic the sidechains in these His analogues, the worse ligands they are for coordinating to Cu(II). This weaker coordination results in a greater proportion of EPR spectral features resembling those of the T2Cu reported in His117Gly Az [13].

To probe the effect of these His substitutions on the $E^{\circ\prime}$, cyclic voltammetry (CV) was performed using a pyrolytic graphite edge (PGE) electrode as described previously [20,41]. At pH 4, WT-Az displays an $E^{\circ\prime}$ of 352 ± 2 mV, consistent with literature [10,41]. For His117MeH-Az, His117SHis-Az and His117Ntr-Az, $E^{\circ\prime}$ was determined to be 380 ± 4, 313 ± 5 and 276 ± 4 mV, respectively (Fig. S2). When plotting these $E^{\circ\prime}$ values against the pK_a of Nδ of these variants, we found a positive trend (Fig. 5). When the CV data were collected at pH 7, the same trend was observed, but with a steeper slope. Although both T1Cu and T2Cu species were observed in the EPR spectra of His117SHis-Az, and His117Ntr-Az, we could not identify more than one set of peaks by CV. The EPR spectra of T2Cu species of His117SHis-Az, and His117Ntr-Az closely resemble that of His117Gly-Az, whose $E^{\circ\prime}$ has been determined to be 670 mV [42], much higher than $E^{\circ\prime}$ for either His117SHis-Az and His117Ntr-Az. Based on the measured values, we reasoned that $E^{\circ\prime}$ of His117SHis-Az and His117Ntr-Az should be attributed to the T1Cu rather than T2Cu species.

The $E^{\circ\prime}$ of WT azurin is pH dependent, with an apparent pK_a of around 7 [43]. Such pH dependence is believed to be related to the deprotonation of His35 and His85 of $E^{\circ\prime}$ [43,44]. In the present work, although Az variants with different His analogues at the 117th position show a similar trend at pH 4, the $E^{\circ\prime}$- pK_a slope is steeper at pH 7. It suggests that the $E^{\circ\prime}$ could be related to the protonation state of His, as His and its analogues have different protonation states at pH 7, while they are all protonated at pH 4.

From a theoretical perspective, ligands of a coordination complex with lower pK_a values, which are more electron withdrawing, should preferably stabilize the reduced state over the oxidized state, leading to a higher $E^{\circ\prime}$. This trend has been tested and confirmed in inorganic complexes, such as copper complexes with phenanthrolines and bipyridyls as the ligands, in which the 5-nitrophenanthroline-copper complex with a lower pK_a (less than 3.0) displayed a higher $E^{\circ\prime}$ [45]. On the other hand, many complexes do not follow this pK_a–reduction potential trend as effects other than electronic properties, such as the stability of Cu(I) state and ligand sterics contribute more to $E^{\circ\prime}$ [46,47]. Interestingly, $E^{\circ\prime}$ of Az and its His analogue variants shows the opposite trend as predicted from the simple electron withdrawing or donating ability. The pK_as used in the correlation are based on results obtained either experimentally or calculated for the free amino acids outside the protein scaffold [29–31]. The pK_as may change when they are in azurin, but it is unlikely that the pK_as change in the opposite direction when they are in azurin, because they are isostructural to each other in the same location. Assuming the pK_as of these His analogues in azurin follow the same trend as those outside the protein, this observation suggests that other factors may determine $E^{\circ\prime}$ of these variants. While the UV–vis and EPR can be readily used to characterize the Cu(II) state of these variants, probing the electronic properties of the Cu(I) state is much harder, because they do not display any UV–vis or EPR spectra related to the Cu(I) state. The Cu (II) and Cu(I) states of WT-Az exhibit the same coordination environment, with slightly longer distances between Cu(I) and its ligands [48]. As for the Az variants with His analogues studied in this work, coordination change is more likely to occur at the Cu(I) state. It is possible that protonation of His analogues at the 117th position is more likely to occur at the Cu(I) state, causing greater disparities between the Cu(I) species that could lead to the unexpected trend of the $E^{\circ\prime}$- pK_a. The hypothesis could be tested by a careful examination of structure of the reduced azurin using X-ray absorption spectroscopy.

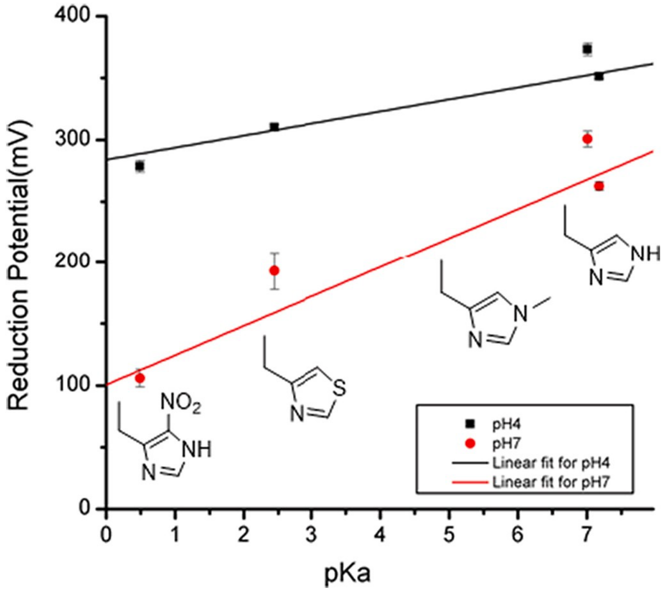

Fig. 5. $E^{\circ\prime}$ of Az vs. pK_a of histidine. Black square: $E^{\circ\prime}$ of Az at pH 4, red circle: $E^{\circ\prime}$ of Az at pH 7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Conclusions

In summary, the His117 of Az has been substituted by a series of isostructural analogues with varying δ nitrogens pK_as using expressed protein ligation. These mutants retain T1Cu UV–vis and EPR characteristics, suggesting that isostructural replacement results in minimal structural changes to the Cu(II) state. The lower pKa of the δ nitrogens also resulted in a higher proportion of T2Cu species resembling that robserved in His117Gly-Az, indicating some population lacking δ nitrogen coordination due to the lower pKa. Surprisingly, we have found a positive trend between $E^{\circ'}$ and pK_a at both pH 4 and pH 7, i.e., a variant with higher pK_a also has a higher $E^{\circ'}$, which is opposite from theoretical predictions based on electron withdrawing/donating ability that is often associated with the pK_a of the ligand. This counter-intuitive relationship between $E^{\circ'}$ of the protein and pK_a of the sidechain group suggests there are additional factors, such as a change in coordination sphere upon reduction. Further studies of these and other His analogues in both azurin and other cupredoxins will result in deeper insight into the roles of histidine in metalloproteins.

Author statement

Yang Yu: Design and data collection and interpretation; manuscript writing

Nicholas M. Marshall: helpful discussions and ideas

Dewain K. Garner: Initial design and investigations

Mark J. Nilges: advise on EPR data collection and interpretation

Yi Lu: Design and data interpretation; manuscript revisions

Declaration of Competing Interest

The authors declare no financial interests/personal relationships which may be considered as potential competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2022.111863.

References

[1] E.T. Adman, Copper Protein Structures, Adv. Protein Chem. 42 (1991) 145–197.
[2] V. Kofman, O. Farver, I. Pecht, D. Goldfarb, Two-dimensional pulsed EPR spectroscopy of the copper protein azurin, J. Am. Chem. Soc. 118 (1996) 1201–1206.
[3] H.B. Gray, B.G. Malmström, R.J.P. Williams, Copper coordination in blue proteins, J. Biol. Inorg. Chem. 5 (2000) 551–559.
[4] L. Banci, R. Pierattelli, A.J. Vila, in: J.S. Valentine, E.B. Gralla (Eds.), Adv. Protein Chem., 60, Nuclear Magnetic Resonance Spectroscopy Studies on Copper Proteins, Academic Press, Washington, DC, 2002, pp. 397–449.
[5] E.I. Solomon, R.K. Szilagyi, S. DeBeer George, L. Basumallick, Electronic structures of metal sites in proteins and models: contributions to function in blue copper proteins, Chem. Rev. 104 (2004) 419–458.
[6] J.J. Warren, K.M. Lancaster, J.H. Richards, H.B. Gray, Inner- and outer-sphere metal coordination in blue copper proteins, J. Inorg. Biochem. 115 (2012) 119–126.
[7] J. Liu, S. Chakraborty, P. Hosseinzadeh, Y. Yu, S. Tian, I. Petrik, A. Bhagi, Y. Lu, Metalloproteins containing cytochrome, iron–sulfur, or copper redox centers, Chem. Rev. 114 (2014) 4366–4469.
[8] P. Hosseinzadeh, Y. Lu, Design and fine-tuning redox potentials of metalloproteins involved in electron transfer in bioenergetics, Biochim. Biophys. Acta 2016 (1857) 557–581.
[9] S. Yanagisawa, M.J. Banfield, C. Dennison, The role of hydrogen bonding at the active site of a cupredoxin: the Phe114Pro Azurin variant, Biochemistry 45 (2006) 8812–8822.
[10] N.M. Marshall, D.K. Garner, T.D. Wilson, Y.-G. Gao, H. Robinson, M.J. Nilges, Y. Lu, Rationally tuning the reduction potential of a single cupredoxin beyond the natural range, Nature 462 (2009) 113–116.
[11] P. Hosseinzadeh, N.M. Marshall, K.N. Chacón, Y. Yu, M.J. Nilges, S.Y. New, S. A. Tashkov, N.J. Blackburn, Y. Lu, Design of a single protein that spans the entire 2-V range of physiological redox potentials, Proc. Natl. Acad. Sci. U. S. A. 113 (2015) 262–267.
[12] S.M. Berry, M.H. Baker, N.J. Reardon, Reduction potential variations in azurin through secondary coordination sphere phenylalanine incorporations, J. Inorg. Biochem. 104 (2010) 1071–1078.
[13] T. den Blaauwen, M. Van de Kamp, G.W. Canters, Type I and II copper sites obtained by external addition of ligands to a His117Gly azurin mutant, J. Am. Chem. Soc. 113 (1991) 5050–5052.
[14] K.M. Lancaster, S.D. George, K. Yokoyama, J.H. Richards, H.B. Gray, Type-zero copper proteins, Nat. Chem. 1 (2009) 711–715.
[15] T.W. Muir, D. Sondhi, P.A. Cole, Expressed protein ligation: a general method for protein engineering, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 6705–6710.
[16] Y. Yu, C. Cui, J. Wang, Y. Lu, Biosynthetic approach to modeling and understanding metalloproteins using unnatural amino acids, SCIENCE CHINA Chem. 60 (2017) 188–200.
[17] K.M. Clark, Y. Yu, N.M. Marshall, N.A. Sieracki, M.J. Nilges, N.J. Blackburn, W. A. van der Donk, Y. Lu, Transforming a blue copper into a red copper protein: engineering cysteine and homocysteine into the axial position of Azurin using site-directed mutagenesis and expressed protein ligation, J. Am. Chem. Soc. 132 (2010) 10093–10101.
[18] K.M. Clark, Y. Yu, W.A. van der Donk, N.J. Blackburn, Y. Lu, Modulating the copper–sulfur interaction in type 1 blue copper azurin by replacing Cys112 with nonproteinogenic homocysteine, Inorg. Chem. Front. 1 (2014) 153–158.
[19] S.M. Berry, M. Ralle, D.W. Low, N.J. Blackburn, Y. Lu, Probing the role of axial methionine in the blue copper center of azurin with unnatural amino acids, J. Am. Chem. Soc. 125 (2003) 8760–8768.
[20] D.K. Garner, M.D. Vaughan, H.J. Hwang, M.G. Savelieff, S.M. Berry, J.F. Honek, Y. Lu, Reduction potential tuning of the blue copper center in Pseudomonas aeruginosa azurin by the axial methionine as probed by unnatural amino acids, J. Am. Chem. Soc. 128 (2006) 15608–15617.
[21] S.M. Berry, M.D. Gieselman, M.J. Nilges, W.A. Van der Donk, Y. Lu, An engineered azurin variant containing a selenocysteine copper ligand, J. Am. Chem. Soc. 124 (2002) 2084–2085.
[22] M. Ralle, S.M. Berry, M.J. Nilges, M.D. Gieselman, W.A. van der Donk, Y. Lu, N. J. Blackburn, The selenocysteine-substituted blue copper center: spectroscopic investigations of Cys112SeCys Pseudomonas aeruginosa azurin, J. Am. Chem. Soc. 126 (2004) 7244–7256.
[23] H. Xiao, F.B. Peters, P.-Y. Yang, S. Reed, J.R. Chittuluru, P.G. Schultz, Genetic incorporation of histidine derivatives using an engineered pyrrolysyl-tRNA synthetase, ACS Chem. Biol. 9 (2014) 1092–1096.
[24] T. Hayashi, M. Tinzl, T. Mori, U. Krengel, J. Proppe, J. Soetbeer, D. Klose, G. Jeschke, M. Reiher, D. Hilvert, Capture and characterization of a reactive haem–carbenoid complex in an artificial metalloenzyme, Nat. Catalysis 1 (2018) 578–584.
[25] J. Hirst, F.A. Armstrong, Fast-scan cyclic voltammetry of protein films on pyrolytic graphite edge electrodes:â€‰ characteristics of electron exchange, Anal. Chem. 70 (1998) 5062–5071.
[26] T. den Blaauwen, G.W. Canters, Creation of type-1 and type-2 copper sites by addition of exogenous ligands to the Pseudomonas aeruginosa azurin His117Gly mutant, J. Am. Chem. Soc. 115 (1993) 1121–1129.
[27] G. van Pouderoyen, C.R. Andrew, T.M. Loehr, J. Sanders-Loehr, S. Mazumdar, H.A. O. Hill, G.W. Canters, Spectroscopic and mechanistic studies of type-1 and type-2 copper sites in Pseudomonas aeruginosa azurin as obtained by addition of external ligands to mutant His46Gly, Biochemistry 35 (1996) 1397–1407.
[28] G. Van Pouderoyen, S. Mazumdar, N.I. Hunt, A.O. Hill, G.W. Canters, The introduction of a negative charge into the hydrophobic patch of Pseudomonas aeruginosa azurin affects the electron self-exchange rate and the electrochemistry, Eur. J. Biochem. 222 (1994) 583–588.
[29] A. Albert, R.J. Goldacre, J. Phillips, Strength of heterocyclic bases, J. Chem. Soc. (Resumed) (1948) 2240–2249.
[30] C.S. Cassidy, L.A. Reinhardt, W.W. Cleland, P.A. Frey, Hydrogen bonding in complexes of carboxylic acids with 1-alkylimidazoles: steric and isotopic effects on low barrier hydrogen bonding, J. Chem. Soc. Perkin Trans. 2 (1999) 635–642.
[31] ACD/Labs , version 8.14, Advanced Chemistry Development, Inc., Toronto, ON, Canada, 2009. www.acdlabs.com.
[32] M.R. Seyedsayamdost, S.Y. Reece, D.G. Nocera, J. Stubbe, Mono-, di-, tri-, and tetra-substituted fluorotyrosines: new probes for enzymes that use tyrosyl radicals in catalysis, J. Am. Chem. Soc. 128 (2006) 1569–1579.
[33] Y. Yu, X. Lv, J. Li, Q. Zhou, C. Cui, P. Hosseinzadeh, A. Mukherjee, M.J. Nilges, J. Wang, Y. Lu, Defining the role of tyrosine and rational tuning of oxidase activity by genetic incorporation of unnatural tyrosine analogs, J. Am. Chem. Soc. 137 (2015) 4594–4597.
[34] Y. Yu, Q. Zhou, L. Wang, X. Liu, W. Zhang, M. Hu, J. Dong, J. Li, X. Lv, H. Ouyang, H. Li, F. Gao, W. Gong, Y. Lu, J. Wang, Significant improvement of oxidase activity through the genetic incorporation of a redox-active unnatural amino acid, Chem. Sci. 6 (2015) 3881–3885.
[35] W. Tautz, S. Teitel, A. Brossi, Nitrohistidines and nitrohistamines, J. Med. Chem. 16 (1973) 705–707.
[36] W.S. Hancock, J.E. Battersby, A new micro-test for the detection of incomplete coupling reactions in solid-phase peptide synthesis using 2,4,6-trinitrobenzene-sulphonic acid, Anal. Biochem. 71 (1976) 260–264.
[37] E.I. Solomon, Spectroscopic methods in bioinorganic chemistry: blue to green to red copper sites, Inorg. Chem. 45 (2006) 8012–8025.
[38] A.I. Vokin, L.V. Sherstyannikova, I.G. Krivoruchka, T.N. Aksamentova, O. V. Krylova, V.K. Turchaninov, Solvatochromism of Heteroaromatic compounds: XX. 4(5)-nitroimidazole, Russ. J. Gen. Chem. 73 (2003) 973–984.

[39] N.A. Sieracki, H.J. Hwang, M.K. Lee, D.K. Garner, Y. Lu, A temperature independent pH (TIP) buffer for biomedical biophysical applications at low temperatures, Chem. Commun. 823-825 (2008).

[40] M.J. Nilges, K. Matteson, R.L. Bedford, in: M.A. Hemminga, L. Berliner (Eds.), ESR Spectroscopy in Membrane Biophysics 27, Springer, New York, 2007.

[41] L.J.C. Jeuken, F.A. Armstrong, Electrochemical origin of hysteresis in the electron-transfer reactions of adsorbed proteins: contrasting behavior of the \"blue\" copper protein, azurin, adsorbed on pyrolytic graphite and modified gold electrodes, J. Phys. Chem. B 105 (2001) 5271–5282.

[42] L.J.C. Jeuken, P. van Vliet, M.P. Verbeet, R. Camba, J.P. McEvoy, F.A. Armstrong, G.W. Canters, Role of the surface-exposed and copper-coordinating histidine in blue copper proteins: the electron-transfer and redox-coupled ligand binding properties of His117Gly Azurin, J.Am.Chem.Soc. 122 (2000) 12186–12194.

[43] T. Pascher, B.G. Karlsson, M. Nordling, B.G. Malmström, T. Vänngård, Reduction potentials and their pH dependence in site-directed-mutant forms of azurin from Pseudomonas aeruginosa, Eur. J. Biochem. 212 (1993) 289–296.

[44] E.T. Adman, in: P.M. Harrison (Ed.), Metalloproteins: Part 1: Metal Proteins with Redox Roles, Structure and Function of Small Blue Copper Proteins, Palgrave Macmillan UK, London, 1985, pp. 1–42.

[45] B.R. James, R.J.P. Williams, Oxidation-reduction potentials of some copper complexes, J. Chem. Soc. (Resumed) (1961) 2007–2019.

[46] C.-C. Su, H.C. Jan, H. Kuo-Yih, L. Shyh-Jiun, W. Shion-Wen, W. Sue-Lein, L. Sheng-Nan, Bonding properties of cu(II)-imidazole chromophores: spectroscopic and electrochemical properties of monosubstituted imidazole copper(II) complexes. Molecular structure of [cu(4-methylimidazole)4(ClO4)2], Inorg. Chim. Acta 196 (1992) 231–240.

[47] J. Prasad, E.J. Ukpong, K. Srivastava, Cyclic voltammetric investigations of copper (II) complexes with various imidazoles in dimethylformamide, J. Indian Chem. Soc. 85 (2008) 32–35.

[48] L.J.C. Jeuken, M. Ubbink, J.H. Bitter, P. van Vliet, W. Meyer-Klaucke, G. W. Canters, The structural role of the copper-coordinating and surface-exposed histidine residue in the blue copper protein azurin, J. Mol. Biol. 299 (2000) 737–755.